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Biocatalysis and Bioenergy

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CHING T. HOU • JEI-FU SHAW

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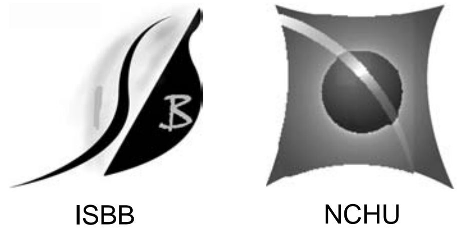

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PREFACE

This book was assembled with the intent of bringing together current advances and in-depth reviews of biocatalysis and bioenergy, with emphasis on biodiesel, bioethanol, biohydrogen, and industrial products. The book consists of selected papers presented at the International Symposium on Biocatalysis and Biotechnology held at the National Chung Hsing University, Taichung, Taiwan, on December 8–10, 2006. At this symposium, 47 distinguished international scientists shared their valuable research results. Additionally, there were 16 selected posters, 12 bioenergy exhibitions, and over 400 attendees. A few chapters contained in this book were contributed by distinguished scientists who could not attend this symposium. The meeting was a great success, and we greatly appreciate the contribution of local organization committee members at NCHU, including Dr. C. H. Yang, Director of NCHU Biotechnology Center, Dr. T. J. Fang, Dr. C. S. Wang, Dr. C. C. Huang, Dr. S. W. Tsai, Dr. Fuh-Jyh Jan, Dr. C. Chang, and their colleagues and students.

Biocatalysis and bioenergy as defined in this book include enzyme catalysis, biotransformation, bioconversion, fermentation, genetic engineering, and product recovery. Bioenergy includes energy derived from biomass, and all kind of biological resources. Due to the high cost of petroleum products, biofuels have drawn great attention recently. There is no comprehensive book on bioenergy or biofuels. The authors are internationally-recognized experts from all sectors of academia, industry, and governmental research institutes. This is the most current book on bioenergy and industrial products. Production of biofuels in the United States is forecast to exceed 16 billion gallons by 2015; ethanol will account for 9.4% of gasoline consumption, and biodiesel will be approximately 4% of the total estimated diesel consumption. Global production of ethanol is expected to exceed 120 billion gallons by 2020, while the worldwide production of biodiesel is expected to reach 3.2 billion gallons by the end of 2010.

Biocatalysis presents the advantages of high specificity, efficiency, energy conservation, and pollution reduction. Therefore, Biocatalysis and biotechnology are increasingly important for bioenergy production.

This book is composed of 32 chapters divided into three sections. The first 10 chapters describe the world's newest biodiesel research. Included is biodiesel research at NCAUR, USDA, production of biodiesel fuel through bioprocesses, a biodiesel cost optimizer-least cost raw material blending for standard quality biodiesel, new catalytic systems for vegetable oil transesterification

based on tin compounds, noncatalytic alcoholysis of vegetable oils for production of biodiesel fuel, improvement to the biodiesel batch process and impact on low temperature performance, development of new products from biodiesel glycerin, industrial products from biodiesel glycerols, optimization of lipase-catalyzed biodiesel through a statistical approach, and the production of biofuel from lipids and alternative resources. Five chapters in the second section are for bioethanol, and include biotechnology of holocellulose-degrading enzymes, from biogas energy to keratinase technology, emerging technologies in dry grind ethanol production, Gram positive bacteria as biocatalysts to convert biomass-derived sugars into biofuel and chemicals, and biological hydrogen production by strict anaerobic bacteria.

The final seventeen chapters discuss industrial products by biocatalysis and include the catalytically self-sufficient cytochrome P-450 monooxygenase system from *Bacillus megaterium* ALA2, the biocatalysis-based development of oligosaccharides in Japan, the synthesis of chiral intermediates for drugs, the screening of novel microbial enzymes and their application to chiral compound production, hydrogenation technologies for the production of high quality of biobeneficiary conjugated fatty acids, biotechnology of mannitol production, the physiological function of DHA phospholipids, the conversion of fishery by-products and waste into value-added products, the chemoenzymatic synthesis of structured lipids, the regiospecific analysis of castor triacylglycerols by ESI-MS, composition, functionality and potential applications of seaweed lipids, the enzymatic production of marine-derived protein hydrolysates and their bioactive peptides, bioengineering and application of glucose polymers, peroxidase-catalyzed polymerization of phenolic compounds containing carbohydrate residues, the production of lipase and oxygenated fatty acids from vegetable oils, production of biologically active hydroxyl fatty acids by *Pseudomonas aeruginosa* PR3, and the biotransformation of oils to value-added compounds.

This book serves as reference for teachers, graduate students, and industrial scientists who conduct research in biosciences and biotechnology.

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PART I

BIODIESEL

CHAPTER 1

Fuel Properties and Performance of Biodiesel

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1.1. INTRODUCTION

When being used as "alternative" diesel fuel, the mono-alkyl esters of vegetable oils or animal fats are referred to as biodiesel. Biodiesel is playing an

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increasingly important role in the fuel landscape, with production and use growing exponentially and standards being established around the world. It is produced by transesterifying the oil or fat in the presence of a catalyst with an alcohol—usually methanol—to the corresponding mono-alkyl esters. The reduced viscosity of the mono-alkyl esters in comparison to the parent oil or fat is critical for the production of biodiesel.

Biodiesel is technically competitive with petroleum-derived diesel fuel (petrodiesel). Correspondingly, research on biodiesel has continued to expand. Advantages of biodiesel include biodegradability, safer handling (as documented by a higher flash point), inherent lubricity, reduction of most regulated exhaust emissions, renewability, domestic origin, and compatibility with the existing fuel distribution infrastructure. Technical problems with biodiesel include oxidative stability, low-temperature properties, and an increase in NOx exhaust emissions. Accordingly, this chapter reviews some recent research results related to cetane numbers and exhaust emissions, cold flow, oxidative stability, and the viscosity and lubricity of biodiesel, besides providing a brief historical introduction.

1.2. HISTORY

At the Paris World Exposition in 1900, one of five diesel engines exhibited ran on peanut oil (Knothe, 2005), which is the first known use of a vegetable oil as a diesel fuel. The French government at that time was interested in a local energy source for its African colonies, as Rudolf Diesel (1858–1913), the inventor of the engine that bears his name, states in some of his writings (Diesel, 1912a, 1912b). The common statement that Diesel invented "his" engine to specifically use vegetable oils as fuel is therefore incorrect. Diesel's primary objective was to develop a more efficient engine, as he states in the first chapter of his *Die Entstehung des Dieselmotors (The Development [or Creation or Rise or Coming] of the Diesel Engine)* (Diesel, 1913). However, Diesel conducted later experiments with vegetable oils as fuels.

Considerable interest existed in some European countries from the 1920s through the 1940s in the use vegetable oils as diesel fuel, especially in countries with African colonies (Knothe, 2001, 2005). The objective was similar to the original demonstration in 1900 and to current background, namely to provide these colonies a local and renewable source of energy. There also was interest in countries such as Brazil, China and India. Especially in China, pyrolyzed vegetable oils were studied as fuel.

This early work documented results that are still valid today. For example, the high viscosity of vegetable oils was identified as a major problem causing engine deposits, and the fact that exhaust emissions of diesel engines are "cleaner" when running on vegetable oils than with petroleum-based diesel fuel was observed visually, although no quantitative exhaust emissions studies were performed (Knothe, 2001, 2005).

Walton (1938) also recognized that the glycerol moiety has no fuel value and suggested splitting it off and running the engine on the residual acids. However, the first documentation of esterified vegetable oil, biodiesel, as a fuel is the Belgian patent 422877 issued August 31, 1937, to Chavanne (Chavanne, 1937). Several other publications discuss the use of these esters as fuel (Chavanne, 1943; van den Abeele, 1942). The fuel was ethyl esters of palm oil. A commercial passenger bus apparently used this fuel on the Brussels-Louvain route (Leuven). In this work, the first cetane number testing of biodiesel, again in the form of ethyl esters of palm oil, was carried out (van den Abeele, 1942). The biodiesel fuel possessed a higher cetane number than the petroleum-based reference fuels.

The energy crises of the 1970s and early 1980s sparked interest in renewable and domestic sources of energy around the world. In this context, vegetable oils were remembered as potential feedstocks for alternative diesel fuels. In 1980, Bruwer *et al.* (1980) reported that diesel engines running on sunflower oil methyl esters were less prone to the build-up of engine deposits. Together with work in other countries, this research eventually led to the now-existing interest in biodiesel. Later developments included the development of standards and legislation and regulations in many countries around the world promoting the use of biodiesel.

1.3. COMBUSTION: CETANE NUMBERS AND EXHAUST EMISSIONS

1.3.1. Cetane Numbers

The cetane number (CN) is an indicator of the ignition quality of a diesel fuel. It is conceptually similar to the octane number (ON) used for gasoline. Generally, a compound that has a high ON tends to have a low CN and vice versa. The CN of a diesel fuel is related to the ignition delay (ID) time, *i.e.*, the time that passes between injection of the fuel into the cylinder and onset of ignition. A shorter ID corresponds to a higher CN and vice versa.

American Society for Testing and Materials (ASTM) method D 613 and International Organization for Standardization (ISO) method 5165 exist for determining the CN. Hexadecane ($C_{16}H_{34}$; trivial name cetane, giving the cetane scale its name) is the high quality standard on the cetane scale with an assigned CN of 100. A highly branched compound, 2,2,4,4,6,8,8,-heptamethylnonane (HMN, also $C_{16}H_{34}$), a compound with poor ignition quality, is the low-quality standard and has an assigned CN of 15. The two reference compounds on the cetane scale show that the CN decreases with decreasing chain length and increasing branching. Aromatic compounds, which occur in significant amounts in petrodiesel, have low CNs, but their CNs increase with the increasing size of *n*-alkyl side chains (Clothier *et al.*, 1993; Puckett and Caudle, 1948). The cetane scale is arbitrary and compounds with CN > 100 or CN < 15 have been identified. The standard specification for petrodiesel, ASTM D

975 (Anon., 2003a), requires CN ≥ 40, while those for biodiesel prescribe minimums of 47 for ASTM standard D 6751 (Anon., 2007a) and 51 for European Committee for Standardization (CEN) standard EN 14214 (Anon., 2003b). Due to the high CNs of many fatty compounds, which can exceed the cetane scale, the term "lipid combustion quality number" for these compounds was suggested (Freedman *et al.*, 1990).

Higher CN have been correlated with reduced nitrogen oxides (NOx) exhaust emissions (Ladommatos *et al.*, 1996), which has led to efforts to improve the CN number of biodiesel fuels by means of cetane-improving additives (Knothe *et al.*, 1997). Despite the inherently relatively high CNs of fatty compounds, NOx exhaust emissions usually increase slightly when operating a diesel engine on biodiesel. The connection between structure of fatty esters and exhaust emissions was investigated (McCormick *et al.*, 2001) by studying the exhaust emissions caused by enriched fatty acid alkyl esters used as fuel. NOx exhaust emissions increase with increasing unsaturation and decreasing chain length, which can also lead to a connection with the CNs of these compounds. Particulate emissions, on the other hand, were hardly influenced by the aforementioned structural factors. The relationship between the CN and emissions is complicated by many factors including the technological sophistication of the engine.

The influence of compound structure on CNs of fatty compounds was discussed (Harrington, 1986) and hypotheses confirmed by practical cetane tests (Freedman *et al.*, 1990; Ladommatos *et al.*, 1996; Klopfenstein, 1985; Knothe *et al.*, 2003). CNs of neat fatty compounds are given in Table 1.1. In summary, CNs decrease with increasing unsaturation and increase with increasing chain length, *i.e.*, uninterrupted CH_2 moieties. However, branched esters derived

TABLE 1.1. Cetane number (CN) of biodiesel and selected fatty acid alkyl esters.

Material	CN
Rapeseed (canola) oil methyl esters	47.9–56
Methyl soyate	48.7–55.9
Sunflower oil methyl ester	54–58
Methyl laurate	61.4; 60.8
Methyl myristate	66.2; 73.5
Methyl palmitate	74.5; 74.3; 85.9
Methyl stearate	86.9; 75.6; 101
Methyl oleate ($C_{18:1}$; Δ9 *cis*)	55; 59.3
Methyl linoleate ($C_{18:2}$; Δ9,Δ12-all *cis*)	42.2; 38.2
Methyl linolenate ($C_{18:3}$; Δ9,Δ12,Δ15-all *cis*)	22.7
Triolein	45
Ethyl oleate	53.9; 67.8
Propyl oleate	55.7; 58.8
Butyl oleate	59.8; 61.6

Source: Knothe *et al.*, 2005.

from alcohols such as *iso*-propanol have CNs competitive with methyl or other straight-chain alkyl esters (Knothe *et al.*, 2003; Zhang and Van Gerpen, 1996). Thus, one long, straight chain suffices to impart a high CN even if the other moiety is branched. Branched esters are of interest because they exhibit improved low-temperature properties. The CNs of most biodiesel fuels are in the range of about 48–60, as can be seen in Table 1.1 (Knothe *et al.*, 2005).

The CNs of fatty compounds have been investigated with an instrument termed the Ignition Quality Tester™ (IQT™) (Knothe *et al.*, 2003), which is a further, automated development of a constant volume combustion apparatus (CVCA) (Aradi and Ryan, 1995; Ryan and Stapper, 1987). The CVCA was originally developed for determining CNs more rapidly, with greater experimental ease, better reproducibility, and reduced use of fuel and therefore less cost than ASTM method D 613 utilizing a cetane engine. The IQT™ method, the basis of the standard ASTM D 6890 (Anon, 2007b), was shown to be reproducible and the results fully competitive or more reliable than those derived from ASTM D 613. Some results from the IQT™ are included in Table 1. For the IQT™, ID and CN are related by the following equation (Knothe *et al.*, 2003):

$$CN_{IQT} = 83.99 \times (ID - 1.512)^{-0.658} + 3.547 \qquad \text{[Eq. 1.1]}$$

In ASTM D 6890, only ignition delay times of 3.6–5.5 milliseconds, corresponding to 55.3 to 40.5 derived-CN (DCN), are covered since precision outside that range may be affected. However, the results for fatty compounds with the IQT™ are comparable to those obtained by other methods (Knothe *et al.*, 2003). Generally, the results of cetane testing for compounds with lower CNs, such as the more unsaturated fatty compounds, show better agreement over the various related literature references than the results for compounds with higher CNs. The reason is the non-linear relationship (Equation 1) between the ID and the CN. The non-linear relationship between the ignition delay time and the CN was observed previously (Allard *et al.*, 1996). Thus, small changes at shorter ignition delay times result in greater changes in CN than at longer ignition delay times. This would indicate a leveling-off effect on emissions—such as NOx discussed above—once a certain ignition delay time with corresponding CN has been reached, as the formation of certain species depend on the ignition delay time. However, for newer engines, this aspect must be modified.

1.3.2. Exhaust Emissions

Generally, four kinds of regulated exhaust emissions are analyzed when operating an engine. These species are particulate matter (PM), NOx, hydrocarbons (HC), and carbon monoxide (CO). Besides these regulated species, a host of other exhaust emissions are generated but they currently remain unregulated.

Biodiesel can lead to reductions of PM, HC and CO of 50% and more, with these results being summarized in a report by the United States Environmental Protection Agency (USEPA, 2002). However, more unregulated pollutants may be generated by the use of biodiesel, but without an increase in the total toxic emissions. Despite this effect of biodiesel operation on most regulated exhaust emissions species, biodiesel generally causes a slight increase (approximately 10%) of NOx exhaust emissions compared to petrodiesel. Since NOx exhaust emissions are precursors of ozone, a prime component of urban smog, this has led to considerable research efforts to identify the cause of this increase as well as to mitigate it. When blending biodiesel with petrodiesel, the effect of biodiesel on these blends is approximately linear to the blend level. Thus the common "B20" blend (20 vol% biodiesel in petrodiesel) displays reduced NOx increase but also less advantageous PM, HC and CO emissions. Also, the technology level of the engine has a strong effect on the levels of the exhaust emissions species (USEPA, 2002; McGeehan, 2004; Sharp *et al.*, 2000), as do engine load conditions (Krahl *et al.*, 2002, 2001).

During the combustion of fuels, it has been postulated that radicals react with atmospheric nitrogen to form NOx (Miller and Bowman, 1989). Otherwise, several causes have been postulated for the increase in NOx exhaust emissions when using biodiesel. One issue is the more widespread high-temperature distribution areas in the combustion chamber (Yuan *et al.*, 2005a), complemented by research postulating an increase in flame temperature due to the double bonds in biodiesel (Ban-Weiss *et al.*, 2006). Biodiesel also has a higher speed of sound and isentropic bulk modulus than petrodiesel, which can lead to changes in the fuel injection timing of diesel engines with resulting higher combustion pressures and temperatures, which in turn cause higher NOx exhaust emissions (Tat *et al.*, 2000). Other work discusses a similar influence of the bulk modulus of compressibility of biodiesel on NOx exhaust emissions (Boehman *et al.*, 2004). Changing the injection timing of the engine has been a method for reducing NOx exhaust emissions when using biodiesel.

Biodiesel in the United States successfully completed USEPA Tier II toxicity testing requirements for registering methyl soyate as a fuel or fuel additive under Title II, Section 211(b) of the Clean Air Acts (amended 1990) in a subchronic inhalation study designed in accordance with guidelines contained in 40 Code of Federal Regulations (CFR) 79, Subpart F (Finch *et al.*, 2002). Other health effects studies on exhaust emissions generated by the use of biodiesel have been conducted and the need for more research in this area has been discussed recently (Swanson *et al.*, 2007). It has been shown, however, that soot reduction when using biodiesel is connected to lower mutagenicity of the PM generated by biodiesel (Krahl *et al.*, 2001, 2002, 2003). This may be due to a lower content of polyaromatic hydrocarbons (Krahl *et al.*, 2002). Particle size plays a significant role in health effects of PM emissions, with small particles being especially problematic: biodiesel tended to cause more larger particles than petrodiesel under most load conditions (Krahl *et al.*,

2001). Other studies have also shown the favorable effect of biodiesel on PM and resulting health effects (Bagley *et al.*, 1998; Carraro *et al.*, 1997). Aldehydes, which are unregulated, tend to increase with biodiesel use (Krahl *et al.*, 2002).

Related to the discussion above, the effect of compound structure on exhaust emissions of biodiesel has been studied (McCormick *et al.*, 2001; Knothe *et al.*, 2006). Increasing unsaturation generally leads to an increase in NOx exhaust emissions, with saturated esters showing NOx exhaust emissions even slightly below the level of the petrodiesel reference fuel. The same effect was also reported for decreasing chain length in tests conducted with a 1991 model year engine (McCormick *et al.*, 2001) but hardly observed in tests with a 2003 model year engine (Knothe *et al.*, 2006). Biodiesel and its components reduced PM emissions more than neat hexadecane and dodecane, prime alkane components of "ultra-clean" petrodiesel fuels. The saturated species methyl palmitate and methyl laurate were especially effective, with reductions over 80% relative to the petrodiesel reference fuel (Knothe *et al.*, 2006). HC and CO emissions increased with shorter chain lengths. Furthermore, biodiesel and the neat esters tested in the 2003 model year engine almost met 2007 PM exhaust emissions standards of 0.01 g/hp h without any emissions control technologies.

The antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate, ascorbyl palmitate and citric acid were evaluated for potential to reduce NOx emissions from a single cylinder, direct-injection, air-cooled, naturally aspirated Yanmar engine (Hess *et al.*, 2005). BHA and BHT reduced NOx emissions by 4.4 and 2.9%, respectively, but the other antioxidants evaluated did not exhibit any beneficial effects (see Table 1.2). Antioxidants may impede NOx formation by inhibiting the formation of combustion-derived radicals.

1.4. COLD FLOW PROPERTIES OF BIODIESEL

In spite of its many advantages, performance during cold weather will impact the year-round commercial viability of biodiesel in moderate temperature climates. Field trials have demonstrated that methyl esters of soybean oil fatty acids (SME) cause performance issues when ambient temperatures approach 0 °C. As overnight temperatures decrease into this range, saturated fatty acid methyl esters (FAME) within SME solidify and form crystals that plug or restrict flow through fuel lines and filters.

All diesel fuels eventually cause start-up and operability problems when subjected to sufficiently low temperatures. As the ambient temperature cools, high-molecular weight paraffins present in petrodiesel nucleate and form solid wax crystals which, suspended in liquid, are composed of short-chain *n*-alkanes and aromatics (Chandler *et al.*, 1992; Owen and Coley, 1990; Lewtas *et al.*, 1991; Brown *et al.*, 1989; Zielinski and Rossi, 1984). Left unattended overnight

TABLE 1.2. Effect of antioxidants on nitrogen oxides (NOx) exhaust emissions.[a]

Ratio	Antioxidant	Loading ppm	Change in NOx[b] %
B0	None	—	−6.6 ± 1.7
B20	BHA	1000	−4.4 ± 1.0
B20	BHT	1000	−2.9 ± 1.5
B20	Ascorbyl palmitate	500[c]	−1.3 ± 0.9
B20	Citric acid	1000	−0.7 ± 0.5
B20	Propyl gallate	1000	−0.4 ± 2.8
B20	TBHQ	1000	−0.3 ± 1.6
B20	α-tocopherol	1000	+0.3 ± 0.2

Source: Hess *et al.*, 2005.
[a]Ratio = blend ratio soybean oil fatty acid methyl esters (SME) in low-sulfur (≤500 ppm) No. 2 petrodiesel (LSD) where "B0" is 0% SME and "B20" contains 20 vol% SME; BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; TBHQ = tert-butylhydroquinone.
[b]Relative to B20 blends without added antioxidant.
[c]500 ppm used instead of 1000 ppm due to limited solubility of ascorbyl palmitate in B20.

in these conditions, growth and agglomeration of solid wax crystals in the fuel cause engine starvation or failure during start-up the next morning.

Cold weather performance of diesel fuels is determined by measuring the tendency of a fuel to solidify or gel at low temperature. Cloud point (CP) is defined as the temperature where crystals become visible ($d \geq 0.5$ μm) (Chandler *et al.*, 1992; Own and Conley, 1990; Westbrook, 2003; Nadkarni, 2000; Botros, 1997). At temperatures below CP, crystals continue to grow and agglomerate eventually causing operability problems (Chandler *et al.*, 1992; Lewtas *et al.*, 1991; Botros, 1997; Chandler and Zechman, 2000; Heraud and Pouligny, 1992; Brown and Gaskill, 1990; Coley, 1989; Brown *et al.*, 1988; Brown *et al.*, 1989; Zielinski and Rossi, 1984). Pour point (PP) is the temperature where agglomeration prevents free movement of fluid (Owen and Conley, 1990; Westbrook, 2003; Nadkarni, 2000; Botros, 1997). Both parameters may be measured easily and rapidly; however, CP generally underestimates the temperature limit to avoid start-up or operability issues while PP tends to be over-optimistic (Chandler *et al.*, 1992; Owen and Coley, 1990; Coley, 1989; Rickeard *et al.*, 1991; McMillan and Barry, 1983).

The cold filter plugging point (CFPP) test was developed to predict more accurately low-temperature limits. After the oil is cooled at a specified rate to the test temperature, a 20 mL volume is drawn through a 45 μm wire mesh filter screen under 0.0194 atm vacuum. The method is repeated in 1 °C increments and CFPP is recorded as the lowest temperature where oil safely passes through the filter within 60 s (Chandler *et al.*, 1992; Owen and Coley, 1990; Westbrook, 2003; Nadkarni, 2000). Although CFPP holds nearly world-wide acceptance as a standard test, in North America more stringent test conditions are necessary to correlate with performance in the field. Thus, the less user-

friendly low-temperature flow test (LTFT) method was developed (Chandler *et al.*, 1992, Owen and Coley, 1990, Zielinski and Rossi, 1984; Brown *et al.*, 1988; McMillan and Barry, 1983; Chandler and Zechman, 2000). The oil is cooled at a rate of 1 °C/hr before 180 mL is drawn through a 17 μm wire mesh screen filter under 0.197 atm vacuum. Similar to CFPP, this method is repeated in 1 °C increments and LTFT is recorded as the lowest temperature where oil safely passes through the filter within 60 s (Chandler *et al.*, 1992, Owen and Coley, 1990, Westbrook, 2003, Rickeard *et al.*, 1991).

1.4.1. Effects of Biodiesel

Biodiesel made from feedstocks containing large concentrations of long-chain saturated fatty acids will have very poor cold flow properties. Less expensive feedstocks such as palm oil or tallow (see Table 1.3) may not be feasible in moderate temperature climates. In contrast, feedstocks with lower concentrations of long-chain saturated fatty acids yield biodiesel with more attractive cold flow properties. For example, biodiesel from canola, linseed, olive, rapeseed, and safflower oils have CP and PP close to or below 0 °C (Table 1.3).

Blending petrodiesel with SME significantly increases CP and PP at relatively low blend ratios (vol% SME) in No. 1 petrodiesel fuel and jet fuel (Dunn, 2001; Dunn and Bagby, 1995). For blends in No. 2 petrodiesel, increasing blend ratio increases CP and PP linearly (R^2 = 0.99 and 0.96). Blending petrodiesel with SME also increases CFPP and LTFT (Dunn and Bagby, 1995). Similar results were reported for blends with biodiesel derived from coconut oil, rapeseed oil, tallow and waste grease (see Table 1.4).

Nearly linear correlations for CFPP *versus* CP and LTFT *versus* CP were reported for neat biodiesel and its blends with petrodiesel (Dunn and Bagby, 1995, 1996; Dunn *et al.*, 1996). For LTFT, the correlation was essentially LTFT ~ CP, suggesting the labor- and time-intensive LTFT test could be spared by simply measuring CP. A major conclusion from these studies was that development of approaches to improve cold flow properties of biodiesel should focus on technologies that decrease CP.

Trace concentrations of contaminants are also known to influence cold flow properties of biodiesel. One study (Yu *et al.*, 1998) examined the effects of residual contaminants arising from refining and transesterification on cold flow properties of neat SME and its blends with No. 1 petrodiesel. Results showed that CP increases with increasing concentration of saturated monoacylglycerols and diacylglycerols. Unsaturated monoolein did not significantly affect CP or PP. Unsaponifiable matter increases CP and PP of SME at concentrations of 3 wt% but had no effect on a 20 vol% SME blend. More recent field data suggests that after long-term storage in cold weather monoacylglycerols and sterol glucosides may cause problems with insoluble matter buildup and plugged filters. Other contaminants arising from conversion processing that are known to influence cold flow properties include alcohol, free fatty acids, and triacylglycerols.

TABLE 1.3. Cold flow properties of biodiesel (B100) derived from various feedstock lipids and alcohols.[a]

Lipid	Alcohol	CP °C	PP °C	CFPP °C	LTFT °C
Canola oil	Methyl	1	–9		
Coconut oil	Ethyl	5	–3		
High oleic (HO) sunflower oil[b]	Methyl			–12	
Linseed oil	Methyl	0	–9		
Olive oil	Methyl	–2	–3	–6	
Palm oil	Methyl	13	16		
Rapeseed oil	Methyl	–2	–9	–8	
	Ethyl	–2	–15		
Safflower oil	Ethyl	–6	–6		
Soybean oil	Methyl	0	–2	–2	0
	Ethyl	1	–4		
	Isopropyl	–9	–12		
	n-Butyl	–3	–7		
	2-Butyl	–12	–15		
Soybean oil/tallow[c]	Methyl	2	1		
Tallow	Methyl	17	15	9	20
Tallow	Ethyl	15	12	8	13
Used cooking oil[d]	Methyl			–1	
Used hydrogenated soybean oil[e]	Ethyl	7	6		
Waste grease[f]	Ethyl	9	–3	0	9

Sources: Clark *et al.*, 1983; Dunn and Bagby, 1955; Foglia *et al.*, 1997; Fukuda *et al.*, 2001; González Gómez *et al.*, 2002; Kalligeros *et al.*, 2003; Lang *et al.*, 2001; Lee *et al.*, 1995; Masjuki *et al.*, 1993; Neto da Silva *et al.*, 2003; Peterson *et al.*, 2000; Peterson *et al.*, 1987; Wu *et al.*, 1998; Zhang and Van Gerpen. 1996.

[a]CP = cloud point; PP = pour point; CFPP = cold filter plugging point; LTFT = low-temperature flow test.

[b]Oleic acid content = 77.9 wt% before conversion to biodiesel.

[c]4 : 1 v/v soybean oil methyl ester/tallow methyl ester.

[d]Total saturated fatty acid methyl ester content = 19.2 wt%.

[e]Hydrogenated to iodine value = 65 g I_2/100 g oil.

[f]Free fatty acids ~9 wt% before conversion to biodiesel.

1.4.2. Improving Cold Flow Properties, Operability and Performance of Biodiesel

Many approaches for improving the cold flow properties of biodiesel have been explored. These include blending with petrodiesel, transesterification with medium or branched-chain alcohols instead of methanol or ethanol, crystallization fractionation, and treatment with cold flow improver (CFI) additives.

1.4.2.1. Blends with Petrodiesel Cold flow properties of FAME and fatty acid ethyl esters of various feedstocks in blends with petrodiesel are summa-

TABLE 1.4. Cold flow properties of biodiesel/petrodiesel blends.[a]

Lipid	Alcohol	Grade	Ratio	CP °C	PP °C	CFPP °C	LTFT °C
None	—	No. 1	B0	–31	–46	–42	–27
Soybean oil	Methyl	No. 1	B10	–22	–42		
Soybean oil	Methyl	No. 1	B20	–17	–30	–27	–19
Soybean oil	Methyl	No. 1	B30	–14	–25	–20	–16
Soybean oil/tallow[b]	Methyl	No. 1	B20	–21	–29	–21	–18
Soybean oil/tallow[b]	Methyl	No. 1	B30	–13	–24	–18	–14
None	—	No. 2	B0	–16	–27	–18	–14
Coconut oil	Ethyl	No. 2	B20	–7	–15		
Rapeseed oil	Ethyl	No. 2	B20	–13	–15		
Soybean oil	Methyl	No. 2	B20	–14	–21	–14	–12
Soybean oil	Methyl	No. 2	B30	–10	–17	–12	–12
Soybean oil/tallow[b]	Methyl	No. 2	B20	–12	–20	–13	–10
Soybean oil/tallow[b]	Methyl	No. 2	B30	–10	–12	–11	–9
HO sunflower oil[c]	Methyl	No. 2	B30		–12		
Tallow	Methyl	No. 2	B20	–5	–9	–8	
Tallow	Ethyl	No. 2	B20	–3	–12	–10	1
Used hydrogenated soybean oil[d]	Ethyl	No. 2	B20	–9	–9		
Waste grease[e]	Ethyl	No. 2	B20	–12	–21	–12	–3

Sources: Anon., 2003a; Dunn and Bagby, 1995; Foglia *et al.*, 1997; Neto da Silva *et al.*, 2003; Peterson *et al.*, 2000; Wu *et al.*, 1998.

[a]Grade = petrodiesel grade according to ASTM fuel specification D 975; Ratio = blend ratio where 'B0' = 0 vol% biodiesel, 'B10' = 10%, 'B20' = 20% and 'B30' = 30%. See Table 1.3 for other abbreviations.

[b]4 : 1 v/v soybean oil methyl ester/tallow methyl ester.

[c]Oleic acid content = 77.9 wt% before conversion to biodiesel.

[d]Hydrogenated to iodine value = 65 g I_2/100g oil).

[e]Free fatty acids ~9 wt% before conversion to biodiesel.

rized in Table 1.4. Properties of non-blended petrodiesel ("B0") are included for comparison and those for corresponding neat biodiesel ("B100") are in Table 1.3. An earlier study (Dunn and Bagby, 1995) reported that blends in No. 1 petrodiesel exhibit better CP, PP, CFPP and LTFT than blends in No. 2 petrodiesel largely because No. 1 petrodiesel generally has better cold flow properties than No. 2 petrodiesel. As discussed above, CP and PP increased nearly linearly with respect to increasing SME blend ratio. Although blends with 20–30 vol% SME in No. 2 petrodiesel did not greatly increase CFPP or LTFT, some exceptions were reported for LTFT of blends with tallow and waste grease ethyl esters. Blends in No. 1 petrodiesel had significant increases in CP, PP, CFPP and LTFT at blend ratios as low as 20 vol% SME. However, under some conditions blends with up to 30 vol% SME in No. 1 petrodiesel may be allowable (CFPP = –20 °C and LTFT = –16 °C as shown in Table 1.4).

As discussed above, CFPP and LTFT of biodiesel and its blends in petrodiesel demonstrate nearly linear correlation with respect to CP. Results from linear regression analyses yielded the following equations:

$$CFPP = 1.02(CP) - 2.9 \quad \text{[Eq. 1.2]}$$

and

$$LTFT = 1.02(CP) - 0.4 \quad \text{[Eq. 1.3]}$$

where $R^2 = 0.90$ and 0.95 and $\sigma_y = 2.5$ and 1.8, respectively (Dunn and Bagby, 1995). Statistical analysis indicated high probabilities ($P = 0.81$ and 0.78, respectively) that slope was unity for both equations. Analysis also yielded $P = 0.60$ favoring an intercept = 0 hypothesis for Equation 1.3, suggesting LTFT may be estimated reasonably accurately by measuring CP. This work also demonstrated that the key to developing effective approaches for improving cold flow properties and performance of biodiesel and biodiesel/petrodiesel blends is to identify ways to first significantly reduce CP.

1.4.2.2. Transesterification with Medium or Branched Chain Alcohols

Biodiesel made from conversion of oils or fats with medium (C_3—C_8) or branched chain alkyl alcohols generally possesses better cold flow properties than the corresponding FAME. For example, melting points (MP) for ethyl palmitate (19 °C) and stearate (31 °C) are significantly below those of their corresponding methyl esters (30 and 39.1 °C) (Lyde, 1990). Large or bulky alkyl headgroups disrupt spacing between individual molecules resulting in formation of nuclei with less stable packing during the early stages of crystallization.

Soybean oil fatty acid methyl, ethyl and *n*-butyl esters were reported to have CP = 0, 1 and –3 °C, respectively (Dunn and Bagby, 1995, Foglia *et al.*, 1997, Clark *et al.*, 1983). Similar decreases in CP with respect to increasing alkyl headgroup chainlength were reported for fatty acid alkyl esters of canola and linseed oils (Lang *et al.*, 2001). Transesterification of these oils with *n*-butanol decreased PP by 4–7 °C with respect to their corresponding methyl esters. Decreases in CP, PP, CFPP and LTFT of tallow alkyl esters with respect to corresponding methyl, ethyl, *n*-propyl and *n*-butyl head groups were also reported (Foglia *et al.*, 1997). Soybean oil fatty acid isopropyl and 2-butyl esters were reported to have CP = –9 and –12 °C and PP = –12 and –15 °C (Zhang and Van Gerpen, 1996; Lee *et al.*, 1995), compared with CP = 0 °C and PP = –2 °C for SME (Dunn and Bagby, 1995). Similar results were reported for CP, PP, CFPP and LTFT of tallow fatty acid isopropyl and isobutyl esters (Foglia *et al.*, 1997).

Transesterification of fats or oils with medium-chain alcohols may increase CN, a parameter that can influence ignition quality and exhaust emissions (Klopfenstein, 1985). On the other hand, increased branching in saturated hydrocarbon chains also decreases CN. Two studies comparing isopropyl

esters of soybean oil fatty acids in blends with petrodiesel reported that these esters had similar performance and reduced exhaust emissions (Zhang and Van Gerpen, 1996; Chang *et al.*, 1996). Results showed that some emissions were further reduced when compared to analogous blends with SME (Chang *et al.*, 1996). Blends with *n*-butyl esters of soybean oil yielded performance and emission results that were also comparable to blends with SME (Clark *et al.*, 1983).

Disadvantages of this approach include increases in kinematic viscosity and overall fuel costs. Viscosity generally increases with the molecular weight of the hydrocarbon chain, though branching in alkyl headgroups may mitigate this effect. However, a major portion of the total fuel cost for biodiesel is the cost of acquiring feedstock and reactant alcohols. Thus, substituting higher alcohols for methanol or ethanol may significantly increase total fuel costs. One possible compromise may be to prepare mixtures of FAME or fatty acid ethyl esters with small portions of esters of higher alcohol.

1.4.2.3. Crystallization Fractionation (Winterization) Biodiesel made from most common feedstocks may be considered a pseudo-binary mixture of components categorized by a relatively large MP differential. Saturated long-chain FAME have very high MP (*e.g.*, 30 °C for methyl palmitate) while unsaturated and polyunsaturated FAME have very low MP (−21 °C for methyl oleate; −35 °C for methyl linoleate) (Lyde, 1990). This differential suggests that an effective approach for improving cold flow properties would be to reduce the total concentration of high-MP components by crystallization fractionation.

The traditional process consists of two stages, crystallization followed by separation. The crystallization stage consists of selective nucleation and crystal growth under controlled cooling and gentle agitation. Once well defined crystals are formed, the resulting slurry is transferred to the second stage for separation into solid and liquid fractions, typically by filtration or centrifugation (Illingworth, 2002; Kellens and Hendrix, 2000; O'Brien, 1998; Anderson, 1996; Krishnamurthy and Kellens, 1996; Rajah, 1996; Duff, 1991; Bailey, 1950). Product yield as defined by separation of high- and low-melting fractions depends greatly on maintaining control of both stages of the process (Anderson, 1996; Krishnamurthy and Kellens, 1996; Rajah, 1996; Duff, 1991).

Dry fractionation is defined as crystallization from a melt without dilution with solvent (O'Brien, 1998; Anderson, 1996; Bailey, 1950). This process is the simplest and least expensive process for separating high and low-MP fatty derivatives (Illingworth, 2002; Kellens and Hendrix, 2000; O'Brien, 1998; Anderson, 1996; Krishnamurthy and Kellens, 1996). It is also the most commonly practiced form of fat fractionation technologies currently in use (Illingworth, 2002; O'Brien, 1998).

Cold flow properties of liquid fractions obtained from dry fractionation of SME and FAME derived from tallow (TME) and used cooking oil (UCOME) are summarized in Table 1.5. These results showed that dry fractionation

significantly improved cold flow properties in comparison with those of non-fractionated biodiesel (Table 1.3). Earlier reports (Dunn *et al.*, 1996; Dunn *et al.*, 1997; Dunn, 1998) demonstrated that application of dry fractionation to SME significantly reduces CP, PP, CFPP and LTFT of SME. These reductions required six steps where bath temperature of each step was decreased in 2 °C increments. Results in Table 1.5 also show that dry fractionation may reduce CP and LTFT of SME to temperatures below those reported for No. 2 petrodiesel (Table 1.4). Although total concentration of saturated FAME decreased to from 18.1 to 5.6 wt%, liquid yields were very small (≤33.4%) relative to mass of starting material (Dunn *et al.*, 1996, 1997).

Lee *et al.* (1996) applied step-wise crystallization fractionation to SME and reported similar results (Table 1.5). The liquid fraction had a total saturated FAME content = 5.5 wt% and crystallization onset temperature = –7.1 °C (determined by differential scanning calorimetry (DSC), compared to values of 15.6% and 3.7 °C before fractionation. Liquid product yield was also relatively low (25.5%).

González Gómez *et al.* (2002) applied crystallization fractionation to UCOME (Table 1.5). Following crystallization at a cooling rate = 0.1 °C/min, fractionation reduced CFPP from –1 to –5 °C and total saturated FAME content from 19.2 to 14 wt%. Again, liquid yields were low (25–30 wt%). Krishnamurthy and Kellens (1996) reported that application of crystallization fractionation to TME increases iodine value (IV) from 41 to 60 and reduces CP from 11 to –1 °C with liquid yields of 60–65%.

Crystallization fractionation of biodiesel modified by the use of crystallization modifiers and organic solvents was also investigated. Results from some of these studies are summarized in Table 1.5. Treating SME with crystallization modifiers commonly used in petrodiesel, such as PP-depressants (PPD) DFI-200 and Winterflow, increased liquid product yields to 80.0–87.0% and decreased CP and CFPP to temperatures below –10 °C (Dunn, 1998; Dunn *et al.*, 1997). Analogous to dry fractionation, fractionation using crystallization modifiers required six steps each with incrementally decreasing bath temperatures. Single-step crystallization fractionation from two organic solvents was investigated (Dunn *et al.*, 1997). Fractionation from hexane at –25 °C resulted in liquid product yields up to 78.4% and decreased CP to –10 °C. Fractionation from isopropanol at –15 °C resulted in yields up to 80.6 wt% and decreased CP to –9 °C.

Lee *et al.* (1996) also investigated crystallization fractionation of SME from several solvents. Fractionation from hexane in three sequential steps with a final bath temperature of –28.4 °C resulted in a liquid product yield of 77% and total saturated FAME content of 6.0 wt%. Crystallization onset temperature by DSC of the liquid fraction was –5.8 °C. Fractionation of SME from methanol solvent separated into two liquid layers as cooling temperatures approached –1.6 °C. Acetone did not reduce crystallization onset temperature of the liquid fraction, and chloroform failed to form crystals at temperatures below –25 °C. Hanna *et al.* (1996) studied fractionation of TME

TABLE 1.5. Cold flow properties of fractionated biodiesel.[a]

Biodiesel	Solvent	Modifier[b]	B/S g/g	Steps	T_{Bath} °C	Yield g/g	Sats. wt%	CP °C	PP °C	CFPP °C
SME	None	None	—	6	–10	0.334	6.3	–20	–21	–19
SME	None	None	—	11	–10	0.255	5.5	–7.1[c]		
UCOME[d]	None	None	—	1	–1	0.25–0.30	14			–5
SME	None	DFI-200, 2000 ppm	—	6	–10	0.801	9.8	–11		–12
SME	None	Winterflow, 2000 ppm	—	6	–10	0.870	9.3	–11		–11
SME	Hexane	None	0.284	1	–25	0.784	16.2	–10	–11	–10
SME	Hexane	None	0.217	3	–28.4	0.77	6.0	–5.8[c]		
SME[e]	Hexane	DFI-200, 2000 ppm	0.200	1	–34	0.992	13.5	–5	–12	
SME[e]	Hexane	Winterflow, 2000 ppm	0.200	1	–34	1.029	11.1	–5	–12	
SME	Isopropanol	None	0.228	1	–15	0.860	10.8	–9	–9	–9
SME[e]	Isopropanol	DFI-200, 2000 ppm	0.200	1	–20	0.952	12.8	–6	–9[f]	
SME[e]	Isopropanol	Winterflow, 2000 ppm	0.199	1	–20	0.989	13.3	–5	–9[f]	

Sources: Dunn, 1998; Dunn *et al.*, 1997; Lee *et al.*, 1996; González Gómez *et al.*, 2002.

[a]UCOME = used cooking oil methyl esters; B/S = biodiesel/solvent mass ratio; T_{Bath} = bath coolant temperature (final step); Yield = mass ratio liquid fraction to starting material; Sats = total concentration of saturated fatty acid methyl esters in liquid fraction (determined by GC). See Tables 1.2 and 1.3 for other abbreviations.

[b]Crystallization modifiers DFI-200 and Winterflow are pour point depressants (PPD) for petrodiesel, added before first fractionation step.

[c]Crystallization onset by differential scanning calorimetry (DSC).

[d]Total saturated FAME content = 19.2 wt% (before conversion of used cooking oil to biodiesel).

[e]Liquid fractions contained small quantities of residual solvent following evaporation.

[f]Results for samples fractionated at –15 °C.

from ethanol, petrodiesel and ethanol/petrodiesel solvents. Fractionation from ethanol/petrodiesel blends in five steps with a final bath temperature of –16 °C produced a liquid fraction with CP reduced to –5 °C.

Crystallization fractionation of biodiesel carries a number of disadvantages. Manipulating the FAME profile in SME affected kinematic viscosity, acid value (AV), specific gravity, IV, and the oxidation induction period (Dunn, 1998). Reducing total saturated FAME concentration had a profound impact on oxidative stability as determined from significant decreases in the oil stability index. Increasing the total unsaturated and polyunsaturated FAME concentration may increase ignition delay time as measured by CN, an effect that worsens performance and increases harmful exhaust emissions (Knothe *et al.*, 1997; Harrington, 1986; Knothe *et al.*, 1996). Fractionation will also increase fuel cost. Solvent-based fractionation will increase costs for safety, handling, and recovery of solvents (Illingworth, 2002; Kellens and Hendrix, 2000; O'Brien, 1998; Anderson, 1996; Krishnamurthy and Kellens, 1996; Rajah, 1996; Duff, 1991; Bailey, 1950). Separating and recycling crystallization modifiers or solvents and decontaminating collected fractions from residual solvent traces may also prove difficult. Finally, unless uses are developed for solid fraction(s) collected from the process, crystallization fractionation will make biodiesel production more costly.

1.4.2.4. Cold Flow Improver (CFI) Additives Performance and economic benefits of utilizing CFI additives to improve cold flow properties of petrodiesel have been recognized for more than 40 years. Many additives effective in modifying wax crystal nucleation, growth, or agglomeration mechanisms are reported in the scientific and patent literature. While exact chemical structures are proprietary, the most effective additives are generally composed of low molecular weight copolymers similar in melting point to *n*-alkanes found in petrodiesel. These additives include a variety of polymers such as ethylene vinyl acetate copolymers, fumarate vinyl acetate copolymers, alkenyl succinic amides, long-chain polyalkylacrylates, polymethacrylates, polyalkylmethacrylates, polyethylenes, chlorinated polyethylenes, copolymers of linear α-olefins with acrylic, vinylic and maleic compounds, copolymers containing esterified derivatives of maleic anhydride, styrene-maleate esters and vinyl acetate-maleate esters, secondary amines, random terpolymers of α-olefin, stearylacrylate and *N*-alkylmaleimide, copolymers of acrylate/methacrylate with maleic anhydride partially amidated with *n*-hexadecylamine, itaconate copolymers, polyethylene-polypropylene block copolymers, polyamides of linear or branched acids and copolymers of α-olefins, maleic anhydride copolymers, carboxy-containing interpolymers, styrene-maleic anhydride copolymers, polyoxyalkylene compounds, and copolymers with long chain alkyl groups from fatty alcohols as pendant groups (Chandler *et al.*, 1992; Owen and Coley, 1990; Lewtas *et al.*, 1991; Heraud and Pouligny, 1992; Brown and Gaskill, 1990; Machado and Lucas, 2002; Hipeaux *et al.*, 2000; Monkenbusch *et al.*, 2000; El-Gamal *et al.*, 1998; Davies *et al.*, 1994; Lal, 1994; Lal *et al.*, 1994; Böhmke and

Pennewiss, 1993; Lewtas and Block, 1993; Demmering *et al.*, 1992; Bormann *et al.*, 1991; Denis and Durand, 1991; Desai *et al.*, 1991; Müller *et al.*, 1991; Beiny *et al.*, 1990; Damin *et al.*, 1986.).

CFI additives mitigate the effect of wax crystals on fuel flow by modifying their shape, size, growth rate and degree of agglomeration. Their combined effect promotes formation of a very large number of smaller, more compact needle-shaped crystals. When trapped by fuel filters these crystals form a semi-permeable cake layer allowing some fuel flow through the filter (Chandler *et al.*, 1992; Owen and Coley, 1990; Lewtas *et al.*, 1991; Brown *et al.*, 1989; Zielinski and Rossi, 1984; Brown and Gaskill, 1990; Coley, 1989; Rickeard *et al.*, 1991; Damin *et al.*, 1986; Holder and Thorne, 1979). Some additives allow operation of diesel engines at temperatures as low as 10 °C below CP of the fuel (Brown *et al.*, 1989; Zielinski and Rossi, 1984; Botros, 1997; Richeard *et al.*, 1991.). Polymer-wax interactions are generally specific, so a particular additive may not perform equally well in all fuels.

Effects of six commercial petrodiesel CFI additives on SME and its blends with No. 1 and No. 2 petrodiesel were studied previously (Dunn and Bagby, 1996; Dunn *et al.*, 1996). Results from these studies are summarized in Table 1.6. These petrodiesel CFI additives decreased PP by up to 18–20 °C for B30 blends in No. 1 petrodiesel and B20 blends in No. 2 petrodiesel. Similarly, these additives decreased PP of neat SME ("B100") by as much as 6 °C. When applied to unblended petrodiesel ("B0"), these additives reduced PP by 7 °C for No. 1 petrodiesel and by 23 °C for No. 2 petrodiesel (Dunn *et al.*, 1996). These results suggested that mechanisms associated with crystalline growth and agglomeration in neat biodiesel were similar to those for petrodiesel (Dunn and Bagby, 1996; Dunn *et al.*, 1996).

Comparison of data in Tables 1.3, 1.4 and 1.6 demonstrates that increasing CFI additive loading (0 to 2000 ppm) decreases PP of SME and its blends in petrodiesel. Although reductions in PP tended to be proportionate to loading, some additives were more efficient than others. Additionally, effectiveness diminished with increasing blend ratio of SME in the blends, with respect to constant additive loading (Dunn *et al.*, 1996). The CFI additives listed in Table 1.6 were also effective in decreasing CFPP. Nylund and Aakko (2000) reported similar results when studying the effects of a CFI additive on cold flow properties of FAME from rapeseed oil (RME) blends in No. 2 petrodiesel. Biodiesel producers and sellers in the United States are presently treating their products with CFPP improvers during cooler weather.

In contrast to the PP data listed in Table 1.6, none of the CFI additives greatly affected CP of SME or its blends with No. 1 or No. 2 petrodiesel. In terms of wax crystallization, CFI additives designed for treating petrodiesel did not selectively modify crystal nucleation in biodiesel (Dunn *et al.*, 1996).

Regression analysis of CFPP and LTFT *versus* CP data sets combining results for biodiesel and biodiesel/petrodiesel blends with those for formulations treated with CFI additives yielded the following equations:

TABLE 1.6. Cold flow properties of SME and its blends in No. 1 and low-sulfur (500 ppm) No. 2 petrodiesel treated with commercial petrodiesel CFI additives.[a]

Ratio	Grade	Additive[b]	Loading, ppm	CP, °C	PP, °C
B100	—	DFI-100	1000	–2	–6
			2000	–1	—
B100	—	DFI-200	1000	–1	–8
			2000	–1	–16
B100	—	Hitec 672	1000	–2	–6
			2000	–2	—
B100	—	OS 110050	1000	–1	–7
			2000	–3	—
B100	—	Paramins	1000	0	–5
			2000	–1	—
B100	—	Winterflow	1000	0	–5
			2000	–1	–17
B30	No. 1	DFI-100	1000	–14	–49
B30	No. 1	DFI-200	1000	–21	–45
B30	No. 1	Hitech 672	1000	–13	–44
B30	No. 1	OS 110050	1000	–17	–46
B30	No. 1	Paramins	1000	–14	–29
B30	No. 1	Winterflow	1000	–19	–39
B20	No. 2	DFI-100	1000	–14	–26
B20	No. 2	DFI-200	1000	–14	–32
B20	No. 2	Hitech 672	1000	–14	–27
B20	No. 2	OS 110050	1000	–15	–18
			2000	—	–21
B20	No. 2	Paramins	1000	–14	–27
B20	No. 2	Winterflow	1000	–13	–39
			2000	—	–59

Sources: Dunn and Bagby, 1996; Dunn *et al.*, 1996, 1997.
[a]Ratio = blend ratios in petrodiesel as defined in Table 1.4; Grade = see Table 1.4; CFI = cold flow improver. See Tables 1.2 and 1.3 for other abbreviations.
[b]Vendors: Du Pont (DFI-100, DFI-200); Ethyl Corp. (Hitech 672); Exxon Chemical (Paramins); SVO/Lubrizol (OS 110050); Starreon Corp. (Winterflow).

$$CFPP = 1.03(CP) - 2.2 \quad \text{[Eq. 1.4]}$$

and

$$LTFT = 0.81(CP) - 2.4 \quad \text{[Eq. 1.5]}$$

where $R^2 = 0.82$ and 0.90 and $\sigma_y = 3.5$ and 4.0, respectively (Dunn and Bagby, 1996; Dunn *et al.*, 1996). Similar to Equations 1.2 and 1.3, Equations 1.4 and 1.5 exhibit a nearly linear correlation between CFPP and LTFT with respect to CP. Although scatter in the data resulted in relatively low R^2 coefficients,

graphical evidence showed that generally decreasing CP is coupled with decreases in CFPP and LTFT. Statistical analysis showed $P = 0.743$ that slope was unity for Equation 1.4, though the slope of Equation 1.5 was decidedly not ($P < 0.0001$). Overall, results from this comparison of CFPP and LTFT data with corresponding CP data were consistent with those discussed above for biodiesel and biodiesel/petrodiesel blends. Addition of CFI additives did not alter the aforementioned conclusion that the most effective approaches for improving cold flow properties of biodiesel will be those that significantly decrease CP.

Chiu *et al.* (2004) investigated four commercial CFI additives at 0.1–2.0 wt% in SME and blends thereof in No. 1 and No. 2 petrodiesel (500 ppm S). In agreement with the studies discussed above, two additives significantly decreased PP of SME blends, but all four additives had little effect on CP, as can be seen from Tables 1.7 and 1.8. Also in agreement with a previous study (Dunn and Bagby, 1995) was the determination that PP changed in a linear fashion with blend composition, which is consistent with freezing point depression from the blending of miscible liquids. Lastly, the additive mixing procedure (addition of additives to SME either pre- or post-blending of SME with diesel fuels) did not appear to impact either CP or PP, as shown by Table 1.7.

Shrestha *et al.* (2005) conducted a study in which SME, mustard seed oil methyl and ethyl esters and used peanut oil methyl esters were blended (B0, B5 and B10) with No. 2 petrodiesel and treated with six commercial petrodiesel CFI additives. It was found that at 100, 200, and 300% of the specified loading rate, CP and PP were reduced by an average of 2.2 °C and 14.1 °C, respectively. Mustard seed oil ethyl esters exhibited the highest average reduction in CP and PP and SME exhibited the lowest, as shown by Table 1.9 for CP. Furthermore, a significant decrease in CP was noticed when additive concentration was increased from 100% of the specified loading rate to 200%; however, the difference between 200% and 300% was not significant. The authors conclude that the effect of fuel additive is not only different for different feedstocks but also some fuel additives worked better for a specific blend of biodiesel with No. 2 petrodiesel.

1.4.2.5. Development of CFI Additives for Treating Biodiesel It was noted when comparing CFPP and LTFT with corresponding CP data (above) that the best approach for improving cold flow properties and performance of biodiesel would be to significantly decrease CP. Advances in the development of CP-depressants (CPD) specifically designed for biodiesel and blends thereof in ultra-low sulfur petrodiesel fuel (ULSD) will be required for additive strategies to be maximally effective in improving the low temperature operability of biodiesel fuel. Such strategies should entail the design of new materials whose molecular structures impart a greater degree of selectivity toward high melting point alkyl esters to allow for modification of the crystalline growth behavior of biodiesel.

TABLE 1.7. CP and PP of SME blended with low-sulfur (500 ppm) No. 2 petrodiesel treated with OS-110050 commercial petrodiesel CFI additive.[a]

OS 110050[b] wt%	Ratio vol%	Mix method 1[c]		Mix method 2[d]	
		CP, °C	PP, °C	CP, °C	PP, °C
0	B100	–4	–7	—	—
0	B40	–12	–15	—	—
0	B30	–12	–15	—	—
0	B20	–15	–21	—	—
0	B0	–18	–26	—	—
0.1	B100	–7	–12	—	—
0.1	B40	–12	–15	—	—
0.1	B30	–15	–21	—	—
0.1	B20	–15	–18	—	—
0.1	B0	–18	–29	—	—
0.2	B100	–7	–12	—	—
0.2	B40	–12	–18	—	—
0.2	B30	–12	–18	—	—
0.2	B20	–15	–21	—	—
0.2	B0	–15	–32	—	—
0.5	B100	–4	–7	—	—
0.5	B40	–15	–21	–15	–21
0.5	B30	–15	–23	–15	–23
0.5	B20	–15	–26	–12	–26
0.5	B0	–15	–32	—	—
0.75	B100	–7	–15	—	—
0.75	B40	–12	–26	—	—
0.75	B30	–15	–29	—	—
0.75	B20	–15	–29	—	—
0.75	B0	–18	–29	—	—
1.0	B100	–4	–7	—	—
1.0	B40	–15	–29	–15	–29
1.0	B30	–15	–32	–15	–29
1.0	B20	–15	–31	–15	–29
1.0	B0	–12	–32	—	—
2.0	B100	–7	–18	—	—
2.0	B40	–15	–18	–15	–26
2.0	B30	–15	–29	–15	–31
2.0	B20	–15	–31	–15	–26
2.0	B0	–12	–31	—	—

Source: Chiu *et al.*, 2004.

[a]Ratio = blend ratio in No. 2 petrodiesel where "B40" = 40 vol% SME, "B0" = 100% No. 2 petrodiesel and others are as defined in Table 1.4. See Tables 1.2, 1.3 and 1.6 for other abbreviations.

[b]0.1 wt% = 1000 ppm.

[c]SME treated with OS-110050 before being blended with petrodiesel.

[d]SME blended with petrodiesel before being treated with OS-110050.

TABLE 1.8. CP and PP of SME and its blends in No. 1 petrodiesel treated with 0.1 wt% (1000 ppm) commercial CFI petrodiesel additives.[a]

Additive[b]	Ratio, vol%	CP, °C	PP, °C
None	B0	–1	–6
None	B10	–3	–12
None	B20	–5	–15
Bio Flow-875	B0	–1	–9
Bio Flow-875	B10	–3	–18
Bio Flow-875	B20	–5	–33
Bio Flow-870	B0	0	–18
Bio Flow-870	B10	–4	–24
Bio Flow-870	B20	–5	–30
Diesel Fuel Anti-Gel	B0	0	–15
Diesel Fuel Anti-Gel	B10	–4	–21
Diesel Fuel Anti-Gel	B20	–5	–24

Source: Chiu *et al.*, 2004.
[a]Ratio = blend ratio in No. 1 petrodiesel as defined in Tables 1.4 and 1.7. See Tables 1.2, 1.3 and 1.6 for other abbreviations.
[b]Vendors: Octel Starreon, LLC. (Bio Flow-870, Bio Flow-875); Dawn Chemical Corp. (Diesel Fuel Anti-Gel).

The first step in pursuit of this approach is to identify and test compounds for selectivity towards modifying nucleation and disrupting crystalline growth and agglomeration in biodiesel formulations. Analogous to petrodiesel CFI additives, compounds for treating biodiesel should possess some CPD characteristics. Soriano *et al.* (2006, 2005) investigated the utility of ozonized vegetable oil as a PPD for neat biodiesel. Ozonized vegetable oils (1.0–1.5 wt%) were effective in reducing the PP of biodiesel prepared from sunflower, soybean and rapeseed oils to –29, –11 and –31 °C, respectively, as shown in Table 1.10. In agreement with previously mentioned studies, CP was essentially unaffected. The lowest reduction in PP was observed in cases where the biodiesel and ozonized samples were prepared from the same vegetable oil. Furthermore, DSC and polarized light microscopy indicated that adding ozonized vegetable oil affects both nucleation and crystal growth of biodiesel. It was discovered that treatment of biodiesel with ozonized vegetable oil leads to formation of considerably smaller, more numerous crystals at subzero temperatures.

Two earlier studies (Kenar *et al.*, 2005; Knothe *et al.*, 2000) were conducted on the effects of compounds synthesized with hydrocarbon tail-group structures resembling those of FAME with attached bulky moieties. These studies examined novel fatty diesters made from reaction of diols with acids and diacids with 2-octanol in toluene solvent with *p*-toluene sulfonic acid catalyst and branched-chain esters of carbonic acid (carbonates) synthesized in nonyl alcohol with *n*-dibutyltin oxide catalyst. None of the synthesized compounds demonstrated effectiveness in decreasing CP or PP of SME.

TABLE 1.9. CP depression at manufacturer's recommended additive loading for biodiesel from various feedstocks and blend levels in low-sulfur (500 ppm) No. 2 petrodiesel.[a]

Additive[b]	MEE			MME			SME			UPEME		
	B5	B20	B100	B5	B20	B100	B5	B20	B100	B5	B20	B100
BioFlow 875	−2.0	0.0	−2.3	−1.5	−1.5	−1.3	−1.5	−1.0	−0.6	−1.0	−2.0	−1.7
Flozol 503	−0.5	−1.5	−0.1	−1.0	−1.5	−0.5	−1.0	−1.0	−1.3	−0.5	−1.0	−1.8
MCC P205	−3.0	−2.5	−6.3	−2.5	−2.0	−0.5	−1.7	−1.8	−1.3	−1.5	−1.5	−2.1
Arctic Express 0.25%	−3.5	−3.0	−6.4	−2.0	−2.5	−1.0	−0.3	0.0	−0.8	−2.5	−1.0	−2.3

Source: Shrestha *et al.*, 2005.

[a]MEE = mustard seed oil ethyl esters; MME = mustard seed oil methyl esters; UPEME = used peanut oil methyl esters; B5 = 5 vol% biodiesel in No. 2 petrodiesel blends; 'B20' and 'B100' are defined in Tables 1.4 and 1.6. See Tables 1.2 and 1.3 for other abbreviations.

[b]Vendors: Octel Starreon, LLC (Bioflow875); Lubrizol Corp. (Flozol 503); Midcontinental Chemical (MCC P205); Power Service (Arctic Express 0.25%).

TABLE 1.10. CP and PP of biodiesel derived from sunflower, soybean, palm and rapeseed oils blended with ozonized sunflower oil (OSFO).[a]

Fuel formulation	CP, °C	PP, °C
Neat SFME	1.0	–5.0
1.0% OSFO A[b] in SFME	0.0	–24.0
1.5% OSFO A in SFME	1.0	–25.0
1.0% OSFO B[b] in SFME	1.0	–29.0
1.5% OSFO B in SFME	1.0	–26.0
Neat SME	1.0	–2.0
1.0% OSFO A in SME	1.0	–9.0
1.5% OSFO A in SME	–1.0	–10.0
1.0% OSFO B in SME	2.0	–11.0
1.5% OSFO B in SME	1.0	–11.0
Neat PME	18.0	12.0
1.0% OSFO A in PME	13.0	11.0
1.5% OSFO A in PME	13.0	11.0
1.0% OSFO B in PME	13.0	11.0
1.5% OSFO B in PME	13.0	11.0
Neat RME	–4.0	–13.0
1.0% OSFO A in RME	–6.0	–30.0
1.5% OSFO A in RME	–4.0	–30.0
1.0% OSFO B in RME	–5.0	–30.0
1.5% OSFO B in RME	–5.0	–31.0

Source: Soriano *et al.*, 2006.
[a]SFME = sunflower oil methyl esters; PME = palm oil methyl esters; RME = rapeseed oil methyl esters. See Tables 1.2 and 1.3 for other abbreviations.
[b]OSFO A and OSFO B contain 0.183 and 0.206 g O_3/mL oil, respectively.

Two previous studies (Moser *et al.*, 2007, 2006) explored the effects of synthetic compounds with hydrocarbon tail-group structures resembling those of FAME with attached bulky moieties on the CP and PP of SME. These studies examined novel fatty ethers made from the reaction of various alcohols (C_2—C_{10}) with epoxidized alkyl oleates in the presence of sulfuric acid catalyst. Bulky esters (isopropyl and isobutyl) were chosen to further enhance the low temperature fluidity of the synthetic adducts produced. As the chain length of the ether moiety attached to the fatty backbone increased in length, a corresponding improvement in low temperature performance was noticed. Although the materials had improved low temperature properties over that of neat SME, none of the synthesized compounds demonstrated effectiveness in decreasing CP or PP when added to SME.

Two reports in the patent literature claim the invention of CFI additives specifically designed to improve the cold weather performance of biodiesel (Scherer and Souchik, 2001; Scherer *et al.*, 2001). Block copolymers of long-chain alkyl methacrylates and acrylates were effective as PPD and flow improv-

ers for lubricant oils and biodiesel fuel additives. Another patent reported that methacrylate copolymers decrease CFPP of biologically-derived fuel oils and biodiesel fuels made from rapeseed oil (Auschra *et al.*, 1999).

Efforts have also been made to employ glycerol in synthesis of CFI agents. Two patents reported that glycerol co-products from biodiesel production can be reacted with isobutylene or isoamylene in the presence of a strong acid catalyst to produce glycerol ether derivatives (Noureddini, 2001, 2000). Adding the derivatives back to biodiesel was shown to improve fuel characteristics, though very large quantities (>1%) were generally required to significantly reduce CP.

1.5. OXIDATIVE STABILITY

The storage stability of a liquid fuel is defined by its relative resistance to physical and chemical changes brought about by interaction with its environment (Westbrook, 2003). Stability takes into account interactions of olefins, dienes and nitrogen-, sulfur- and oxygen-containing compounds that can lead to sediment formation and changes in color depending on type and quantity of unstable materials present. Cleanliness of the fuel with respect to the presence of water, particulate solids, fuel degradation products and microbial slimes also influences stability (Giles, 2003).

Fuel properties degrade during long-term storage by *i)* oxidation or autoxidation from contact with ambient air, *ii)* thermal or thermo-oxidative decomposition from excess heat, *iii)* hydrolysis from contact with water or moisture in tanks and fuel lines, and *iv)* microbial contamination from migration of dust particles or water droplets containing bacteria or fungi into the fuel (Westbrook, 2003, Giles, 2003). In terms of fuels derived from fatty compounds such as biodiesel, effects of oxidative degradation during storage on overall fuel quality have been identified as a major concern by fuel distributors, suppliers and consumers.

Biodiesel derived from oils or fats is typically a mixture of relatively high concentrations of long-chain monounsaturated and polyunsaturated compounds to promote more compatible cold flow properties. Thus, biodiesel such as SME is significantly more prone to oxidative degradation than petrodiesel. Unsaturated organic compounds are significantly more susceptible to autoxidation than saturated compounds. Autoxidation occurs at allylic methylene positions along the backbone of olefinic compounds progressing at different rates depending on number and location of allylic methylene positions (Frankel, 1998). With respect to long-chain fatty acid alkyl esters such as biodiesel, the polyunsaturated esters are particularly vulnerable to autoxidation because they contain more allylic methylene positions than monounsaturated esters. The relative rates of autoxidation of the "C_{18}" unsaturates given in the literature (Frankel, 1998, and references therein) clearly demonstrate this point: 1:41:98 for oleates:linoleates:linolenates.

Oxidative degradation during long-term storage may affect fuel quality of biodiesel and its blends with petrodiesel. Oxidation under accelerated conditions such as elevated temperatures or purging with dry air or oxygen affects kinematic viscosity, AV, and peroxide value (PV) (Dunn, 2002; Monyem *et al.*, 2000; Bondioli *et al.*, 1995; Thompson *et al.*, 1998; Du Plessis *et al.*, 1985, 1983). Increases in kinematic viscosity may indicate presence of high-molecular weight polymers while increases in AV generally indicate presence of secondary degradation products. Increases in PV may result in an increase in CN, a parameter that can affect ignition delay time (Clothier *et al.*, 1993; Van Gerpen, 1996). Factors known to influence oxidation of fatty derivatives include presence or exclusion of air, temperature, light, oxidation inhibitors (antioxidants), oxidation promoters (pro-oxidants) such as hydroperoxides and metal catalysts (Bondioli *et al.*, 1995; Du Plessis *et al.*, 1985; Knothe and Dunn, 2001; Canakci *et al.*, 1999; Graboski and McCormick, 1998; Knothe and Dunn, 1997). Consequently, a great deal of study has gone into developing test methods for monitoring and improving the oxidative stability of biodiesel.

An earlier study (Dunn, 2002) examined effects of oxidation under controlled accelerated conditions on fuel properties of SME. SME samples from four different sources with varying storage histories were oxidized at elevated temperatures with continuous stirring under a $0.5\,cm^3$/min air purge. Antioxidants *tert*-butylhydroxyquinone (TBHQ) and α-tocopherol at 2000 ppm loadings retarded oxidative degradation of SME as determined by analysis of kinematic viscosity, AV, PV, specific gravity, CP, and PP results. It was shown that kinematic viscosity and AV had the best potential as parameters for timely and easy monitoring of biodiesel fuel quality during long-term storage.

1.5.1. Monitoring Oxidative Stability

Stavinoha and Howell (2000, 1999; Stavinoha, 1998) reviewed many standard tests and recommended modification of ASTM methods D 2274 (Oxidation Stability of Distillate Fuel Oil [Accelerated Method]), D 4625 (Diesel Storage Stability at 43 °C), or D 6468 (High Temperature Stability of Distillate Fuels). Method D 2274 showed poor precision and little correlation with field study data or with parameters utilized by the fats and oils industry (Westbrook, 2003; Giles, 2003; Monyem *et al.*, 2000). Bondioli *et al.* (2002) and Canakci *et al.* (1999) reported that D 2274 was not suitable for biodiesel because its degradation led to formation of soluble polymers that could not be efficiently isolated or quantified by washing and filtration. Although method D 4625 demonstrates good correlation with field study data, this method has a relatively long (4–16 week) test period that precludes it from being useful in monitoring oxidative stability of stored fuels (Westbrook, 2003; Giles, 2003; Monyem *et al.*, 2000; Stavinoha and Howell, 2000; Bondioli *et al.*, 2002). Method D 6468 was also problematic due to its inability to cope with difficult to isolate soluble polymers during degradation (Giles, 2003; Bondioli *et al.*, 2002).

The Biodiesel Stability (BIOSTAB) project, supported by the European Commission, was initiated in 2001 to establish clear criteria and analytical methods for the monitoring biodiesel fuel stability (Various, 2003; Prankl, 2002). The resulting unified method, EN 14112 (Anon., 2003c) established a means for measuring oxidative stability utilizing the Rancimat or oxidation stability instruments. This test method was essentially developed from standards employed in the fats and oils industry to measure isothermally the induction period for oxidation of fatty derivatives. At present, both biodiesel fuel standards ASTM D 6751 (Anon., 2007a) and EN 14214 (Anon., 2003b) include an oxidative stability specification based on measurement by method EN 14112.

In general, the test is based on analysis of the oil stability index (OSI) in accordance with American Oil Chemists' Society (AOCS) method Cd 12b-92 (Anon., 1999). An oil sample is placed in a glass test tube and held stationary in an electrically controlled block heater. The tube is capped and dry air is continuously purged at a constant flow rate through the sample. Effluent air containing volatile degradation products is then swept out of the test tube and transferred into a second test tube containing deionized water. Among the degradation products from thermo-oxidation of fatty compounds are volatile organic acids which are subsequently dissolved in the water. The conductivity in the water reservoir is continuously monitored by a probe with resultant data being recorded in 3 min (0.05 h) increments. OSI (the oxidation induction period) is the point where the second derivative of the conductivity *versus* time data curve equals zero. For analysis of biodiesel, block temperature must be set to 110 °C and sample mass is constant at 3 g for Rancimat and 5 g for the oxidative stability instrument. Biodiesel fuel specifications ASTM D 6751 and EN 14214 specify a minimum OSI = 3 h and 6 h, respectively (Anon., 2007a, 2003b).

As part of the aforementioned BIOSTAB framework, studies by Lacoste and Lagardere (2003) and Mittelbach and Schober (2003) applied analysis of OSI by Rancimat instrument to RME, TME, UCOME and FAME derived from sunflower oil. The former study also reported good correlations between OSI and AV, ester content and linolenic acid content. Bondioli *et al.* (Bondioli *et al.*, 2002) reported that OSI may be used to monitor degradation of biodiesel samples aged under storage conditions simulating ASTM method D 4625 (Diesel Storage Stability at 43 °C). Earlier applications of the Rancimat instrument to measure effects of aging on biodiesel were also reported by Du Plessis *et al.* (1985), Bondioli *et al.* (1995) and Mittelbach and Gangl (2001). Canakci *et al.* (1999) observed that conducting OSI measurements at 110 °C was not suitable for evaluating biodiesel derived from high IV feedstocks including SME. Biodiesel with IV > 110 tends to undergo very rapid thermo-oxidation under these conditions. Based on fuel tank temperature with normal return rates, this report recommended a more appropriate test temperature of 60 °C for SME. This result agreed with those reported in another study on the effects of block temperature on OSI of neat SME and its blends with JP-8 jet fuel

(Dunn and Knothe, 2003). That study also recommended block temperatures of 75-90 °C for SME/JP-8 jet fuel blends.

An earlier study (Dunn, 2005a) examined OSI analyses at 60 °C of SME from five separate sources by oxidative stability instrument. Samples had varying histories of storage and handling. Results showed that OSI was an effective means for monitoring oxidative stability of SME. This study also recommended utilizing a control or reference material such as methyl oleate to facilitate comparison of OSI analyses conducted periodically over a long period of time, such as the monitoring of biodiesel during storage. Finally, this work suggested that OSI was more sensitive than IV for detecting effects of oxidative degradation in its early stages when monitoring SME during storage.

Another work (Knothe and Dunn, 2003) investigated effects of fatty acid structure on OSI of SME. Despite having equivalent IV, OSI results at 70 °C reported for methyl petroselinate ($C_{18:1}$; Δ6) and vaccenate ($C_{18:1}$; Δ11) were significantly higher than for methyl oleate ($C_{18:1}$; Δ9). Besides position of the double bond, variations in alkyl headgroup chain length, hydrocarbon tail-group chain length, and fatty acid or ester group functionality more significantly affected OSI than IV. Small quantities of unsaturated compounds containing bis-allylic carbon positions may have a disproportionately large effect on OSI compared to compounds containing just allylic carbon positions.

The IV is defined as an average total number of double bonds per mole in a mixture of fatty materials. It is not a good descriptor for correlating physical and chemical properties with fatty acid composition in biodiesel. For example, the IV does not provide any information on structural factors such as number of allylic or bis-allylic methylene groups or location of double bond(s) within the hydrocarbon chain. Knothe (2002) recommended alternative indices termed allylic position equivalents (APE) and *bis*-allylic position equivalent (BAPE) based on the total number of allylic and bis-allylic positions present in the fatty acid chains.

Thermal analytical techniques such as thermogravimetric analysis (TGA), conventional DSC and pressurized-DSC (P-DSC) have been extensively developed for studying oxidation reactions of many types of materials. Thermal analysis has advantages related to acceleration of rates of reaction at elevated temperatures and continuous purging with fresh dry air or oxygen. P-DSC has the added advantage of providing elevated pressures which increases the total number of moles of oxygen available for reaction. These techniques have been applied to the analysis of petrochemical and synthetic lubricants, biodegradable lubricants, aviation turbine oils and polymers (Sharma and Stipanovic, 2003; Gamelin *et al.*, 2002; Riga *et al.*, 1998; Yao, 1997; Zeman *et al.*, 1995; Patterson and Riga, 1993; Zeman *et al.*, 1993; Kaufman and Rhine, 1988; Zeman *et al.*, 1984; Walker and Tsang, 1980; Noel, 1972) as well as edible fats and oils, air-blown vegetable oils, epoxidized soybean oil, genetically modified soybean oil and alcohol-based fuels (Adhvaryu and Erhan, 2002; Kenar *et al.*, 2002; Tan *et al.*, 2002; Adhvaryu *et al.*, 2000; Kowalski *et al.*, 2000; Perez, 2000;

Litwinienko and Kasprzycka-Guttman, 1998; Litwinienko *et al.*, 1995; Kasprzycka-Guttman *et al.*, 1995; Kowalski, 1995; Hassel, 1976; Cross, 1970).

Perhaps the first study to examine oxidation of FAME was performed by Raemy *et al.* (1987). Results showed that increasing temperature or degree of unsaturation decreased induction period in isothermal DSC curves. Induction periods also demonstrated a direct correlation with results from analysis by Rancimat instrument. Several studies have reported on the use of non-isothermal (temperature-ramping) DSC and P-DSC to study (Litwinienko *et al.*, 2000; Litwinienko and Kasprzyska-Guttman, 2000; Litwinienko *et al.*, 1999) oxidation kinetics of C_{18} fatty acids and their ethyl esters and reported that increasing the degree of unsaturation increases reactivity by decreasing activation energies for oxidation.

Dunn (2000) demonstrated that increasing pressure from 1000 to 5000 kPa does not significantly affect oxidation onset temperature (OT) of SME from non-isothermal static mode (zero air-purge) P-DSC curves scanned at heating ramp rate (β) = 5 °C/min. A more recent work by Dunn (2006a) compared analysis of OT by non-isothermal P-DSC in static and dynamic (positive air-purge) modes, TGA and (conventional) DSC. Results from TGA analyses at β = 5 °C/min and under ambient pressure failed to detect measurable oxidation. On the other hand, results from dynamic mode P-DSC curves were consistently lower than those obtained from conventional DSC curves, with respect to constant β = 5 °C/min. This was attributed to the increased quantity of air (oxygen) present in the P-DSC cell (P = 2000 kPa) compared to air in the conventional DSC cell (ambient pressure). Increasing the quantity of oxygen available for reaction accelerated the oxidation component of the thermo-oxidative reaction mechanism resulting in a decrease in OT. Similar comparison of OT results from dynamic and static mode P-DSC showed that the positive air-purge further accelerates oxidation by replacing oxygen as it was consumed during the reaction. Thus, OT data from dynamic mode P-DSC were consistently lower than those from static mode P-DSC curves.

Similar to studies reported by Litwinienko and co-workers discussed above, a recent report (Dunn, 2006b) demonstrated that non-isothermal (conventional) DSC, static mode P-DSC and dynamic mode P-DSC may be employed to study kinetics of the oxidation of SME. OT results obtained at ambient pressure for DSC and P = 2000 kPa for P-DSC and with varying β = 1–20 °C/min were analyzed by the Ozawa-Flynn-Wall method to calculate activation energies and rate constants. This work concluded that rates of the oxidation reaction could be calculated at any temperature based on accurate measurement of kinetic parameters from analysis of non-isothermal dynamic mode P-DSC scans.

Many advances have been made in development of P-DSC and other thermal analysis techniques in the study of oxidation reactions in fatty derivatives such as biodiesel. Kinetic parameters and phase transitions associated with oxidative degradation may be rapidly and accurately determined. However, the applicability of P-DSC may be limited in analysis of fuel formu-

lations since it does not allow direct measurement of effects of oxidation on kinematic viscosity, AV, PV and other important fuel properties (Stavinoha and Kline, 2001).

Other techniques to be investigated for analyzing effects of oxidation on fatty derivatives include monitoring PV, conjugated dienes, anisidine value, and carbonyls. Spectroscopic methods include electron spin resonance, infrared, fluorescence, chemiluminescence, and NMR (Shahidi and Wanasundara, 1998).

1.5.2. Improving Oxidative Stability of Biodiesel

A common strategy to combat autoxidation of biodiesel, aside from minimizing storage time, is the use of oxidation inhibitors (antioxidants). Derived from both natural and synthetic sources, antioxidants are diverse in structure and mode of action (metal chelators, free radical scavengers, etc.). In general, the biodiesel industry prefers synthetic antioxidants owing to their increased efficacy over natural antioxidants. Loadings (concentrations) are kept relatively low, 200–1000 ppm, to minimize effects on costs. Furthermore, synergism among antioxidants in the edible fats and oils industry is well documented (Frankel, 1998). Thus, many commercial antioxidant formulations are mixtures of two or more oxidation inhibitors.

Although there are numerous publications on the effect of natural and synthetic antioxidants on the stability of oils and fats used as food and feed, until recently relatively little publicly available information was available on the effect of antioxidants on the oxidative stability of biodiesel. One of the earliest studies reporting of the effects of antioxidants on biodiesel was that of Du Plessis *et al.* (1985), which examined storage stability of sunflower oil methyl esters (SFME) at various temperatures for 90 d. Effects of air temperature, presence of light, addition of TBHQ (see Figure 1.1) and contact with steel were evaluated by analysis of free fatty acid content, PV, kinematic viscosity, anisidine value, and induction period. Addition of TBHQ delayed oxidation of samples stored at moderate temperatures (≤30 °C). In contrast, under "unfavorable" (50 °C) conditions, TBHQ was ineffective.

Canacki *et al.* (1999) tested the influence of TBHQ on PV of SME during storage and found good improvement in stability. Simkovsky and Ecker (1999) investigated several antioxidants at relatively low loading (300 ppm) on the induction period of RME at different temperatures using the active oxygen method (AOM) but did not find significant improvement.

Working within the aforementioned "BIOSTAB" framework, Bondioli *et al.* (2003) studied the stability of RME under commercial storage conditions over a one year period using the Rancimat method (EN 14112, Anon., 2003c) with and without the addition of TBHQ and pyrogallol (Figure 1.1). Both additives, as can be seen in Table 1.11, imparted significant improvement in the oil stability index (OSI) compared with RME not treated with added antioxidant. This study also reported significant decreases in storage stability

BHT TBHQ BHA TBHT Trolox

Ethoxyquin

Pyrogallol	R = H
Propyl Gallate	R = COOPr
Octyl Gallate	R = COOOc
Dodecyl Gallate	R = COODo

Ascorbyl Palmitate

Figure 1.1. Selected Synthetic Antioxidants. BHT = Butylated hydroxytoluene; TBHQ = *tert*-butylhydroxyquinone; BHA = butylated hydroxyanisole; TBHT = *tert*-butyl hydroxytoluene; Trolox = 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

caused by removal of natural antioxidants (tocopherols) by distillation of RME. The authors conclude by suggesting, in part, that the minimum concentration of antioxidant be used for optimum effects since higher concentrations may have negative impacts on other parameters.

In 2003, Mittelbach and Schober, also working as part of the 'BIOSTAB' program, investigated the influence of ten commercially available synthetic antioxidants and ten mixtures of natural antioxidants on the OSI of undistilled and distilled RME, SFME, UCOME, and TME. The greatest enhancement in OSI was obtained from the synthetic antioxidants pyrogallol, propyl gallate, TBHQ, and butylated hydroxyanisole (BHA). These four antioxidants plus butylated hydroxytoluene (BHT), due to its widespread use in the food industry, were selected for further study at loadings from 100 to 1000 ppm. In agreement with results reported by Bondioli *et al.* (2003), undistilled biodiesel samples exhibited increased oxidative stability as measured by OSI than their distilled counterparts, with the exception of TME (2003). Furthermore, OSI of RME, UCOME, and TME could be improved significantly with pyrogallol, propyl gallate, and TBHQ, whereas BHT was not as effective.

Conversely, SFME exhibited relatively poor improvement in oxidative stability with the use of antioxidants, presumably due to the higher concentrations of linoleic acid methyl esters in sunflower oil in comparison to the other biodiesel samples evaluated by the authors. Therefore, a good correlation was found between the improvement in oxidative stability as measured by OSI when antioxidants are used and the fatty acid composition of the biodiesel sample (Mittelbach and Schober, 2003).

Schober and Mittelbach (2004) also investigated the impact of concentration of eleven synthetic phenolic antioxidants on oxidative stability of RME, UCOME and TME) by EN 14112 (Anon., 2003c) and on other relevant fuel

TABLE 1.11. Rancimat induction peroid (RIP = OSI) during a 12-month period of storage of RME with and without TBHQ and pyrogallol.[a]

			Monthly RIP [OSI], h							
Biodiesel	Antioxidant	Loading ppm	0	1	2	3	5	7	9	12
Distilled RME	None	—	4.16	4.21	4.23	4.25	4.11	4.02	4.01	3.89
Undistilled RME	None	—	9.20	8.84	8.35	7.65	7.37	7.22	7.08	6.83
Undistilled RME	TBHQ	400	36.00	35.85	35.00	34.17	33.05	33.18	33.73	32.77
Undistilled RME	Pyrogallol	250[b]	22.42	22.25	nd	22.25	22.33	21.82	21.54	20.85

Source: Bondioli *et al.*, 2003.
[a]OSI = oil stability index; nd = not determined. See Tables 1.2 and 1.10 for other abbreviations.
[b]250 ppm used instead of 400 ppm due to limited solubility of pyrogallol in RME.

properties such as kinematic viscosity, density, carbon residue, CFPP, sulfated ash, and AV. At 1000 ppm, an improvement in oxidative stability was achieved for all antioxidants studied, as can be seen from Table 1.12. In particular, DTBHQ (di-tertbutylhydroquinone), Ionox 220, Vulkanox ZKF, Vulkanox BKF, and Baynox were able to improve significantly the OSI of biodiesel samples, even at lower concentrations. Variation of antioxidant loading from 100–1000 ppm showed that antioxidant efficacy varied depending on the type of biodiesel treated. For instance, the oxidative stability of RME at antioxidant loadings < 250 ppm could only be improved to a satisfactory level by Baynox. DTBHQ exhibited the strongest benefit on RME and UCOME, whereas Vulkanox BKF imparted highest OSI values for distilled UCOME and TME (see Table 1.12). Evaluation of the influence of antioxidants on other important biodiesel fuel parameters showed no negative impacts on kinematic viscosity, density, carbon residue, CFPP, and sulfated ash of the various biodiesel samples. In contrast, a noticeable increase in AV was observed at antioxidant levels of 1000 ppm. At lower antioxidant concentrations, the increase was much lower and AV remained within the prescribed EN 14214 limit of 0.50 mg KOH/g (Anon., 2003b). Lastly, the authors advise that to minimize the potential negative effect of antioxidants on other fuel properties that they be employed at the lowest possible useful loading, as also recommended by Bondioli *et al.* (2003).

TABLE 1.12. Effect of added synthetic antioxidant (1000 ppm) on OSI of RME, UCOME, Distilled-UCOME and Tallow methyl esters (TME).[a]

	OSI, h			
Antioxidant[b]	RME	UCOME	Distilled-UCOME	TME
None	4.56	7.50	2.03	4.74
DTBHQ	9.37	34.11	16.62	27.54
Ionox 220	8.62	21.89	19.77	45.73
Vulkanox ZKF	7.49	14.43	11.51	18.23
Vulkanox BKF	8.63	21.89	26.53	52.24
Baynox[c]	9.36	11.85	11.02	15.85
Ionox 103	8.35	11.10	7.09	10.86
Ionox 99	5.54	9.99	7.12	13.11
Ionox 75	5.72	9.65	7.46	13.71
Inol K65	6.00	9.83	8.21	13.12
MBP-K	5.67	8.89	5.71	12.03
MBM-K	4.97	8.16	4.35	9.41

Source: Schober and Mittelbach, 2004.

[a]DTBHQ = 2,5-di-*tert*-butylhydroquinone. See Tables 1.2, 1.5, 1.10 and 1.11 for other abbreviations.

[b]Vendors: Degussa Sant Celoni SA (Ionox 75, Ionox 99, Ionox 103, Ionox 220, Inol K65); Bayer AG (Vulkanox BFK, Vulkanox ZKF, MBP-K, MBM-K, Baynox); Fine Chemicals (DTBHQ).

[c]Loading = 5000 ppm to achieve effective antioxidant concentration of 1000 ppm (Baynox is 20% BHT in biodiesel).

Dunn (2005a) examined OSI of SME samples from five different sources with varying storage histories with and without added antioxidants. Both TBHQ and α-tocopherol increased OSI in comparison to untreated SME (see Table 1.13). TBHQ at 500 ppm exhibited greater efficacy than α-tocopherol at 1000 ppm at reducing oxidative degradation of biodiesel in all but one SME tested. Variations in OSI response of the various SME samples treated with antioxidants were likely due to aging and other effects sustained prior to acquisition and treatment with additives.

Liang *et al.* (2006) investigated the effect of natural and synthetic antioxidants on oxidative stability of crude and distilled palm oil FAME (PME). Crude palm oil contains minor components such as carotenes and α-tocopherols that upon transesterification, yields crude PME that exhibit superior oxidative stability (OSI > 25 h) compared to distilled PME (OSI ~ 3.5 h), which does not contain minor components. Liang and colleagues therefore endeavored to improve the oxidative stability of distilled PME so that it meets the minimum OSI = 6 h as specified in EN 14214 (Anon., 2003b). Natural (α-tocopherol) and synthetic (BHT and TBHQ) antioxidants were investigated in distilled PME. It was discovered that both natural and synthetic antioxidants exhibited beneficial effects on the oxidative stability of distilled PME

TABLE 1.13. Effect of TBHQ and α-tocopherol on OSI of SME.[a]

Ester	Antioxidant	Loading ppm	OSI[b] h	IV[c] g I_2/100 g oil
Methyl oleate	None	0	140 ± 3.5	84.7
SME-A	None	0	9.4 ± 0.25	133.4
	α-Tocopherol	1000	24 ± 1.0	
	TBHQ	500	54 ± 1.0	
SME-B	None	0	9.5 ± 0.22	120.4
	α-Tocopherol	1000	17.8 ± 0.53	
	TBHQ	500	20.2 ± 0.39	
SME-C	None	0	4.1 ± 0.59	130.7
	α-Tocopherol	1000	21 ± 4.3	
	TBHQ	500	17.2 ± 0.48	
SME-D	None	0	7.2 ± 0.30	134.5
	α-Tocopherol	1000	36.4 ± 0.30	
	TBHQ	500	146 ± 1.7	
SME-E	None	0	53 ± 1.3	130.3

Source: Dunn, 2005a.

SME from five different sources: SME-A, B and C were obtained from the ADEPT Group (Los Angeles, CA), SME-D from the National Biodiesel Board (Jefferson City, MO) and SME-E directly from its producer; Methyl oleate (99+%) was from Nu Chek Prep (Elysian, MN).

[a]IV = iodine value. See Table 1.2 for other abbreviations.

[b]OSI analyses by oxidative stability instrument: Block temperature = 60 °C, air flow rate = 150 mL/min.

[c]Calculated based on fatty acid composition determined by GC.

TABLE 1.14. Effect of natural and synthetic antioxidants on OSI of crude and distilled palm oil biodiesel (PME).[a]

		Dosage of Antioxidant, ppm			
Biodiesel	Mixture	α-Tocopherol	BHT	TBHQ	OSI h
Crude PME	654[b]	—	—	—	25.70
Distilled PME	0	—	—	—	3.52
Distilled PME	0	1000	—	—	6.17
Distilled PME	0	—	50	—	6.42
Distilled PME	0	—	—	50	8.85

Source: Liang *et al.*, 2006.
[a]See Tables 1.2, 1.10 and 1.11 for abbreviations.
[b]Mixture = α-Tocopherol (119 ppm)/α-tocotrienol (113 ppm)-tocotrienol (352 ppm)/δ-tocotrienol (70 ppm).

(see Table 1.14). In accordance with previously mentioned studies, synthetic antioxidants BHT and TBHQ were more effective than natural antioxidants as lower concentrations (17 times less) were necessary to achieve OSI > 6 h. Another study by Loh *et al.* (2006) investigated the effect of natural and synthetic antioxidants on oxidative stability of PME produced from used cooking oil. The order of increasing antioxidant effectiveness, determined by OSI, was found to be: α-tocopherol < BHT < TBHQ < BHA < PG.

Litwinienko *et al.* (1997) reported that non-isothermal (conventional) DSC and P-DSC analyses can evaluate antioxidant activity in linolenic ($C_{18:3}$) acid doped with phenolic antioxidants. Activation energies were inferred by applying the Ozawa-Flynn-Wall analytical method. Results showed that increasing BHT loading from 0.3 to 4.0 mM increased the activation energy of oxidation from 73.0 to 97.8 kJ/mol.

Stavinoha and Kline (2001) adapted ASTM method D 6186 (Oxidation Induction Time of Lubricating Oils by Pressure Differential Scanning Calorimetry [P-DSC]) for analyzing the oxidative stability of SME treated with antioxidants. This report concluded that isothermal P-DSC analysis is suitable for screening the effectiveness of antioxidants for treating biodiesel.

An earlier study (Stavinoha and Howell, 2000) examined the effects of TBHQ and α-tocopherol on oxidative stability of SME from four different sources by non-isothermal P-DSC in static (zero air-purge) mode. P-DSC curves were analyzed by measuring the OT where P = 2000 kPa, initial temperature = 25 °C, and β = 5 °C/min. Results for two of the SME samples showed that addition of 2000 ppm α-tocopherol increased OT by ~20 °C while addition of 2000 ppm TBHQ increased OT by ~30 °C. Addition of the same concentration of α-tocopherol and TBHQ to the other two SME samples increased OT by ~30 °C and ~40 °C, respectively. Interpretation of these results suggested TBHQ was more effective at increasing relative resistance to oxidation of SME than α-tocopherol, a conclusion that was in accordance with those by Mittelbach and Schober (2003) for the isothermal Rancimat method.

Although P-DSC results were consistent with corresponding OSI at 50 °C, no correlation for predicting OSI directly from OT results was evident (Dunn, 2000).

A recent study (Dunn, 2005b) evaluated the effectiveness of TBHQ, BHA, BHT, propyl gallate, and α-tocopherol in mixtures with SME by non-isothermal P-DSC analyses in static and dynamic (positive air-purge) modes. Antioxidant activity was interpreted from OT results derived from P-DSC curves conducted under P = 2000 kPa and at β = 5 °C/min. Synthetic antioxidants propyl gallate, BHT, and BHA were most effective and α-tocopherol least effective in terms of increasing OT with respect to untreated SME (see Table 1.15). Increasing antioxidant concentration showed sharp increases in OT for loadings up to 1000 ppm, followed by smaller increases in OT at higher loading levels. This study recommended BHA or TBHQ (concentrations up to 3000 ppm) for safeguarding biodiesel from effects of autoxidation during storage. BHT was also found to be suitable at relatively low concentrations (210 ppm). Propyl gallate showed some physical compatibility problems with SME during phase equilibrium studies and therefore may not be readily soluble in blends with petrodiesel at larger SME ratios. Although α-tocopherol exhibited good solubility in SME/No. 2 petrodiesel blends, it was significantly less effective than the synthetic antioxidants.

TABLE 1.15. Conventional DSC and pressurized-DSC (P-DSC) results showing effect of antioxidants on oxidation onset temperature (OT) of SME.[a]

Ester	Antioxidant	Loading ppm	Conventional DSC[b] OT, °C	Static mode[c] OT, °C	Dynamic mode[d] OT, °C
Methyl oleate	None	0	168 ± 3.9	159.2 ± 0.56	164 ± 2.4
SME	None	0	136 ± 2.0	126 ± 1.2	116 ± 1.4
SME	α-Tocopherol	500	—	134.2± 0.74	125.1 ± 0.31
		2000	143 ± 3.4	146.2 ± 0.37	128 ± 1.6
SME	TBHQ	500	—	139 ± 1.2	129 ± 1.1
		2000	167 ± 1.7	157.76 ± 0.078	137 ± 1.0
SME	BHA	500	—	146.3 ± 0.63	136.9 ± 0.16
		2000	—	161 ± 2.2	149.4 ± 0.24
SME	BHT	500	—	141 ± 1.7	138.52 ± 0.078
		2000	—	157.7 ± 0.46	151.3 ± 0.30
SME	Propyl gallate	500	—	142 ± 2.2	139.5 ± 0.75
		2000	—	161 ± 2.4	151.2 ± 0.84

Sources: Dunn, 2005b, 2006a.

Onset oxidation temperature (OT) from non-isothermal heating curve analyses as ramp rate (β) = 5 °C/min.

[a]See Tables 1.2 and 1.5 for abbreviations.

[b]Air-purge = 75 mL/min, cell pressure (P) = ambient, equilibration temperature = 25 °C.

[c]Zero air-purge (closed system), P = 2000 kPa (290 psig), equilibration temperature = 25 °C.

[d]Air-purge = 35 mL/min, P = 2000 kPa (290 psig), equilibrium temperature = 30 °C.

The effects of antioxidants on OT of SME by non-isothermal (conventional) DSC, static mode P-DSC, and dynamic mode P-DSC were investigated by Dunn (2006a), which is summarized in Table 1.15. Results from all three methods consistently showed that treating SME with antioxidants TBHQ and α-tocopherol increased OT with respect to untreated SME. Statistical comparison of P-DSC results with those from isothermal analysis of OSI at 60 °C was facilitated by calculation of the corresponding response factors (defined ratios of OT of the sample to that of methyl oleate, and of OSI of the sample to that of methyl oleate). Data for the sample and reference material (methyl oleate) were measured under the same experimental conditions. Results showed the highest degree of correlation ($P = 0.79$) between dynamic-mode P-DSC and isothermal OSI analyses.

1.6. VISCOSITY

Viscosity is the major reason why oils and fats are transesterified to biodiesel. The viscosity of vegetable oils is about an order of magnitude greater than that of petrodiesel while that of biodiesel is only slightly greater than that of petrodiesel. Kinematic viscosity is the form of viscosity prescribed in biodiesel standards. The ASTM fuel specification D 6751 (Anon., 2007a) requires the viscosity of biodiesel to be in the range of 1.9–6.0 mm^2/s at 40 °C while European specification EN 14214 (Anon., 2003b) prescribes a narrower range of 3.5–5.0 mm^2/s at 40 °C, which has the effect of excluding some oils or fats as sources of biodiesel. The kinematic viscosity of methyl soyate is typically in the range of 4.1–4.2 mm^2/s at 40 °C while that of the parent soybean oil is 32 mm^2/s at 37.8 °C. The kinematic viscosity of methyl esters of other vegetable oils is in a similar range of about 4.0–4.8 mm^2/s, including PME, SFME, and RME (canola oil methyl esters). Biodiesel from greases of used cooking oils tend to have higher viscosity, often above 5.0 mm^2/s due to their higher content of saturated or *trans* fatty acids. In comparison, the kinematic viscosity of petrodiesel fuels is typically in the range of 2.0–3.0 mm^2/s at 40 °C. Kinematic viscosity is typically determined with methods such as ASTM D 445 or ISO 3104 (Anon., 2003d). Viscosity is strongly temperature-dependent, increasing significantly with decreasing temperature.

The kinematic viscosity of alkyl esters or biodiesel and its components has been the subject of several studies (Krisnangkura *et al.*, 2006; Tate *et al.*, 2006; Knothe and Steidley, 2005a; Yuan *et al.*, 2005b; Kerschbaum and Rinke, 2004; Allen *et al.*, 1999; Tat and Van Gerpen, 1999; De Filippis *et al.*, 1995; Gouw *et al.*, 1966). The viscosity of a fatty acid ester depends on chain length as well as the number and nature of double bonds. The more CH_2 groups in the chain the higher the viscosity, the greater the number of *cis* double bonds, the lower the viscosity. Compounds with one *trans* double bond have nearly the same viscosity as the corresponding saturated compounds, thus *trans* double bonds impart higher viscosity than *cis* (Knothe and Steidley, 2005a). Consequently,

the kinematic viscosity of biodiesel depends strongly on its fatty acid profile. Thus the kinematic viscosity at 40 °C of saturated methyl esters increases from 2.43 mm^2/s for methyl laurate to 3.30 mm^2/s for methyl myristate to 4.38 mm^2/s for methyl palmitate to 5.85 mm^2/s for methyl stearate. Increasing the size of the ester group has a similar effect. Thus ethyl oleate, propyl oleate and butyl oleate display kinematic viscosities of 4.78, 5.44 and 5.69 mm^2/s, respectively. Methyl oleate, on the other hand, has a kinematic viscosity at 40 °C of 4.51 mm^2/s while the its *trans* isomer methyl elaidate exhibits a value of 5.86 mm^2/s, virtually identical to that of methyl stearate. The polyunsaturated esters methyl linoleate and methyl linolenate have values of 3.65 mm^2/s and 3.14 mm^2/s, respectively. For sake of comparison, triolein has a kinematic viscosity at 40 °C of 32.94 mm^2/s. Methyl ricinoleate, the major component of castor oil methyl esters, which corresponds to methyl oleate with an OH group at C12 in the chain, has a kinematic viscosity at 40 °C of 15.44 mm^2/s.

1.7. LUBRICITY

The diesel engine has historically relied on petrodiesel components to lubricate parts such as fuel pumps and injectors. The advent of low-sulfur petrodiesel fuels and, more recently, ULSD (≤15 ppm S) as required by regulations in the United States, Europe and elsewhere, has led to failure of such engine parts since these fuels possess poorer lubricity than the high sulfur fuels used previously. The reason is that the hydrodesulfurization process that eliminates the sulfur species from petrodiesel fuel also removes the polar oxygen- and nitrogen-containing species which had been responsible for this lubricity (Dimitrakis, 2003; Barbour *et al.*, 2000; Wei and Spikes, 1986). The poor lubricity of low-sulfur petrodiesel (Dimitrakis, 2003; Wei and Spikes, 1986; Wall *et al.*, 1999; Lacey and Westbrook, 1995; Tucker *et al.*, 1994; Wang and Reynolds, 1994; Nikanjam and Henderson, 1993; Lacey and Lestz, 1992a, 1992b) requires additives or blending with another fuel of sufficient lubricity.

Currently, the most common test method for assessing the lubricity of diesel fuel is the high-frequency reciprocating rig (HFRR) lubricity tester (standards are ASTM D 6079 and ISO 12156 (Anon., 2003e). During this test, usually carried out at 60 °C, a wear scar is generated on a steel ball by rubbing against a steel disk. The size of the wear scar correlates with the lubricity of the sample. The smaller the wear scar, the better the lubricity and vice versa. Lubricity specifications are not included in biodiesel standards. However, petrodiesel standards contain lubricity specifications, with a maximum wear scar of 460 µm prescribed in the European petrodiesel standard EN 590 and a maximum wear scar of 520 µm in the American standard ASTM D 975 (Anon., 2003a).

Biodiesel inherently possesses good lubricity (Lang *et al.*, 2001; Lacey and Westbrook, 1995; Drown *et al.*, 2001; Van Gerpen *et al.*, 1999; Galbraith and

TABLE 1.16. HFRR data (60 °C; duplicate determinations) of commercial biodiesel, petrodiesel, hydrocarbons and neat fatty compounds.

Material	Wear scar, μm	Material	Wear scar, μm
Biodiesel	129; 134	Hexadecane	572; 571
ULSD	651; 636	1-Hexadecene	475; 477

Neat fatty compounds

Material	Wear scar, μm	Material	Wear scar, μm
Methyl laurate	416; 408	Methyl 9,12-linolelaidate	175; 177
Methyl palmitate	357; 362	Methyl ricinoleate	191; 174
Methyl palmitoleate	246; 228	Linoleic acid	0; 0
Methyl stearate	322; *277*	Monoolein	139; 123
Oleic acid	0; 0	Diolein	186; 163
Methyl oleate	290; 342	Triolein	143; 154
Methyl linoleate	236; 219	Oleyl alcohol	301; 289
Methyl linolenate	183; 185	Ricinoleyl alcohol	148; 162

Source: Knothe and Steidley, 2005b.

Hertz, 1997; Waynick, 1997). As Table 1.16 demonstrates, commercial biodiesel displays better lubricity than its neat major components, fatty acid esters, and its parent vegetable oil (see entries with triolein). This observation has been described several times in the literature. However, in the neat form some minor components of biodiesel, especially monoacylglycerols, free fatty acids, and glycerol display lubricity equal to or better than commercial biodiesel (Knothe and Steidley, 2005b). Lubricity is also enhanced with increasing unsaturation of the fatty acid chain. When more than one lubricity-enhancing moiety is present in a molecule, for example, the two OH groups in monoacylglycerols and ricinoleyl alcohol or three OH groups in glycerol, then lubricity of the neat compound is even more improved. On the other hand, excellent lubricity of a neat compound does not necessarily translate into lubricity enhancement by this compound when used at additive level in a petrodiesel fuel with poor lubricity as exemplified by glycerol, whose poor solubility in petrodiesel negatively affects its lubricity enhancement capabilities.

Thus biodiesel is a better lubricity enhancer than its parent vegetable oil because of the presence of high-lubricity species, some of which, such as monoacylglycerols, can arise during biodiesel production. Biodiesel is required at levels such as 2% for lubricity enhancement in order to achieve a sufficient additive level of high-lubricity materials in petrodiesel. Table 1.17 gives data showing the effect of minor components of biodiesel on its lubricity.

Results related to those discussed above (Knothe and Steidley, 2005b) are available in the literature (Hu *et al.*, 2005; Hillion *et al.*, 1999). The results in these publications do not agree on all aspects, however, there is agreement that low-level “contaminants” significantly affect biodiesel lubricity and its low-level blends with petrodiesel.

TABLE 1.17. Effect of blending or additization on HFRR data (60 °C) of ultra-low sulfur petrodiesel fuel (ULSD). For data of the neat petrodiesel fuels, see Table 1.16.

Blend / additive w/ ULSD	Wear scar, µm
1% Biodiesel	292; 292
2% Biodiesel	281; 258
1% Methyl oleate	597; 515
2% Methyl oleate	384; 368
5% Methyl oleate	365; 359
10% Methyl oleate	289; 298
0.01% Oleic acid	234; 233
1% Monoolein	134; 161
1% Diolein	237; 251
1% Triolein	385; 370
2% Triolein	287; 314
1% Methyl linoleate	573; 573
2% Methyl linoleate	536; 551
1% Glycerol	641; 649
1% Methyl oleate, 0.01% monoolein	335; 303
1% Methyl oleate, 0.01% diolein	533; 485
1% Methyl linoleate, 0.01% linoleic acid	437; 413
1% Methyl linoleate, 0.01% glycerol	571; 580
1% Methyl linoleate, 0.01% monolinolein	301; 274
1% Methyl linoleate 0.01% dilinolein	527; 533

Source: Knothe and Steidley, 2005b.

1.8. CONCLUSIONS

Biodiesel is a renewable alternative fuel for combustion in compression ignition (diesel) powered trucks, automobiles and farm vehicles, and in many other applications. It is environmentally innocuous and safe to handle due to relatively high flash points. It has many fuel characteristics that are compatible to petrodiesel including combustible energy content, specific gravity, kinematic viscosity, and CN. Biodiesel has inherent lubricity characteristics that make it an ideal anti-wear additive when blended with ULSD (≤15 ppm S content) No. 2 petrodiesel. Combustion of biodiesel can significantly reduce exhaust emissions including HC, CO, PM, smoke opacity, sulfur oxides, and polycyclic aromatic hydrocarbons. Finally, almost no modification of modern diesel engines is necessary to adapt them for burning biodiesel or biodiesel/petrodiesel blends.

Despite its many advantages as a renewable alternative fuel, biodiesel presents a number of problems that must be resolved for it to be attractive for more widespread applications. These problems include improving relatively poor cold flow properties, monitoring and maintaining fuel quality

against oxidative degradation during long-term storage, and reducing NOx levels in exhaust emissions.

The cold flow properties of biodiesel may cause issues when blended with petrodiesel during cooler months in moderate climates. SME begins to 'gel' or solidify very rapidly as its temperature approaches freezing (0 °C). This problem is more pronounced for biodiesel made from feedstock lipids possessing higher saturated fatty acid contents, such as palm oil and used cooking oils. Two promising approaches form improving cold flow properties include converting feedstock to biodiesel using medium or branched chain alcohols, and fractionation to reduce total saturated fatty acid content in biodiesel. Both of these approaches may be effective in decreasing CP, and they may also significantly increase costs of the final fuel product. Commercial CFI additives may be more economical. However, despite their effectiveness in depressing PP, these additives do not significantly reduce CP.

Maintaining fuel quality during long-term storage is a major concern for biodiesel producers, marketers and consumers. Again the most cost-effective means for improving oxidative stability of biodiesel is to treat it with antioxidants. Care must be exercised in cleaning tanks before filling them with biodiesel, and in paying attention to storage conditions such as temperature, purging out air with a nitrogen "blanket," and keeping the fuel away from direct lighting. Biodiesel stored over long periods should be monitored regularly for signs of degradation.

The use of additives to address a great number of fuel performance issues is ubiquitous in the biodiesel and petrodiesel industries. Unless the fuels themselves are enhanced through compositional modification, the employment of additives is likely to continue for the foreseeable future. As such, in spite of the impressive technological advances that have been made over the last 50 years or more in the field of fuel additives, a great deal of research remains to be accomplished to fully address technical deficiencies inherent in fuels, in particular the comparatively new arena of biodiesel and blends thereof in ULSD. With the conversion from low-sulfur (≤500 ppm) No. 2 petrodiesel to ULSD (≤15 ppm) in the U.S. in 2006, many additive treatment technologies that were previously successful with No. 2 petrodiesel may not yield similar results in ULSD. By extension, additives used for blends of SME/low-sulfur petrodiesel may not impart the same level of beneficial effect in blends of SME/ULSD, which once again emphasizes the need for continued research and development into the field of fuel additives.

1.9. ABBREVIATIONS

β	Ramp rate, °C/min
AV	Acid value, mg KOH/g oil
BHA	Butylated hydroxyanisole (*tert*-butyl-4-hydroxyanisole)
BHT	Butylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol)

CFI	Cold flow improver
CFPP	Cold filter plugging point, °C
CN	Cetane number
CO	Carbon monoxide
CP	Cloud point, °C
CPD	Cloud point-depressant
CVCA	Constant volume combustion apparatus
DSC	Differential scanning calorimetry
DTBHQ	Di-*tert*-butylhydroquinone
FAME	Fatty acid methyl esters
HC	Hydrocarbons
HFRR	High frequency reciprocating rig
ID	Ignition delay, ms
IQT	Ignition quality tester
IV	Iodine value, g I_2/100 g oil
LSD	Low-sulfur (≤500 ppm) petrodiesel
LTFT	Low-temperature flow test, °C
MP	Melting point, °C
NOx	Nitrogen oxides (emissions)
OSI	Oil stability index (induction period), h
OT	Oxidation onset temperature, °C
P-DSC	Pressurized-differential scanning calorimetry
PM	Particulate matter
PME	Palm oil fatty acid methyl esters
PP	Pour point, °C
PPD	Pour point-depressant
PV	Peroxide value, meq/kg
RME	Rapeseed oil fatty acid methyl esters
SME	Soybean oil fatty acid methyl esters
SFME	Sunflowerseed oil fatty acid methyl esters
TBHQ	*tert*-Butylhyroquinone
TGA	Thermogravimetric analysis
TME	Tallow fatty acid methyl esters
UCOME	Used cooking oil fatty acid methyl esters
ULSD	Ultra-low sulfur (≤15 ppm) petrodiesel

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CHAPTER 2

Enzymatic Reactions for Production of Biodiesel Fuel and Their Application to the Oil and Fat Industry

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2.1. INTRODUCTION

Reduction and recycle of waste materials are becoming big social concerns for suppressing the destruction of the global environment, and for building sustainable societies. In addition, the signatories to the Kyoto Protocol / United Nations Framework Convention on Climate Change in 1997 agreed to work toward the limitation or reduction of greenhouse gases. To reduce the emission of greenhouse gases, the use of biomass fuels [methane, ethanol (EtOH), and fatty acid methyl ester (FAME)] instead of fossil fuels is effective because a CO_2 circulatory system (photosynthesis) does not increase the total amount of CO_2. Hence, a great deal of attention is focused currently on production of biomass fuels from unutilized resources.

Biodiesel fuel (BDF) is defined as fatty acid (FA) alcohol esters produced from vegetable oil (triacylglycerols, TAGs) and short-chain alcohol, and generally refers to esters of FAs and methanol (MeOH) which is the cheapest component. TAGs are high viscous and can be used as a fuel only in diesel engines with heavy oil. On the other hand, conversion of TAGs to FAMEs decreases their viscosity and flash point, and FAMEs can be used as a fuel in diesel engines with light oil.

BDF is eco-friendly fuel because of its non-toxicity, biodegradability, low concentration of small particulate matter and SOx in exhaust gas, and because it does not add to the amount of carbon in the total environment. In addition, conversion of waste edible oil to BDF contributes to the reduction and recycle of the waste material. These advantages have attracted attention all over the world: European demand for BDF was 3.2 million tons in 2005, and estimated to double in 2006. In United States, which firstly proposed BDF, the demand was 260 thousand tons in 2005. In Japan, meanwhile, several local governments including Kyoto city produce BDF from waste edible oil to use as a fuel for public transportation, but the total demand in 2005 was only several thousand tons because of difficulty of collecting used frying oils from households.

BDF is produced currently by a chemical process with an alkaline catalyst, which has some drawbacks, such as the energy-intensive nature of the process, the interference of the reaction by free fatty acids (FFAs) and water, the need for removal of alkaline catalyst from the product, the difficulty in recovering glycerol, and the treatment of alkaline wastewater. To overcome these problems, the processes using ion-exchange resins (Shibasaki-Kitakawa *et al.*, 2007), supercritical MeOH (Kusdiana and Saka, 2004), MeOH vapor (Ishikawa *et al.*, 2005), and immobilized lipases (Mittelbach, 1990; Nelson *et al.*, 1996; Selmi and Thomas, 1998) have been proposed. In this paper, enzyme processes for production of BDF from waste edible oil, waste FFAs, and acid oil recovered from soapstock are described. In addition, applications of the element reactions to the oil and fat industry are introduced.

2.2. CONVERSION OF VEGETABLE OIL TO BDF

Lipases catalyze not only hydrolysis, but also esterification and transesterification. Hence, methanolysis of TAGs with a lipase is considered to be one of the effective reactions for production of BDF from waste edible oil and surplus oil. Conversion of TAGs to FAMEs using lipases was reported first by Mittelbach (1990). The early stage of studies was carried out using refined vegetable oils as starting materials. For the industrial production of BDF, waste edible oil, restaurant grease, and acid oil recovered from soapstock by-produced in vegetable oil refining also have been used as the raw materials. In addition, a continuous process with immobilized lipase is effective because of the high cost of the enzyme, and an organic solvent-free system is advantageous to avoid the problems of toxicity and flammability. It has been reported so far that immobilized lipases from *Rhizomucor miehei* (Mittelbach, 1990; Nelson *et al.*, 1996; Selmi and Thomas, 1998), *Candida antarctica* (Mittelbach, 1990; Nelson *et al.*, 1996; Shimada *et al.*, 2002), and *Burkholderia cepacia* (Hsu *et al.*, 2004) are effective for production of BDF, but only the immobilized *C. antarctica* lipase among them could be reused for long periods (Shimada *et al.*, 2002). Hence, production of BDF using immobilized *C. antarctica* lipase is described here.

2.2.1. Inactivation of Lipase by Insoluble MeOH

There were several reports that TAGs can be converted to FAMEs using an immobilized lipase. But the conversion in the reaction systems was not high, and the immobilized enzyme was not used repeatedly. Hence, we first attempted to resolve these problems.

In general, lipases efficiently catalyze transesterification when the substrates dissolve each other. Our first experiment was to investigate the solubility of fatty alcohols in vegetable oil. Fatty alcohols, with carbon lengths of 3 or more, completely dissolve in oil in an equimolar amount to total FAs in TAGs (stoichiometric amount), but the solubility of MeOH and EtOH was 1/2 and 2/3 of the stoichiometric amount, respectively. Disregarding the low solubility, all alcoholysis of TAGs so far reported were conducted with more than the stoichiometric amount of MeOH or EtOH. We were therefore aware that proteins generally are unstable in short-chain alcohols, and hypothesized that low methanolysis (ethanolysis) might be due to the inactivation of lipase by contact with insoluble MeOH (EtOH), which exists as micelle in the oil. Actually, MeOH was completely consumed in methanolysis of vegetable oil with less than 1/3 molar equivalent of MeOH to the stoichiometric amount using immobilized *C. antarctica* lipase, but the methanolysis was decreased significantly when adding more than 1/2 molar equivalent of MeOH (Fig. 2.1). In addition, the decreased activity did not restore in the subsequent reaction with 1/3 molar equivalent of MeOH, showing that the immobilized lipase was

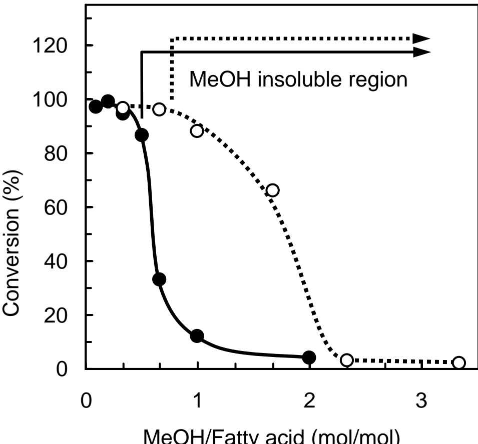

Figure 2.1. Irreversible inactivation of immobilized *C. antarctica* lipase by contact with MeOH micelles. One reaction mixture (●) was composed of vegetable oil and MeOH, and the other mixture (○) was composed of acylglycerols/33 wt% FAMEs mixture and MeOH. The reaction mixture (10 g) was shaken at 30 °C for 24 h with 4 wt% immobilized lipase. The amount of MeOH was expressed as the molar ratio to the amount of total FAs in the system. The conversion was expressed as the amount of MeOH consumed for the ester conversion of acylglycerols (when the amount of MeOH is less than that of FAs in acylglycerols), and as the molar ratio of FAMEs to total FAs in the system (when the amount of MeOH is more than that of FAs in acylglycerols). Arrows indicate the region in which a part of MeOH exists as micelles.

irreversibly inactivated by contact with insoluble MeOH in the oil (Shimada *et al.*, 1999).

2.2.2. Stepwise Methanolysis of Vegetable Oil

At least an equal mol of MeOH to FAs in TAGs is required for the complete conversion of TAGs to FAMEs. Immobilized *C. antarctica* lipase, however, was inactivated by adding more than 1/2 mol MeOH for the stoichiometric amount. To avoid the inactivation, methanolysis of TAGs was conducted by three successive addition of 1/3 mol MeOH for FAs in TAGs (Fig. 2.2) (Shimada *et al.*, 1999). The first-step methanolysis was conducted in a mixture of a vegetable oil (TAGs), 1/3 mol MeOH, and immobilized *C. antarctica* lipase. The conversion of TAGs to FAMEs reached 33% at 7 h, showing that MeOH was consumed almost completely. The addition of a second 1/3 mol MeOH at 10 h converted 66% of TAGs to FAMEs after 24 h. Finally, a third 1/3 mol MeOH was added, and the reaction was continued. After 48 h, the conversion reached 98%, showing that three-step methanolysis was effective for nearly complete conversion of TAGs.

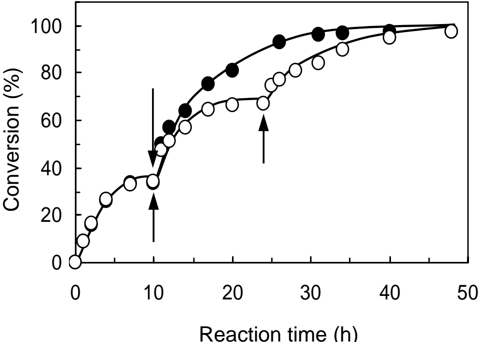

Figure 2.2. Time courses of two- and three-step methanolyses of vegetable oil using immobilized *C. antarctica* lipase. Two-step reaction (●): A mixture of vegetable oil, 1/3 mol MeOH for total FAs, and 4 wt% immobilized liapse was shaken at 30 °C. After 10 h, 2/3 mol MeOH was added to the reaction mixture. Three-step reaction (○): The first-step reaction was conducted under the same conditions as those of two-step reaction, and 1/3 mol MeOH was added at 10 and 24 h. Downward and upward arrows indicate the addition of 2/3 and 1/3 mol MeOH, respectively.

Solubility of MeOH in TAG is low, but high in FAME. The first-step reaction product was composed of 67 wt% acylglycerols and 33 wt% FAMEs, and 2/3 mol MeOH for the total FAs was completely soluble in the reaction mixture. Immobilized *C. antarctica* lipase did not inactivate even in the mixture of acylglycerols/FAMEs and 2/3 mol MeOH (Fig. 2.1) (Watanabe *et al.*, 2000). This finding led to success in a two-step methanolysis of TAGs (Fig. 2). The first-step methanolysis was started in a mixture of a vegetable oil, 1/3 mol MeOH, and immobilized *C. antarctica* lipase. After the conversion reached 33%, a second 2/3 mol MeOH was added to the reaction mixture and the reaction was continued. The two-step methanolysis converted 97% of TAGs to FAMEs after 36 h.

To study the durability of immobilized lipase, the two- and three-step methanolyses were repeated by transferring the enzyme to each fresh substrate mixture every 36 and 48 h, respectively. Consequently, more than 95% conversion was maintained during 70 cycles (105 days) in the two-step reaction, and during 52 cycles (104 days) in three-step reaction (Shimada *et al.*, 1999; Watanabe *et al.*, 2000). These results showed that the lipase can be used at least for 100 days without significant loss of the activity.

2.3. CONVERSION OF WASTE EDIBLE OIL TO BDF

Fresh vegetable oil and its waste differ significantly in the contents of water and FFA. Only 0.1 wt% water decreases the velocity of methanolysis of TAGs with immobilized *C. antarctica* lipase but does not affect the equilibrium of

the reaction (Shimada *et al.*, 1999). A waste edible oil containing 0.2 wt% water and 2.5 wt% FFAs underwent methanolysis with 1/3 mol MeOH for total FAs. When the reaction was repeated using the same enzyme, the reaction velocity increased gradually and reached a constant value after several cycles, although the conversion in the equilibrium state was 33% even in the first-cycle reaction (Watanabe *et al.*, 2001). This result shows that the inhibition of methanolysis by a very small amount of water in the waste oil is eliminated by performing the reaction in cycles.

The increase of velocity in methanolysis of the waste edible oil can be explained as follows. Water in the oil is attracted to the glycerol layer generated by methanolysis. Because the water goes out of the field of enzymatic methanolysis (oil layer), the reaction velocity gradually increased. Actually, the content of water in the acylglycerols/FAMEs layer (oil layer) decreased from 0.2 to 0.05 wt% and that of the glycerol layer was 4.1 wt% after five cycles (Watanabe *et al.*, 2001).

A waste edible oil was used as a raw material without pretreatment, and was converted to FAMEs by three-step methanolysis with immobilized *C. antarctica* lipase. The conversion reached 93% after 48 h (Watanabe *et al.*, 2001), although that of refined vegetable oil was 98% (Shimada *et al.*, 1999). In general, when a vegetable oil is used for frying, some FAs are converted to epoxides, aldehydes, and polymers by oxidation or thermal polymerization (Nawar, 1984; Cuesta *et al.*, 1993). Because the lipase does not recognize these oxidized compounds, the conversion of waste oil is assumed to decrease a little compared with that of refined oil. In addition, the content of FFAs in the product after the reaction (0.3 wt%) was lower than that in waste oil (2.5 wt%), indicating that methyl esterification of FFAs occurred along with methanolysis of the oil (Watanabe *et al.*, 2001).

To study the stability of immobilized *C. antarctica* lipase in methanolysis of waste edible oil, the three-step methanolysis was repeated by transferring the enzyme to a fresh substrate mixture. The conversion was maintained during 50 cycles (100 days) (Watanabe *et al.*, 2001), showing that contaminants in waste oil do not affect the stability of the lipase preparation. In a chemical alcoholysis with an alkaline catalyst, FFAs in a waste edible oil convert to alkaline soap; the water present disturbs an efficient reaction. Hence, FFAs and water should be removed before the reaction, and a small amount of alkaline soap generated must be removed by washing with water after the reaction. But the enzymatic process does not need the pretreatment and downstream purification.

2.4. CONVERSION OF WASTE FFAS TO BDF

Industrial processes for production of oils/fats and their related materials by-produce FFAs. Although parts of FFAs have been used as raw materials for producing paints, lubricants, and soaps, their oversupply has caused a decrease in the market price. An alternative usage is therefore desired to adjust to the

fluctuation of market demand, and an idea is surfacing in which FFAs are recycled as a fuel by their change to FAMEs.

FFAs can be converted to FAMEs by a chemical process with an acid catalyst. It is, however, necessary to remove the acid catalyst and to treat acid wastewater. Meanwhile, an enzymatic process has advantages of avoiding the problems associated with the chemical process. Conversion of waste FFAs to FAMEs by immobilized *C. antarctica* lipase is introduced here.

Docosahexaenoic acid (DHA; 22:6n-3) plays a role in prevention of a number of human diseases including inflammation, cardiovascular disease, cancer, and mental diseases (Hung, 2007). DHA has also been reported to accelerate the growth of preterm infants (Carlson *et al.*, 1993; Lanting *et al.*, 1994). These valuable physiological activities have been attracted attention, and tuna oil containing DHA has been used as a food material, an ingredient in infant formulas, and a nutraceutical lipid. In addition, an oil containing high concentration of DHA has been commercialized as a neutraceutical since 1994 in Japan (Maruyama and Nishikawa, 1995). DHA-rich oil is produced industrially by selective hydrolysis of tuna oil with *Candida rugosa* lipase which acts very weakly on the ester bond of DHA (Shimada *et al.*, 2005a). Because more than 70% of tuna oil was by-produced as FFAs by the hydrolysis, conversion of the FFAs to FAMEs was studied.

FFAs from tuna oil were esterified with 1 to 10 mol MeOH using immobilized *C. antarctica* lipase. The reaction velocity decreased with increasing the amount of MeOH. However, the degrees of esterification at 24 h (nearly equilibrium state) were 88 and 95% when using 1 and 2 mol of MeOH for FFAs, respectively. Although more than 2 mol of MeOH were used, the degree did not increase owing to the equilibrium of esterification and its reverse reaction (hydrolysis of FAMEs) (Watanabe *et al.*, 2002).

An equilibrium state in esterification is shifted to the direction of ester synthesis by removal of the water generated. To increase the degree of esterification, a two-step reaction was attempted (Table 2.1). First, FFAs from tuna oil were esterified with 1 or 2 mol MeOH for FFAs using immobilized *C. antarctica* lipase. After the removal of water in the reaction mixture under reduced pressure, remaining FFAs were esterified with 1 to 10 mol MeOH for unesterified FFAs. When the first-step esterification was conducted with an equal mol of MeOH to FFAs and the second-step esterification was conducted with 5 mol MeOH for unesterified FFAs, the total degree of esterification reached 97%. Also, when the first-step reaction was performed with 2 mol MeOH and the second-step reaction was performed with 5 mol MeOH for unesterified FFAs, the total degree of esterification was 98%. The former two-step reaction was repeated by transferring immobilized lipase to a fresh substrate mixture. Accordingly, the degrees of the first- and second-step reactions were maintained during 45 cycles (Watanabe *et al.*, 2002).

In addition, the velocity of esterification with the immobilized lipase sped up with increasing the reaction temperature, but the stability of the lipase decreased (Watanabe *et al.*, 2002). It is, therefore, necessary to keep the temperature at 30 °C or less in order to use the lipase for a long period.

TABLE 2.1. Two-step methyl esterification of waste FFAs with immobilized *C. Antarctica* lipase.

First-step reaction[a]		Second-step reaction[b]		
FFA/MeOH (mol/mol)	Esterification (%)	FFA/MeOH (mol/mol)	Esterification (%)	Total esterification (%)
1:1	82.5	1:1	53.8	91.9
		1:5	82.1	96.9
		1:10	81.4	96.7
1:2	95.2	1:1	26.5	96.4
		1:5	52.0	97.7
		1:10	54.8	97.8

[a]A 80-g mixture of waste FFAs and MeOH was shaken at 30 °C for 24 h with 1.0 wt% immobilized *C. antarctica* lipase.

[b]The first-step reaction was separated to the oil and water layers, and the oil layer was dehydrated at 50 °C for 3 mm Hg for 1 h. A 10-g mixture of the dehydrated oil layer and 1, 5, and 10 mol MeOH for unesterified FFAs was shaken at 30 °C for 5 h with 1.0 wt% immobilized *C. antarctica* lipase.

2.5. CONVERSION OF ACID OIL BY-PRODUCED IN VEGETABLE OIL REFINING TO BDF

Vegetable oils are refined through pressing/extraction, degumming, alkali deacidification, decolorization, and deodorization. Alkali deacidification by-produces soapstock. Acidulation of soapstock prepares acid oil which contains FFAs, acylglycerols, and other lipophilic compounds. It is reproduced currently as FFAs, which are used as raw materials for production of soaps, lubricants, and paints. But the demand for FFAs is almost in saturation. Hence, conversion of acid oil to BDF is expected to avoid an oversupply of the industrial FFAs and subsequent price decrease.

Acid oil can be converted to BDF by a chemical process (Haas *et al.*, 2003) that requires excessive amounts of MeOH and acid catalyst to achieve a high degree of conversion. To avoid this, acylglycerols in acid oil must be hydrolyzed completely prior to FAME production. It is also necessary to remove the acid catalyst from the product (BDF) and to treat acid wastewater which is generated in the downstream process. To overcome the problems associated with the chemical process, enzymatic methods have been considered.

2.5.1. Conversion of Acid Oil Model to BDF

Immobilized *C. antarctica* lipase is effective for conversion of TAGs and FFAs to FAMEs; thus, a system with lipase can be expected to produce BDF from acid oil. A mixture of equal weights of vegetable oil (TAGs) and FFAs was, therefore, treated with an equal mol of MeOH to total FAs in the oil mixture

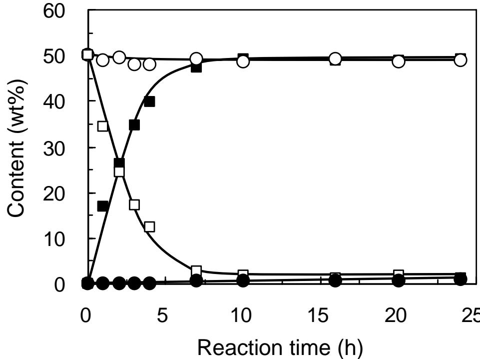

Figure 2.3. Time course of treatment of acid oil model using immobilized *C. antarctica* lipase. A mixture of equal weights of vegetable oil and FFAs was allowed to react at 30 °C with an equimolar amount of MeOH to total FAs in the reaction system using 0.5 wt% immobilized lipase. ○, Content of TAGs; ●, DAGs; □, FFAs; ■, FAMEs.

using the immobilized lipase. Contrary to the expectation, only FFAs in acid oil were converted to FAMEs, and most of TAGs were unreactive (Fig. 2.3) (Watanabe *et al.*, 2005b). Even with excess amounts of MeOH added to reaction mixture, the TAGs did not undergo methanolysis.

Attention was given to water generated by methyl esterification of FFAs. Methanolysis of TAGs with immobilized *C. antarctica* lipase decelerated slightly in the presence of only 0.05 wt% water, and did not proceed when adding 3 wt% water to the reaction mixture (Shimada *et al.*, 1999; Watanabe *et al.*, 2005b). In addition, methyl esterification of FFAs reached 95% when conducting for 3 h using 1 wt% immobilized lipase (Watanabe *et al.*, 2002), while 95% methanolysis of TAGs needed 30 h with 4 wt% immobilized lipase (Shimada *et al.*, 1999). These results indicate that the velocity of the methyl esterification is about 40 times faster than that of the methanolysis, to formulate the following hypothesis (Fig. 2.4). In the reaction of a mixture of FFAs/TAGs/MeOH with immobilized *C. antarctica* lipase, methyl esterification of FFAs proceeds preferentially and by-produces water. The water inhibits methanolysis of TAGs, resulting in the remains of TAGs. Based on this hypothesis, a two-step process was planned: first, is the methyl esterification of FFAs in the mixture of FFAs and TAGs. The resulting product is composed of mainly FAMEs and TAGs. Because the mixture contains water generated by the esterification, the water is removed by evaporation. The second step was methanolysis of TAGs in the mixture of TAGs and FAMEs.

The first-step reaction was conducted in a mixture of FFAs/TAGs (1:1, wt/wt) with an equal mol of MeOH to total FAs (2 mol to FFAs) using 0.5 wt% immobilized lipase. FFAs were converted efficiently to FAMEs, and the content of FFAs decreased to 1.3 wt%, although most of TAGs remained in the reaction mixture. The oil fraction and immobilized enzyme were separated, and MeOH and water in the oil fraction was removed under reduced

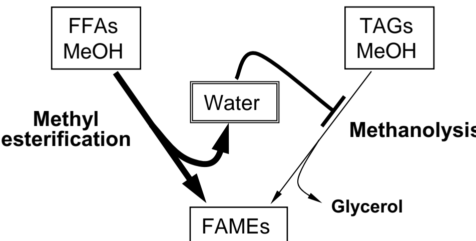

Figure 2.4. Reactions in a mixture of FFAs, TAGs, and MeOH with immobilized *C. antarctica* lipase.

pressure. To the resulting oil was added an equal mol of MeOH to unreacted FAs (FFAs and acylglycerols) and 6wt% immobilized lipase, and the second-step reaction was performed. Consequently, the content of FAMEs reached 99wt%. The first- and second-step reactions were recycled by transferring immobilized enzyme to each fresh substrate every 24h. The immobilized enzyme was very stable in each step of reaction, and the content of FAMEs in the oil fraction obtained by the two-step reaction maintained more than 96wt% during 100 cycles (100 days) (Watanabe *et al.*, 2005b).

2.5.2. Conversion of Acid Oil By-Produced in Vegetable Oil Refining to BDF

2.5.2.1. First-Step Reaction: Methyl Esterification of FFAs A two-step reaction established using an acid oil model as a material was applied to conversion of an acid oil prepared from soapstock by-produced in vegetable oil refining to BDF. The composition of acid oil was 78wt% FFAs, 8wt% TAGs, 3wt% DAGs, 1wt% phytosterols (referred to as sterols), 2wt% FA steryl esters (FAStEs), and 8wt% unknown lipophilic compounds. The first-step reaction, methyl esterification of FFAs, was conducted with an equal mol of MeOH to total FAs in acid oil using immobilized *C. antarctica* lipase. The degree of esterification of FFAs in acid oil reached 91% (the content of FAMEs, 73wt%) after 24h. But repetition of the reaction resulted in the significant decrease in the activity (half life, 3 days) of the lipase (Watanabe *et al.*, 2007).

An enzymatic process cannot be adopted for industrial production of BDF, if immobilized lipase cannot be used for long period. Studies on the stability of immobilized *C. antarctica* lipase revealed that it was the most stable when the reaction was conducted with 5 to 8mol MeOH for total FAs in acid oil prepared from soapstock (Watanabe *et al.*, 2007). The phenomenon, that there is the optimum region of MeOH concentration, may be explained as follows: *i)* immobilized lipase inactivates in the presence of large amounts of MeOH, *ii)* acid oil includes an unknown compound or compounds which inactivate

the lipase, *iii)* addition of large amounts of MeOH suppresses the inactivation by reducing interaction of the lipase and unknown compound(s), and *iv)* the inactivation of lipase in the presence of more than 8 mol MeOH is caused by MeOH itself.

Based on the results, the first-step reaction was conducted with 5 mol MeOH for total FAs in acid oil using 1 wt% immobilized *C. antarctica* lipase. The degree of esterification of FFAs reached 96% (FAME content, 77 wt%). In addition, when the reaction was repeated by transferring the lipase to a fresh substrate every 24 h, the degrees of esterification after 60 and 100 cycles were 94% (FAME content, 77 wt%) and 88% (FAME content, 71 wt%), respectively (Watanabe *et al.*, 2007).

2.5.2.2. Second-Step Reaction: Methanolysis of TAGs After the first-step reaction, immobilized lipase was removed and the oil fraction recovered. MeOH and water in the oil fraction were then removed by evapolation. The resulting mixture was named dehydrated first-step product, which was composed of 80 wt% FAMEs, 2 wt% FFAs, 10 wt% acylglycerols, 1 wt% sterols, 2 wt% FAStEs, 5 wt% unknown lipophilic compounds. The second-step reaction is methanolysis of acylglycerols in the dehydrated first-step product.

A mixture of dehydrated first-step product and 5 mol MeOH for unreacted FAs (FAs in acylglycerols and FFAs) was allowed to react with immobilized *C. antarctica* lipase. After 24 h, the content of FAMEs increased from 80 to 91 wt%, and the contents of acylglycerols and FFAs decreased from 10 to 0.9 wt% and from 2 to 0.8 wt%, respectively. Repetition of the reaction, however, decreased the activity of immobilized lipase (half life, 5 days). In spite of further addition of MeOH (which was effective for the increase in the stability of immobilized lipase in the first-step reaction), the stability of lipase was not improved and its inactivation promoted adversely. This instability was subsequently avoided by adding vegetable oil to the reaction mixture. Hence, vegetable oil was added to give the acylglycerols content of 50 wt%, and the second-step reaction was conducted with an equal mol of MeOH to unreacted FAs. Acylglycerols were converted to FAMEs, and the content of FAMEs reached 91 wt% after 24 h. However, when the reaction was repeated every 24 h using the same immobilized lipase, the lipase activity decreased rapidly after 30 cycles (Fig. 2.5A) (Watanabe *et al.*, 2007).

The stability of *C. antarctica* lipase was increased by the addition of glycerol. The second-step reaction was repeated every 24 h using a mixture of dehydrated first-step product, an equal mol of MeOH to unreacted FAs, and 10 wt% glycerol for the mixture of the dehydrated product. A significant inactivation of the lipase observed after 30 cycles was suppressed by addition of glycerol, and the content of FAMEs reached 90 wt% even after 100 cycles (Fig. 2.5B). The composition of the oil layer produced by 60 cycles of the second-step reaction was 91 wt% FAMEs, 0.6 wt% FFAs, 0.8 wt% TAGs, 2 wt% DAGs, 0.6 wt% sterols, 1 wt% FAStEs, and 3 wt% unknown lipophilic compounds (Watanabe *et al.*, 2007).

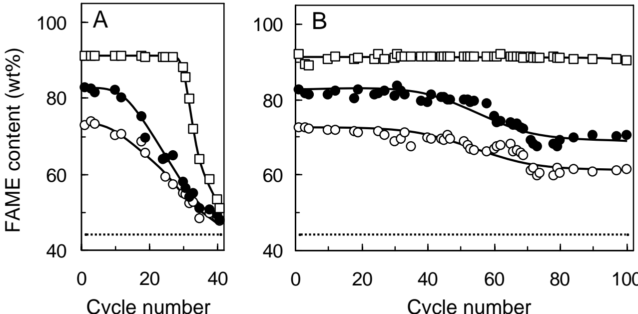

Figure 2.5. Stabilization of immobilized *C. antarctica* lipase in the second-step reaction by addition of vegetable oil and glycerol. The reaction was repeated at 30 °C with 6 wt% immobilized lipase by transferring the lipase to a fresh substrate mixture every 24 h. **A**, A reaction mixture was composed of dehydrated first-step product, rapeseed oil, and MeOH. Rapeseed oil was added to give the acylglycerol content of 50%, and the amount of MeOH was an equimolar amount to unreacted FAs. **B**, A reaction mixture was prepared by adding 10 wt% glycerol to the mixture used in Figure A. ○, The content of FAMEs at 2 h; ●, at 4 h; □, at 24 h. Dotted lines indicate the content of FAMEs before the reaction (44.1 wt%).

2.6. APPLICATION OF REACTIONS THAT CONVERT FAS TO THEIR METHYL (ETHYL) ESTERS

Lipase-catalyzed methanolysis of TAGs and methyl esterification of FFAs achieve high degree of conversion even with an equal mol of MeOH to FAs. In addition, methyl esterification of FAs reaches nearly 90% in the presence of water and 98% on removing generated water. These reactions, therefore, become superior tools when only FAs in the raw materials (including unstable compound(s)) are converted to their esters with short-chain alcohols. There are several processes, including lipase-catalyzed conversion of FAs to their methyl (ethyl) esters, to accomplish this.

2.6.1. Conversion of Fish Oil to Its Corresponding FA Ethyl Esters

Polyunsaturated fatty acids (PUFAs) have various physiological functions and are widely used as pharmaceuticals, neutraceuticals, and as food additives. The ethyl ester of eicosapentaenoic acid (EPA) has been used for the treatment of arteriosclerosis and hyperlipemia since 1991 in Japan (Hara, 1993). DHA possesses not only similar physiological activities to those of EPA, but also an important function in the brain and retina (Hung, 2007). In addition, DHA accelerates the growth of preterm infants as does arachidonic acid (Carlson *et al.*, 1993; Lanting *et al.*, 1994). From these reasons, DHA ethyl ester (DHAEE) is currently expected to be used as a medicine, and the development of the purification methods is desired.

Enzymatic process is one of the effective techniques for purification of DHAEE from fish oil, such as tuna oil. The first step for purifying DHAEE is conversion of tuna oil to FA ethyl esters (FAEEs) (Fig. 2.6). Of course, a chemical reaction with an alkaline catalyst can convert tuna oil to the mixture of FAEEs containing DHAEE, but an enzymatic process under mild conditions is preferable because heating under alkaline conditions often results in the isomerization of DHA. Therefore, stepwise alcoholysis, which was established in production of BDF from vegetable oil, was applied to ethanolysis of tuna oil.

When tuna oil underwent ethanolysis with more than 2/3 mol EtOH for FAs in tuna oil using immobilized *C. antarctica* lipase, the lipase inactivated because a part of EtOH existed as micelles in the oil. To avoid such inactivation, first-step ethanolysis was conducted in a mixture of tuna oil and 1/3 mol EtOH using 4 wt% immobilized lipase. After complete consumption of EtOH, the second and third 1/3 mol of EtOH were added to the reaction mixture. The three-step ethanolysis achieved the conversion of more than 95% of tuna oil to its corresponding FAEEs, and maintained the high degree of conversion for 54 cycles (108 days) (Watanabe *et al.*, 1999).

DHAEE can be purified from the mixture of FAEEs produced by the stepwise ethanolysis (Fig. 2.6) (Shimada *et al.*, 1997c, 1998, 2005a; Maruyama *et al.*, 2000). The mixture of FAEEs was first subjected to short-path distillation. The fractionation enriched DHAEE from 24 to 57 wt% without significant loss of DHAEE. When the DHAEE concentrate underwent alcoholysis with lauryl alcohol (LauOH) using a lipase (immobilized *R. miehei* or *Rhizopus oryzae* lipase) which acts weakly on DHA, all FAEEs except DHAEE were preferentially converted to their lauryl esters. The selective alcoholysis

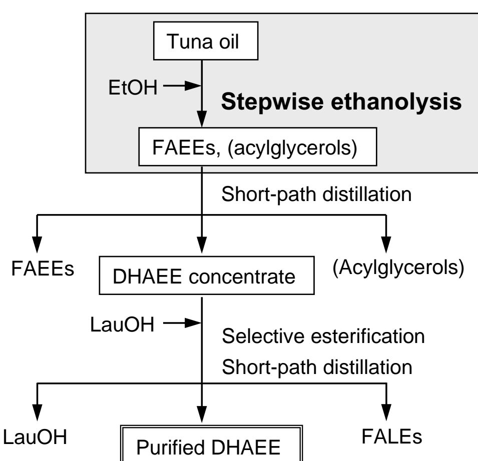

Figure 2.6. Purification process of DHAEE from tuna oil. Reaction for conversion of tuna oil to FAEEs is surrounded with shaded box.

increased the DHAEE content in the FAEE fraction to 90 wt% (Shimada *et al.*, 1997c, 1998; Maruyama *et al.*, 2000). Finally, the reaction products were separated to LauOH, FAEEs rich in DHAEE (purified DHAEE preparation), and FA lauryl esters (FALEs) by short-path distillation (recovery of DHA, 52%) (Maruyama *et al.*, 2000).

2.6.2. Conversion of PUFA to Its Ethyl Ester

PUFAs can be purified by processes including not only selective alcoholysis, but also selective esterification (Fig. 2.7) (Hills *et al.*, 1990; Shimada *et al.*, 2001a, 2001c, 2005a, 2005b). An oil containing desired PUFA is first hydrolyzed non-selectively using a lipase from *Pseudomonas, Burkholderia*, or *Alcaligenes*, which acts on all FAs strongly. The resulting FFAs are esterified selectively with LauOH using a lipase from *Candida rugosa* or *R. oryzae*, which acts weakly on the desired PUFA. Accordingly, all FAs except PUFA are converted to FALEs, and PUFA is enriched in the FFA fraction. LaOH, FFAs rich in PUFA, and FALEs in the reaction mixture can be purified by short-path distillation, or by a process comprising distillation and *n*-hexane fractionation. This process resulted in highly purified DHA (Shimada *et al.*, 1997a), arachidonic acid (Yamauchi *et al.*, 2005), γ-linolenic acid (Shimada *et al.*, 1997b), and dihomo-γ-linolenic acid (Nagao *et al.*, 2007).

Purified PUFAs can be converted to their ethyl esters by adopting lipase-catalyzed esterification. For example, the ethyl esterification of DHA (Shimada *et al.*, 2001b, 2003d) with an equal mol of EtOH using immobilized *C. antarc-*

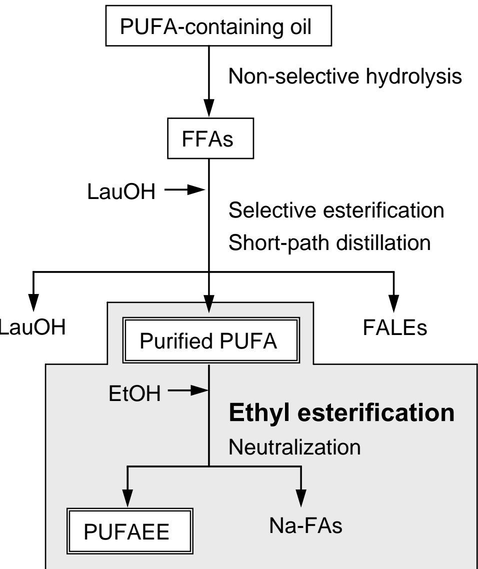

Figure 2.7. Purification process of PUFA from PUFA-containing oil. Reaction for conversion of PUFA to its ethyl ester is surrounded with shaded box.

tica lipase converted nearly 90% DHA to its ethyl esters. The degree of esterification did not increase although more amounts of EtOH were added, because water generated by this reaction prevented a higher degree of esterification. After water in the reaction mixture was removed by evaporation, ethyl esterification was conducted again with 5 mol EtOH for unesterified DHA. This two-step esterification achieved 96% esterification of DHA. In addition, DHAEE synthesized was purified efficiently from the reaction mixture by alkali deacidification (Shimada *et al.*, 2001b).

2.6.3. Purification of Tocopherols and Sterols

Tocopherols have antioxidant activity and are used widely as a pharmaceutical substance, an ingredient in cosmetics, and a neutraceutical food. Also, sterols and FAStEs reduce blood cholesterol level (Moreau, 2003), and salad oil, margarine, and mayonnaise, to which sterols or FAStEs are added, are marketed as Food for Specified Health Uses in Japan. Tocopherols, sterols, and FAStEs are major components in deodorizer distillate by-produced in the deodorization step of vegetable oil refining. At present, tocopherols are purified from vegetable oil deodorizer distillate (VODD) by a combination of chemical methyl esterification of FFAs, molecular (short-path) distillation, MeOH (EtOH) fractionation, ion exchange chromatography, and so on. Sterols are also purified from by-products in the purification of tocopherols by fractionation with organic solvents, but the yield is not high. Meanwhile, a procedure for purifying FAStEs has not been developed, and all FAStEs are wasted. Hence, a process including lipase-catalyzed reactions was proposed for a facile purification of tocopherols, sterols, and FAStEs. One of the reactions is methyl esterification of FFAs.

An outline of the purification process is shown in Figure 2.8 (Shimada *et al.*, 2000, 2001c, 2003c, 2005a, 2005b; Watanabe *et al.*, 2004; Nagao *et al.*, 2005). Main components in VODD are tocopherols, sterols, FAStEs, FFAs, and acylglycerols. These components were fractionated into low and high boiling points. The main components in the low boiling point fraction were tocopherols, sterols, FFAs, and partial acylglycerols (named VODD tocopherol/sterol concentrate; VODDTSC), and those in the high boiling point fraction were FAStEs and TAGs (named VODD steryl ester concentrate; VODDSEC). When purifying tocopherols and sterols from VODDTSC, if sterols are converted to FAStEs and partial acylglycerols are hydrolyzed to FFAs, the reaction mixture consists of FFAs, tocopherols, and FAStEs. Because the molecular weights of these compounds are completely different (FFAs, 280; tocopherols, 430; FAStEs, 670), they can be purified easily by short-path distillation. In addition, the purification becomes much easier by converting FFAs to FAMEs, since the boiling point of FAMEs is lower than that of FFAs.

To conduct three reactions (hydrolysis of acylglycerols, esterification of sterols with FFAs, and methyl esterification of FFAs), two lipases from *C.*

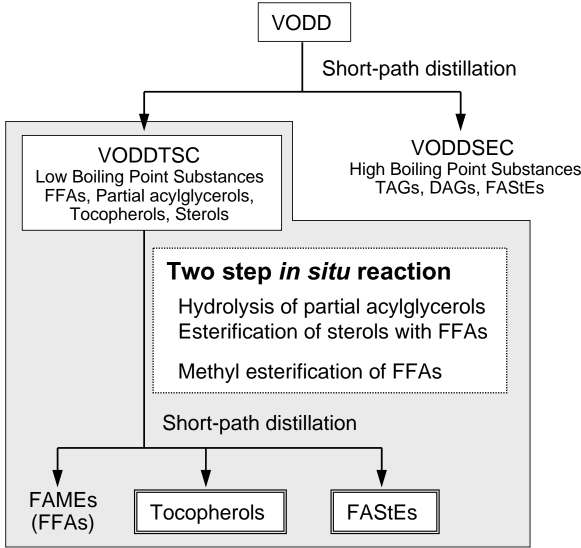

Figure 2.8. Process of purifying tocopherols and sterols from VODD. Reactions including methyl esterification of FFAs are surrounding with shaded box.

rugosa and *Alcaligenes* sp. were selected (Nagao *et al.*, 2005). *C. rugosa* lipase: *i)* did not recognize tocopherols, *ii)* catalyzed esterification of sterols with FFAs, and the degree of esterification in the presence of water and under dehydration were 80 and 95%, respectively, *iii)* catalyzed esterification of FFAs with MeOH in the presence of water, but did not achieve more than 80% esterification, *iv)* converted FAStEs to free sterols in the presence of large amounts of MeOH and small amounts of FFAs, and *v)* did not show activity when the water content in the reaction mixture was ≤0.05 wt%, and the activity did not restore immediately even though water was added. In addition, *Alcaligenes* lipase: *i)* did not recognize tocopherols, *ii)* catalyzed esterification of FFAs with MeOH, and the degree of esterification reached 95% even in the presence of water, *iii)* did not catalyze methyl esterification of FFAs without addition of water, and *iv)* did not recognize sterols and FAStE.

The properties of the two lipases led to establish a two-step *in situ* reaction system, in which hydrolysis of acylglycerols, esterification of sterols with FFAs, and methyl esterification of FFAs were performed in one batch. The time course of the reaction is shown in Figure 2.9 (Nagao *et al.*, 2005). First, agitation of a mixture of VODDTSC and 5 wt% water with *C. rugosa* lipase hydrolyzed partial acylglycerols rapidly and the sterols were esterified with FFAs. By conducting this treatment with dehydration under reduced pressure, 95% of sterols were converted to FAStEs at 20 h. The content of water in the reaction mixture decreased to 0.03 wt% after 24 h, showing that *C. rugosa* lipase does not work. Then, *Alcaligenes* lipase, MeOH, and water were added to the

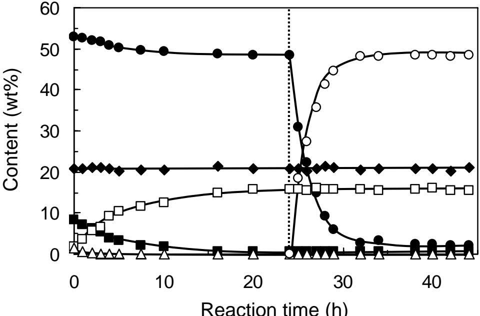

Figure 2.9. Time course of two-step *in situ* reaction for purification of tocopherols and sterols in VODDTSC. A mixture of VODDTSC, 5 wt% water, and 250 U/g *C. rugosa* lipase was agitated with dehydration at 20 mm Hg. At 24 h indicated with dotted line, 20 wt% water, 7 mol MeOH for FFAs, and 25 U/g *Alcaligenes* lipase were added to the reaction mixture, and the reaction was continued at ordinary pressure. ■, The content of sterols; □, FAStEs; ○, FAMEs; ●, FFAs; △, acylglycerols; ◆, tocopherols.

reaction mixture, and agitation was continued. Consequently, 95% FFAs were converted to FAMEs after 34 h, and FAStEs synthesized were not reconverted to free sterols. Through this reaction, the content of tocopherols did not change because the two lipases did not recognize tocopherols. After the reaction, the oil layer was subjected to short-path distillation, and the tocopherol and FAStE fractions were recovered. Tocopherols were purified to 72 wt% (yield, 88%) and sterols were purified as FAStEs to 97 wt% (yield, 90%) (Nagao *et al.*, 2005).

In addition, FAStEs in VODDSEC can be purified easily by adopting lipase reaction. *C. rugosa* lipase strongly hydrolyzed TAGs, but weakly hydrolyzed FAStEs. Hence, VODDSEC was hydrolyzed using this lipase. After the reaction, the oil layer was subjected to short-path distillation, and the FAStE fraction was recovered. FAStEs were purified to 97% (yield, 88%) (Hirota *et al.*, 2003).

2.6.4. Conversion of Steryl Esters and Astaxanthin Esters to Their Free Forms

Sterols and FAStEs in VODD were purified as FAStEs through the process described above. As the oil and fat industry demands both sterols and FAStEs, conversion of FAStEs to sterols was attempted.

Hydrolysis of FAStEs with various lipases was conducted but their reactions reached steady state at low degree of hydrolysis (<50%), owing to the reversible reaction (Fig. 2.10A). Meanwhile, as noted earlier, lipases act more weakly on FAME than FFA. If FFAs, in the hydrolysis of FAStEs, are converted to FAMEs, the reverse reaction (esterification of sterols) will scarcely occur because FAMEs do not participate in esterification of sterols with FFAs.

(A) FA steryl ester + H_2O ⇌ Sterol + FFA

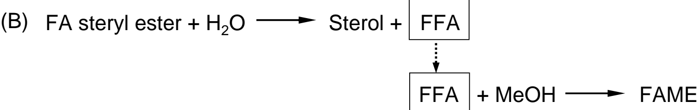

Figure 2.10. Lipase-catalyzed reaction on a mixture of FAStE and water in the presence or in the absence of MeOH. **A**, Hydrolysis of FA steryl ester and esterification of sterol with FFA. **B**, Lipase-catalyzed reactions in a mixture of FAStE, water and MeOH.

Consequently, the equilibrium of the reaction shifts to accumulation of free sterols (Fig. 2.10B).

According to this strategy, conversion of oleic acid steryl esters (OAStEs) to free sterols was studied. The screening tests showed that *Pseudomonas aeruginosa* lipase was suitable for this reaction, and that MeOH, butanol, octanol, and decanol were effective substrates. Additionally, the velocity reached a constant value in the presence of more than 10 wt% water, and the degree of conversion was maximum at 10 wt% water. Based on these facts, a mixture of OAStEs/MeOH (1:2, mol/mol) and 10 wt% water was stirred with *P. aeruginosa* lipase, resulting in conversion of 98% OAStEs to sterols (Shimada *et al.*, 2003a).

Free sterols were purified from the reaction mixture, which mainly contained sterols and FAMEs, by *n*-hexane fractionation because the sterols were poorly soluble in *n*-hexane but FAMEs and FFAs were readily soluble in the organic solvent. The process comprising lipase reaction and *n*-hexane fractionation purified sterols with 87% recovery of the initial content in OAStEs (purity of sterols, 99%) (Shimada *et al.*, 2003a).

Lipase-catalyzed conversion of FAStEs to sterols in the presence of MeOH was applied to astaxanthin FA esters to free astaxanthin, because a screening test showed that commercial lipases did not hydrolyze astaxanthin FA esters. A mixture of astaxanthin and its mono- and diesters (5:80:15, by mol) was treated in the presence of 50 wt% water with 5 mol EtOH for FAs in astaxanthin esters using *P. aeruginosa* lipase. After the reaction, the molar ratio of astaxanthin and its mono- and diesters was 89:10:1, showing that the lipase efficiently converts astaxanthin esters to free astaxanthin. The free form was also purified by *n*-hexane fractionation with 69% recovery of the initial content of astaxanthin in the mixture of its free and ester forms (Nagao *et al.*, 2003).

2.6.5. Regiospecific Analysis of TAG

2.6.5.1. Regiospecific Reaction Catalyzed by Immobilized C. antarctica Lipase Irimescu *et al.* (2001, 2002) recently established a reaction system:

2-eicosapentaenoyl (docosahexaenoyl) monoacylglycerol was produced by ethanolysis of trieicosapentaenoyl (tridocosahexaenoyl) glycerol using immobilized *C. antarctica* lipase. The resulting 2-MAG was used as an intermediate for production of structured TAGs, 1,3-capryloyl 2-eicosapentaenoyl (docosahexaenoyl) glycerol. The ethanolysis for production of 2-MAG was conducted with more than 12 mol EtOH for total FA in the simple TAG. On the other hand, as described earlier, insoluble MeOH (EtOH) micelles in TAGs inactivated irreversibly the immobilized lipase. Because the two phenomena were inconsistent, ethanolysis of TAG with excessive amounts of EtOH was recycled by transferring the lipase to a fresh substrate mixture. It was consequently confirmed that the lipase was stable in the mixture of TAG and over excessive amounts of EtOH (Shimada *et al.*, 2003b).

The phenomenon, that a little excess amount of short-chain alcohols inactivates immobilized *C. antarctica* lipase but their overly excessive amounts do not, has not yet been clarified. However, because it is well known that lipase is active and very stable in water-immiscible organic solvents (Zaks and Klibanov, 1984), this fact may be explained: the enzyme has bound water which it needs for the expression of its activity and which affects the maintenance of its structure. The structure is not maintained in the mixture including a small excessive amount of EtOH, but is strengthened in the mixture including large excessive amounts of EtOH owing to removal of bound water which does not participate in the expression of the activity.

Additionally, immobilized *C. antarctica* lipase showed non-positional specificity in alcoholysis with MeOH (EtOH) soluble in TAGs (Shimada *et al.*, 1999, 2001b, 2002), but 1,3-positional specificity in ethanolysis of TAGs in the presence of largely excessive amounts of EtOH (Irimescu *et al.*, 2001, 2002; Shimada *et al.*, 2003b). It was also reported that *Penicillium camembertii* lipase catalyzes conversion of MAGs to DAGs when it has enough amounts of bound water, but that it does not catalyze the conversion when the amount of bound water is reduced by dehydration of water in the reaction mixture under reduced pressure (Watanabe *et al.*, 2005a). The hypothesis, that the stability and substrate specificity of immobilized *C. antarctica* lipase are affected by the amount of bound water, may therefore be right to the point.

2.6.5.2. Application of 1,3-Position Specific Ethanolysis to Regiospecific Analysis Ethanolysis of TAGs in the presence of largely excessive amounts of EtOH using immobilized *C. antarctica* lipase prepared 2-MAGs in a high yield (Shimada *et al.*, 2003b). A typical time course is shown in Figure 2.11. The ethanolysis generated an enantiomeric mixture of 1,2- and 2,3-DAGs in the early stage, but not 1,3-DAGs. The content of 2-MAGs reached a maximum value at 4 h (31 wt%; the content of FAs in 2-MAGs was 28 mol% based on total FAs in the reaction mixture), and 1(3)-MAGs were not detected during the first 4 h although negligible amounts of 1(3)-MAGs were detected after 7 h. The reaction mixture at 4 h consisted mainly of 2-MAGs and FAEEs. In addition, *C. antarctica* lipase acted on ester bonds of saturated and unsatu-

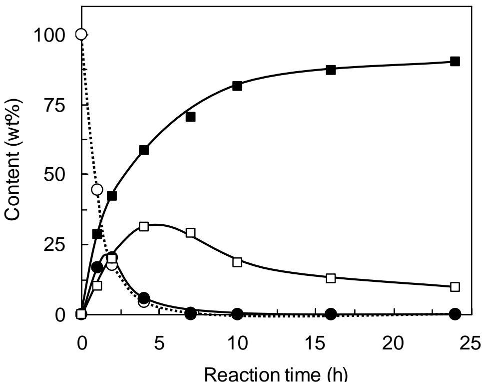

Figure 2.11. Time course of ethanolysis of TAGs using immobilized *C. antarctica* lipase in the presence of largely excessive amounts of EtOH. A single cell-cell oil containing 40 wt% arachidonic acid was used as a substrate. A mixture of oil/EtOH (1:3, wt/wt) was shaken at 30 °C with 4 wt% immobilized lipase. ○, Content of TAGs; ●, mixture of 1,2- and 2,3-DAGs; □, 2-MAGs; ■, FAEEs.

rated C_{14}-C_{24} FAs to a similar degree (Shimada *et al.*, 2003b). The ethanolysis converted TAGs efficiently to 2-MAGs and does not show FA specificity; therefore, FA composition at the 2-position in TAGs can be determined by analysis of FAs in 2-MAGs generated by the ethanolysis (Shimada *et al.*, 2003b; Kawashima *et al.*, 2004). The scale of reaction can be reduced to mg-order, and 2-MAGs can be recovered not only by silica gel column chromatography, but also by thin layer chromatography on a silica gel plate impregnated with boric acid (Shimada *et al.*, 2003b).

So far, the regiospecific analysis has been conducted by the Grignard reaction (Becker *et al.*, 1993) or by hydrolysis with a 1,3-position specific lipase, such as those from the pancreas, *R. oryzae*, and *R. miehei* (Luddy *et al.*, 1964). After reaction of a desired oil, FA compositions at the 2- and 1,3-positions can be determined by analysis of FA composition in 2-MAGs or 1,3-DAGs, which are isolated from the reaction mixture. However, the two methods have some drawbacks. The Grignard reaction requires close attention to technique because even a small amount of moisture greatly impedes the reaction. Also, the lipase method can not provide an exact analysis because the 1,3-specific lipases reported thus far do not act on all FAs to a similar degree, and because 2-MAGs are hydrolyzed easily after migration of FA at the 2-position to the 1(3)-position. In particular, regiospecific analysis of PUFA-containing oils cannot be achieved by this enzymatic method. On the other hand, the method by ethanolysis with immobilized *C. antarctica* lipase eliminated these drawbacks, and was confirmed to be reliable by other researchers (Shen and Wijesundera, 2006). Considering these circumstances, the ethanolysis with lipase may be adopted widely for regiospecific analysis of TAGs.

2.7. CONCLUSIONS

BDF has attracted attention as an eco-friendly fuel, and its demand is expected to increase further. We have introduced enzymatic processes which eliminate the drawbacks of chemical process. Not only the enzymatic process, but also processes using supercritical MeOH, ion-exchange resins, and MeOH vapor will likely be used in the future, and a company will decide the most suitable process for its own production environment. We hope that an enzyme process will be selected as a candidate.

Some element reactions for BDF production can be applied widely to oil and fat processing. Since enzyme-catalyzed reactions proceed efficiently under mild conditions, they are suitable for the treatment of materials including unstable compounds. Furthermore, enzymes can convert only a desired compound to its other molecular form because of the strict substrate specificity compared with chemical catalysts. We hope that much attention will be focused on the superiority of enzyme, and that lipase reactions will be applied more and more as the practical process in the oil and fat industry.

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CHAPTER 3

Biodiesel Cost Optimizer: Least-Cost Raw Material Blending for Standardized Biodiesel Quality

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3.1. INTRODUCTION

Biodiesel production around the world is a booming business. Many drivers for this strong growth can be identified. European governments want to reduce carbon dioxide emissions: Kyoto Agreement targets have to be met. Many governments also wish to support their own farmers by creating new markets for agriculture products. To realize these non-technical objectives, governments around the world have set up tax exemption systems, tax incentives, or other energy credit systems, making startups in biodiesel production feasible and economically sustainable. Because other markets have plenty of raw materials available for making fatty acid methyl esters for fuel, biodiesel trade

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becomes a new commodity business. Biodiesel for transport energy is an opportunity from technical point of view. Bringing high performance biodiesel with outstanding cetane numbers and flow characteristics can generate new flexibility when blended with fossil diesel. Adding a few percent of biodiesel to Ultra Low Sulfur Diesel (Diesel Type IV or higher) also brings extra lubricity lost by the sulfur reduction.

Ten years of heavy investments in biodiesel resulted in a worldwide market where three main types of biodiesel producers can be identified:

- Large integrated units combining "virgin" oil extraction from rapeseed, or soybean, or palm with downstream refining and transesterification with methanol. The minimum step-in size for such plants seems to be 100,000 MT a year (30 million gallon).
- Small-scale container-size production units can easily be fitted to an existing oil processing business or operate as stand-alone units. These are ideal for producing biodiesel in remote areas with limited demands. Such units can also be combined and even integrated with oil refining to create flexible production units.
- Finally, multi-process units are being built that can deal with both alkaline and acid transesterification. Such biodiesel producers are well positioned to develop an expanding supply of fuel based on both recycled and virgin vegetable oils.

These different businesses may have diverse backgrounds, rooted in vegetable oil business or the petrol business, or based on investment financing, however, they all have one thing in common: they produce biodiesel, or better fatty acid methyl esters (FAME) to be used by petrol companies for blending with fossil diesel to B2, B5, B10 or B100. These fatty acid methyl esters must comply with the Biodiesel Standard in use in the region of commercialization. And most importantly, this biodiesel needs to be produced at a market competitive price.

Without the Biodiesel Standard, there would be no sustainable biodiesel market. The Biodiesel Standard guarantees that products are properly tested for blend properties, performance in engine use, and functionality with injection systems used in the modern common rail diesel engines. Many car manufacturers state that FAME used in commercial fuel must meet both standard references: EN14214, the European Biodiesel Standard, and ASTM D 6751, the U. S. Biodiesel Standard (Table 3.1). Making biodiesel that complies with the applicable Biodiesel Standard helps guarantee the functionality and performance in transport engines.

This, however, is not sufficient if the pricing of the product isn't right. At off-market prices, sellers will never find buyers for the product. Biodiesel price depends on several non-related independent price systems, such as fossil diesel and (vegetable) oil price, methanol and glycerin price, the local-currency-to-

TABLE 3.1. Biodiesel standards.

	EN 14214	ASTM D-6751–03a	Units
Application	FAME	FAMAE	
Density at 15 °C	0.86–0.90	—	g/cm^3
Viscos. 40 °C	3.5–5.0	1.9–6.0	mm^2/sec.
Distillat.Temperature, AET, 95%	—	90% @ 360 °C	°C
Flashpoint	>120	>130 (150 av.)	°C
CFPP	*country specific	—	°C
Pour point	—	—	°C
Cloud point	—	report customer	°C
Sulfur	<10 mg/kg	<0.0015 (S15), <0.05 (S500)	% mass
CCR 100%	—	—	% mass
10% dist. resid.	<0.3	—	% mass
Sulfated ash	<0.02	<0.02	% mass
(Oxid) Ash	—	—	% mass
Water mg/kg	<500	<0.050% vol. (water & sediment)	mg/kg
Total contam. mg/kg	<24	—	mg/kg
Cu-Corros. 3h/50 °C	1	<No.3	
Oxidation stability hrs; 110 °C	6 hours min	—	h
Cetane No.	>51	>47	
Neutral. No. (Acid Value)	<0.5	<0.80	mg KOH/g
Methanol	<0.20	—	% mass
Ester content	>96.5	—	% mass
Monoglyceride.	<0.8	—	% mass
Diglyceride	<0.2	—	% mass
Triglyceride	<0.2	—	% mass
Free glycerol	<0.02	<0.020	% mass
Total glycerol	<0.25	<0.240	% mass
Iodine No.	<120	—	
Linolenic acid ME	<12	—	% mass
C18:3 and high. unsat.acids	—	—	% mass
C(x:4) & greater unsaturated esters	<1	—	% mass
Phosphor	<10	<0.001% mass	mg/kg
Ramsbottom carbon residue	—	<0.050	% mass
Carbon residue	—	<0.050% by mass	
Gp I metals (Na,K) mg/kg	<5	—	mg/kg
Gp II metals (Ca,Mg) mg/kg	<5	—	mg/kg
Alkalinity mg/kg	—	—	mg/kg

U.S. dollar exchange rate, and finally on a favorable tax environment. In practice, the raw materials account for more than 70% of the final biodiesel price. It is therefore of utmost importance having these raw materials at the lowest cost possible, while properly managing production and logistics costs.

When looking at the specifications listed in the Biodiesel Standards (Table 3.1), it is easy to identify the specific items that are exclusively linked to the FAME composition of the product. We may indeed suppose that free glycerine, free methanol, tri-, di- and mono-glyceride content, residual water, traces of sulfur and phosphorous, and the presence and level of other minor components should normally all be within the limits set by proper processing and product clean-up technology. Good Manufacturing Practice (GMP) will guarantee compliance for all these specifications linked to remaining ingredients fractions and intermediates. Any plant capable of making FAME should have these items right; there can be no discussion on this.

The specification lines affected by a changing FAME composition are different: the cetane number or cetane index, the oxidation stability, the viscosity, the Cloud Point (CP), Pour Point (PP), or Cold Filter Plugging Point (CFPP). These are of major importance for the functionality of the biodiesel, and for performance under difficult working conditions, such as use at reduced temperature in northern winter conditions. The same specification requirements will also be found in the World-Wide Fuel Charter, which. sets the acceptable levels of biodiesel addition to the different grades of diesel and are tied to increasing environmental requirements: (grades I, II, III, now IV and coming, grade V).

Let's have a look at these specifications and evaluate how they relate to functionality. We can then assess how they can be related to FAME composition, and how they could be used to calculate the Lowest Cost Blend of FAME, while still complying with the requirements of the applicable Biodiesel Standard.

3.2. TECHNICAL BACKGROUND OF CRITICAL ITEMS IN THE BIODIESEL STANDARD

The Cetane Number (CN), or sometimes Cetane Index, is directly linked to the cold start ability of the engine, *i.e.*, the ease of a fuel's auto-ignition. It translates also in reduced exhaust emission, optimized fuel consumption, and reduced combustion noise. The definition of CN is based on the volume% of cetane (n-hexadecane $C_{16}H_{34}$ = fast ignition; CN = 100) in α-methyl naphthalene ($C_{11}H_{10}$ = ignition delay; CN = 0), or in blends with hepta-methyl nonane ($C_{16}H_{34}$; CN = 15) that provides the specified standard of 13 degrees (crankshaft angle) ignition delay at the identical compression ratio to that of the fuel sample. The CN is the reference specification directly linked to the performance level. In the European Biodiesel Standard, the CN should be 51 or higher; this is in-line with Diesel Type IV requirements, but it might need to be adjusted upwards when Diesel Type V is implemented. In the U. S. Biodiesel Standard, CN should only be 47; this is good for old-fashioned diesel technology. It can be expected to rise to higher values when higher performance diesel engines break through in the U. S. market.

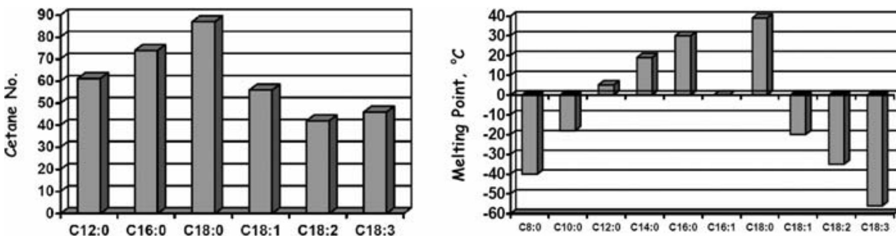

Figure 3.1. Cetane number, fatty acid type, and melting point of FAME (U. S. Department of Energy, 2004).

Engine performance is one of the most important properties of quality biodiesel. FAMEs have a lower energy content than fossil diesel (11% on volumetric basis; 5–6% on gravimetric basis), but a slightly higher chemical efficiency due to the presence of oxygen in the molecule, facilitating overall combustion. Biodiesel also brings significant emission reductions: a slight reduction in carbon dioxide and hydrocarbon emissions, and a reduction in polyaromatics and Particulate Matter, are sometimes gained at the cost of a slight increase in NOx (7–20%), depending on the FAME composition. Chain length and presence or absence of double bounds will directly affect the Cetane Number (Fig. 3.1).

Fuel *density* is important for blending characteristics, but also relates to emission levels, fuel consumption, and emission control systems. Similarly, the viscosity of the fuel is important. *Kinematic Viscosity* is included in the Biodiesel Standard because it relates directly to the injection system performance. In the Biodiesel Standards, viscosity is often set at a specific temperature point. With most fatty acid methyl esters this is never a problem, but viscosity changes at low temperature can be much more problematic. Biodiesel tends to thicken faster than fossil diesel. Specific additives might be required to deal with this.

Cold flow properties are of major importance in winter diesel. Cold performance limits are set to guarantee lack of crystallization. These are expressed as Cloud Point (CP), Pour Point (PP), or as Cold Filter Plugging Point (CFPP). For B100 biodiesel these have to be fully met with the biodiesel, and, if needed, with extra crystal retardation additives. In B2-B30 blends, though, the tendency is rather towards two types of Biodiesel, one for summertime (for example with CFPP of 0 °C), and one for wintertime (for example with CFPP −10 °C).

Diesel Type III or higher will have very low sulfur levels; less than 3 ppm is the target in Diesel Type IV. Reducing the sulfur level is needed to comply with strict environmental requirements, and it not only improves engine life, but comes with considerable reduction in Particulate Matter emission, especially when combined with diesel after-treatment (NOx absorbers, particulate matter filters). FAME normally doesn't add extra sulfur. Addition of 2% biodiesel to low sulfur fossil diesel normally brings back the required lubricity

essential for proper pump performance and limiting wear and tear. For simple technical reasons B2 is an absolute must. However, many targets for biodiesel addition aim at much higher blending levels: B5, B10, B20 or B30.

Oxidation Stability (OSI or Rancimat) has been introduced in many ways. In EN14214 it exists as OSI and as a maximum acceptable *Iodine Value*, or maximum level of linolenic acid or of poly-unsaturated fatty acids. Oxidation stability is of importance when it comes to polymerization and oxidation during storage as well as during use in the engine. Oxidation is directly related to the presence of unsaturated bonds in the FAME, and probably because of this the EN 14214 includes a cap on Iodine Value.

3.3. HOW ARE THESE SPECIFICATIONS LINKED TO COMPOSITION?

In the different Biodiesel Standards the specifications are linked to the fatty acid composition itself. Some characteristics may be affected by the presence of minor components, but most variation is directly linked to the FAME composition. Two questions emerge: how are these relationships built, and how can we take advantage of these when making blends with the lowest possible raw material price?

Is the Iodine Value found in EN 14214 based on science? It is certainly not so in an absolute way, but some relation cannot be denied. Frankel (2005) for example clearly states that oxidation stability is a function of two things: the number of double bounds, and their position towards one another in the fatty acid. Oleic acid with one double bound oxidizes 40 times slower than linoleic acid with two double bounds, and one bis-allylic position in-between both. Linolenic acid with three double bounds separated with two bis-allylic positions oxidizes only 2.5 times faster than linoleic acid. Oxidation is a radical driven reaction, and the bis-allylic positions are a much more favorable point of attack than the allylic positions next to the double bound.

Similarly, Dijkstra *et al.* (1996) have shown that the OSI of triglycerides is a non-linear relationship determined by the fatty acid composition, and further promoted by the presence of pro-oxidants such as metal ions, and inhibited by the presence of antioxidants such as tocopherols. A similar relationship can be found for FAME:

- Poly-unsaturated (PUFA) methyl esters have a much lower oxidation stability than mono-unsaturated (MUFA) or saturated (SAFA) methyl esters,
- Distilled FAME are very low in oxidation stability, since all natural antioxidants are removed.

This is also the mechanism and relationship found when adding synthetic antioxidants to biodiesel, as has been demonstrated by Ingendoh (2007).

Though FAME has limited oxidation stability, they remain a valid alternative for diesel. Conventional diesel fuel has a boiling range of 180–340 °C, with a composition of n-alkanes, cycloalkanes, alkyl benzenes, and polyaromatic compounds. Fossil diesels have a CN in the range 40–100. FAME has properties that are close to all these basic diesel properties. FAME can also easily blend with fossil diesel at any level due to their similar solvent behavior; the viscosity of fossil diesel and biodiesel are also in the same range.

The CN indicates which oils could be suitable as alternative diesel. CN by definition is based on a linear set of blends of cetane in α-methyl naphthalene: cetane or hexadecane ($C_{16}H_{34}$) shows fast ignition, *i.e.*, CN 100; alpha-methyl naphthalene ($C_{11}H_{10}$) has a strong ignition delay, *i.e.*, CN 0.

CFPP, PP, or CP is also related to the FAME composition. As is clearly shown in the "2004 Biodiesel Handling and Use Guidelines" published by the U. S. Department of Energy (2004), longer saturated fatty acids have a higher melting point (Fig. 3.1). More double bounds in a same fatty acid chain reduce the melting point of the fatty acid methyl ester. The U. S. Department of Energy report (2004) also describes a similar relationship between CN and fatty acid type (Fig. 3.1): increasing chain length yields a higher CN or vice versa; less double bounds in a higher CN; and double bond closer to the end of the fatty acid also improve the CN, although this also increases the risk of benzene formation during combustion.

In other words: major high-CN components in the triglycerides will lead to higher CN of the resulting FAME. Problem is that some of the very high CN components such as palmitic acid methyl ester and stearic acid methyl ester have very poor crystallization characteristics. And more unsaturated compounds may be excellent to prevent crystallization, but will perform poorly on CN and on oxidation stability. There is a clear trade-off between both characteristics. A good FAME composition will inevitably be a mixture of higher and lower melting FAME with higher and lower CN, and optimized oxidation stability.

3.4. MODELING THE CFPP AND COLD FLOW PROPERTIES

When it comes to predicting the point of crystallization, it gets more complicated. Crystallization of oils or fats or of FAME is a thermodynamic process. FAME crystallization by itself is probably much simpler than the complex crystallization of oils and fats as seen in food products from chocolate to margarine. Basic research on FAME crystallization is in progress, but currently not readily available to put in a simple and useful mathematical model sufficient to describe the CP, PP, or CFPP.

The problem is that it is almost impossible to predict nucleation, the start of the crystallization process. Nucleation is also a thermodynamic process. It requires extra energy to form a crystal nucleus from liquid molecules. This nucleus, once formed, can then grow. In practice, significant supercooling will

be needed to start this nucleation. The amount of supercooling needed will be dependent on local conditions such as the solvent composition of the FAME, the form and surface structure of the recipient, and the presence or absence of insoluble and specific soluble impurities.

Once nucleation has started, crystallization will immediately proceed with release of crystallization energy. FAME crystallization is an exothermal process, with the total amount of crystallization depending on the total amount of crystallizable FAME and the amount of supercooling. The amount of crystallizable FAME can be estimated from the total content of palmitic and stearic acid, and corrected for the presence of trans fatty acids, if any. Long chain trans fatty acids will be found in recycled oils and fats containing hydrogenated components, or in overheated oils from high temperature deodorization or from heavy frying.

It is clear that an accurate prediction of the CP, PP, or CFPP is almost impossible, certainly since presence of crystallizable minor components can have a significant effect. Some of these components, such as phytosterol glycoside esters in soy-based FAME. can speed nucleation and crystallization; other molecules creating steric hindrance in the growing nucleus will retard crystallization. Such components are often added to biodiesel to reduce the size of crystals or inhibit crystal formation by preventing nucleation.

Two approaches are possible to deal with CFPP in a more pragmatic way: *i)* by capping the amount of acceptable crystallizable FAME, and *ii)* by trying to predict the melting point of a specific composition.

The first approach is the most generic one, and probably the most realistic. The second approach, modeling melting points based on a series of measurements on binary or ternary blends, has already been tried. AGQM Biodiesel has shown that by multivariance modeling a linear relationship can be calculated between melting point and FAME composition (2006). The problem with such multivariate model is that it only works within the borders provided during the development of the model. In this case only a small number of binary mixes of soybean oil methyl esters in rapeseed methyl esters were used, and only one additional ternary mix with palm oil methyl esters. The variation of melting points described by Haupt (2006) for the pure rapeseed methyl esters range from –11 °C to –17 °C. This should already be a warning for people wanting to predict melting points: any prediction made with such model will never have a better precision than what you put in, *i.e.*, ±3–4 °C. More recently a more solid CFPP simulation model has been integrated in the Biodiesel Cost Optimizer; this proprietary model is based on multivariate mathematics. This opens the choice for a double strategy in predicting crystallization behaviour.

The more pragmatic approach used in the *Biodiesel Cost Optimizer* (see below) is based on the work of Dr. Lee and colleagues (1996). Soy methyl esters were winterized on lab scale at –21.5 °C, –25.0 °C and –28.4 °C. This yielded “FAME olein” with 11.2%, 7.4% respectively 6.0% saturated FAME (palmitic acid and stearic acid methyl esters), and “FAME stearin” with 46.6%, 42.2% respectively 33.1% saturated FAME. This simple lab test con-

firms that 11.2% saturated FAME (in this case 8.5% palmitic acid methyl ester and 2.7% stearic acid methyl ester) stay in the liquid phase at –20 °C. This obviously is not an absolute guarantee that any combination of palmitic and stearic acid methyl esters will stay liquid at –20 °C, but it indicates that they certainly will remain liquid at –10 °C. For winter blends of biodiesel with fossil diesel (B2-B30) this is probably more than good enough as a target value for mathematical modeling.

3.5. BIODIESEL COST OPTIMIZER

The *Biodiesel Cost Optimizer* (Fig. 3.2), built as a mathematical tool, has been developed to help the biodiesel industry find a least cost formula for making FAME compositions that perform within the specifications of the applicable Biodiesel Standard. The Excel Workbook *Biodiesel Cost Optimizer* contains three separate sheets:

i) *Introduction Page.* This sheet contains the start-up screen and all formulas for calculating IV, CN, viscosity, OSI, and heat of combustion.
ii) *Blend Calculation ($ optimized).* This is the sheet used for simulation and changing raw material input or simulation constraints. The sheet is fully modifiable: up to 11 different raw materials can be studied simultaneously.
iii) *Raw Material Input Data.* This sheet contains a database of standard raw materials that could be selected for use in the simulation model. Using copy and paste, columns can easily be introduced in the Blend Calculation sheet.

New compositions and updated prices can be added at all time.

3.6. BACKGROUND OF THE CALCULATIONS

All simulations in the model are based on the assumption that the different characteristics described above can be mathematically modeled. Linear models are used where possible:

i) A linear model is used for calculation of *Fatty Acid Composition* data, *Iodine Value* calculation (AOCS Method of Analysis Cd 1c-85), *Kinematic Viscosity* (methyl esters of fatty acids behave as Newtonian liquids), *Heat of Combustion*, and *Price*.
ii) The *Cetane Number (CN).* A series of simulations reveals that the calculated CN is very close to the measured value, be it somewhat at a slightly lower value. According to Annex A of EN14214, the measured Cetane Number method has a rather poor reproducibility with R of 5.0 units and a repeatability r of 2.4 units.

Fatty Acid	Fatty acid	Mol. wt. FAME	SBO Soybean	RSO Canola Rapeseed Oil	SFO Sun-flower	Palm oil crude	Palm Stearin RBD	Palm Olein 56 RBD	CSO Cotton-seed Oil	Corn oil	Lard	Tallow	Recycled Oil "G"	BLEND	ASTM 6751
8:00	caprylic	158.24													
10:00	capric	186.30													
12:00	lauric	214.35				0.2	0.1	0.2			0.1	0.1	0.2	0.1	
14:00	myristic	242.41	0.1	0.0	0.1	1.1	1.2	1.1	1.2	0.1	1.5	3.5	0.5	0.4	
14:1w5															
16:00	palmitic	270.46	10.6	4.4	6.5	44.8	58.2	38.5	23.0	11.0	24.8	26.0	14.0	20.9	
16:1w7	palmitoleic		0.2	0.3		0.3	0.2	0.3	4.0	0.2	3.1	3.0	0.6	0.2	
18:00	stearic	298.51	3.5	2.0	3.3	4.3	4.7	4.6	19.0	2.0	12.3	19.0	4.2	3.7	
18:1w9	oleic	296.49	23.7	58.2	24.5	38.4	28.8	42.4	51.0	28.1	45.1	41.0	49.1	28.1	
18:2w6	linoleic	294.48	53.4	21.2	64.4	10.1	6.2	12.2		57.2	9.9	3.0	24.2	40.4	
18:3w3	linolenic	292.46	8.1	10.2	0.3	0.4	0.2	0.5		1.0	0.1	1.0	5.5	5.8	
20:00	arachidic		0.3	0.6			0.3	0.2		0.3	0.2	0.2	0.4	0.2	
20:1w9	gadoleic	352.60		1.5							1.3	0.5	0.7		
20:2w6				0.1											
20:3w6															
20:4w6	arachidonic														
20:3w3													0.3		
22:00	behenic		0.1	0.3			0.0			0.1			0.3	0.1	
22:1w9	erucic			0.5											
24:00	lignoceric			0.2											
22:6w3	DHA			0.2											
24:1w9	nervonic														
TFA (C18:n)			0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	4.0	4.0	4.0	0.2	
		SUM	99.9	99.9	99.1	99.6	99.9	100.0	98.2	100.0	98.4	97.3	100.0	99.8	
		C16+18+20 + TFA	14.7	7.5	10.0	49.2	63.3	43.5	42.2	13.6	41.3	49.2	22.9	25.0	SAFA+TFA=OK
A. Polymer content & refinery loss			2.0	1.5	1.5	5.0	0.5	0.5	2.5	2.0	4.0	4.0	5.0	3.0	
		Measured CN	46.2	50.0	46.6	63.0							53.0		
		Measured IV	134.0	116.0	135.0	53.0	38.1	61.1		126.0	62.0	48.0	100.0		
$/MT		CIF-FOB R'dam (crude)	713	769	715	613	625	650	820	790	560	565	500	703	-77 $ per MT biodiesel
		Antioxidant factor	95.4	57.0	53.6	60.0	60.0	60.0	60.0	107.0	60.0	60.0	30.0	84.8	
		Calculated CN	46.9	50.2	46.2	64.5	67.8	62.8	66.7	47.7	63.3	67.0	53.9	52.2	CN = OK
		Calculated Kinematic Viscosity	3.99	4.22	3.99	4.40	4.44	4.41	4.70	4.03	4.58	4.68	4.30	4.11	Viscosity=OK
		Calculated Heat of Combustion	2776	2815	2788	2692	2658	2710	2750	2778	2748	2731	2780	2750	99%
		Calculated IV	134.2	116.0	134.6	52.1	36.2	59.2	48.5	126.0	61.1	47.6	100.4	109.6	IV = OK
		O.S.I. (estimate)	5.6	6.3	3.0	19.7	31.2	16.4	316.0	6.7	20.6	46.6	3.5	6.6	OSI = OK
		BLEND	72.0		0.0	31.0								103.0	vs.RME

price factor (0-100)	C16+18+20 + TFA limit	This cell is minimized	SBO Soybean	RSO Canola Rapeseed Oil	SFO Sun-flower	Palm oil crude	Palm Stearin RBD	Palm Olein 56 RBD	CSO Cotton-seed Oil	Corn oil	Lard	Tallow	Recycled Oil "G"	BLEND
100%	25.0	-66.6		1	1	1			1					
			This column is maximized											

Light Blue open for INPUT CHANGE — Yellow BLEND Composition in % (= result of SOLVER)
Green SOLVER values for CN, Kinematic viscosity, IV, OSI, $/MT and SAFA+TFA (limit or optimum)
Orange set on ZERO before using SOLVER — RED cell to be minimized: column to be maximized
INPUT: Copy Raw Material columns directly from "Raw Mateiral Input Data" worksheet, or input data in Rows 1 till 28

V. 070326 ID&A Ignace Debruyne — 0.0 — 29.4

Figure 3.2. The Biodiesel cost optimizer: blend calculation screenshot.

iii) *Raw Material Prices* in the spreadsheet are market prices for crude or RBD oil (for some palm oil fractions) C&F, or FOB Rotterdam or Northern Europe, *i.e.*, not yet containing additional neutralization and transesterification costs. If needed "U. S. prices" in $/lb can be introduced.

Several other non-linear elements and specific constraints are taken into account:

i) The *$ or Price factor* can be switched on. The price line is added to get an immediate feedback on what a certain blend would cost compared pure to rapeseed methyl esters (RME), or costed against methyl esters made from one single feedstock.
ii) The maximum amount of crystallizable fatty acids can be set: *Saturated (C16 + C18 + C20)* and *trans-fatty acids* (*SAFA* + *TFA*). This value is used in a completely different way. As explained above, these FAME could initiate crystallization in winter formulas, directly modifying the CP and the CFPP. This is of importance for B100, but also affects the performance of lower concentration mixes of biodiesel in fossil diesel. For blends with fossil diesel, biodiesel with CFPP of −10 °C is a good target for "winter diesel."
    a) For winter diesel it is recommended to set this value close to around 10% or slightly higher.
    b) In summer, or in warmer areas on the globe, much higher limits for SAFA + TFA must be possible. Using a value up to 25% supposedly will not create problems under such conditions. In tropical areas, even higher values might be acceptable.
iii) *Oxidation Stability Index (OSI)* modeling is based on a non-linear model. FAME oxidation stability is directly linked to origin of raw materials, *i.e.*, fatty acid composition and presence of natural antioxidants and pro-oxidants (Dijkstra *et al.*, 1996) as demonstrated by this equation:

$$OSI = \frac{\sum_i x_i a_i}{\sum_i x_i v_i} \qquad \text{[Eq. 3.1]}$$

with x_i the mass fraction of each component
a_i an antioxidant factor different for different raw materials
v_i the oxidation rate which is different for saturated, mono-, di- and tri-unsaturated fatty acids.

Data for a_i and v_i are derived from oil data available in literature and adjusted with a factor to give a good match with measurements obtained

with specific methyl ester products. This model assumes that fair average quality raw materials are used. Strongly oxidized products such as recycled oils or fats are probably very different from this. Also "virgin" oils may differ significantly in stability, depending on the quality of the oilseeds used for producing the oil.

iv) Finally, the model allows an estimate of refining loss for unrefined raw materials, or recycled oils and fats having a high content of oxidized and polymerized material that will be lost in the preliminary refining needed before the transesterification.

Eleven different raw materials can be presented simultaneously, and used for simulation (Fig. 3.2). New raw materials can directly be input in the master sheet, or they can first be saved in the *Raw Material Input Data* sheet and then copied to the master *Blend Calculation* sheet. During input, the sum line will automatically show the sum of Fatty Acids. This number should be very close to 100% after correct data input.

In practice a lower number of raw materials will be used for calculations with the *Biodiesel Cost Optimizer* model. The columns to be used can be selected by putting a "1" (*i.e.*, this column must be used) or a "0" (which means: "don't use this column) in the cells [E39:N39]. The model assumes that the raw material in "Column D" is to be maximized. Any raw material of choice can be copied in this column from the Raw Material Input Data sheet. Normally, the lowest cost component should be placed here.

3.7. SIMULATION RESULTS

Calculations in the *Biodiesel Cost Optimizer* are done with the built-in Solver function of Excel. The model has been fully set up to deal with all measurements in above list. The results of the calculation will be found in the BLEND line above the selected columns. The final composition is given in the BLEND column at the right end of the table. More results describing the BLEND are given in the cells below the composition:

- The SAFA + TFA value
- Price difference of the blend raw materials as compared to pure rapeseed methyl esters
- The calculated Cetane Number (CN)
- The Kinematic Viscosity
- The Heat of Combustion (in kg-cal/mole) as% compared to rapeseed methyl esters
- The Iodine Value
- The Oxidation Stability Index (OSI), which is an estimate based on the assumption that fair average quality raw materials are used.

With an easy click the *Biodiesel Cost Optimizer* can select for European Biodiesel Standard EN 14214, or U. S. Biodiesel Standard ASTM D 6751. If needed other target values can be input manually.

3.8. RECOMMENDATIONS

Operation of the *Biodiesel Cost Optimizer* is fast and easy, making it possible to make large sets of simulations in a short time. This helps better understanding the different value of the different fatty acid methyl esters. It will quickly become clear, when using the *Biodiesel Cost Optimizer*, that oleic acid methyl esters are the preferential FAME in every biodiesel formula. Oleic acid methyl esters bring a relatively high oxidation stability (50h or more), combined with a more than acceptable CN of around 56, and excellent melting point at –19 °C. Unfortunately, pure oleic acid methyl esters are not available in the market.

Table 3.2 displays some examples for ternary blends of palm oil, soybean oil and rapeseed oil methyl esters. Under the price conditions given in the table, a typical winter formula would contain 80–90% rapeseed methyl esters, with the balance mainly soy methyl esters and maybe a few percent of palm methyl esters. In summertime no rapeseed would be used. For Europe 70 to 80% soy methyl esters would combine with palm methyl esters as the balance. For Biodiesel in the U. S., the palm oil methyl ester content would be equally high, making the formula considerably cheaper than pure soy methyl esters.

The *Biodiesel Cost Optimizer* can easily be used for evaluating the value of more exotic components such as lauric fats, or recycled oils and fats. Recycled oils are interesting for price reasons, although availability of a solid supply and

TABLE 3.2. Some ternary blend calculations (prices of May 2006).

SAFA + TFA	$442 PaO	$541 SBO	$721 RSO	IV	CN	$MT	$/MT Profit
7.2	0	0	100	116.2	50.3	732	
8.0	0.9	6.1	94.6	116.7	50.2	719	13
9.0	2.3	11.9	87.5	116.9	50.2	705	27
10.0	3.7	17.7	80.3	117.0	50.2	691	41
12.0	6.5	29.4	66.0	117.3	50.2	664	68
14.0	9.3	41.1	51.7	117.6	50.2	636	96
16.0	12.1	52.8	37.3	117.9	50.2	608	124
19.0	16.4	70.5	15.6	118.4	50.2	566	166
22.0	22.0	80.7	0.0	116.7	50.6	534	198
25.0	30.0	72.0	0.0	109.6	52.2	527	205
30.0	46.1	57.5	0.0	97.7	54.7	514	218

PaO = palm oil; SBO = soybean oil; RSO = rapeseed oil; target is EN 14214.

the possible presence of high amounts of SAFA (C16 or C18 from palm oil components or hydrogenated fats) and/or trans fatty acids (from hydrogenation processes) could be limiting. Similarly, recycled oils and fats might be heavily loaded with oxidized and polymerized compounds, restricting the use, and indirectly increasing the price of the refining. However, as indicated above, the *Biodiesel Cost Optimizer* model has the built-in option to correct for these losses automatically.

3.9. CONCLUSIONS

The *Biodiesel Cost Optimizer* combines linear and non-linear mathematical relationships to identify the least cost blend composition for making quality biodiesel. The result of the calculation will not be an absolute answer. The *Biodiesel Cost Optimizer* is rather developed as a tool for fast identification of interesting blends with the least cost that should perform to specifications close to the target. A final test with the real mix will indicate if this is a feasible solution. In the extraordinary case where larger differences with the model should show, a directed search for the odd behavior can be organized looking at special (minor) components that might be at the origin of this odd behavior.

However, in most practical situations, the calculated composition will very closely match the target specifications.

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CHAPTER 4

New Catalytic Systems for Vegetable Oil Transesterification Based on Tin Compounds

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4.1. INTRODUCTION

During the Paris Exposition in 1900 peanut oil was successfully used as liquid fuel in a diesel engine (Shay, 1993). However, because of its low cost and easy availability at that time, petroleum became the dominant energy source, and petroleum diesel was developed as the primary fuel for diesel engines. Nonetheless, petroleum and its derived fuels have periodically been in short supply and, as a consequence, a search for alternative energy sources has emerged. Thus, in the 1930s and 1940s neat vegetable oils were used in diesel engines in emergency situations (Ma and Hanna, 1999). At that time, mixtures of fatty acids ethyl esters or hydrocarbons, obtained, respectively, from the alcoholysis or pyrolysis of vegetable oils, were also envisioned as fuels. Indeed, a mixture of fatty acid ethyl esters was produced in Belgium by the ethanolysis of

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palm-tree oil and used in diesel engines (Chavanne, 1938) and a mixture of hydrocarbons was produced in China by a tung oil pyrolysis batch system and used as a liquid fuel (Chang and Wan, 1947).

More recently, with the petroleum shortage during the 1970s and 1990s (due to political problems, as well as the depletion of the world's non-renewable fuels resources and an increasing demand for energy), imperative concerns have emerged related to energy supply security. On the other hand, an increasing environmental awareness is leading researchers to search for alternative fuels from renewable resources that are environmental friendly (Muniyappa *et al.*, 1996). In this context, the use of fatty acids methyl or ethyl esters (FAME or FAEE), largely known as biodiesel, has been highlighted in different countries as the main alternative for a large-scale substitution of diesel fuel.

Despite the differences in the chemical composition, biodiesel has physical-chemical properties that are similar to diesel, showing a decrease in the emission of CO_2, SO_X and unburned hydrocarbons during the combustion process when compared to fossil fuels (Crabbe *et al.*, 2001). For these reasons it is possible to use biodiesel blended with diesel without significant changes to engines, with important decrease in the emission of pollutant gases.

Different methods of synthesizing biodiesel have been proposed. Among these methods, the most common is the catalytic transesterification of vegetable oils with a short chain alcohol, usually methanol or ethanol, affording glycerin as a byproduct. Indeed, transesterification is well studied and established, especially using main-metal hydroxides or alkoxides and mineral acids, such as sulfuric acid and sodium hydroxide, as catalysts (Wright *et al.*, 1944; Freedman *et al.*, 1984). Reviews on this subject are available (Lang *et al.*, 2001; Schuchardt *et al.*, 1998). These catalytic systems have some technological problems, one being the acid system associated with corrosion, and, more fundamentally, with soaps formation as side-products. Those are known to emulsify the biodiesel with glycerin, especially if ethanol is used.

Different attempts have been done in order to develop alternative ways to minimize these problems, such as removing the water from the reaction media, from non-catalytic supercritical condition and from other catalytic systems (Schuchardt *et al.*, 1998). Concerning the developing of alternative catalytic systems, it was related to the use of *i)* homogeneous catalysts, like enzymes (Fukuda *et al.*, 2001), organic bases (Schuchardt *et al.*, 1998), and Lewis acid metal compounds (Abreu *et al.*, 2003; Di Serio *et al.*, 2005), *ii)* homogeneous catalysts anchored in ionic liquids or supported in organic polymers or inorganic solids (Abreu *et al.*, 2005; Neto *et al.*, 2007; Schuchardt *et al.*, 1996), and *iii)* heterogeneous catalysts, such as metal oxides (Abreu *et al.*, 2005), metal carbonates (Suppes *et al.*, 2001), aluminum-silicates (Leclercq *et al.*, 2001), and acid organic polymers (Schuchardt *et al.*, 1996). In the last few years, exhaustive studies on the use of different tin compounds in homogeneous (Abreu *et al.*, 2003, 2004; Ferreira *et al.*, 2007), multi-phase and heterogeneous conditions (Abreu *et al.*, 2005; Macedo *et al.*, 2006; Neto *et al.*, 2007) have been conducted. In this work, we wish to review and discuss these studies.

4.2. TIN COMPLEXES IN HOMOGENEOUS CATALYTIC SYSTEMS

To study the catalytic performances of the Lewis acid metal compounds for the soybean methanolysis in homogeneous conditions, we prepared complexes using the ligand 3-hydroxy-2-methyl-4-pyronate (maltolate) and the cations Sn^{+2}, Zn^{+2}, Pb^{+2} and Hg^{+2}, as depicted in Figure 4.1 (Abreu *et al.*, 2003).

Initially, the different complexes were tested in the methanolysis of soybean oil at 80 °C using the molar ratio 400:100:1 (methanol:oil:catalyst). It is worth mentioning that these conditions are probably not optimal to obtain the highest reaction yields for all systems. However, this procedure was useful to compare the catalytic activities of the different catalysts. After 3 h, conversions up to 90%, 28%, 15%, and 2%, respectively for the complexes **1, 4, 2**, and **3** were observed. Using similar conditions (temperature and molar ratio) other Sn^{+4} complexes were also studied, showing lower activities when compared to complex **1** (Meneghetti *et al.*, 2007).

Different vegetable oils, such as andiroba (*Carapa guianensis*), babassu (*Orbignia sp.*), palm tree (*Elaeis sp.*), piqui (*Caryocar sp.*) and soybean (*Glycine max*), whose fatty acids have different alkyd-chain length and number of double bonds, were methanolyzed using the catalytic precursor **1**, and Table 4.1 presents the obtained reaction yields (Abreu *et al.*, 2004). Since all catalytic experiments were carried out using similar reaction conditions and the same reaction time (1 hour), it is possible to compare their activities directly from the reaction yields. It became clear from Table 4.1 that the reaction activities

Figure 4.1. Metal complexes **1** (M = Sn^{+2}), **2** (M = Pb^{+2}), **3** (M = Hg^{+2}), and **4** (M = Zn^{+2}).

TABLE 4.1. Methanolysis of different vegetable oils catalyzed by (1) using the same alcohol/vegetable oil/catalyst molar ratio (400/100/1).

Vegetable Oil	Yield (%)*	Fatty acid composition	
		Unsaturated (%)	Chain size ≤ 16 C (%)
Soybean	37.1	76	14
Andiroba	23.3	66	28
Babassu	35.6	19	74
Palm tree	16.2	58	35
Piqui	9.6	51	40

Source: Abreu *et al.*, 2004.

*Percentage of the recovered methyl esters after 1 h of transesterification per initial mass of vegetable oil.

TABLE 4.2. Alcoholysis of soybean oil using different alcohols catalyzed by 1 using the same alcohol/vegetable oil/catalyst molar ration (400/100/1).

Alcohol	Yield (%)*
Methanol	37.1
Ethanol	8.3
n-Propanol	4.2
iso-Propanol	0.2
n-Butanol	1.1
terc-Butanol	0.3
cyclo-Hexanol	4.3

Source: Abreu *et al.*, 2004.
*Percentage of the recovered short-chain alcohol esters after 1 h of transesterification per initial mass of vegetable oil.

are strongly influenced by the nature of the vegetable oil. Comparing palm tree and soybean fatty acids that have carbonic chains of similar sizes but different saturation degrees, it can be inferred that the methanolysis reaction is favored by the presence of unsaturations in the carbonic chain. This hypothesis is corroborated by the behavior of piqui oil fatty acids, which shows a saturation degree slightly superior to those of palm tree oil and presented lower activity. On the other hand, comparing the activities using fatty acids with similar saturation degrees, but different alkyl-chain lengths like those from babassu, palm tree and piqui oils, it can be assumed that the activity of the tin catalyst is higher for shorter chains. It is important to highlight that, among all the substrates used, babassu and soybean oils presented the best activities, indicating that both saturation degree and alkyl-chain length are the dominating factors affecting the catalytic activity.

The alcoholysis of soybean oil using different alkyl-chain alcohols, like methanol, ethanol, propanol, *iso*-propanol, *n*-butanol, *terc*-butanol and *cyclo*-hexanol, was carried out using the catalytic precursors and the main results obtained are shown in Table 4.2. Considering that similar reaction conditions were used, it is also possible to assume a direct relationship between activity and reaction yield. As depicted in Table 4.2, the catalytic activities are strongly dependent on the nature of the alcohol. When the alcohol alkyl chain is linear the reaction activities decrease with increasing chain length and when branched ones are used the activity decreased drastically. These results strongly suggest that a steric effect is controlling the catalytic activity.

4.3. ANCHORING TIN COMPLEXES IN MULTI-PHASE SYSTEMS

Although tin complexes exhibit very good activity for vegetable oil alcoholysis, their technological potential in homogeneous conditions is very poor. Indeed, as long as these complexes remain dissolved in the reaction medium,

it is difficult to recover and reuse them. For this reason, immobilization of the tin complexes was attempted to obtain similar catalytic activity allied with the advantages of a heterogenous system (Abreu *et al.*, 2005; Neto *et al.*, 2007). To achieve this goal, two different strategies were followed: *i)* supporting the tin complex in an organic solid phase, and *ii)* anchoring it on ionic liquids (biphasic catalysis).

One attempt to obtain a recyclable catalytic system for the transesterification reaction using the tin complex **1** was to support it in a DOWEX® acid resin, preparing an organic solid containing tin complexed in the acid sites (Abreu *et al.*, 2005). When this solid was tested as catalyst for soybean methanolysis, using the same amount of tin and similar conditions as used in the homogeneous system, only 0.5% reaction yield was observed. It is worth mentioning that this reaction yield was even lower than the value observed when pure resin was used (1 %). This reduction in the reaction yield can be explained by assuming that the interaction of the resin with the tin complex probably deactivated the acid sites of both the resin and the complex **1** (Abreu *et al.*, 2005).

Another attempt to immobilize **1** was a two-phase system obtained by preparing a solution of the tin complex in the ionic liquids 1-butil-3-methylimidazolium hexafluorophosphate ($BMI.PF_6$) (Abreu *et al.*, 2005) and 1-butil-3-methylimidazolium tetrachloroindate ($BMI.InCl_4$) (Neto *et al.*, 2007). The reactions yields in these two-phase systems were similar to those obtained in homogeneous conditions using comparable conditions. However, recovering and reusing the ionic liquid phases, the reaction yields drastically decreased with each new substrate charge, dropping to almost zero after the fourth one. Electronspray ionization mass spectrometry (ESI-MS) analysis of the $BMI.InCl_4$ ionic liquid phase and the organic phase before and after catalysis, indicate that the tin catalyst remains in the IL phase and no leaching of the catalyst occurs using $BMI.InCl_4$ (Neto *et al.*, 2007). However, a clear decomposition of the complex was observed that is probably the cause of the decrease in the catalytic activity during the recycle experiments.

4.4. MECHANISTIC ASPECTS OF TIN COMPLEX CATALYSTS

The mechanism proposed for vegetable oils alcoholysis promoted by Lewis acids based on tin and others bivalent pirone complexes is shown in Figure 4.2 (Abreu *et al.*, 2005; Neto *et al.*, 2007). The catalytic active species is formed when the metal complex reacts with alcohol, affording an alkoxyde complex with a vacant site. Then, the glyceride carbonyl coordinates to the vacant site in the catalytic active species, increasing the carbonyl's normal polarization and thus enhancing the alcohol's nucleofilic attack, probably occurring *via* a four-membered ring transition state. After breaking and making C-O and O-H bonds to produce free glyceryn and biodiesel, the dissociation of the ester carbonyl completes the cycle.

Figure 4.2. Mechanism proposed for vegetable oils alcoholysis promoted by Lewis acid based on tin and others bivalent pirone complexes.

4.5. HETEROGENEOUS TIN CATALYSTS

Tin oxide was tested as a catalyst for the soybean oil transesterification reaction (Abreu *et al.*, 2005). Conversion of up to 56 % and 94.7 % were obtained, respectively, after one and five hours. At the end of the reaction it was possible to recover the catalyst by simple filtration of the mixture. The recovered solid was used three more times, under the same reaction conditions, and the catalytic activity was conserved.

Metal-oxides of the type $(Al_2O_3)_X(SnO)_Y(ZnO)_Z$ were also studied as heterogeneous catalysts for the transesterification reaction of soybean oil (Macedo *et al.*, 2006). It was observed that these materials are active for soybean oil alcoholysis with different alkyl-chain alcohols using several alcohols, including branched ones. The best result was achieved using methanol, with conversion yields up to 80% in 4 h. As observed for the complex **1** in homogenous conditions, the catalytic activities are strongly dependent on the nature of the alcohol. For alcohols with a linear chain, the reaction activities decrease with

increasing chain length and decrease drastically when branched alcohols are used. These results strongly suggest that steric effects control the catalytic activity and probably a similar mechanism takes place. As pure tin oxide, these solids were also recovered and reused under the same conditions, reaching similar yields after recycling the catalysts four times (Macedo *et al.*, 2006).

4.6. CONCLUSIONS

Excellent results were obtained using tin complexes in homogeneous conditions. It is important to highlight that, apart from the high activities achieved, no soap formation as side-product resulted, and an easily separation of the biodiesel and the glycerin at the end of the reaction was observed. Unfortunately, it was not possible to obtain a recyclable multi-phase system anchoring the tin complex to an ionic liquid or to a solid phase. However, the ESI-MS analysis of the ionic liquid phase and the organic phase before and after catalysis indicate that the tin catalyst remains in the IL phase, that the biodiesel phase is almost pure, and that a simple decantation is enough to separate the desired product. The fact that no leaching of the tin complex takes place and the biodiesel obtained is free of tin is particularly interesting because of the toxicity of this metal.

On the other hand, heterogeneous systems using tin oxide and alumina doped with tin and zinc oxides are active for soybean oil methanolysis, with the advantage of no emulsion formation at the end of the reaction. These catalysts not only present high conversion yields (up to 93% in 3 hours) but it is also possible to recycle them without any loss in catalytic activity. For this reason, it is reasonable to assume that these catalysts could be economically viable for use in large scale biodiesel production.

4.7. ACKNOWLEDGMENTS

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CHAPTER 5

Non-Catalytic Alcoholysis of Vegetable Oils for Production of Biodiesel Fuel

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5.1. INTRODUCTION

Biodiesel fuel (BDF)—biofuel made from natural oils and fats—is currently regarded as a promising diesel replacement fuel. An alkali-catalyzed

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alcoholysis process, forming fatty acid methyl esters (FAME) from vegetable oils, is widely used to produce BDF. This is, however, a high-cost process, considering the refinement of products or the removal of catalysts. To reduce the cost, some Japanese groups are developing non-catalytic alcoholysis reaction processes for production of FAME from vegetable oil. In this paper, Japanese policy on biomass energy and current state of BDF in Japanese society are described, and some research works on non-catalytic alcoholysis reaction for production of BDF in Japan are introduced.

5.2. JAPANESE POLICY ON BIOMASS ENERGY

Petroleum, coal and natural gas are globally limited, and they will be exhausted in the future. On the other hand, biomass resources are renewable and can be produced continuously by photosynthesis. Therefore, biomass resources can be utilized as renewable energy without disturbing carbon balance of ecosystem on the earth. The amount of biomass is estimated about 830 billion tons as carbon. It accounts for the total amount of petroleum, coal and natural gas, and 80 times as much as the amount of the energy consumed in a year in the world.

In the Kyoto Protocol at the Third Session of the Conference of the Parties to the United Nations Framework Convention on Climate Change (COP3), Japan decided to reduce the amount of discharge of CO_2 by and average of 6% between 2008 and 2012 compared to the amount in 1990. "Improvement in energy efficiency," "absorption by forest," and "promotion of utilization of new energy and renewable energy" are thought to be important measures to achieve the goal. That is, in order to reduce the amount of discharge of CO_2 and prevent global warming, the consumption of fossil resources must be reduced by utilizing biomass resources as energy and materials.

To promote utilization of biomass resources and contribute to the prevention of global warming, to create recycling society and to revitalize rural areas, a Japanese comprehensive strategy called "Biomass Nippon" was drawn up by six ministries including the Ministry of Agriculture, Forestry and Fisheries (MAFF), and endorsed by the Japanese Cabinet in December 2002. The strategy was revised in March 2006. In this strategy, utilization of BDF is pivotal.

The situation of biomass energy in Japanese society is different from that in western countries to some degree. In western countries, biomass energy is tied to the utilization of surplus agricultural products. On the other hand, in Japan, local utilization of organic waste is brought into focus, because of difficulty of intensive production of energy crops.

In Japan, the ratio of biomass energy to total energy is currently only 0.8%, a figure that needs to be increased.

5.3. CURRENT STATE OF BDF IN JAPAN

It is not feasible to use virgin vegetable oil as material for BDF in Japan because the country must import much of it. However, it might be possible to utilize waste edible oil as material for BDF.

In Japan, about 2,500,000 tons of edible oil is consumed in a year, and about 450,000 tons of waste edible oil is discarded from food factories, and as household garbage. About 200,000 tons is collected and utilized mainly as materials for soap and animal feed. Some of the collected waste edible oil is converted into FAME and used as BDF.

"Someya-shoten," a collection trader company in Tokyo, produces about 1500 L of FAME from waste edible oil each day through an alkaline catalyst method, and sells it at a price of 80 yen/L (about 67 cents/L). This company has been collecting 20–30 tons of waste edible oil daily and selling it as animal feed. By selling a part of the waste edible oil as BDF, the company could increase its profit by one-million yen per month (almost 10 thousand U.S. dollars/month).

In Kyoto city where the COP3 was held, the city government has been collecting waste edible oil from households and restaurants in the city to convert it into FAME since 1997. The city government produces FAME from the waste edible oil by use of an alkaline catalyst. The FAME is used as fuel for garbage trucks and buses. About 220 garbage trucks are using 100% FAME as their fuel. In the case of the city buses, B20 (which consists of 20% FAME and 80% ordinary diesel fuel) is used because 100% FAME does not give them enough power. About 80 city buses are running on B20. The amount of BDF currently used in Kyoto city is about 1500 kL/year. The total amount of BDF used in Japan each year is estimated at about 3000 kL, while about 46 million kL of diesel fuel is consumed.

5.4. PROBLEMS WHICH INHIBIT UTILIZATION OF BDF

5.4.1. The Tax System in Japan

In Japan, prefectural governments do not charge any tax on BDF which consists of 100% FAME. However, in the case of B20, prefectural governments charge the same tax as ordinary diesel fuel: 32 yen/L (about 27 cents/L). This tax system is one factor which inhibits wide spread of BDF in Japanese society.

5.4.2. Technical Problems Involved in Conventional Alkaline Catalyst Method

To promote utilization of BDF in Japan, not only the problem due to the tax system, but also some technical problems involved in conventional alkaline catalyst method need to be solved.

(a)

$$\begin{array}{l} CH_2OOCR_1 \\ | \\ CHOOCR_2 \\ | \\ CH_2OOCR_3 \end{array} + 3CH_3OH \rightleftharpoons \begin{array}{l} CH_3COOR_1 \\ CH_3COOR_2 \\ CH_3COOR_3 \end{array} + \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array}$$

Triglyceride Methanol Fatty Acid Methyl Esters Glycerol

(b)

$$TG + CH_3OH \rightleftharpoons DG + R_1COOCH_3$$

$$DG + CH_3OH \rightleftharpoons MG + R_2COOCH_3$$

$$MG + CH_3OH \rightleftharpoons GL + R_3COOCH_3$$

Figure 5.1. Alcoholysis of triglyceride with methanol. (a) overall reaction; (b) three consecutive and reversible reactions. TG: triglyceride; DG: diglyceride; MG: monoglyceride; GL: glycerol.

In an alcoholysis reaction for production of BDF from vegetable oil, triglyceride (the main component of vegetable oil) is reacted with methanol and fatty acid methyl ester (FAME) and glycerol are formed (Fig. 5.1), and the FAME is used as BDF. In a conventional process for production of BDF, alkaline catalysts such as NaOH and KOH are used to promote the reaction.

In a FAME production process with an alkaline catalyst method, the catalyst has to be removed from products after the reaction. Otherwise, the by-product glycerol cannot be utilized in other industries. However, the removal of the alkaline catalyst increases total cost for BDF production.

When waste edible oil is converted into FAME by use of an alkaline catalyst, free fatty acid has to be removed prior to the reaction to maintain the activity of the catalyst. This however reduces the yield of the process.

To solve the technical problems involved in the conventional alkaline catalyst method, some Japanese groups are working on the development of a non-catalytic alcoholysis reaction process for BDF production.

5.5. ADVANTAGES OF NON-CATALYTIC ALCOHOLYSIS REACTION FOR PRODUCTION OF BDF

A non-catalytic alcoholysis reaction process has some advantages over a conventional alkaline catalyzed reaction process for production of BDF.

In a system with non-catalytic alcoholysis reaction, the purification process to remove catalyst after reaction is not required. Therefore, the configuration of the total system can be simplified and the by-product, glycerol, can be directly utilized in other industries, reducing the total cost for production of the BDF.

In a system with a non-catalytic alcoholysis reaction, not only triglycerides but also free fatty acid might be converted into FAME. Therefore, neutralization to remove free fatty acid is not required prior to the reaction process, improving the yield of the total system.

5.6. RESEARCH ON NON-CATALYTIC ALCOHOLYSIS REACTION FOR PRODUCTION OF BDF IN JAPAN

5.6.1. Supercritical Methanol Method

Saka and Kusdiana (2001) at the University of Kyoto investigated methyl-esterification in supercritical methanol without using any catalyst. The experiment was carried out in a batch-wise reaction vessel preheated at 350 and 400 °C at a pressure of 45–65 MPa. In a preheating temperature of 350 °C, 240 s of supercritical treatment with methanol was sufficient to convert the rapeseed oil to FAME, and, although the prepared FAME was basically the same as those of common method with an alkaline catalyst, the yield of FAME obtained at 350 °C was found to be higher than that obtained at 400 °C. The supercritical methanol process required a shorter reaction time and a simpler purification procedure. In addition, by using the supercritical methanol method, FAME was produced not only from triglycerides but also from free fatty acids.

Then, they tried to combine the methyl-esterification reaction in supercritical methanol with the hydrolysis reaction in subcritical water (Kusdiana and Saka, 2004). In this process, triglycerides are first hydrolyzed into free fatty acids, which are then converted into FAME in supercritical methanol. They reported that content of monoglycerides in the product was reduced and the quality of the final product was improved with this two-step process.

5.6.2. Simultaneous Reaction of Transesterification and Cracking

Recently, Iijima *et al.* (2005) at the National Agricultural Research Center in Japan proposed a new method, called STING, based on the simultaneous reaction of transesterification and cracking. In this method, transesterification and cracking proceed simultaneously under a supercritical condition, and triglycerides, diglycerides, monoglycerides and FAME, which consist of medium chain fatty acid, and higher alcohols, lower alcohols and other hydrocarbons, are formed. These components make one phase and are used as a diesel fuel replacement. Therefore, no byproducts are formed in this process.

In a process with the STING method, no glycerol is formed as byproduct, improving the yield of the process.

The product has a lower viscosity and lower pour point (PP) compared with FAME formed by a conventional alkaline catalyzed method. Therefore, quality of the product is regarded to be higher.

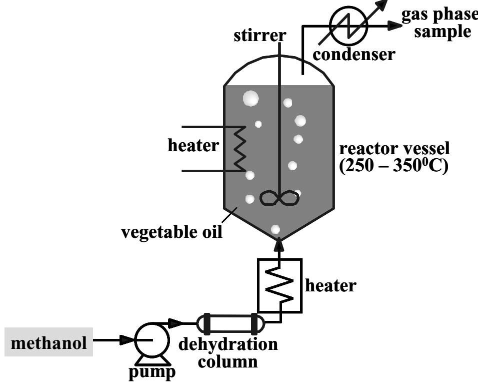

Figure 5.2. A schematic flow diagram of a bench scale reactor, based on the superheated methanol vapor bubble method.

5.6.3. Superheated Methanol Vapor Bubble Method

To further decrease the cost required for production of BDF, Yamazaki *et al.* proposed a superheated methanol vapor bubble method (2007).

Figure 5.2 shows a schematic flow diagram of a reactor based on the supercritical methanol vapor bubble method. In the reactor, superheated methanol vapor is blown into oil continuously, and reacted with triglyceride to form FAME. The formed FAME flows out from the reactor with unreacted methanol vapor and is collected by a condenser. No catalyst is used in this method.

Reaction with the superheated methanol vapor bubble method can be conducted under atmospheric pressure conditions. Therefore, both initial and running costs for the process can be reduced, and applicability of the superheated methanol vapor bubble method might be high.

Effects of reaction conditions on the efficiency of the reactor were investigated with a bench scale reactor: the outflow rate of fatty acid methyl esters from the reactor vessel reached the maximum at 290 °C.

In the case that waste edible oil is used as raw material, diglyceride, monoglyceride, and fatty acid might be contained in the raw material. Therefore, the effects of these impurities on the outflow rate of FAME from the reactor were also investigated. Not only triglycerides but also free fatty acids could be converted into fatty acid methyl esters, and the reaction rate constant obtained with free fatty acids was several times higher than that with triglycerides. These results imply that the superheated methanol vapor method is suitable for waste edible oil and crude vegetable oils rich in free fatty acids.

Based on the data obtained with the bench scale reactor, an industrial scale reactor which could produce 1000 kg of FAME in an hour was designed, and material balance and economical efficiency of the reactor were estimated (Ishikawa *et al.*, 2005). In the designed process (Fig. 5.3), 154 kg of heavy oil needs to be burned as heat source of the reactor, to produce 1000 kg of FAME.

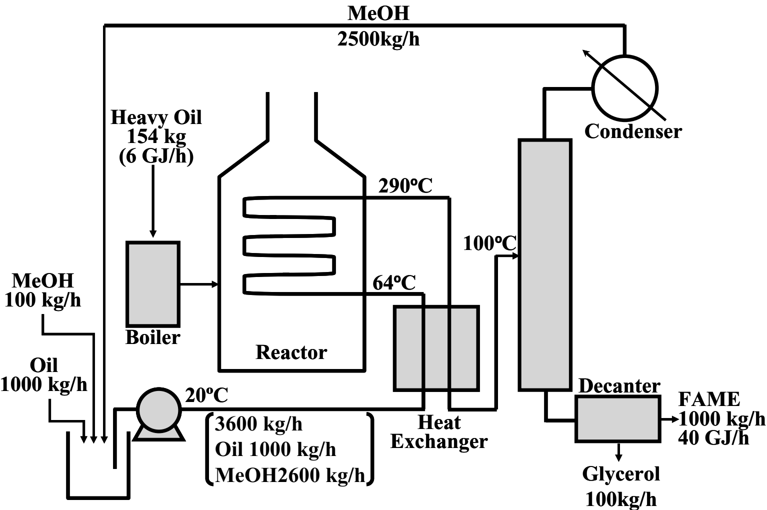

Figure 5.3. Flowchart of energy and materials of large scale non-catalytic reactor, based on the superheated methanol vapor bubble method.

Figure 5.4 Pilot-scale reactor, which can produce 40 L of fatty acid methyl ester based on the superheated methanol vapor bubble method.

Cost to produce 1 litter of FAME with the designed reactor was estimated to be about 40 yen (33 cents), a figure much lower than the cost required with a conventional alkaline catalyst method.

A pilot scale reactor which can produce about 40 L of fatty acid methyl ester in a day by use of superheated methanol vapor bubble method has been constructed (Fig. 5.4). The feasibility of the process will be demonstrated by using the pilot scale reactor.

5.7. CONCLUSIONS

The current state of research on the conversion of vegetable oil, especially waste edible oil, into BDF has been introduced. In Japan, about 46 million kL of diesel fuel is consumed in a year. The waste edible oil can support only about 1% of that total consumption, even if all the waste edible oil in Japan is utilized as materials for BDF. However, the technologies introduced in this paper can be applied for wide range of botanical resources such as waste effluent, or by-products from oil refining factories in the Southeast Asia which do not compete with resources for edible use.

Results from these research works should reduce the cost of production of BDF, contribute to the prevention of global warming, reduce the consumption of fossil resources, and revitalize Japanese rural areas.

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CHAPTER 6

Biodiesel from Acidulated Soapstock (Acid Oil)

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6.1. INTRODUCTION

Biodiesel as an alternative fuel has gained significant importance; increasing oil prices, the promise of independence from foreign oil imports, the Kyoto Protocol directive to reduce overall CO_2 emissions, as well as governmental incentive programs have all contributed. According to the American Soybean Association (2007), approximately 250 MM gallons of biodiesel was produced in the U. S. in 2006, constituting a significant increase over just a two-year period (Fig. 6.1). Overall production capacity in 2007 was estimated at 1.2 Bil gal/y (National Biodiesel Board, 2007).

A significant proportion of the overall U.S. biodiesel capacity is located in the Midwest. Major suppliers in the Midwest with at least 20 MM gal production are Minnesota Soybean Processors in Brewster, MN (30 MM), Peter Cremer in Cincinnati, OH (30MM), SoyMor in Glenville, MN (30 MM),

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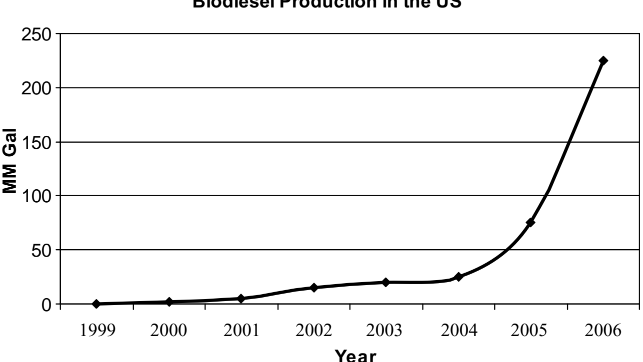

Figure 6.1. Biodiesel volumes. Source: www.soystats.com 2007.

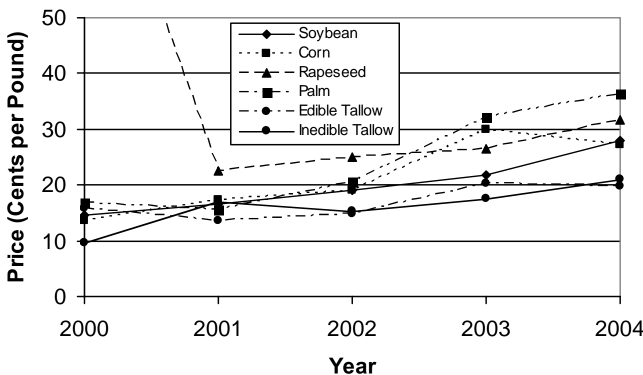

Figure 6.2. Prices for fats and oils. Source: *Chemical Economics Handbook* (SRI Consulting), November 2005.

Stepan Company in Joliet, IL (21 MM), and Western Iowa Energy in Wall Lake, IA (30 MM) (BBI International 2007). Although soybean oil still constitutes the bulk of biodiesel production, more manufacturers are either moving toward, or already have incorporated, multiple feedstock capability. Alternative feedstocks include beef tallow and other animal fats, yellow grease, and other waste oils (Kulkarni, 2006). A look at a recent price index for fats and oils (Fig. 6.2) shows that virgin oil prices, as well as those of animal fats, have steadily increased. This economic reality has manufacturers increasingly looking at opportunities to find alternative feedstocks—such as acidulated soapstock (acid oil)—which may require more processing but are also substantially cheaper than fully or partially refined oils and fats.

6.2. BIODIESEL PROCESS TECHNOLOGY OVERVIEW

The majority of manufacturing processes rely on base catalyzed transesterification of refined or partially refined oils whose free fatty acid (FFA) content is typically <0.1%, with methanol (Meher, 2006; Ma, 1999). The catalyst of choice is sodium methoxide as a solution in methanol: it contains little moisture, although it is more expensive than other alternatives. Sodium hydroxide and, more rarely, potassium hydroxide are also used, as are heterogeneous catalysts. The latter typically require much higher temperatures (approximately 170 °C to 250 °C) and pressures (up to 750 psi) in the presence of metal oxides such as a mixture of zinc oxide, aluminum oxide and zinc aluminates (Unilever, 1950; Stern *et al.*, 1999; Suppes *et al.*, 2004; Bournay and Baudot 2006; Di Serio *et al.*, 2006). Aside from the inherent advantages of using heterogeneous catalysts, these processes also claim highly pure (up to 99%) glycerin as a by-product. Currently, Axens is the only company to have commercialized a procedure, the Esterfip-H process, that is based on mixed metal oxides developed by the Institute Francais Du Petrol (Stern *et al.*, 1985).

For homogeneous base catalyzed processes, reaction conditions are generally at ambient or slightly higher pressure, and a temperature of 65 °C–70 °C, in the presence of approximately 0.5% catalyst, with a 6:1 molar ratio of methanol to oil (Freedman *et al.*, 1986). The process can be operated continuously (Noureddini *et al.*, 1998) or in batch mode. Examples of continuous industrial processes include: Ballestra, Connemann CD, and the Lurgi PSI process.

Reaction times for a typical alkali catalyzed batch transesterification are 60 min, and yields of 99% can be achieved. Batch operations may include two reaction and two settling stages during which glycerin is removed. Additional catalyst may be added to the second transesterification step to compensate for losses incurred during the first glycerin separation. Reaction times can be shortened considerably by intense mixing through high shear pumps (Lichtenberger, 2007). Such cavitation type processes are capable of achieving cycle times in the order of several minutes (Gogate *et al.*, 2006). Other process options include the use of co-solvents (Boocock, 1998; Mahajan, 2006) or enzymatic technology (Du *et al.*, 2003).

Feedstocks high in free fatty acid (>1%), however, are not easily converted by direct transesterification because of concurrent soap formation of the free fatty acids with the catalyst. Excessive amounts of soap significantly interfere with the washing process by forming emulsions, thus leading to substantial yield losses. Water in the feedstock also leads to soap formation through saponification (Freedman *et al.*, 1984; Canakci and Van Gerpen, 1999). One potential approach in these cases is to pre-esterify the fat/oil in the presence of an acid catalyst such as sulfuric acid or equivalent, and methanol, followed by base catalyzed transesterification (Scheme 6.1) (Lepper and Friesenhagen 1986; Jeromin *et al.*, 1987; Canakci and Van Gerpen, 2001).

$$\text{Triglyceride }(CO_2R)_3 + RCO_2H + MeOH \xrightarrow[\text{Acid Cat.}]{60-75^{\circ}C} RCO_2Me + H_2O + \text{Triglyceride }(CO_2R)_3$$

Neutralize Acid → Salt + H_2O

$$\xrightarrow[\text{Base Cat.}]{65^{\circ}C,\ MeOH} RCO_2Me + \text{Glycerol }(OH)_3$$

Scheme 6.1. The two-step process with pre-esterification.

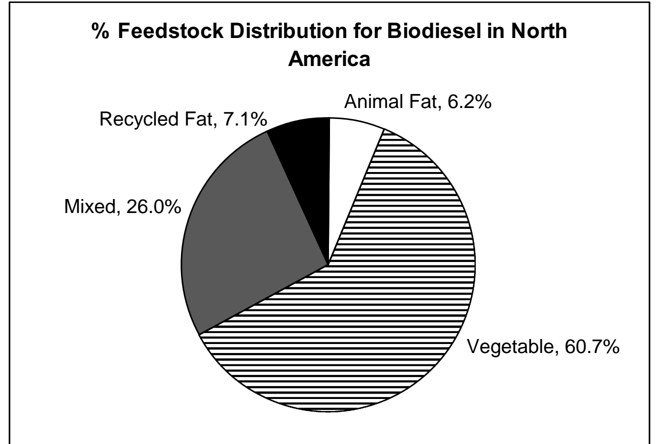

Figure 6.3. Types of feedstock for biodiesel production. Source: *Chemical Economics Handbook* (SRI Consulting), November 2006.

Although many manufacturers still rely on refined feedstocks from soybean oil or rapeseed (Fig. 6.3) for ease of processing and other advantages such as cold flow properties, the increasingly high cost of these feedstocks is not sustainable. Therefore, the trend to use alternative oils, animal fats and waste oils, in part or exclusively to lower production cost, can be found throughout the industry. Acidulated soapstock (acid oil) is just one example of a by-product or waste oil (Kulkarni, 2006) high in free fatty acids, which maybe useful in the production of biodiesel. The following discussion, however, will concentrate on technology using acid oil as a raw material.

6.3. ACIDULATED SOAPSTOCK AS BIODIESEL RAW MATERIAL

Approximately 80% of the cost to manufacture biodiesel consists of the raw material (Van Gerpen, 2005). Comparing the cost of "low quality" feedstocks

TABLE 6.1. Prices and availabilities of fats and ils.

Fat or oil	US volume (1000 metric tons)	Price ($/lb) on Aug 10, 2007
Yellow grease, Missouri River	620	0.200
Brown -gGrease Chicago	—	0.110
Other greases	562	—
Poultry gat, Delmarva	518	0.228
BFT, gelivered Chicago	—	0.285
Inedible tallow	1748	—
Acidulated soapstock	**130**	**0.225**
Crude degummed soybean oil, CBOT	—	0.372

containing varying concentrations of free fatty acid it becomes clear that these feedstocks present an economic opportunity (Table 6.1).

Acidulated soapstock is a raw material available from the alkali refining process of crude oils. Natural oils contain a number of impurities (free fatty acids, minerals, phospholipids and others) which are removed in the refining process and end up as soapstock, consisting mostly of water and soap with excess alkali. Soapstock is a pasty to semi-solid material which may require storage at temperatures above 60 °C in order to stay liquid (Woerfel, 1983). Haas has published a procedure to produce biodiesel directly from soapstock by the complete saponification of any residual glycerides and phosphoglycerides, followed by water removal and esterification of the acid salts in the presence of sulfuric acid and methanol at 35 °C and ambient pressure (Haas *et al.*, 2000). Alhough fatty acid conversions were nearly complete, a high methanol to fatty acid ratio of 30:1 was necessary. In addition, the high water content of soapstock makes this procedure less appealing for industry, as it leads to increased transportation cost as well as to additional cost for discharging the significant volumes of water. Haas subsequently published a procedure using greatly reduced methanol to fatty acid ratios, based on acidulated soapstock as feed (2003).

Most manufacturers use soapstock to spray on meal for animal feed, or ship the material to acidulators. Some seed oil producers treat soapstock on site with sulfuric acid at a temperature of 90–95 °C to produce acidulated soapstock (Dijkstra and Segers, 2007). Acidulated soapstock is very dark in color with a strong, rancid, burned odor from the free fatty acids and neutral oils. Free fatty acid content varies and can be in excess of 90%. Moisture content as well as unsaponifiables can be substantial and the pH (based on samples provided to Stepan Company) may vary from 3 to 4.5. An example of a typical analysis of an acid oil sample is listed below (Table 6.2).

Several approaches to the conversion of acid oil into biodiesel have been explored at Stepan and by many other researchers.. An overview of these studies is discussed below.

TABLE 6.2. Composition of exemplary cid oil.

Component	Weight percent
Moisture	2%
Impurities	0.5%
Unsaponifiables	2–3.5%
Free fatty acids	60–93%
Iodine value	118–128
Peroxide value	2.0 meq
Total fatty acids	91.5%

TABLE 6.3. Effectiveness of pretreatment of acid oil to lower phosphorous content.

Treatment of acid-oil at 80 °C	Phosphorous (ppm)	Phosphorous reduction (%)	Recovered acid-oil (%)
No treatment	704	0	n.a.
Paper-filter 20 μm	491	30.3	98
Celite filtration 1 wt% Loading	598	15.1	96
Centrifugation 3000 rpm, 5 m at 20 °C	488	30.7	93

6.4. BIODIESEL FROM ACID OIL USING PRE-TREATMENT

Since acid oil is a by-product of the oil refining process, the fatty acid distribution of acid oil may depend on how many different raw materials are processed at the crusher; mixtures of oils are often the result. Therefore, for the biodiesel manufacturer, raw material management requires care and flexibility when dealing with acidulated soapstock. Some acid oils may require storage conditions at temperatures of at least 65 °C to prevent sediment formation. Impurities such as phospholipids are likely to be present in acidulated soapstocks. Phosphorous compounds may present a challenge during water washing, as they tend to act as emulsifiers. Table 6.3 examines the effectiveness of several options to remove these compounds from a particular batch of untreated acid oil containing 704 ppm phosphorus (Scott and Furman, 1961). Simple filtration using a 20 micron paper filter or centrifugation at 80 °C were most successful at reducing the amount of phosphorous components. The concentrated solids from the centrifugation contained 0.54% phosphorous.

Pretreatments such as filtration can have a direct impact on the quality of the final ester product, as well as on yields. Table 6.4 shows the effect on product quality of the ester from acid oil, which had been filtered to remove solids and other components. The filtered acid oil was pre-esterified under reflux with methanol (12:1 ratio MeOH to FFA) in the presence of 0.56 wt% of 98% sulfuric acid as catalyst, based on the acid oil charge. The reaction was continued until the acid value was less than 5 mg KOH/g, followed by neutral-

TABLE 6.4. Product quality and yield of esters from pre-filtered and unfiltered acid oil.

Pretreatment	Overall yield [%]	Final AV [meq KOH/g]	Free glycerin [ppm]	Total glycerin [ppm]	Phosphorous [ppm]	Sediment-final-ester
Unfiltered	79	0.1	110	1190	14	Yes
Paper filtration	75	0.1	75	179	<10	None
Centrifugation	85	0.1	230	2144	<10	None
Celite-filtration	86	0.1	307	1302	<10	None

ization of the catalyst with sodium methoxide. The crude ester product was then subjected to base catalyzed transesterification with sodium methoxide under standard conditions of 65 °C and a 6:1 methanol to oil ratio (Freedman *et al.*, 1984). The final phosphorous content is only somewhat higher for the unfiltered material, suggesting that the majority of phosphorous components are removed during the process (Van Gerpen, 2005). Phosphorous levels for the filtered or centrifuged samples were measured at less than 10 ppm. In addition, the unfiltered acid oil sample was the only one to develop sediment upon standing, after conversion to methyl ester. This sediment development suggests that some form of pretreatment, such as filtration or centrifugation, may be necessary for an industrial process, depending on the quality of acid oil available. Overall yields were calculated based on Ester Product Out / Acid Oil In, including all the steps from filtration to pre-esterification and transesterification.

Paper filtered acid oil did not have the best overall yield, but showed significant differences during processing. Distinct splits during glycerin drops and water washes were observed, resulting in low free and total glycerin values for the final ester.

Pre-esterification with sulfuric acid as the catalyst presents some disadvantage in that it requires neutralization before proceeding with a base catalyzed transesterification. This step can be avoided by utilizing heterogeneous acids, such as resin sulfonic acids (Tesser *et al.*, 2005; Shibasaki-Kitakawa *et al.*, 2007; Santacesaria *et al.*, 2007). Acid oil was subjected to several resin catalysts, and their activity was compared (Figure 6.4) (Luxem *et al.*, 2006). All reactions were performed at 65 °C with 20 wt% resin on a dry basis and a MeOH to FFA ratio of 3.8:1, converting 87%–92% of the free fatty acid in about 3.5 hrs (except Dowex® C211, possibly due to residual moisture).

In summary, pre-esterification presents a viable strategy in dealing with high FFA feedstocks. Homogeneous acid catalyzed esterification of FFA proceeds readily and under mild conditions. A disadvantage of this approach is that the acid catalyst will have to be neutralized before proceeding to the transesterification step. In addition, methanol from the esterification step will have to be dried before it can be recycled. Heterogeneous catalysts also

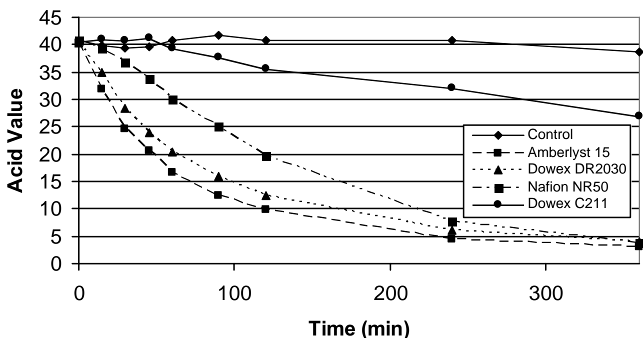

Figure 6.4. Acid value in acid oil as function of resin catalyst and time during esterification with methanol.

$$\text{Triglyceride (3} \times CO_2R) + RCO_2H + MeOH \xrightarrow[\text{H}_2\text{SO}_4,\ 200-500\ \text{psi}]{130-180^\circ\text{C}} RCO_2Me + H_2O + \text{Glycerol (3} \times OH)$$

Scheme 6.2. Reaction of FFA and neutral oils under pressure.

displayed sufficient activity for FFA conversion to esters, and they have the advantage of rendering the neutralization step unnecessary. Recently Omota reported the esterification of fatty acids by reactive distillation using sulfated zirconia as heterogeneous catalyst (Omota, 2003).

However, each approach will add, at least initially, to the capital expenditures of a plant due to the added complexity of the operation.

6.5. ACID CATALYZED CONVERSION OF FFA IN ACID OIL UNDER PRESSURE

Although acid catalyzed transesterifications exhibit slow kinetics (Ataya *et al.*, 2007), acid catalysts have the advantage of concurrently converting FFA to esters. The process can be performed under pressure in the presence of sulfuric acid and excess methanol (Luxem, 2004), in which case esterification of FFA and transesterification of free oils proceed simultaneously, though at different rates (Scheme 6.2).

To achieve this, soapstock was reacted under pressure with methanol and sulfuric acid as the catalyst at 130 °C–180 °C and up to 500 psi. Without the intermittent removal of the by-products water and glycerol, the reaction equilibrated at about 10 AN (acid number), or approximately an 82% conversion

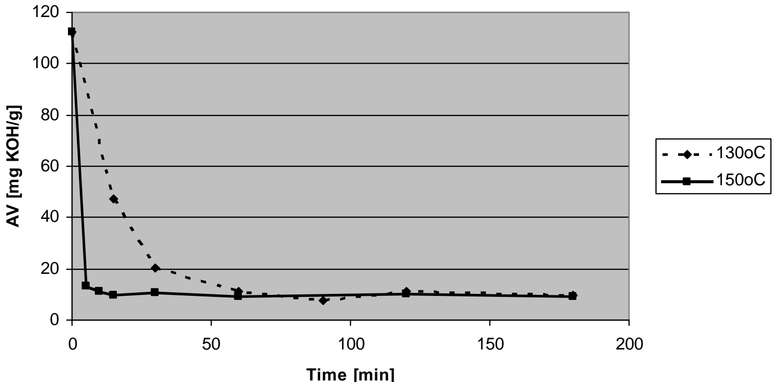

Figure 6.5. Sulfuric acid catalyzed esterification of FFA at 130 °C and 150 °C.

TABLE 6.5. Ester yields from acid oil during acid catalyzed reaction under pressure.

Temp [°C]	Reaction-time [hrs]	Acid oil [g]	MeOH [g]	H_2SO_4 98% [g]	AV Ester [mgKOH/g]	Yield [%] ester/-acid-oil
150	2.6	100.0	35.0	0.25	2.5	89.2
150	4.5	100.1	35.0	0.125	0.8	92.5
180	1.5	100.0	35.0	0.25	6.3	90.6

of acid, Ssimilar results were obtained by Eaves *et al.* (1959). Equilibrium was reached after 60 min at 130 °C, whereas the same conversion point was reached in 15 min at 150 °C (Figure 6.5). The overall reaction kinetics are such that a continuous type process would be possible to develop.

In batch mode, conversions of up to 92.5% were achieved under the above mentioned conditions (Table 6.5). More complete conversions for a continuous reactor design would require intermittent removal of excess methanol and water, possible by flashing the wet methanol. To simulate a continuous process with removal of water a batch experiment was subjected to three repeat cycles of flashing water and methanol, resulting in an acid value 0.5 mg KOH/g for the crude ester product.

Without catalyst, transesterification of rapeseed oil proceeds in supercritical (Demirbas, 2003) methanol between 350 °C and 400 °C and pressures of 45–65 MPa (6500–9400 psi) and in short reaction times of 240 seconds (Saka and Kusdiana, 2001; Kusdiana, 2004).

Though the obvious advantages of such a process is that a variety of feedstocks can be processed and a “one catalyst system” can be used, the

conditions also require specialized equipment due to the high pressures involved and would therefore likely increase the cost of such an approach.

6.6. CONVERSION OF FFA IN HIGH ACID FEEDS USING GLYCEROL

Using glycerin (Scheme 6.3) to convert fatty acids into mono-, di-, and triglycerides offers an additional route to treat high FFA containing oils (Luxem and Mirous, 2006). The reactions of glycerol or polyols with fatty acids are well documented in the literature, but severe reaction conditions of up to 250 °C are required to drive the reaction to completion (Sonntag, 1979). Therefore it would be advantageous to develop a catalyst system that would allow a lower operating temperature. Osman reports on the direct esterification of glycerin and fatty acids in the presence of various catalysts at temperatures between 180–200 °C with or without solvent present (Osman, 1968). Pouilloux and coworkers reported reactions of glycerin with oleic acids using anion exchange resins, such as Amberlyst® 31, with over 75% conversion of the acid at 90 °C, but requiring 24 hours of reaction time (Pouilloux *et al.*, 1999).

To achieve better conversions in less time, several common acid, base and transition metal catalysts were screened for the purpose of lowering the reaction temperature in the glycerin process. The results for each category of catalyst are represented in Figure 6.6. All reactions were performed with crude (neutralized) glycerin. The activity of the catalysts is compared based on a standard reaction time at 180 °C for 4 hours and normalized based on equal equivalents of metal content per moles of acid. The organo-metal catalysts, tetrabutyltitanate (TBT), dibutyltin oxide (DBTO), and tinoxalate (SnOx) were the most efficient catalysts. Overall, while tin compounds are effective catalysts, they can be problematic because of their potential toxicity, unless they can be removed from the product in a cost effective manner.

Broensted acids proved less effective as catalysts and scale-up considerably larger (~60%) amounts of distillate than expected were noted, consisting

CO_2R, CO_2R', CO_2R'' + R^1CO_2H + OH, OH, OH $\xrightarrow{220 - 230^oC}$ CO_2R, CO_2R', CO_2R'' + CO_2R^1, CO_2R^1, OH + CO_2R^1, OH, OH

80°C, MeOH, MeONa $\longrightarrow$ RCO_2Me + OH, OH, OH

Scheme 6.3. Biodiesel formation of FFA containing oils with glycerin.

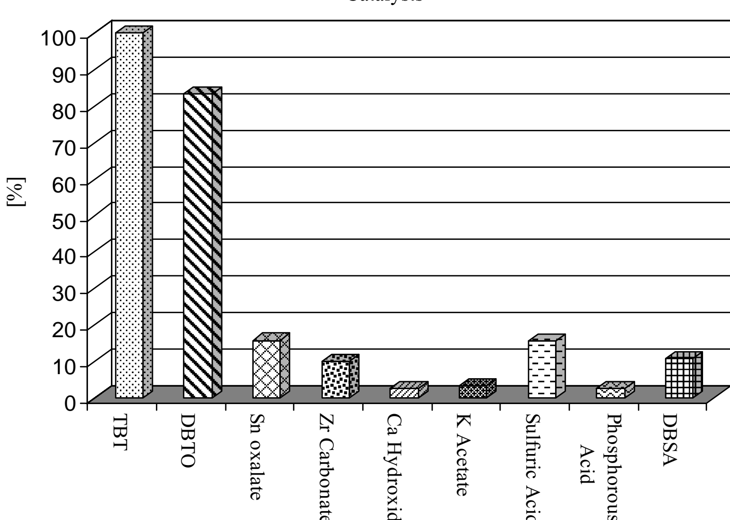

Figure 6.6. Catalyst screen for the glycerol esterifcation of acid oil.

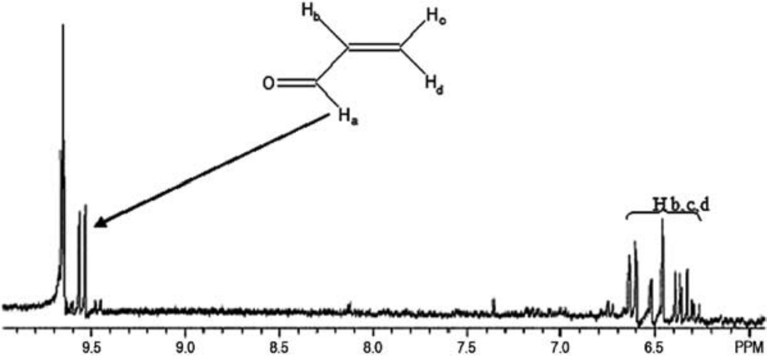

Figure 6.7. Acrolein in distillate from acid catalyzed reaction of glycerol and FFA in acid oil.

mainly of water and minor amounts of low boiling fatty acid. Upon further analysis not only additional water but also acrolein was present (Figure 6.7). The additional water can therefore be attributed to the dehydration of glycerin (formation of acrolein) and possibly etherification (formation of polyglycerides). Acrolein formation of particular concern on scale up because of potential health risks (Merck Index, 2006). The formation of acrolein as a by-product was also reported using anion exchange catalysts during direct esterifications of glycerol and fatty acids (Pouilloux *et al.*, 1999). This side reaction of glycerol is well known and can occur uncatalyzed at high temperatures (Sonntag, 1982).

TABLE 6.6. Conversion of FFA with glycerol at 180 °C.

Catalyst Type	Concentration [wt% / acid oil]	Conversion [%]
TBT	0.2	71.2
DBTO	0.2	81.2
Tin oxalate	1.0	92.5
Zirconium carbonate	2.0	79.4
Montmorrillonite K-10	2.0	70.8
Nafion SAC-13	2.0	74.1

The highest conversion of FFA achieved was 93% at 180 °C after 4 hours using tin oxalate at 1 wt%, whereas DBTO achieved 81% conversion at 0.2 wt% loading, without using solvents to azeotrope water from the reaction (Table 6.6).

Dibutyl tin oxide was ultimately chosen for a scale-up over the titanate catalyst, mainly because of handling issues and the high moisture content of the acid oil raw material. On an >100,000 lb scale, the acid conversion was nearly complete to an AV of 0.5 mg KOH/g, using recovered glycerin. However, cycle times were significantly longer than on the laboratory scale because of equipment limitations. The esterification product was then converted directly via base catalysis into biodiesel, which was subsequently distilled to give an ASTM spec product. The overall yield was 95% (lbs Ester Out / lbs Acid Oil in) before distillation. However, like all procedures described here, the final ester is highly colored and required clean-up, distillation in this case, which dropped the final recovery closer to 92%.

Only two heterogeneous catalysts were evaluated; Montmorillonite K-10 and SAC-13 (a Nafion catalyst), gave FFA conversions of 71% and 74% respectively after 4 hrs at 180 °C at a 2 wt% loading, but suffered from the same issue of acrolein formation as the homogeneous acids.

6.7. CONCLUSIONS

Several options to convert free fatty acids in acid oil and subsequently into biodiesel were presented. Though all described methods were ultimately successful in converting acid oil into biodiesel, it also became clear in our work, as well as that of other researchers, that cheaper feedstocks can come at a cost. Specifically, additional processing steps such as filtration, distillation, or heated storage tanks, as well as logistics around raw material variability can add considerable capital cost. Successful implementation of these high free fatty acid materials into a production facility depends very much on existing equipment, raw material receiving, and down stream processing capabilities, all of which are probably more available to larger manufacturers or highly integrated chemical plants. Additionally, feedstock prices are moving targets,

and at the time this work was performed (2001) acidulated soapstock was available for \$0.12–0.15/lb. In general, alternative feedstock prices have risen substantially, erasing some of the economic advantages. A decision to use lower grade raw materials will ultimately have to be weighed against the feedstock prices and the capital investment necessary to produce in-spec biodiesel in a reliable manner.

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CHAPTER 7

Industrial Products from Biodiesel Glycerol

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7.1. INTRODUCTION

Because of environmental concerns, "eco-friendly" products and processes are gaining momentum as potential substitutes in many existing applications. Currently, much of the impetus for these alternatives comes from innovative uses of agricultural products. For centuries agriculture has been an obvious source of food and shelter; however, more recently, with the emerging awareness of the non-renewable nature of petroleum oil and the adverse environmental impacts of its utilization, interest has focused on agriculture as a source of environmentally benign renewable resources to help sustain present and future energy requirements, particularly in the areas of automotive and home heating fuels.

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As the world's energy sources gradually gain momentum towards sustainability, triacylglycerols derived from animal fats and oilseed crops are being examined as possible precursors for the manufacture of inexpensive, renewable diesel and heating fuels. Biodiesel (defined as the fatty acid methyl or ethyl esters of animal fats and vegetable oils) is one renewable fuel whose use is advancing rapidly throughout the world. Biodiesel is an attractive alternative to petroleum diesel because it burns cleaner (with reduced emissions) and is sustainable through agricultural efforts. Biodiesel is synthesized by the chemical transesterification of triacylglycerols with any short chain alcohol (primarily methanol or ethanol), as shown in Scheme 7.1.

Recent figures indicate that the United States produced an average of 4.6 million metric tons of animal fat and grease and 10.4 million metric tons of vegetable oil annually between the years 2001 and 2003. These numbers continue to fluctuate slightly from year to year; however, recent trends confirm nominal increases in animal fat and vegetable oil production, making it progressively more important to provide additional outlets for these commodity materials. The large production numbers for fats and oils in connection with federal tax incentives, subsidies, state legislation, a petroleum diesel shortage, and increased environmental awareness all contribute to the rising demand for biodiesel. According to the National Biodiesel Board (NBB), U. S. biodiesel production in 2006 was estimated at 250 million gallons and, while this is a 230% increase from 2005, it represents only a fraction of the total capacity of the existing, operating plants with another 1.7 billion gallons of new biodiesel capacity planned over the next 18 months (Tullo, 2007). While the expectation is that biodiesel production and use will continue to climb, in reality, the rate of biodiesel growth will depend largely upon the price and availability of substrates (soybean oil and rapeseed oil are the preferred feedstocks in the

Triacylglycerol (100 lbs) + 3 ROH (10 lbs) —Alkali→ Fatty Acid Esters (100 lbs) + Glycerol (10 lbs)

Scheme 7.1. Base-catalyzed transesterification of triacylglycerols (TAGs) to produce fatty acid esters (biodiesel). Methyl esters (shown) are the most common but others, such as ethyl esters, can be produced depending on the alcohol used in the reaction. R_1, R_2 and R_3 represent unique fatty acids attached to the glycerol backbone of the TAG.

U. S. and Europe, respectively), petroleum diesel and methanol and may also be affected by the emphasis on food or fuel applications. Presently, rising soybean oil and declining diesel fuel prices have shaved about 25 cents per gallon off biodiesel profits and in actuality any fluctuation in the price of soybean oil, methanol, or petroleum diesel will affect the profit margins and ultimately the rate at which biodiesel can be produced economically. If oil prices rise to $65 per barrel again, biodiesel production could grow by another 250 million gallons, but if oil prices fall to $45 per barrel, companies may reconsider plans to enter the market (Tullo, 2007).

Additionally, the large amount of biodiesel projected to be produced may ultimately become self-limiting due to feedstock limitations. Another factor that needs consideration is the increased crude glycerol stream that results from biodiesel production. For every 100 pounds of biodiesel produced, 10 pounds of crude glycerol is generated, the disposal of which must be addressed to make large production capacities economically and environmentally sound. The absolute content of the crude glycerol streams may vary from reaction to reaction, but essentially all are composed of glycerol and alcohol (usually methanol) with small concentrations of free fatty acids (FFA), fatty acid alkyl esters, and tri-, di- and monoglycerides. The compositional differences that do occur in the crude glycerol stream are by and large the result of the point of biodiesel synthesis from which it was obtained (midstream *vs.* endstream), the efficacy of the transesterification process, and the recovery efficiency of the biodiesel. This glycerol surplus has caused some synthetic glycerol producers, such as Dow Chemical and Procter & Gamble, to amend their position in the glycerol market and in some instances has caused entire plants to be closed. In contrast, some companies are benefiting from increased glycerol markets by restructuring their operations to consume glycerol rather than produce it. For example, Solvay has so modified conventional protocols at their plant in Tavaux, France: glycerol is used in the production of epichlorohydrin, rather than using epichlorohydrin to produce glycerol (McCoy, 2006a). Archer Daniels Midland (ADM), Dow Chemical, Cargill, and Huntsman Corp. are currently exploring opportunities to use glycerol, rather than propylene oxide, as a precursor in the production of propylene glycol (McCoy, 2006b): in fact, Dow Chemical has recently announced its intention to open a plant in Houston to carry out this conversion and anticipates making limited commercial quantities available in mid-2007.

In our studies, we have found that the amount of glycerol in the biodiesel coproduct stream tends to vary between roughly 40% (crude glycerol obtained from a mid-process stream) and 80% (semi-refined glycerol obtained at the end of the process). In this paper, we discuss our work involving the use of both pure glycerol and biodiesel glycerol as fermentation substrates for the microbial synthesis of poly(hydroxyalkanoates) (bio-polyesters) and sophorolipids (glycolipid biosurfactants) and as chemical precursors for the synthesis of hyperbranched polymers and reactive intermediates.

7.2. BASIC GLYCEROL METABOLISM

For any compound to be used by microorganisms as a fermentation substrate, a mechanism must exist by which the compound can be incorporated into the organism's central metabolic systems for the production of energy as well as all key intermediates. This is no different for glycerol. Luckily, a large number of unrelated microorganisms possess means by which this can be accomplished.

In most instances intrinsic membrane proteins (IMPs) are used to control what enters and what leaves microbial cells. Some of the IMPs are involved in regulating the uptake of small molecules including water (to control the osmotic conditions of the cell) and other small uncharged molecules that can be used for survival. These IMPs can be classified into 3 distinct subgroups: *i)* homotetramer aquaporins (AQPs), each containing six transmembrane helices, are highly specific for water (Ren *et al.*, 2000; Murata *et al.*, 2000); *ii)* glycerol facilitator proteins (GlpFs), which have a close structural similarity to the AQPs but whose amino acid sequences in the channel-lining side chains at the narrowest points of the channel create two different environments that regulate channel selectivity (Froger *et al.*, 2001), and *iii)* aquaglyceroporins, such as AQP3, AQP7 and AQP9, proteins that allow both water and glycerol (with less affinity than GlpF) to pass the cytoplasmic membrane of the cell. Figure 7.1 illustrates the reactions necessary for bacterial glycerol assimilation. The first step is the transport of glycerol across the cell membrane into the cytoplasm of the cell. This is accomplished in *E. coli* by facilitated diffusion through GlpF proteins; however, there has been some question recently

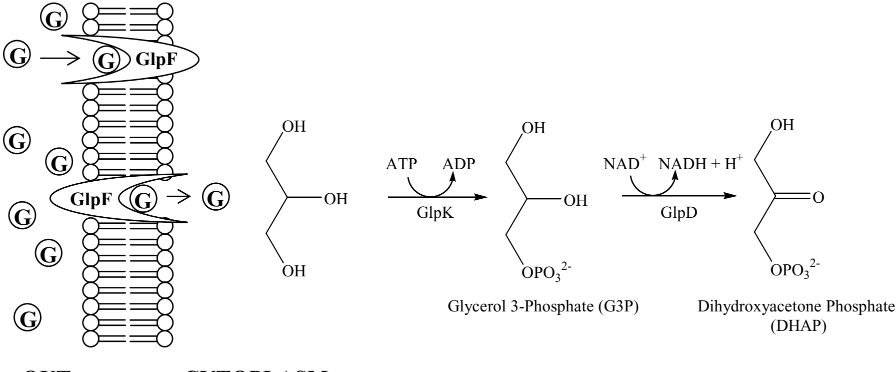

Figure 7.1. Mechanism of uptake and initial enzymatic reactions in the microbial metabolism of glycerol (G). *Note:* The gene products GlpF, GlpK, GlpD correspond to a glycerol facilitator protein, glycerol kinase, and glycerol 3-phosphate dehydrogenase, respectively.

whether *Pseudomonas* utilizes analogous systems for glycerol transport. Although data originally demonstrated an energy-dependent transport system in *Pseudomonas* (Siegel and Phibbs, 1979) subsequent experiments suggested that, although there is an 80% identity between the *glpF* genes of *E. coli* and *Pseudomonas*, glycerol may be transported in *Pseudomonas* by a high-affinity, binding protein-independent facilitated diffusion system (Williams *et al.*, 1994). Either way, as the glycerol passes the membrane it is sequentially acted upon by glycerol kinase (EC 2.7.1.30; the gene product of the *glpK* gene) and glycerol 3-phosphate (G3P) dehydrogenase (EC 1.1.99.5; the gene product of the *glpD* gene) both of which are closely associated with the cytoplasmic membrane (Williams *et al.*, 1994). The action of glycerol kinase, along with energy in the form of ATP, phosphorylates one of the primary hydroxy groups on the glycerol molecule to produce glycerol 3-phosphate (G3P), which is then oxidized through the action of an NAD-independent G3P dehydrogenase to form dihydroxyacetone phosphate (DHAP) (Heath and Gaudy, 1978), which is in turn isomerized to glyceraldehyde 3-phosphate (GA3P). Both DHAP and GA3P are intermediates in both the Embden-Meyerhof and Entner-Doudoroff metabolic pathways and thereby can be converted either to pyruvate (through a series of five well known enzymatic reactions), or to glucose, a key intermediate in sophorolipid biosynthesis.

7.3. POLY(HYDROXYALKANOATE) SYNTHESIS

Poly(hydroxyalkanoates) (PHAs) embody a complex class of naturally occurring bacterial polyesters that are synthesized as intracellular carbon and energy reserves. Numerous bacterial species have been studied for their capacity to synthesize PHA under suitable growth conditions. This has resulted in no less than 150 known structural variations of PHA polymers, each reliant upon the individual bacterial strain and the growth conditions utilized for production (Steinbuchel and Valentin, 1995). Because of their broad structural variations, each PHA is commonly classified into one of three categories. The first, the so-called short-chain-length (*scl*-) PHAs, are the most studied of all PHA polymers and generally include those polymers made up of β-hydroxy fatty acids containing 3–5 carbon atoms (*i.e.*, poly-3-hydroxybutyrate, PHB; or PHB-*co*-3-hydroxyvalerate, PHB/V) and behave as semicrystalline thermoplastics. PHB is the best understood of all the PHA polymers, but its side-chain methyl group (Fig. 7.2A) results in a polymer that is stiff, brittle and, with a degradation temperature only marginally higher than its melting temperature, difficult to process. These properties made it apparent that industrial application of *scl*-PHA would necessitate chemical adaptations for property enhancement. In 1981 Holmes and colleagues at Imperial Chemical Industries (ICI) developed a controlled-fermentation protocol to naturally synthesize copolymers by feeding an assortment of carbon substrates (Holmes *et al.*, 1981). The most promising copolymer produced from this technology was

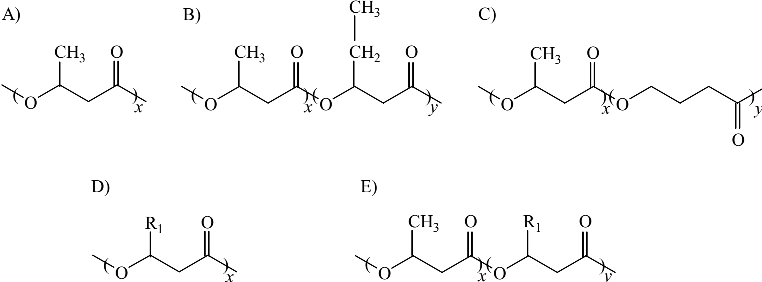

Figure 7.2. Chemical structures of poly-3-hydroxybutyrate (PHB; **A**), PHB-*co*-3-hydroxyvalerate (PHB/V; **B**), PHB-*co*-4-hydroxybutyrate (PHB/4HB; **C**), Medium-chain-length polyhydroxyalkanoate (*mcl*-PHA; **D**), NODAX (**E**). *Note:* The R_1 side chain is generally composed of a straight chain aliphatic hydrocarbon from 3 to 11 carbons in length (*mcl*-PHA) or 3, 5, 7 or 9 carbons in length (NODAX).

PHB/V, an *scl-* copolymer (Fig. 7.2B) produced by *Alcaligenes eutrophus* (since reclassified as *Ralstonia eutropha*) from propionic acid and glucose. This new polymer was tougher than PHB and had a lower processing temperature making it more appealing to industry. Ultimately, this polymer was marketed under the trade name Biopol . In 1988, Doi and coworkers found that a new *scl*-PHA copolymer of PHB-*co*-4-hydroxybutyrate (PHB/4HB; Fig. 7.2C) could be synthesized from 4-hydroxybutyric acid or γ-butyrolactone (Doi *et al.*, 1988; Kunioka *et al.*, 1989).

Alternatively, PHA polymers that are composed of either saturated or unsaturated β-hydroxy fatty acid monomer units ranging in length from C6 to C14 have been grouped together and make up the second category for PHA polymers (Fig. 7.2D). These so-called medium-chain-length (*mcl-*) PHA polymers, were first reported in 1983 by De Smet *et al.* who noticed that *Pseudomonas oleovorans* accumulated polyesters containing a 3-hydroxyoctanoate unit as the major component when grown on octane (De Smet *et al.*, 1983). Subsequently, it was determined that many species of *Pseudomonas* belonging to the rRNA homology group I also accumulate *mcl*-PHA polymers (Lageveen *et al.*, 1988; Huisman *et al.*, 1989; Brandl *et al.*, 1988; Gross *et al.*, 1989) and some of those *mcl*-PHA-producing bacterial strains can use triacylglycerols (Doi *et al.*, 1995; Ashby and Foglia, 1998), free fatty acids (Brandl *et al.*, 1988; Gross *et al.*, 1989; Casini *et al.*, 1997; Hazer *et al.*, 1998; Ashby *et al.*, 2002), and/or simple sugars (Huijberts *et al.*, 1992; Ashby *et al.*, 2001) to produce these polymers. These *mcl*-PHA polymers are amorphous and generally exhibit elastomeric properties depending on the specific side-chain length and the degree of unsaturation.

The third class of PHA involves those polymers that are composed of both *scl*-(generally ≥85 mol%) and *mcl*-(generally ≤15 mol%) monomers. These copolymers were developed by Procter & Gamble under the trade name

NODAX (Fig. 7.2E) and have been shown to possess the toughness associated with *scl*-PHA polymers along with the ductile properties of *mcl*-PHA (Noda *et al.*, 2005). Further, these copolymers exhibit reduced melting temperatures to facilitate processing. Since PHAs are biodegradable and biocompatible, they have been comprehensively studied as "environmentally benign" alternatives for petrochemical polymers principally in the areas of medicine, drug-delivery, agriculture, horticulture, the fibers industry, and consumer products.

In the late 1990s, Braunegg *et al.* reported that crude glycerol derived from the transesterification process of biodiesel production could be used as a substrate for PHB synthesis (Braunegg *et al.*, 1999; Koller *et al.*, 2005). Acetyl CoA is the required intermediate for the enzymatic synthesis of PHB and *mcl*-PHA from all substrates including glycerol. The activity of the pyruvate dehydrogenase complex, which is the sequential action of 3 distinct enzymes, catalyzes the synthesis of acetyl CoA from pyruvate. The acetyl CoA, in turn, can be used for PHA polymer synthesis. In our studies, we evaluated six separate strains of *Pseudomonas*, all known to harbor the genes associated with PHA production (Solaiman *et al.*, 2000; Solaiman, 2002), for their ability to synthesize PHA from glycerol. We determined that four of the six strains studied were able to grow on pure glycerol, and of those four, only two (*Pseudomonas oleovorans* NRRL B-14682 and *Pseudomonas corrugata* 388) produced PHA polymers (Table 7.1). Interestingly, *P. oleovorans* and *P. corrugata* synthesized PHB and *mcl*-PHA from glycerol, respectively (Ashby *et al.*, 2004). These results corroborated fermentation as a potentially new application for glycerol in the production of value-added polymers. The crude glycerol used in our studies was derived from a midstream source of soybean oil-based biodiesel production and, once the residual methanol was removed, was composed of 40% glycerol, 34% hexane-solubles (made-up of 92% fatty acids, either in the free acid or methyl ester form, and 6% mono- and diacylglycerols), and 26% water.

By comparison, both pure glycerol and the crude glycerol stream were used as substrates for the production of PHB and *mcl*-PHA from *P. oleovorans* and *P. corrugata*, respectively. Figure 7.3 shows the comparative cell productivities of both polymers (as a function of dry cell weight) from pure and crude glycerol. It is evident that pure glycerol has a widely different effect on the polymer productivities of each organism. *P. oleovorans* was more tolerant of the growth conditions created as the concentration of pure glycerol increased in the media. When the crude glycerol stream was used as substrate, with its free fatty acid (FFA) / fatty acid methyl ester (FAME) fraction, the PHB productivity exhibited the same trends (albeit at values that were 42 ± 9% less) as with pure glycerol. The major difference in polymer productivity using crude glycerol was seen with *P. corrugata*, whose *mcl*-PHA productivities continued to increase up to 3% media content and remained elevated through 5%. In addition to the osmotic effects brought about by increasing glycerol media concentrations, it was thought that the utilization preference for

TABLE 7.1. Bacterial screening for growth, PHA production and polymer composition from glycerol.

				3-Hydroxymethyl esters (mol%)									
Bacterial strain	Cell growth[a] / PHA synthesis[b]	PHA type	Glycerol source	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$	$C_{14:3}$
P. corrugata 388	+/+	*mcl*-PHA	Pure	—	1	10	49	11	28	1	Tr[d]	—	—
			Crude[c]	1	4	39	26	4	3	1	5	15	3
P. oleovorans NRRL B-14682	+/+	PHB	Pure	100	—	—	—	—	—	—	—	—	—
			Crude[c]	100	—	—	—	—	—	—	—	—	—
P. oleovorans NRRL B-14683	+/–	n/a	Pure	n/a									
P. oleovorans NRRL B-778	–/–	n/a	Pure	n/a									
P. putida KT2442	+/–	n/a	Pure	n/a									
P. resinovorans NRRL B-2649	–/–	n/a	Pure	n/a									

[a]Cell growth was determined to be positive upon an increase in the absorbance at 600 nm after 48 h incubation time.
[b]Polymer synthesis was determined to be positive if PHA could be isolated from the bacterial strain after extraction in CHCl3 and reprecipitation into cold methanol.
[c]Crude glycerol was derived from a biodiesel coproduct stream consisting of 40% glycerol, 34% FFA/FAME, and 26% water.
[b]Tr = less than 0.5 mol%.

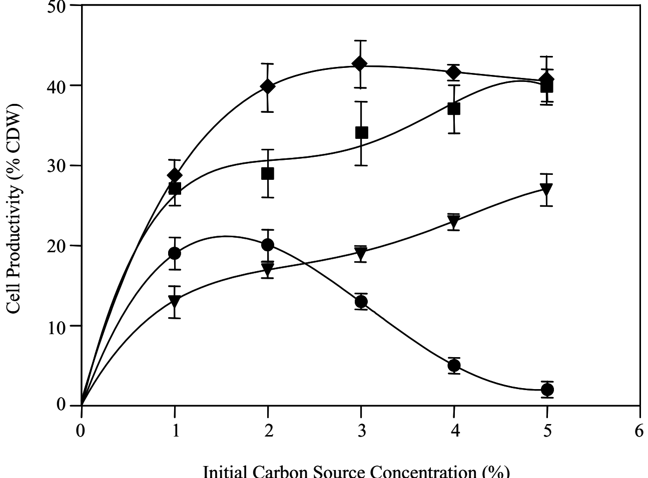

Figure 7.3. PHA cellular productivity of *P. oleovorans* NRRL B-14682 and *P. corrugata* 388 grown on increasing concentrations of pure glycerol and crude glycerol. *P. oleovorans*: pure glycerol (■), crude glycerol (▼); *P. corrugata*: pure glycerol (●), crude glycerol (◆). *Note:* Crude glycerol was obtained from a biodiesel coproduct stream that contained 40% glycerol, 34% FFA/FAME, and 26% water.

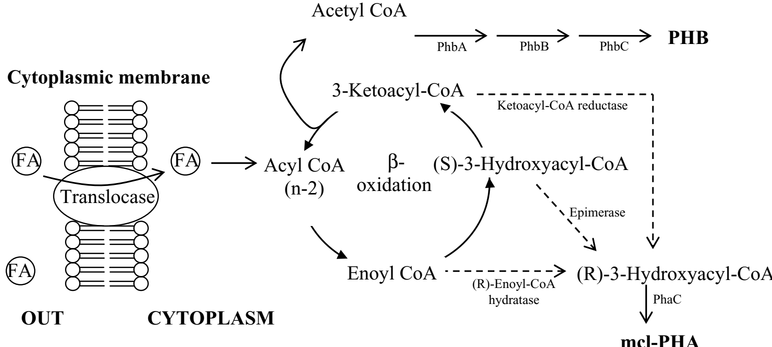

Figure 7.4. Transport of fatty acids (FA) and metabolic reactions in β-oxidation for the formation of acetyl CoA and (R)-3-hydroxyacyl CoA, key intermediates for polyhydroxyalkanoate biosynthesis. *Note:* The gene products PhbA, PhbB, PhbC, and PhaC correspond to the enzymes 3-ketothiolase, acetoacetyl CoA reductase, PHB synthase, and PHA synthase, respectively.

glycerol vs. the FFA/FAME fraction of the crude glycerol stream also played an important role in the polymer productivities (Fig. 7.4).

To comprehend the mechanics behind the synthesis of both PHB and *mcl*-PHA from both bacterial strains, we monitored the utilization of the individual components (glycerol and FFA/FAME) that made up the crude glycerol

TABLE 7.2. Substrate utilization by *P. oleovorans* NRRL B-14682 and *P. corrugata* 388 when grown on crude glycerol.[a]

Crude glycerol conc. (wt%)	Substrate available (g)		Substrate utilized (%)	
	Glycerol	FFA / FAME fraction	Glycerol	FFA / FAME fraction
P. oleovorans NRRL B-14682:				
1%	2.3	1.5	96	40
2%	4.3	3.2	49	16
3%	6.3	5.0	40	10
4%	8.2	6.7	23	4
5%	10.2	8.4	17	0
P. corrugata 388:				
1%	2.3	1.5	78	87
2%	4.3	3.2	44	50
3%	6.3	5.0	19	28
4%	8.2	6.7	15	13
5%	10.2	8.4	9	11

[a]The crude glycerol used was composed of 40% glycerol, 34% FFA/FAME, and 26% water.

stream (Table 7.2). *P. oleovorans* favored glycerol to FFA and FAME for growth and polymer production. The synthesis of acetyl CoA from glycerol is more energetically favorable than its generation from FFA. The following chemical equation demonstrates the net requirements and summed co-products generated as glycerol is converted to acetyl CoA through substrate level phosphorylation:

$$\text{Glycerol} + 3\text{NAD}^+ + \text{ADP} + \text{CoA} \rightarrow \text{Acetyl CoA} + 3\text{NADH} + 3\text{H}^+ + \text{ATP} + \text{CO}_2 + \text{H}_2\text{O} \qquad \text{[Eq. 7.1]}$$

The generation of 3 NADH molecules in this procedure translates into a total of 9 ATP molecules after oxidative phosphorylation. These 9 ATP when added to the ATP formed during substrate level phosphorylation yields a total net gain of 10 ATP molecules per acetyl CoA molecule produced from glycerol. In contrast, one round of fatty acid β-oxidation shows a net gain of only 4 ATP molecules per acetyl CoA formed. Initiation of β-oxidation requires the input of one ATP molecule to activate the FFA. Once activated, a single round of β-oxidation results in the formation of 1 molecule of $FADH_2$ and 1 molecule of NADH which, after oxidative phosphorylation, results in a net increase of 4 ATP molecules. The preference for glycerol over FFA and FAME by *P. oleovorans* was confirmed by comparing the composition of the FFA/FAME fraction before and after bacterial growth. It was established that the FFA/FAME content was in close approximation in both the starting crude glycerol sample and the isolated FFA/FAME fraction after bacterial growth thus effec-

tively demonstrating the minimal utilization of FFA/FAME when glycerol was available.

In contrast, *Pseudomonas corrugata* used glycerol and FFA/FAME at roughly the same rate (Table 7.2). Because *P. corrugata* naturally synthesizes *mcl*-PHA, it was thought that glycerol could be used by the organism as a growth substrate while the FFA or FAME could be used as a source of 3-hydroxyacyl-CoA precursors through β-oxidation. Analysis by GC/MS (see Ashby *et al.* (2004) for protocol) of the culture supernatant following bacterial growth revealed that, unlike *P. oleovorans*, the FFA/FAME content was statistically different from the starting material. The increased variability between the initial and the post-fermentative FFA/FAME content supports the contention that the FFA/FAME fraction of the crude glycerol stream was utilized more efficiently by *P. corrugata*, than by *P. oleovorans*, both for growth and synthesis of precursors for polymer formation.

Analysis of the polymers produced by *P. oleovorans* and *P. corrugata* revealed the synthesis of PHB and *mcl*-PHA, respectively (Table 7.1). The major components (those present in concentrations greater than 10 mol%) of the *mcl*-PHA from *P. corrugata* grown on pure glycerol consisted of the following: 3-hydroxyoctanoic acid ($C_{8:0}$), 10 mol%; 3-hydroxydecanoic acid ($C_{10:0}$), 49 mol%; 3-hydroxydodecanoic acid ($C_{12:0}$), 11 mol%; and 3-hydroxydodecenoic acid ($C_{12:1}$), 28 mol%. In contrast, when grown on the crude glycerol stream, containing the FFA/FAME components, *P. corrugata* synthesized *mcl*-PHA polymers whose major components consisted of 3-hydroxyoctanoic acid ($C_{8:0}$), 39 mol%; 3-hydroxydecanoic acid ($C_{10:0}$), 26 mol%; and 3-hydroxytetradecadienoic acid ($C_{14:2}$), 15 mol%. These variations in polymer content supported the concept that *P. corrugata* uses the FFA/FAME portion of the crude glycerol stream to synthesize at least some of the *mcl*-PHA monomers. The large concentration of unsaturated side-chains (primarily in the form of $C_{12:1}$ and $C_{14:2}$) provides additional possibilities for property control. Specifically, the olefinic groups contained in the side chains can be modified through crosslinking by both chemical (peroxides, sulfur vulcanization, epoxidation and aging) (Gagnon *et al.*, 1994a, 1994b; Ashby *et al.*, 2000) and physical (radiation) means (de Koning *et al.*, 1994; Ashby *et al.*, 1998a, 1998b) to alter the polymer properties.

The molar masses of the PHA polymers also exhibited dissimilar trends when the organisms were grown with crude glycerol (Ashby *et al.*, 2004). In the presence of pure glycerol, the molar masses of both the PHB and *mcl*-PHA polymers decreased as the glycerol concentration increased. The literature documents a number of instances in which PHA molar mass has been controlled through the use of media additives such as polyethylene glycol (Ashby *et al.*, 1997, 1999) and glycerol (Madden *et al.*, 1999; Ashby *et al.*, 2005a). The decline in the PHB molar mass was determined to be the result of glycerol-based chain termination. Studies showed that chain termination was, in fact, the result of the covalent linking of glycerol to the propagating chain causing premature termination. The statistical likelihood of early chain termination

rises as the media concentration of glycerol increases. This causes a sustained decrease in polymer molar mass. Furthermore, it was determined that the preference for glycerol-based termination was through the primary hydroxy groups of the glycerol. In a statistically random event, the expectation is that 66% of the chains would terminate via the primary hydroxy groups and 33% would terminate through the secondary hydroxy group of the glycerol. In reality, 99% of the glycerol-terminated chains were linked through the primary hydroxy groups thus demonstrating an enzymatic preference for primary over secondary hydroxy groups. It is thought that the same mechanism holds for the *mcl*-PHA molar mass decrease however, this has not been definitively deduced owing to the complexity of the NMR spectra for *mcl*-PHA polymers. With crude glycerol the *mcl*-PHA derived from *P. corrugata* was initially smaller ($M_n \cong 109{,}000$ g/mol) than the polymer derived from pure glycerol and the molar mass remained constant regardless of the initial crude glycerol media concentration. In contrast, the molar mass of the PHB produced by *P. oleovorans* grown on 1% crude glycerol had a number average molar mass (M_n) of 656,000 g/mol which decreased as the starting crude glycerol concentration increased. These results further confirmed the preference for glycerol by *P. oleovorans* in the production of PHB, while *P. corrugata* used the FFA/FAME stream for *mcl*-PHA synthesis. While maximum PHA polymer production has yet to be realized with glycerol, the results of our work provide a new potential outlet for the crude glycerol stream for biopolymer production without the need for costly refining or disposal.

7.4. SOPHOROLIPID SYNTHESIS

Surfactant producers are finding it more and more difficult to increase revenue due to the high costs of petroleum and customers who are unwilling to compensate by paying higher prices. This quandary has made some of the leading surfactant producers reorganize their surfactant business as a means of limiting losses. Even the manufacturers of oleochemical surfactants have been vulnerable to increasing cost of feedstock as more and more vegetable oil goes to biodiesel production. This has created increased opportunities for expansion of the "green" surfactant markets through the use of glycerol itself as a starting material for surfactant production. By using glycerol as a starting material, rather than creating it as a byproduct, surfactant producers can offer a supplementary outlet for glycerol (thus helping to maintain its value), as a means of reducing their reliance on petroleum-based raw materials, all the while creating an environmentally friendly product.

Sophorolipids (SLs) are extracellular glycolipids that are normally synthesized from glucose and a fatty acid source (either FFA or triacylglycerol) by a number of yeasts (primarily *Candida bombicola*). They are composed of a disaccharide (sophorose; 2-O-β-D-glucopyranosyl-β-D glucopyranose) attached to a hydroxy fatty acyl moiety at the ω-1 or ω carbon (Fig. 7.5). Typi-

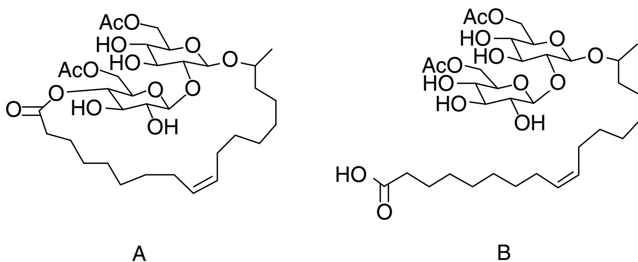

Figure 7.5. Sophorolipid (17-L-(∴2′-*O*-β-glucopyranosyl-β-D-glucopyranosyl]-oxy)-9-cis-octadecenoic acid 1′,4″-lactone 6′,6″-diacetate (**A**) and its free-acid form (**B**).

cally, the 6′ and 6″ hydroxy groups of the sophorose sugar are acetylated and the fatty acid chain varies in length from 16 and 18 carbons and may be saturated or unsaturated (Asmer *et al.*, 1988). In addition, the carboxylic acid group of the fatty acid may be lactonized (preferred) to the disaccharide ring at carbon 4″ or remain in the open-chain form.

The amphiphilic nature of SLs imparts surfactant-like properties to these molecules and has permitted their use as additives in personal care products (Mager *et al.*, 1987), detergents (Hall *et al.*, 1995) and in the lubricant industry. These compounds are non-toxic, biodegradable, and are produced in large quantities, thus making them an attractive target for glycerol utilization. In addition, their distinct configuration has stimulated interest in their utilization as precursors for the production of specialty chemicals such as sophorose, hydroxy-substituted fatty acids and fatty amines (Rau *et al.*, 2001; Zerkowski and Solaiman, 2007). Modification of the SL structure can alter the physical and chemical properties of SLs, such as critical micelle concentration (CMC), and surface active properties. Many strategies have been employed to adjust SL properties including changing the lipidic carbon source in order to alter the SL fatty acid content (Nuñez *et al.*, 2001), using chemo-enzymatic reactions to produce molecules such as glucose lipids (Rau *et al.*, 1999) and SLs with fatty acid chains of varying functionality (Bisht *et al.*, 1999; Carr and Bisht, 2003), and synthesizing amino acid/SL conjugates to improve water solubility and provide additional sites for further chemical modifications (Zerkowski *et al.*, 2006).

One of the economic strategies for the production of any new chemical includes the use of low-cost raw materials in order to improve profit margins. The substitution of crude glycerol (with its accompanying fatty acid component) in place of glucose and pure free fatty acid as the substrate material should result in reduced production costs for SLs and provide a supplementary outlet for crude glycerol, thus eliminating isolation and refinement costs for high purity applications. Our study demonstrated that *C. bombicola* grows and produces SLs from both pure glycerol (at approximately 10 g SL/L of media) and crude glycerol (at approximately 60 g SL/L of media). The difference in productivity was at least partially due to the lack of a fatty acid source and

the fermentation conditions brought about by using pure glycerol. Pure glycerol, when added to the fermentation in the same concentration as crude glycerol, creates an elevated osmotic stress in the media that retards cell growth and SL synthesis. In our study, because of the relatively large FFA/FAME content associated with the crude glycerol, the 10% (w/v) concentration used (typical for most SL fermentations) translated into approximately 4% (w/v) glycerol (when using the 40% crude glycerol stream) compared to 10% (w/v) in the pure glycerol fermentations. This large discrepancy is enough to affect the productivity of the culture. Cell yields from the pure glycerol fermentations were commonly 80% less than for the crude glycerol fermentations. This indicated that higher glycerol concentrations negatively effect the growth of *C. bombicola*. In the pure glycerol experiments, a lack of an exogenous fatty acid source requires that the organism have the ability to synthesize fatty acids from glycerol for survival. However, the energy-intensive nature of fatty acid biosynthesis does not permit the accumulation of large concentrations of fatty acids for use as membrane components and SL biosynthesis. Conversely, one-third of the crude glycerol stream was made up of a hexane-soluble fraction comprised of FFAs and/or FAMEs. When grown on this material, *C. bombicola* can use the glycerol fraction for cellular growth and assimilation to glucose (Fig. 7.6) for sophorose synthesis while, at the same time, using the FFA / FAME fraction as a source for the fatty acid side-chains thus allowing larger synthetic capabilities for SLs from crude glycerol.

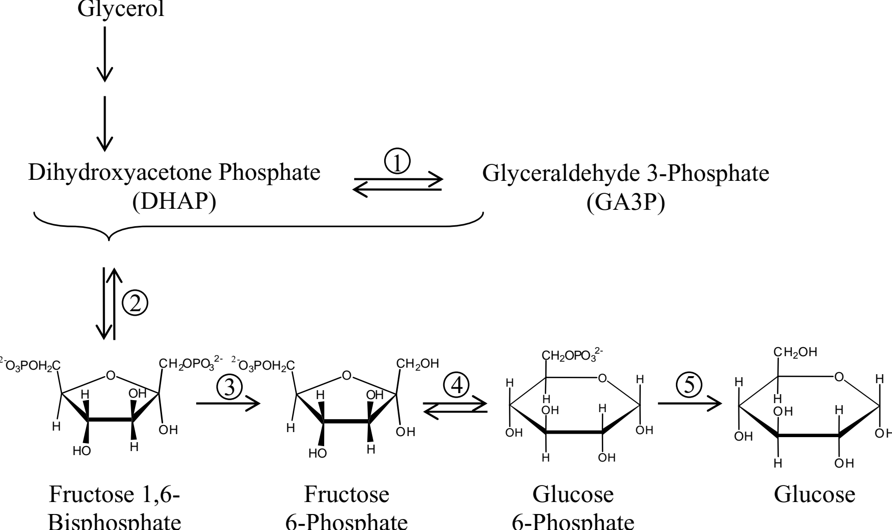

Figure 7.6. The metabolic reactions involved in the conversion of glycerol to glucose, the required precursor in the formation of sophorose. *Note:* Reaction 1 catalyzed by triose phosphate isomerase. Reaction 2 catalyzed by aldolase. Reaction 3 catalyzed by fructose 1,6-bisphosphatase. Reaction 4 catalyzed by phosphoglucose isomerase., Reaction 6 catalyzed by glucose 6-phosphatase.

TABLE 7.3. Fermentation of glycerol (pure vs. crude) to sophorolipids by *C. bombicola* and the effect on predominant structural form.

Feedstock	Product (mol %)[a]		
	FFA	FAME	Lactone
Glucose / Oleic acid	1	—	99
Pure glycerol	1	—	99
Crude glycerol[b]	24	36	41

[a]Conformational forms were determined by LC/MS according to the procedure outlined in reference (Nuñez *et al.*, 2001).
[b]Crude glycerol was composed of 40% glycerol, 34% FFA/FAME, and 26% water.

It was thought that the presence of a methyl group on the carboxyl end of the fatty acid would thwart lactonization and result in a larger concentration of open-chain SLs, thereby facilitating property control and permitting post-synthetic modification of the fatty acid side-chain without the need to break chemically the lactone ring. The structural content in the SL products was determined by liquid chromatography/mass spectrometry (LC/MS) with atmospheric pressure chemical ionization (APCI) (Ashby *et al.*, 2005b). The data proved that the make up of the SLs from pure and crude glycerol was indeed dissimilar (Table 7.3). When the yeast was grown with pure glycerol, LC/MS analysis revealed the formation of the SL lactone forms with the majority of the fatty acids being either palmitic, stearic or oleic as the main products (99%) with negligible amounts of non-lactonized SL. In contrast, the use of crude glycerol resulted in SLs that favored the open-chain form, accounting for 60% (24% free acid and 36% methyl esters) of the total SL content with oleic acid and linoleic acid predominating.

7.5. GLYCEROL AS A CHEMICAL INTERMEDIATE (HYPERBRANCHED POLYMERS AND REACTIVE INTERMEDIATES)

Another focal point for glycerol utilization has been in the synthesis of condensation polymers by esterification of glycerol with difunctional comonomers such as aliphatic dicarboxylic acids. In the past, monomers such as glycidol (Sunder *et al.*, 1999), which is a highly reactive epoxide, or cis-1,3-O-benzylideneglycerol (Carnahan and Grinstaff, 2001) were used as the glycerol-

protected monomer precursor. Until recently, glycerol itself was rarely considered as a polymer precursor, but because of its availability and trifunctionality, glycerol is now drawing more interest as a propagating unit in polymerization reactions. This section will focus on chemical- and enzyme-catalyzed reactions to produce hyperbranched oligomers and other potentially useful glycerol-based synthons in the synthesis of biodegradable polymers from glycerol.

Hyperbranched polymers are randomly branched macromolecules that can be synthesized by chemical or enzymatic condensation reactions. Hyperbranched polymers are related to the family of macromolecules known as dendrimers. Whereas dendrimeric polymers are highly branched, monodisperse molecules meticulously produced by multi-step syntheses, hyperbranched polymers are randomly branched molecules prepared by simple one-step reactions. Recent research has aimed to synthesize glycerol-based oligomers in reaction sequences that can be further optimized to produce hyperbranched polymers of significantly higher molecular weight (Wyatt *et al.*, 2006). The unique physical and chemical properties that often result from the highly branched architecture of polymers of this type make them suitable for numerous industrial and biomedical applications (Hong *et al.*, 2000; Malmstrom *et al.*, 1995). An assortment of hyperbranched polymers, including polyesters (Hawker and Frechet, 1990; Hawker *et al.*, 1991; Kim, 1992), polyphenylenes (Kim and Webster, 1990), polyamides (Spindler and Frechet, 1993), and polyurethanes (Kumar and Ramakrishnan, 1996; Miller *et al.*, 1992, 1993), can be produced by varying the monomer units to yield desired synthetic targets with tailored structure-property relationships. Flory performed the earliest studies on hyperbranched polymers, in which he described the critical gel-point for products produced from trifunctional AB_2 monomers (Flory, 1952). Since that time, condensation reactions involving di-functional A_2 monomers, such as diacids, with tri-functional B_3 monomers, such as glycerol, have been used in the synthesis of branched polymers instead of AB_2 monomers, which are more expensive and have limited availability (Lin and Long, 2003; Stumbe and Bruchmann, 2004; Kulshrestha *et al.*, 2005; Jikei and Kakimoto, 2001; Hao *et al.*, 2003). However, kinetic calculations show that the first condensation reaction, which produces an AB_2 species, is faster than subsequent polymer propagation; thus, the remainder of the reaction still progresses as polycondensations between AB_2- type species prior to the gel point. Gelation is often a problem in the synthesis of hyperbranched polymers. To avoid gelation in $A_2 + B_3$ systems, several synthetic strategies, including performing the reactions in dilute solutions (Fang *et al.*, 2000) or performing reactions in the absence of solvent while monitoring the viscosity of the system (Stumbe and Bruchmann 2004), have been employed. Others have shown that gelation, branching, and molecular weight of the polymers can be controlled in lipase-catalyzed, bulk polycondensations (Kulshrestha *et al.*, 2005).

The potential for using free glycerol to replace monomers commonly used to produce glycerol propagating units in hyperbranched oligomers has been

demonstrated by reacting four structurally and chemically different diacids with glycerol in the presence of an acid catalyst (Wyatt *et al.*, 2006). Additionally, hyperbranched polyester synthesis can be performed using lipase catalysis at mild temperatures (Kulshrestha *et al.*, 2005; Kumar *et al.*, 2003). This allows the advantage of being able to include thermally sensitive co-monomers as feasible alternatives in the synthesis of novel glycerol-based hyperbranched structures. Such glycerol-based polymers are expected to render new surgical materials for orthopedic and ophthalmic applications, reconstructive surgery, and drug delivery agents (Frazza and Schmitt, 1971; Vert and Li, 1992; Shalaby and Johnson, 1994). These biocompatible polymers also have potential uses as cosmetics, food additives, surfactants, lubricants, and azeotrophic phase separators (Seiler *et al.*, 2003).

The hyperbranched oligomers and polymers that result from the copolymerization of glycerol with diacids of varying carbon chain length, molecular structure, and molecular composition can be successfully synthesized under reaction conditions that prevent gelation (Wyatt *et al.*, 2006; Stumbe and Bruchmann, 2004). To optimize the reactions, several conditions must be considered including reaction time, catalyst type, purification techniques, and the solvent used. Due to the uniqueness of each oligomeric product, purification and characterization of the oligomers must be adjusted according to their solubility differences. Products produced from acid-catalyzed reactions in the absence of solvent were obtained on average at 62% yield after purification (Wyatt *et al.*, 2006). On the other hand, equivalent reactions conducted in dilute solutions gave polymer yields on average of 84%. Therefore, it is assumed that lowering the viscosity of the mixture allows for a more complete reaction by increasing the rate of interaction between reactants and the propagating chain. As expected, the water solubility of the oligomers decreased with increasing chain length of the diacid monomer used in preparing each oligomer. The iminodiacetic acid-glycerol oligomers (Fig. 7.7a) were insoluble in non-polar solvents such as hexane and chloroform but were very soluble in water and polar solvents such as methanol and dimethylformamide (DMF). Hydrogen bonding from the hetero-nitrogen atom in the iminodiacetic acid-glycerol oligomers likely contributes to their increased solubility in polar solvents. The oligomers synthesized with azelaic acid, succinic acid, or glutaric acids (Fig. 7.7b) were typically soluble in polar solvents such as acetonitrile and methanol but insoluble in non-polar solvents such as toluene. The succinic acid-glycerol oligomers were only slightly soluble in water while azelaic acid-glycerol and glutaric acid-glycerol derivatives were not soluble in water.

The acid catalyzed oligomers were also synthesized in dilute solutions of dimethylsulfoxide (DMSO) and DMF. DMSO proved to be a less attractive solvent due to its odor and the difficulty of removing it from the products. Additionally, products that were typically clear and colorful when produced in DMF emerged as dark, tar-like materials when synthesized in DMSO. When comparing products of neat reactions to complementary reactions performed in dilute solutions, a 2.5 fold increase in molecular weight and degree

Figure 7.7. Chemical structures of iminodiacetic acid—glycerol prepolymers (A), and diacid—glycerol prepolymers (B). *Note:* $n = 2$ for succinic acid, $n = 3$ for glutaric acid, and $n = 7$ for azelaic acid.

of branching was observed for the latter. The polymer polydispersity was low (average ≈ 1.26) for all acid-catalyzed reactions.

The M_n and M_w values for succinic acid-glycerol oligomers produced in dilute solutions in the presence of an acid catalyst were determined to be 2118 and 2255 daltons, respectively, by gel permeation chromatography (GPC) while the M_n and M_w values of azelaic acid-glycerol oligomers synthesized under the same conditions were 3245 and 5975 daltons, respectively. Kumar *et al.* reported M_n and M_w values for enzyme catalyzed adipic acid-glycerol oligomers determined by size-exclusion chromatography in tetrahydrofuran (THF) to be 2500 and 3700 daltons, respectively (Kumar *et al.*, 2003). Therefore, neither catalyst system, whether enzyme-catalyzed in bulk or acid-catalyzed in DMF, shows a net advantage over the other. The size of each monomer varies directly with the molecular weight of its corresponding oligomer; therefore, each product should have similar degrees of polymerization regardless of catalyst type. However, Kulshrestha described another simple one-pot enzymatic synthesis to prepare both linear and hyperbranched copolymers containing glycerol (Kulshrestha *et al.*, 2005). Terpolymerizations of adipic acid (A_2), 1,8-octanediol (B_2), and glycerol (B_3) were performed without the addition of solvent, which resulted in products with an average molecular weight of 75600 daltons.

NMR spectroscopy supported the presence of branched structures in the glycerol-based oligomers as observed by repeating downfield ^{1}H resonances in their NMR spectra (Wyatt *et al.*, 2006; Kulshrestha *et al.*, 2005; Kumar *et al.*, 2003) and the molecular weight patterns determined from the results of MALDI-TOF analysis. While all reaction conditions have not been evaluated,

neat reactions catalyzed with dibutyltin oxide show a degree of branching (approximately 55%) that is higher than the degree of branching (approximately 25%) that is typical of diacid-glycerol oligomers catalyzed by titanium butoxide (Wyatt *et al.*, 2006).

7.6. CONCLUSIONS

As the interest in biodiesel continues to increase, glycerol is becoming more and more of a concern as the production capacity of glycerol far exceeds the markets. In fact, in 2006 there was an overcapacity of approximately 500,000 metric tons of glycerol in Europe alone based on the growth of the biodiesel industry. This has resulted in a glut of crude glycerol that is expected to worsen as the country continues to distance itself from petroleum products and move towards more renewable industries, especially as they relate to alternative fuels. However, just as the generation of large quantities of glycerol is a tremendous concern, it also presents a great opportunity to develop new uses for glycerol in the production of value-added products. Of course, the possibility exists that the glycerol could be recovered, purified and sold. However, this practice is costly, time-consuming and, with the levels of biodiesel expected, probably would not be a viable or cost-effective option. We have shown that glycerol can be used as a carbon substrate in the microbial synthesis of both PHA polymers and SLs and can be used as a starting material for the chemical- and enzymatic-synthesis of hyperbranched oligomers for biodegradable polymer synthesis.

When glycerol was used as a fermentation substrate, it was evident that pure glycerol and crude glycerol resulted in widely different products. This substrate-dependent variation is advantageous to both PHA and SL synthesis. For example, the presence of FFAs and FAMEs (as is the case with crude glycerol from biodiesel production) resulted in an *mcl*-PHA polymer with increased olefinic functionality, which had an affect on the molar masses of the polymers, and may be beneficial as these sites are modified to improve polymer properties. Sophorolipid synthesis was also affected by the specific glycerol used in the fermentations. Pure glycerol resulted in SLs that were almost entirely in the lactone conformation, while crude glycerol stimulated SL production and induced the formation of SLs in the open form. The high concentrations of open form SLs benefit subsequent post-synthetic modification of the fatty acid side-chain without the need for ring opening reactions that may inadvertently affect the structure (such as the degree of acetylation) and function of the molecule. This helps lower the overall processing costs and alleviate undesired alteration of molecular structure of SL.

Even though low-cost renewable agricultural feedstocks and coproducts can help lower the costs of production of microbial bioproducts, there are technical issues that need to be addressed prior to adoption of the technology on a large scale. One such issue is the variability that exists from one synthetic process to the next. These variations may result in coproduct streams whose

content varies from process to process resulting in non-uniform glycerol streams. Continual research is thus needed to devise innovative bioprocesses to accommodate these crude coproduct streams. The definitive conclusion would be a biobased industry that could utilize agricultural coproduct streams as feedstocks and/or precursors for the manufacture of biomaterials benefiting consumers and the environment.

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CHAPTER 8

Development of New Products from Biodiesel Glycerin

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8.1. INTRODUCTION

Glycerin or glycerol is a polyhydroxy compound that contains one hydroxyl group on each of the three carbons comprising this small organic compound. Glycerine is water soluble, but commonly occurs in biological molecules such as triglycerides or phospholipids which exhibit very limited solubility in aqueous systems. During transesterification of vegetable oils for biodiesel production this solubility behavior can be exploited to separate glycerin from the mixture of alkyl ester products. Glycerin is the principle by-product of the conventional base catalyzed process for biodiesel production and represents approximately 10 wt% of the product stream (Fig. 8.1). Prior to the increase in biodiesel production that occurred over the past decade as a result of the continued interest in renewable fuels, the market demand for glycerin was relatively balanced with the supply. At that time estimates of 5% growth in the glycerin market were predicted. Glycerine was obtained as a by-product of processing vegetable oils and animal fats into soaps, fatty acids, and (non-

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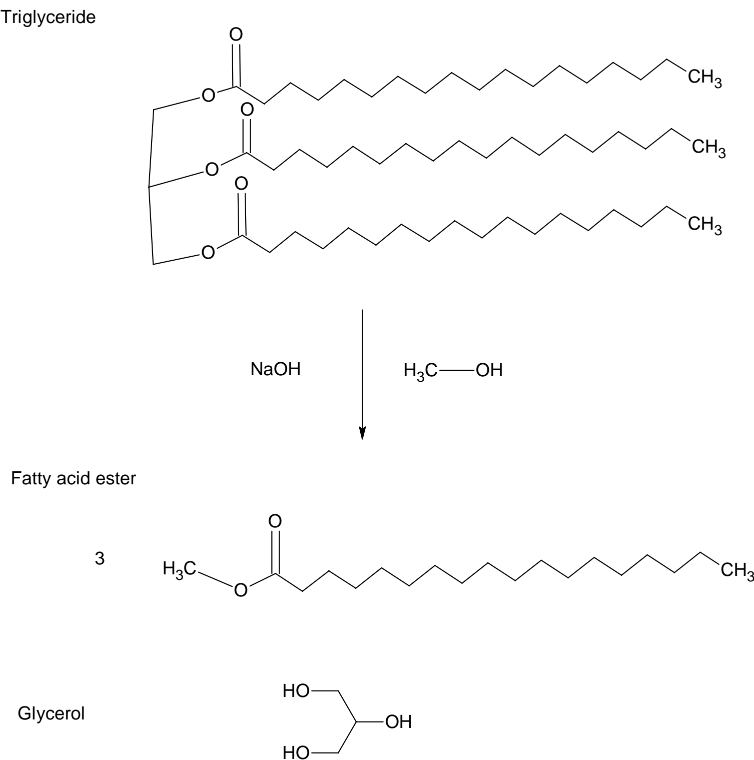

Figure 8.1. Formation of co-product glycerin (glycerol) during transesterification of vegetable oil.

fuel) esters. However, the emergence of the biodiesel industry and the expanding markets for alternative diesel fuels has generated a surplus of glycerin. Due to the additional production of alkyl esters as a biofuel the amount of glycerin available has significantly exceeded the market demand. This has spawned numerous efforts to find applications for this surplus with many investigations focused on identifying new markets and developing products that utilize glycerin.

The glycerin entering the market as a result of biodiesel production is of variable quality depending on the particular facility, what process stream it was taken from, and whether any purification or refining steps were performed. Many small biodiesel producers do not install the additional equipment necessary to produce a USP grade product from the crude glycerin co-product stream. This is based on an economic analysis of the current or projected market value of the purified product versus the cost to purchase, install, and operate a distillation unit that would be used for this purpose. Due to the current surplus of glycerin, newer facilities choose not to install a refining section. This is a rational business decision since such equipment is not necessary to produce biodiesel, the primary marketable product. However,

the possibility exists to retrofit a biodiesel production facility with distillation equipment if the market conditions for glycerin become more favorable in the future.

Production of biodiesel by the standard alkaline catalyzed process uses a molar ratio of 6:1 alcohol:triglyceride (Freedman *et al.*, 1984; Schwab *et al.*, 1987; Fukuda *et al.*, 2001). Process economics indicates that as much of this excess alcohol as possible should be recovered from the product stream for use in subsequent production runs. While the esters (biodiesel) and the glycerol separate relatively easily into two liquid phases due to solubility and density differences, the excess alcohol partitions across both phases. The alcohol is much more volatile than either of the reaction products and can be separated with evaporators or vacuum distillation. Upstream of the evaporator the glycerin phase may be nearly 50% methanol. After evaporation of methanol from the lower glycerin phase the composition of the crude glycerin co-product stream is greater than 85% glycerol. This crude glycerin stream can be purified as discussed above by distillation to yield higher grade products. However, the current surplus of glycerin has depressed the market value of the higher grade glycerin products significantly while the crude glycerin struggles to find a market.

8.2. REACTIONS

Glycerol is a unique polyhydroxy compound because it contains one hydroxyl group per carbon atom in a small three carbon molecule. This results in a relatively large degree of functionality in the molecule, with sites for derivatization at every carbon. However, these hydroxyl groups are not equivalent and therefore react differently.

Generally, the hydroxyl group is chemically stable, but with the appropriate catalyst and reaction conditions can condense with an acid or alcohol to form esters and ethers, respectively (Babayan and McIntyre, 1971; McIntyre, 1979; Wright and DuPuis, 1946; Roach and Wittcoff, 1949; Wittcoff *et al.*, 1949). The ether may be formed within the molecule, as an epoxide, which increases the reactivity of the site (Fig. 8.2). Numerous oxidation products may also be obtained from glycerol (Miyake and Shibamoto, 1995). This includes chemicals such as acrylic acid, glyceraldehyde, hydroxypropionaldehyde, and propionic acid. Of historical interest, the oxidation of glycerol was investigated by Fenton using the iron/peroxide system to produce many structures not previously synthesized in the laboratory. At reducing conditions glycerol yields products of industrial interest such as the 1, 3-propanediol, propylene, and

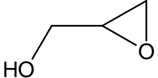

Figure 8.2. Structure of an epoxide.

Figure 8.3. Formation of triacetin from glycerol and acetic acid.

propylene glycol (Lahr and Shanks, 2003; Wang *et al.*, 2003). The following sections discuss reactions involving glycerol that are of current interest in the development of new biobased products.

The formation of acetin or glycerol triacetate is an example of the reaction of glycerol with acetic acid to yield an ester (Fig. 8.3). In this case both reactants are miscible with each other and the reaction is promoted by heating with an acid catalyst. The reaction is reversible, however, the equilibrium may be shifted toward esterification by removing the water of reaction as it is generated. If the co-reactant acid also has multiple groups then the possibility of forming polymers exists. When the co-reactant is a diacid the product may be predominately linear with bond formation through the primary hydroxyl groups. Additional reaction may occur with the involvement of the secondary hydroxyl group of glycerol and lead to a network polymer, or the secondary hydroxyl may be derivatized with another pendant group. The properties of the resulting materials can be controlled through the development of the structure. These materials find applications in the paints and coatings industries, as this chemistry is the foundation of alkyd resins. However, the ester reaction is reversible and the bond that is formed between the alcohol group of one reactant and the acid group of another is susceptible to cleavage. Generally, strong acid or base conditions are required to hydrolyze these ester linkages. Polyester materials prepared from glycerol and an organic diacid exhibit a high degree of biodegradability provided that the amount of branching or the number of pendant groups is not excessive. Such materials have agricultural and biomedical applications where the degradation of the products may be controlled and are either innocuous or even metabolized as a carbon source.

Ethers can be prepared by the reaction of an alkoxide with an alkyl halide. Both of these reactants are synthesized from alcohols so that ethers may be considered to be produced indirectly from the respective alcohols. Due to the number of hydroxyl groups attached to glycerol, the possibility of generating polymers exists. The formation of polyglycerol, the polyether of glycerol, is of commercial interest and finds applications in surfactants when the number of

glycerol units is less than ten. Polyglycerol sulfates are also interesting bioactive compounds. The ether linkage is very stable and requires strongly acidic conditions to cleave.

The formation of epoxides, the intramolecular ether, is useful in synthesis where it is desired to activate a molecule for further reactions. For example, the double bonds of an olefin are an excellent site for the introduction of an epoxide group. The energy stored in the three-member ring of an epoxide leads to higher reactivity than would be observed with an olefin. This technique is used to advantage with oleochemical substrates.

8.3. POLYMER MATERIALS

The development of biobased products is part of the current initiative to support the sustainable agriculture movement and to replace petrochemical substrates with renewable feedstocks. These activities provide the incentive to convert or incorporate agricultural biomass, co-products, and similar low-value materials into more valuable materials that benefit both growers and consumers. Glycerin obtained from the production of biodiesel presents a versatile substrate for the development of new products, and complements the biobased initiative.

Because of the high functionality of glycerol it is not surprising that a significant amount of research has focused on new polymeric materials that incorporate glycerol (Fig. 8.4). This includes polyesters, polyethers, and polycarbonates (Ray and Grinstaff, 2003; Fu *et al.*, 2003). In addition to the linear and network polymers, dendritic or hyperbranched structures have been investigated extensively (Malmstrom *et al.*, 1995; Hawker *et al.*, 1997; Jayaraman and Frechet, 1998; Bosman *et al.*, 1999; Sunder *et al.*, 1999, 2000;

HO OH OH OH OH O O O OH

polyglycerol (n=4)

HO OH O O O O O OH O O O O O OH OH

polyester (glycerol oxalate)

Figure 8.4. Glycerol based polymers.

Carnahan and Grinstaff, 2001a). These materials are designed to be biocompatible and biodegradable which leads to numerous biomedical applications such as controlled release matrices, tissue scaffolds, and carriers for drug delivery systems (Haag *et al.*, 2000; Carnahan and Grinstaff, 2001b).

8.4. SURFACTANTS

Glycerol is a component of natural products, such as phospholipids and monoglycerides, that exhibit surfactant properties. The classical surfactant consists of a hydrophilic group attached to a lipophilic moiety. This provides the molecule with the ability to solubilize both aqueous and nonaqueous phases. Glycerol presents a nonionic head group that may be derivatized with fatty acid groups. If two fatty acid moieties are attached to the primary hydroxyl groups of glycerol, an interesting class of surfactants is formed. Additionally, if glycerol is replaced by a short chain of polyglycerols the hydrophilicity of the resulting compound can be altered. The hydrophilic/lipophilic balance may range from 4 to 13 depending on the number of glycerol units incorporated (McIntyre, 1979). This type of structure allows for further derivatization through the secondary hydroxyls of each glycerol unit. In this manner the polyglycerol segment can serve as a replacement for the ethylene oxide units common to some commercial surfactants. A significant benefit of this approach is in replacing a petrochemically derived material such as ethylene oxide or propylene oxide with a renewable material, glycerol. This promotes the use of biobased products where all components of the product are derived from renewable materials. It should be noted that the preparation of polyglycerol may be catalyzed with acid or base conditions and has the potential to generate acrolein, a toxic compound, if precautions are not taken. Generally, the reaction is followed by the change in viscosity of the mixture. The applications for these materials are numerous in both edible and industrial formulations and include viscosity modifiers, crystallization inhibitors, and emulsifying agents.

Glycerol may be derivatized with a variety of compounds to prepare anionic, cationic, or nonionic types of surfactants. This allows numerous compounds to be designed for diverse applications while incorporating glycerol as a key component. This extends the versatility of glycerol in the synthesis of surfactants and demonstrates the general utility of glycerol as a renewable synthon.

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CHAPTER 9

Optimization of Lipase-Catalyzed Biodiesel by Statistical Approach

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9.1. INTRODUCTION

Biodiesel of fatty acid methyl esters (FAME) has become more attractive recently because, being made from renewable resources, it has environmental benefits. There are four chemical approachs to synthesize biodiesel: direct use and blending, microemulsions, thermal cracking (pyrolysis), and transesterification (Haas *et al.*, 2002). At present, commercial biodiesel is made by alkali-catalyzed transesterification of an oil or fat with an alcohol, usually methanol, in a process that shifts the glyceride fatty acids from glycerol to methanol, producing FAME and glycerol. In contrast to chemical synthesis, biocatalysts (lipases) allow for the synthesis of specific alky esters, the easy recovery of glycerol, and the transesterification of glycerides with high free fatty acid (FFA) content (Nelson *et al.*, 1996). Therefore, enzymatic biodiesels by lipase-catalyzed chemical reactions under mild conditions have garnered much current commercial interest recently. An optimized enzymatic biodiesel improves the conversion yield and reduces the cost of production under the most favorable conditions, benefitting manufacturers and appealing to consumers. This paper discusses a systematic approach, the Response Surface Methodology (RSM), to improving the conversion yield of biodiesel. This is followed by a case study that illustrates the application of RSM.

9.2. ENZYMATIC BIODIESEL

Many studies have been carried out on biodiesel production, using different oils as the raw material, different alcohols (methanol, ethanol, and butanol), and different catalysts (Marchetti *et al.*, 2007). However, chemical-catalyzed transesterification of waste cooking oil has problems centering around the pretreatment of feedstock, recovery of glycerol, removal of the catalyst, and the energy-intensive nature of the process (Wu *et al.*, 1999; Kulkarni and Dalai, 2006). But they also have advantages over traditional chemical-catalyzed reactions: no by-products are generated, the product is easily recovered, the reaction conditions are mild,, and catalyst can be recycled. Enzymatic reactions are insensitive to FFA and water content in waste cooking oil (Wu *et al.*, 1999; Hsu *et al.*, 2004; Kulkarni and Dalai, 2006).

Fukuda *et al.* (2001) reported enzymatic transesterification using lipase has become more attractive for biodiesel production, since the glycerol produced as a by-product can easily be recovered and the purification of FAME is simple to accomplish. Lipases shown in Table 9.1 can effectively catalyze the transesterification of triglycerides and the problems mentioned above can be circumvented by using the enzyme.

Shimada and Bornscheuer (2002) developed two stepwise methanolysis reaction systems with immobilized *Candida antarctica* lipase. System one, the first-step reaction, was conducted in the presence of 1/3 molar equivalent of methanol for the stoichiometric amount, and the second-step reaction was performed by adding 2/3 molar equivalent of methanol. In system two, the

TABLE 9.1. Comparison between commercial chemical-catalysis and lipase-catalysis methods for biodiesel production.

	Chemical-catalysis	Lipase-catalysis
Raw materials used	Previous purification	No effect
Temperature	High	Mild
Reaction time	Longer	Shorter
Operation process	Complex	Simple
By-products amounts	More	Less
Cost	Expensive	Cheaper (in continuously process)
Environmental impact	Acute	Minimum

first-step substrates were waste oil and 1/3 molar equivalent of methanol; in the second-step, the first-step eluate and 1/3 molar equivalent of methanol; in the third-step, the second-step eluate and 1/3 molar equivalent of methanol. The conversion of waste oil to biodiesel was greater than 90% in the two reaction systems. Many reports about enzymatic-catalysis biodiesel have been prepared, and are exhibited in Table 9.2.

9.3. ENZYMATIC-CATALYZED BIODIESEL SYNTHESIS BY RSM

RSM is an efficient statistical technique that optimizes multiple variables to search for the optimal point with minimum number of experiments (Box *et al.*, 1978; Khuri and Cornell, 1987). Recently, optimization of lipase-catalyzed production of various biodiesels (FAME) by RSM has been investigated.

Li *et al.* (2007) reported the use of dry biomass, *Rhizopus oryzae (R. oryzae*) IFO4697, whole cell-catalyzed methanolysis of soybean oil for biodiesel (methyl ester) in *tert*-butanol system. Changing one separate factor at a time (COST), five-level-four-factor Central Composite Design (CCD) were used to evaluate the effects of synthesis conditions, such as *tert*-butanol to oil volume ratio, methanol to oil molar ratio, water content, and dry biomass amount. Biodiesel yields of 72% were obtained under the optimal conditions using the proposed model for prediction.

Demirkol *et al.* (2006) reported that the lipase of Lipozyme® RM IM (*Rhizomucor miehei*) was efficient for the methanolysis of refined soybean oil with methanol to give methyl ester. RSM based on a three-level, three-factor (variable) face-centered cube design was used for the optimization of methanolysis. The independent variables contained enzyme to oil weight ratio, oil to methanol molar ratio, and reaction temperature. Critical conditions for the response at which methyl ester content of the product was 76.9% were determined.

Chang *et al.* (2005) showed the ability of immobilized lipase *C. antarctica* (Novozym® 435) to catalyze the alcoholysis of canola oil and methanol. RSM and a five-level-five-factor CCRD were employed to evaluate the effects of synthesis parameters, such as reaction time, temperature, enzyme concentration, substrate molar ratio of methanol to canola oil, and added water content

TABLE 9.2. Enzymatic-catalysis biodiesel in various lipase, substrates and solvent.

No.	Lipase	Alcohol	Oil	Solvent	Yield	Reference
1	*Candida antarctica* (Novozym® 435)	Methanol	Canola oil	Hexane	98%	Chang *et al.*, 2005
2	*Candida antarctica* (Novozym® 435)	Methanol	Cottonseed oil	Free	92%	Köse *et al.*, 2002
3	*Candida antarctica* (Novozym® 435)	Methanol	Cottonseed oil	*t*-butanol[a]	97%	Royon *et al.*, 2007
4	*Candida antarctica* (Novozym® 435)	Methanol[d]	Degumming soybean oil	Free	94%	Watanabe *et al.*, 2002
5	*Candida antarctica* (Novozym® 435)	Methanol[d]	Soybean oil	Free	97%	Samukawa *et al.*, 2000
6	*Candida antarctica* (Novozym® 435)	Methanol[d]	Vegetable oil	Free	91%	Watanabe *et al.*, 2007
7	*Candida antarctica* (Novozym® 435)	Methyl acetate	Soybean oil	Free	92%	Du *et al.*, 2004
8	*Candida antarctica* (Novozym® 435)	Ethyl acetate	Jatropha oil Karanj oil Sunflower oil	Free	91% 90% 93%	Modi *et al.*, 2007
9	*Candida cylindracea*	Methanol Butanol	Waste activated bleaching earth	Hexane	78% 96%	Lara and Park, 2004
10	*Candida cylindracea*	Methanol	Waste activated bleaching earth	Kerosene	100%	Kojima *et al.*, 2004
11	*Pseudomonas cepacia* (PS-30)	Methanol Ethanol	Soybean oil	Free	67% 65%	Noureddini *et al.*, 2005
12	*Pseudomonas cepacia* (PS-30)	Ethanol	Tallow and grease	Free	95%	Hsu *et al.*, 2001
13	*Pseudomonas fluorescens*	Methanol	Sunflower oil	Free	>90%	Soumanou and Bornscheuer, 2003

14	*Pseudomonas fluorescens*	Propanol	Sunflower oil	1,4-dioxane	>95%	Iso *et al.*, 2001
15	*Rhizomucor miehei* (Lipozyme® IM-77)	Methanol	Soybean oil	Hexane	92%	Shieh *et al.*, 2003
16	*Rhizomucor miehei* (Lipozyme® RM IM)	Methanol	Soybean oil	Hexane	85%	Demirkol *et al.*, 2006
17	*Rhizopus oryzae*	Methanol[d]	Soybean oil	Free	>80%	Kaieda *et al.*, 1999
18	*Rizppus oryzae* (whole-cell)	Methanol	Olive oil	Free	90%	Ban *et al.*, 2001
19	*Rizppus oryzae* (whole-cell)	Methanol	Soybean oil	Free	70–83%	Ban *et al.*, 2002
20	*Rizppus oryzae* (whole-cell)	Methanol	Soybean oil	Free	72%	Li *et al.*, 2007
21	*Thermomyces lanuginoul* (Lipozyme® TL IM)	Methanol[d]	Soybean oil	Free	90%	Du *et al.*, 2005
22	*Thermomyces lanuginoul* (Lipozyme® TL IM) and *Candida antarctica* (Novozym® 435)	Methanol	Soybean oil deodorizer distillate	*t*-butanol[b]	97%	Wang *et al.*, 2005
23	*Thermomyces lanuginoul* (Lipozyme® TL IM) and *Candida antarctica* (Novozym® 435)	Methanol	Rapeseed oils	*t*-butanol[c]	95%	Li *et al.*, 2006

[a]32.5 % of oil (v/v).
[b]*tert*-butanol:oil = 1:1 (v/v).
[c]*tert*-butanol:oil = 1.6:1 (v/v).
[d]Stepwise addition methanol.

on percentage weight conversion of canola oil methyl ester by alcoholysis. Based on the analysis of ridge max, the predicted optimum conversion yield was 99.4%, and the experimental value was 97.9%.

Shieh *et al.* (2003) indicated a biodiesel transesterification using soybean oil and methanol and commercial immobilized lipase from *R. miehei* (Lipozyme® IM-77). The response surface analysis showed that the following variables were important: reaction time, temperature, enzyme amount, molar ratio of methanol to soybean oil, and added water content on percentage weight conversion to soybean oil methyl ester by transesterification. The optimum yield based on ridge max analysis gave a 92.2% weight conversion.

9.4. THE USE OF RESPONSE SURFACE METHODOLOGY (RSM)

An awareness of the influences of various factors on the biodiesel will give a clear picture of the possible independent variables which can be manipulated to improve or optimize production (the dependent variables). Among dozens of possible candidates, use of some is limited by consumer habit or by the unavailability of the required tool, while others may give a higher priority to those variables that are convenient to adjust, and leave those that demand more time and effort for later study. After screening, the remaining few may thus form the basis for a focused study. However, optimization is not easy even with only two or three variables. RSM has been developed particularly for the optimization of sophisticated multivariable systems where the quantitative relationship between key variables is not always clear, as is the case with a complex operation such as transesterification. RSM allows simultaneous consideration of more than one variable at different levels, and of the corresponding interactions between these variables, on the basis of a relatively small number of experiments.

9.4.1. Principles of RSM

The word "response" refers to how a function (to be optimized) reacts to change(s) in its independent variable(s). Take a quadratic function of the form: $f(\mathrm{x}) = 10 - \mathrm{x}^2$, for illustration. Changing the independent variable "x" from 1 to 2, the "response" of "*f*" would be "changing from 9 to 6: the curve of $f(x)$ with "x" (the independent variable) being the abscissa is a concave down parabola. The highest point of the curve can easily be located graphically at $x = 0$, where $f(x) = 10$. Another way of looking at the question of locating the highest point (or optimum point) is to use calculus: equating the first derivate of "*f*" (with respect to x) to zero, and finding the solution is $x = 0$, which is the same answer as was obtained by the graphical method. Graphical and analytical methods are two powerful tools, if not the only two, in optimization.

It is much more complex when the function "*f*" involves two independent variables: $\mathrm{z} = f(\mathrm{x,y})$. The graphical representation of the function will be a

surface in three-dimensional space, on which z, the dependent variable, changes as x and y varyaccording to the relationship defined by f. Standing on the highest point of the curved surface, as is the case with a single variable function, calculus tells us that f_x and f_y (the first partial derivatives of f with respect to x and y) are zero. However, it is necessary to be aware that the reverse is not necessarily true. An analytical solution can be obtained by equating both (f_x and f_y) to zero and solving the simultaneous equations. Since an optimum point is merely one from of critical point—the solution gained may represent a saddle point—its solution should be verified by further mathematical testing. Numerical tools may be required for the solution of the above simultaneous equations when they are non-linear, and it will be even more difficult if non-linear differential equations are involved. However, these are beyond the scope of this paper. Should this be the case, readers are encouraged to turn to references about numerical methods, or computer software packages for numerical solutions. In spite of all the troubles and efforts which may be needed to obtain an analytical or numerical solution, the graphical method may be considered to be the friendlier option.

In practical applications, it is quite likely that quantitative description of the relationship between the dependent variable (*e.g.*, conversion yield) and the independent variable(s) (*e.g.*, reaction time, temperature, enzyme concentration, and substrate molar ratio) is impossible, and the optimum point cannot be found analytically. On such occasions, the graphical method, although less precise, is a practical and straight forward option, providing the graphical version of the description of the function without knowing how the function is mathematically defined.

Should the graphical method be chosen to study the behavior (response) of the unknown function, the next question would be whether the independent variables are to be changed one at a time, or simultaneously. There are times when one can find the optimum point by adjusting one variable at a time while holding other variable(s) constant: the one-variable-at-a-time technique. Consider an example where the amount of enzyme (x%) and the reaction temperature (y °C) are the two variables under investigation. Assume that at present, a temperature (y) of 45 °C is being used. Therefore, while holding y constant at 45 °C, a series of experiments involving changing x from 0 to 50% is carried out and conversion yield (%) is chosen as the dependent variable (z). From the graph of z *vs.* x the optimum x_{opt}, can be decided. In the second run, f is held constant at x_{opt}, and y is changed. A z *vs.* y graph is thus obtained, and y_{opt} can be decided. This procedure is: given the mathematical definition of f, equivalent to solving $f_x = 0$ for x while holding y constant, and solving $f_x = 0$ for y while holding x constant, instead of solving simultaneous equations. Although the method is strategically simple, the potential interaction between independent variables is not considered. In cases of significant interaction, the optimum point located by the one-variable-at-a-time technique could be significantly different from the true optimum point.

All experimentation is prone to human error and, additionally, sampling reproducibility in non-homogenous systems such as enzyme catalyzed trans-

esterification is poor; hence fluctuations of data are not surprising. To counter these inherent errors, scattered data points on a graph need to be smoothed by finding a curve (or surface) that best represents the pattern of obtained experimental data. Regression is a mathematical tool that helps decide the line, or curve, or surface that best fits (represents) given experimental data by minimizing an object function called the "sum of squares," which is the sum of the square of the distances between the data points and the line, curve or surface. Most graphical software packages are capable of performing versatile regressions. Statistically, a larger sampling size (more experiments) will bring us closer to the true solution. However, "how many is enough?" is the question we tend to ask under pressure of increased time and effort. In addition to regression, sampling size is another question which needs to be addressed.

As stated above, regression involves minimizing the "sum of squares," whose calculations are based on the distances (or deviations) between data points and a "curve" (or surface) which best represents scattered data. Therefore, to have a target for measuring distances, we need to assume a function (*e.g.*, a polynomial) whose characteristic shape is similar to the pattern of the data presented. Mathematical theories can then be applied to find the minimum "sum of squares" by adjusting parameters (*e.g.*, coefficients of a polynomial). Typical mathematical models used in such cases are polynomial, sigmoidal, or Gaussian functions, among others. The chosen mathematical functions (or models) for regression do not necessarily by themselves imply any physical or chemical significance. These functions could be adopted solely for the purpose of finding a curve or surface that best response the data.

In RSM, it is assumed that in the neighborhood of an optimum point, the concavity (whether concave up or down, or even twisted) of an arbitrary surface makes it reasonable to use a quadratic function as an approximation of the surface. The assumption may still hold even if the region of concern is not near an optimum point, but is small enough for the approximation to be valid. Most of the time, this simplification is good enough for practical purposes.

Box and Wilson first proposed the concept of RSM in 1951 (Box and Wilson, 1951). RSM uses a quadratic function of the form: $Y = a_0 + a_1X_1 + a_2X_2 + a_3X_1X_2 + a_4X_1^2 + a_5X_2^2$ to approximate the surface in the neighborhood of an optimum point. Where Y is the dependent variable (one of the conversion yield indices in this case), a_0 through a_5 are the coefficients (to be determined by regression) and X_1, X_2 are the factors (or operation variables which can be manipulated in the production process) that influence conversion yield, the dependent variable. The shape of the surface representing the quadratic model is determined by the coefficients. The six adjustable coefficients (a_0 through a_5) together with its quadratic nature make the model sufficient for most practical applications.

Theoretically, manipulating six parameters should enable the shape of the surface to be adjusted to fit any set of data that are smooth and quadratic in nature, given that the data fall either in the vicinity of an optimum point, or

in a small enough region. However, in practice, it is quite likely that one or more of the following situations might occur.

- Experimental data tend to fluctuate, and sometimes the true object function is not quadratic even in the neighborhood of the optimum point (poor degree of fitness for quadratic model).
- Independent variables do not fall in the vicinity of the optimum point.
- One or more of the chosen factors might not affect the dependent variable sufficiently to be included in the model.

While understanding and differentiating amongst the above listed possibilities is crucial, it is just as important to answer the following questions: Is the experimental error within tolerable limits or is the response of the dependent variable quadratic in nature? Is the region covered small enough to be represented by the model? And, are the chosen factors sufficiently significant to be included in the model? Statistical analysis is therefore necessary in order to verify the data, and to reach a conclusion which has a scientific basis. To minimize the time and effort involved in carrying out numerous experiments and, at the same time, have a large enough sample size for statistical validity, careful design of the experiments is important. Experiment design is a technique developed to establish an optimal number of experiments (Mason *et al.*, 1989; Montgomery, 1984; Thompson, 1982) which can simultaneously satisfy the dual criteria of a minimum number of experimental runs and large enough sample size to claim statistical significance. The next section discusses the basics of experiment design.

9.4.2. Experiments Design

As mentioned before, people tend intuitively to turn to the one-variable-at-a-time technique for its conceptual simplicity, and ignore the possible interaction between independent variables. A good example of the interaction between factors is that between enzyme concentration (E) and reaction temperature (T). Assuming E and T are the chosen factors for optimization, one possible interaction will be that T tends to influence the way E affects the conversion yield and *vice versa*. Since reaction temperature increased, enzyme activity was suppressed than at low temperature and the rate of enzyme-catalysis is affected by temperature: this will inevitably affect conversion yield of the product. Should the interaction be minor or negligible, a one-factor-at-a-time search will give a satisfactory result.

An experiment design for one-variable-at-a-time optimization is shown in Table 9.3. The experiment involves five levels for each factor, expressed in coded form which can be linearly transformed back to corresponding true values, so that the arrangement could be applied to other systems. In the coded form, one unit could represent 10 °C difference in reaction temperature,

TABLE 9.3. Experiment design for one-factor-at-a-time optimization (two-factor five-level).

	Independent variables		Dependent variable
Run number	X_1	X_2	Y
1	–2	0	Y_1
2	–1	0	Y_2
3	0	0	Y_3
4	+1	0	Y_4
5	+2	0	Y_5
6	–1	–2	Y_6
7	–1	–1	Y_7
8	–1	+1	Y_8
9	–1	+2	Y_9
Optimum point	–1	+1	$Y_{optimum} = Y_8$

or 10% enzyme content. From run numbers 1 to 5, X_2 was held constant at 0 (the center), and it was assumed that $X_1 = -1$ was found to produce the highest response (Y_2). Then, from run numbers 6 to 9 (the combination of $X_1 = -1$ and $X_2 = 0$ was carried out in run number 2), X_1 was fixed at –1, while X_2 varied from –2 to +2, and it was assumed that $X_2 = +1$ was found to be optimum (Y_8). $X_1 = -1$ and $X_2 = +1$ which produced the highest response (Y_8) was therefore identified as the optimum point. It can not be over-emphasized here that the true optimum point could be elsewhere, should interaction effects be significant.

The CCD (Mason *et al.*, 1989; Montgomery, 1984; Thompson, 1982) is commonly employed for systems with potential interactions effect(s) between factors. For an n-factor-five-level design, five coded levels ($-d$, –1, 0, +1, $+d$) are assigned to each factor, where d is called the extended level and $d = (2)^{n/4}$. An example of two-factor-five-level design is given in Table 9.4. For two-factor design $d = (2)^{2/4} = 1.414$, for three-factor design $d = (2)^{3/4} = 1.682$, and so on. In Table 4, run numbers 1 to 4 correspond to a two-level factorial design, and run numbers 9 to 10 are duplicate experiments so that statistical diagnosis can estimate experimental error. For more detailed explanation of the theories involved in the experiment design, readers are encouraged to go to the cited references. Comparing Table 3 and Table 4, it is clear that CCD can provide more information, and the price is a little as a 10% increase in time and effort. Considering the benefit from verification of the potential interactions between factors, this small price should be worth paying.

9.4.3. Statistical Analysis

After doing experiments according to Table 4, a group of ten observations (Y_1 through Y_{10}) can be used to carry out regression using the quadratic model as

TABLE 9.4. Central composite design (two-factor five-level).

Run number	Independent variable		Dependent variable
	X_1	X_2	Y
1	1	1	Y_1
2	1	−1	Y_2
3	−1	1	Y_3
4	−1	−1	Y_4
5	0	−1.414	Y_5
6	0	1.414	Y_6
7	1.414	0	Y_7
8	−1.414	0	Y_8
9	0	0	Y_9
10	0	0	Y_{10}

stated above. The obtained model, with the six coefficients determined by regression, represents a surface in the $Y - X_1 - X_2$ space. Using a computer graphic tool, one can visually pinpoint the critical point, be it a stationary point (where varying factors will not significantly change the dependent variable) or extrema (maxima or minima). While getting the response surface, the questions about error, interactions, or even whether or not the factor(s) should be included in the model in the first place, remain to be answered. Statistical analysis could provide answers to these questions.

Statistical analysis can be done by "analysis of variance" (ANOVA, Box *et al.*, 1978). If it is thought that interaction may be taking place, then a more complex experiment design should be considered. The price paid will be a minor one in comparison with the benefits in terms of more convincing results and more statistical information. One of the most important ANOVA terms in the application of RSM is R^2 (R-square), which shows the goodness of fit of the mathematical model. R^2 is determined by calculating the ratio of regression sum of square (SS_R) to the total sum of square (SS_T), $R^2 = SS_R/SS_T$. Since SS_T is the sum of SS_R (which comes from lack of fit) and SS_E (the error sum of squares, which comes from pure experimental error), the closer R^2 is to 1 (or 100 on a percentage basis) the smaller SS_E is and, therefore, the more intimate the relationship between the model prediction and the true response.

Another quantity, the P-value, indicates how significant is each term in the model (X_1, X_2, X_1X_2, X_1^2 and X_2^2). The p-value for i-th term in the model reveals that for the term X_1 in the model, the probability that X_1 is not significant to the response is p_i. For example, $p_2 = 0.05$ means that the probability that X_2 is not significant to the response is 0.05. In common language, there is 95% of the chance that X_2 is significant. It can also be side that X_2 is significant 5% level. In practice, depending on the desired level of accuracy, a p-value lower than 0.1 (10% level) is commonly considered acceptable.

Recently, advances in engineering and the sciences have provoked the development of all-in-one computer software packages that merge computer graphics, experiment design, regression, statistical analysis, worksheet and documentation. SAS (SAS, 1989), the Statistical Analysis System, is widely used. Design-Expert (1996) by Stat-Ease is very user-friendly. Similar software packages like STATISTICA (2000) and SPSS (2000) are also popular in the scientific software market. These software packages normally have a powerful tutorial system that will guide new users through the steps of RSM optimization.

RSM optimization has wide applications in various fields such as food technology, chemical technology, material engineering, and so on. Many successful examples can be found in the literature. Shieh *et al.* (2003) showed lipase-catalyzed transesterification of soybean oil and methanol, using RSM. They narrowed down the search domain by the on-factor-at-a-time technique and used the obtained optimum point as the center of the "central composite rotatable design (CCRD)" in subsequent RSM. Köse *et al.* (2002) investigated that the alcoholysis of refined cotton seed oil in the presence of immobilized lipase for *C. antarctica* and the optimum conditions were suggested with maximum methyl esters 91.5%. Hass and Scott (1996) optimized as a solvent for the lipase-catalyzed diesel fuel production by the RSM. Uosukainen *et al.* (1999) applied RSM in combination with principal-component analysis methods for optimizing the enzymatic transesterification of rapeseed oil methyl esters.

9.5. A CASE STUDY: LIPASE-CATALYZED BIODIESEL SYNTHESIZED

As mentioned at the beginning of this paper, there are four chemical approach to synthesize biodiesel: direct use and blending, microemulsions, thermal cracking (pyrolysis), and transesterification (also called alcoholysis) (Ma and Hanna, 1999). Although efficient in the terms of reaction time, the chemical approach to synthesizing biodiesel from triacylglycerols has some disadvantages, such as difficulty in the recovery of glycerol, the need for removal of salt residue, and the energy-intensive nature of the process. In contrast with the chemical synthesis, biocatalysts (lipases) allow for synthesis of specific alky esters, easy recovery of glycerol, and transesterification of glycerides with high FFA content (Nelson *et al.*, 1996). An optimized enzymatic biodiesel improves the conversion yield and reduces the cost of production. The present work focuses on the reaction parameters that affect immobilized lipase from *C. antarctica* (Novozym® 435)-catalyzed the alcoholysis of canola oil with methanol in n-hexane. The main objectives of this work were to better to understand the relationships between the reaction variables (time, temperature, enzyme amount, substrate molar ratio, and added water content) and the response (percent weight conversion), and to obtain the optimum conditions for biodiesel synthesis using CCRD and RSM analysis.

9.5.1. Experimental Design

A five-level-five-factor CCRD was employed in this study, requiring 32 experiments (Cochran and Cox, 1992). The fractional factorial design consisted of 16 factorial points, 10 axial points (two axial points on the axis of each design variable at a distance of 2 from the design center), and 6 center points. The variables and their levels selected for the study of biodiesel synthesis were: reaction time (4–20 h); temperature (25–65 °C); enzyme amount (10%–50% weight of canola oil, 0.1–0.5 g); substrate molar ratio (2:1–5:1; methanol: canola oil) and amount of added water (0–20%, by weight of canola oil). Table 9.5 shows the independent factors (X_i), levels and experimental design coded and uncoded. Thirty-two runs were performed in a totally random order.

9.5.2. Statistical Analysis

The experimental data (Table 5) were analyzed by the response surface regression (Proc RSREG) procedure to fit the following second-order polynomial equation (SAS, 1990):

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki}\chi_i + \sum_{i=1}^{5} \beta_{kii}\chi_i^2 + \sum_{i-1}^{4} \sum_{j=i+1}^{5} \beta_{kij}\chi_i\chi_j \qquad \text{[Eq. 9.1]}$$

where Y is response (percent weight conversion), β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients, and X_i the uncoded independent variables. The option of RIDGE MAX was employed to compute the estimated ridge of maximum response for increasing radii from the center of the original design.

9.5.3. Model Fitting

The RSREG procedure for SAS was employed to fit the second-order polynomial equation 1 to the experimental data—percent weight conversions (Table 5). Among the various treatments, the greatest weight conversion (96.5%) was treatment #22 (12 h, 45 °C, 50% enzyme, substrate molar ratio 4:1, added water 10%), and the smallest conversion (only 22.4%) was treatment #12 (16 h, 55 °C, 20% enzyme, substrate molar ratio 5:1, added water 5%). From the SAS output of RSREG, the second-order polynomial equation is given below:

$$\begin{aligned} Y = {} & -185.209 + 10.414X_1 + 2.838X_2 + 5.828X_3 + 31.402X_4 - \\ & 4.357X_5 - 0.268X_1^2 - 0.006X_2X_1 - 0.027X_2^2 - \\ & 0.019X_3X_1 - 0.055X_3X_2 + 0.002X_3^2 - 0.0238X_4X_1 + \\ & 0.023X_4X_2 - 0.345X_4X_3 - 3.993X_4^2 - 0.184X_5X_1 + \\ & 0.019X_5X_2 - 0.001X_5X_3 + 0.467X_5X_4 + 0.145X_5^2 \end{aligned} \qquad \text{[Eq. 9.2]}$$

TABLE 9.5. Central composite rotatable second-order design and experimental data for 5-factor-5-level response surface analysis.

Treatment#	Random#	Time (h) X_1	Temperature (°C) X_2	Enzyme amount (%) X_3	Substrate molar ratio (methanol/canola oil) X_4	Added H_2O (% by wt of canola oil) X_5	*Observed yield (% weight conversion) Y*
1	8	–1 (8)[a]	–1 (35)	–1 (20)	–1 (3:1)	1 (15)	31.87
2	13	1 (16)	–1 (35)	–1 (20)	–1 (3:1)	–1 (5)	47.37
3	27	–1 (8)	1 (55)	–1 (20)	–1 (3:1)	–1 (5)	24.73
4	11	1 (16)	1 (55)	–1 (20)	–1 (3:1)	1 (15)	23.19
5	24	–1 (8)	–1 (35)	1 (40)	–1 (3:1)	–1 (5)	91.31
6	23	1 (16)	–1 (35)	1 (40)	–1 (3:1)	1 (15)	83.74
7	22	–1 (8)	1 (55)	1 (40)	–1 (3:1)	1 (15)	60.72
8	7	1 (16)	1 (55)	1 (40)	–1 (3:1)	–1 (5)	68.51
9	1	–1 (8)	–1 (35)	–1 (20)	1 (5:1)	–1 (5)	23.05
10	14	1 (16)	–1 (35)	–1 (20)	1 (5:1)	1 (15)	24.28
11	25	–1 (8)	1 (55)	–1 (20)	1 (5:1)	1 (15)	24.85
12	21	1 (16)	1 (55)	–1 (20)	1 (5:1)	–1 (5)	22.43
13	16	–1 (8)	–1 (35)	1 (40)	1 (5:1)	1 (15)	72.77
14	32	1 (16)	–1 (35)	1 (40)	1 (5:1)	–1 (5)	72.19
15	12	–1 (8)	1 (55)	1 (40)	1 (5:1)	–1 (5)	35.44
16	28	1 (16)	1 (55)	1 (40)	1 (5:1)	1 (15)	36.15
17	9	–2 (4)	0 (45)	0 (30)	0 (4:1)	0 (10)	36.91

18	5	2 (20)	0 (45)	0 (30)	0 (4:1)	0 (10)	46.09
19	3	0 (12)	−2 (25)	0 (30)	0 (4:1)	0 (10)	72.19
20	2	0 (12)	2 (65)	0 (30)	0 (4:1)	0 (10)	23.50
21	18	0 (12)	0 (45)	−2 (10)	0 (4:1)	0 (10)	22.50
22	6	0 (12)	0 (45)	2 (50)	0 (4:1)	0 (10)	96.45
23	30	0 (12)	0 (45)	0 (30)	−2 (2:1)	0 (10)	60.77
24	10	0 (12)	0 (45)	0 (30)	2 (6:1)	0 (10)	24.58
25	20	0 (12)	0 (45)	0 (30)	0 (4:1)	−2 (0)	95.71
26	26	0 (12)	0 (45)	0 (30)	0 (4:1)	2 (20)	50.49
27	15	0 (12)	0 (45)	0 (30)	0 (4:1)	0 (10)	50.40
28	4	0 (12)	0 (45)	0 (30)	0 (4:1)	0 (10)	54.50
29	31	0 (12)	0 (45)	0 (30)	0 (4:1)	0 (10)	67.78
30	29	0 (12)	0 (45)	0 (30)	0 (4:1)	0 (10)	59.87
31	19	0 (12)	0 (45)	0 (30)	0 (4:1)	0 (10)	71.48
32	17	0 (12)	0 (45)	0 (30)	0 (4:1)	0 (10)	51.56

[a]Numbers in parenthesis represent actual experimental amounts.

The analysis of variance (ANOVA) indicated that the second-order polynomial model (above) was statistically significant and adequate to represent the actual relationship between the response (percent weight conversion) and the significant variables, with very small *p*-value (0.0001) and a satisfactory coefficient of determination ($R^2 = 0.955$).

9.5.4. Effect of Synthesis Parameters

The effect of varying reaction temperature and substrate molar ratio at constant reaction time (12 h), enzyme amount (30%), and added water content (10%) is shown in Figure 9.1. In general, an increase in substrate molar ratio led to lower yields at any temperature. It was concluded that a great deal of methanol inactivated Novozym® 435 to synthesize the biodiesel. Similar results, that an excess of methanol decreased the enzymatic biodiesel catalyzed by Lipozyme® IM77, was reported by our previous study (Shieh *et al.*, 2003).

The entire relationships between reaction factors and response can be better understood by examining the planned series of contour plots (Fig. 9.2) generated from the predicted model (equation 2) by holding constant the enzyme amount (20, 30, and 40%, weight of canola oil) and substrate molar

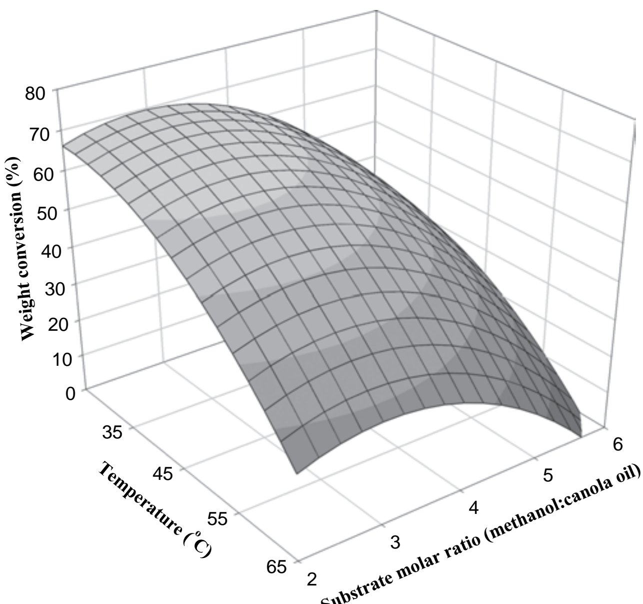

Figure 9.1. Response surface plot showing the effect of substrate molar ratio, reaction temperature, and their mutual interaction on biodiesel synthesis. Other synthesis parameters (reaction time, enzyme concentration, and added water amount) are constant at 0 levels.

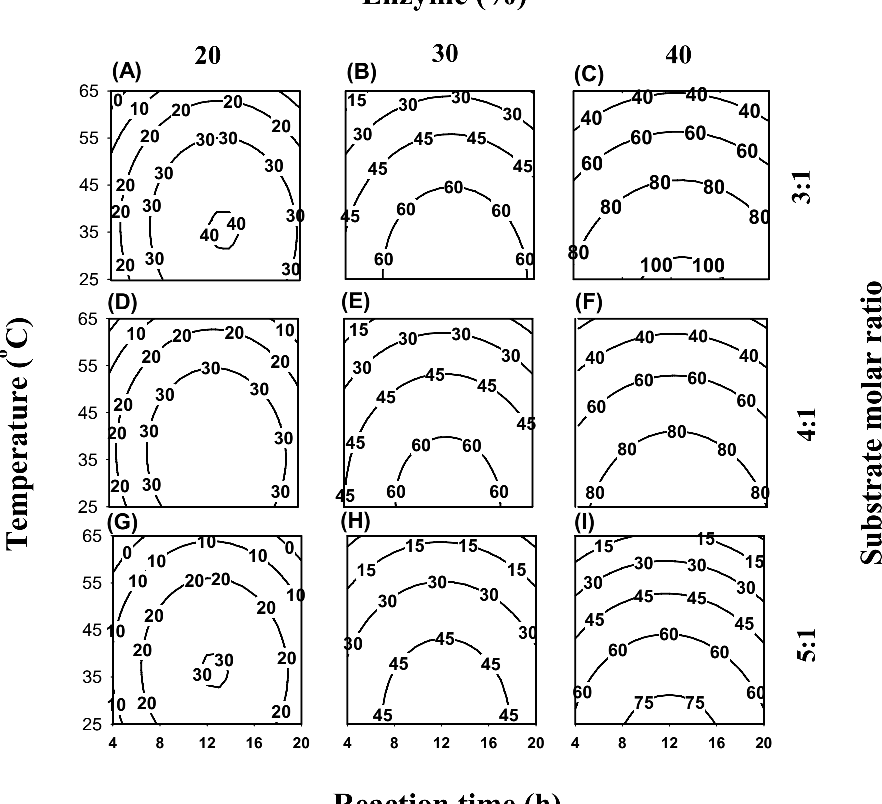

Figure 9.2. Contour plots of percent weight conversion of biodiesel. Enzyme concentration was by weight of canola oil and substrate molar ratio was methanol to canola oil. The numbers inside the contour plots indicate weight conversions at given reaction conditions.

ratio (3:1, 4:1, and 5:1). Figures 9.2A, 9.2B, and 9.2C represent the same substrate (3:1); and A, D, and G represent the same enzyme amount (20%). Such an application could be employed to study the synthesis variables simultaneously in a five-dimensional space and easily to observe the overall effects of synthesis variables on yield conversions. Generally, all nine contour plots in Figure 2 exhibited similar behavior in that predicted weight conversion increased in the beginning and decreased over 12 h. Therefore, a 12-h synthesis gave the highest percent weight conversion compared to the others in the experimental region. Overall, all nine contour plots in Figure 2 indicated that predicted weight conversion increased by the enzyme amount. A reaction with more enzyme amount gave higher weight conversion compared to the others. However, it was decreased by substrate molar ratio because that superabundant methanol inhibited the activity of Novozym® 435. Therefore, the optimum

TABLE 9.6. Estimated ridge of maximum response for variable percent weight conversion.

Coded radius	Estimated response (corporation)	Standard error	X_1 (h)	X_2 (°C)	X_3 (%)	X_4 (methanol / canola oil)	X_5 Added water (%)
0	55.26	3.27	12.00	45.00	30.00	4.00	10.00
0.2	65.00	3.23	12.09	43.23	33.18	3.87	9.51
0.4	75.57	3.24	12.18	41.46	36.31	3.74	8.89
0.6	87.01	3.59	12.30	39.73	39.35	3.62	8.12
0.8	99.40	4.60	12.43	38.03	42.27	3.51	7.20

substrate molar ratio was very important in the production of lipase-catalyzed biodiesel for the alcoholysis reaction.

9.5.5. Attaining Optimum Condition

The optimum synthesis of enzymatic biodiesel was determined by the ridge max analysis (SAS, 1990). The method of ridge analysis computes the estimated ridge of maximum response for increasing radii from the center of original design. The ridge max analysis (Table 9.6) indicated that maximum molar conversion was 99.4 ± 4.6% at 12.4 h, 38.0 °C, 42.3% enzyme amount, 3.5 : 1 substrate molar ratio, and 7.2% added water content at the distance of the coded radius 0.8.

9.5.6. Model Verification

The adequacy of the predicted model here was examined by additional independent experiments at the suggested optimum synthesis conditions. The predicted value was 99.4% molar conversion and the actual experimental value was 97.9%. A chi-square test (p-value = 0.96, degrees of freedom = 5) indicated that observed values were significantly the same as the predicted values and the generated model adequately predicted the percent molar conversion (Ott, 1988). Thus, the optimization of lipase-catalyzed synthesis for biodiesel (canola oil methyl ester) by Novozym® 435 was successfully developed by CCRD and RSM.

9.6. CONCLUSIONS

A systematic search for optimum conditions requires a quantified basis (by instrumentation or panels) and efficient experiment design. RSM optimization is a powerful tool which is particularly useful in areas such as improving the conversion yield of enzymatic-catalysis biodiesel. Among dozens of potential factors, careful screening, based on professional judgment, must be performed

to reduce the number of independent variables, and focus on a few key factors. The window of search should be kept within realistic limits if the quadratic model is to be applicable. Finally, statistical analysis can help to justify a regression result and provide a basis for decision-making.

In mathematical optimization for many applications, it is commonly expected that the response surface will have a bell shape for which the point located at the top of the surface can be picked as the optimum point without any debate. However, in the case study of this paper, such a response surface was not obtained. Other engineering criteria were applied as a compromise between pros and cons, according to implications revealed by the response surfaces. Cross-checking the optimization result based on instrumental measured quantities with that from panel studies is important for validation. Although it is a powerful optimization tool with versatile applications, without professional knowledge and experience, the contribution of RSM is limited.

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CHAPTER 10

Production of Biofuel from Lipids and Alternative Resources

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10.1. INTRODUCTION

There is an increasing need for new energy resources due to growing industrialisation, shrinking petroleum resources, and global climate warming.

Biomass is one of the sustainable sources for energy supply. Its major advantage is the reduction of the CO_2 emission due to the nearby neutral CO_2-balance.

Some of the renewable resources for the production of "green energy" are vegetable oils and animal fats. The use of neat lipids as transportation fuel has not been very successful due to their high viscosity which is leading to engine problems. The production of biodiesel (alkyl esters of fatty acids) is one of the best alternatives, and the blending of biodiesel with petroleum diesel is now widespread in Europe and in the U.S.

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There are two major obstacles for the use of lipids as energy resources. The primary use of vegetable oils will remain tied to food, and the conflict between nutritional use and energy is affecting the global prices of vegetable oils. The second reason is the high manufacturing cost of food-grade and refined canola oil in the E. U., soy-bean oil in the U. S., and palm oil in South-East Asia and Africa.

Therefore there is increased interest in less expensive resources such as waste cooking and frying oils, rendered animal fats, recuperated lipids, and side-streams from refining. Another alternative is the conversion of non-food vegetable oils (such as yatropha) and lipids derived from algae. To reduce the manufacturing costs of the biodiesel, the direct use of crude and non-refined oils is promising.

The relatively higher costs of biodiesel in comparison with diesel fuel is due to the high costs of the starting material, estimated to be between 70 and 85% of the total production costs. Waste oils and other oil resources are less expensive, and generally unrefined oils are 20 to 25% cheaper than refined oils.

However the use of non-refined lipids will involve new processing technologies for the production of biodiesel, which induces an increase in the production costs. Excellent reviews on the use of alternative resources for the production of biodiesel have been published recently by Knothe (2005), Zhang (2003), Kielhaine and Dalai (2006).

In the following study the conversion of non-refined palm oil, waste cooking oil, animal fats, deodorizer distillate, and soap stocks into biodiesel will be reported. Finally, the direct use of animal fat for the production of electricity will be discussed.

10.2. PRODUCTION OF BIODIESEL FROM NON-REFINED VEGETABLE OILS

The conventional method of preparing biodiesel is an alkaline catalysed transesterification reaction of refined vegetable oils with methanol to produce biodiesel according to E.U. (EN14214) or U.S. (ASTM D6751) standards.

The refining of vegetable oils involves a degumming step, using water and/or citric acid to remove mainly phospholipids and other gums. In chemical refining, free fatty acids are neutralized mainly with a sodium hydroxide solution, producing a side stream of soap stock. In a bleaching step, coloured materials, polymeric compounds, free fatty acids, soaps, and metals are removed while during the deodorisation step, oxidation products are eliminated. In the physical refining process, neutralisation is omitted and after degumming, a bleaching process is operated, followed by a deodorisation to remove oxidation products and free fatty acids.

Free fatty acids and phospholipids are harmful components in biodiesel and are removed from crude vegetable oils by a variety of techniques, in order to convert the oils into biodiesel with parameters according to the standards.

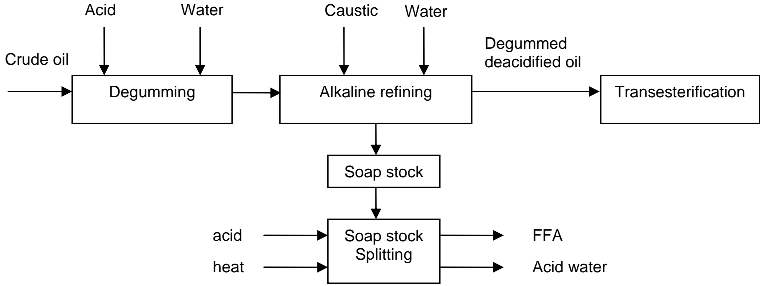


Figure 10.1. Alkaline Refining.

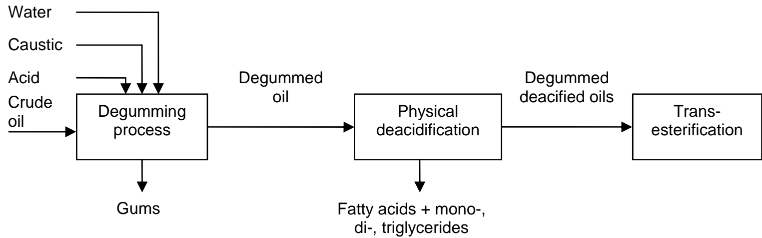


Figure 10.2. Physical refining.

It is generally accepted that the production of biodiesel meeting the E. U. standards is possible from crude oils with a free fatty acid content lower than 2–2.5%, and levels of phospholipids up to 300 ppm (Dorado *et al.*, 2004). However the presence of a high amount of phospholipids results in poor biodiesel-glycerol and biodiesel-water separations which in turn results in a lower yield. Due to the lower concentration of P-compounds in palm oil and animal fat, these raw materials can be used without further degumming treatment.

Elimination of free fatty acids from biodiesel can be performed by separation of the free fatty acids before the transesterification, or by transformation of the acids into esters by acidic esterification. Free fatty acids can be separated from the triglycerides by a pre-treatment, either by an alkaline refining using sodium hydroxide with formation of soap stock (Fig. 10.1), by physical refining (stripping at high temperature in vacuum) (Fig. 10.2) or by extraction of the FFA with the glycerine layer (containing the alkaline catalyst in methanol).

The "alcohol refining" developed by Westfalia Separator (Harten, 2006) is now widely used in a continuous process (Fig. 10.3). Advantages of alcohol refining are that it provides perfect degumming (<5 ppm P without pre-degumming) and that during the transesterification less fouling and emulsion formation is observed. In this way crude palm oil with a FFA content of 3% can be directly converted into biodiesel (Fig. 10.4).

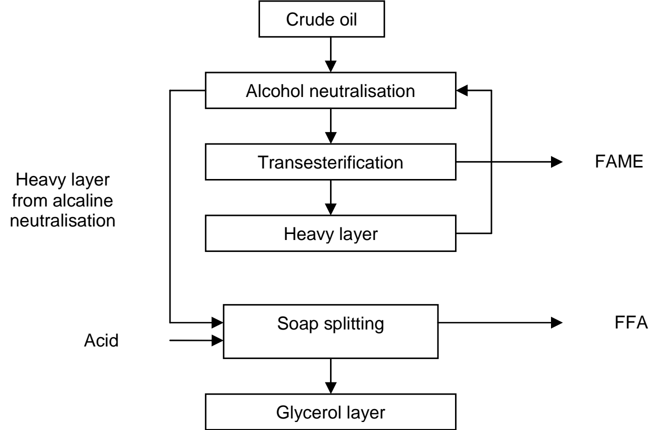


Figure 10.3. Alcohol refining. (Westfalia)

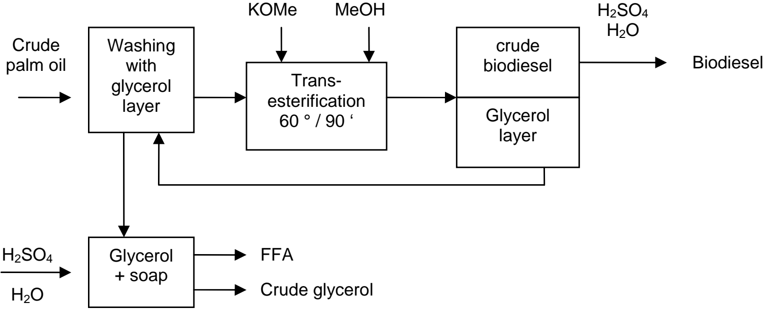
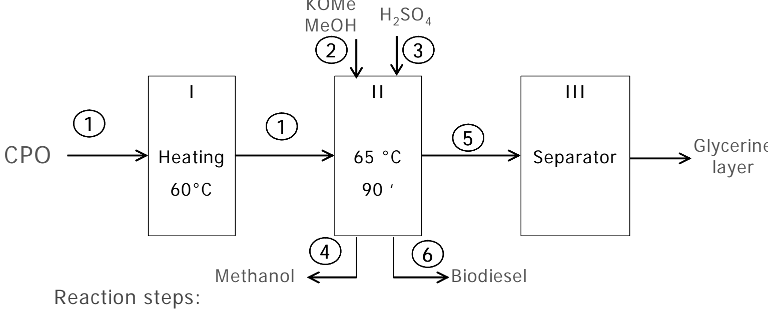


Figure 10.4. Production of biodiesel from crude palm oil.

The disadvantages of alcohol refining are that the free fatty acids are not converted into esters and the glycerol layer, which cannot be purified, has to be discarded. An alternative procedure for the direct conversion of crude palm oil into biodiesel has been developed at Ghent University (Verhé, 2007) (Fig. 10.5). During this process the unrefined palm oil, with a FFA content between 3–8%, is converted into biodiesel using a unique process involving a one vessel operation. First a potassium methoxide catalyzed transesterification is carried out, followed by a sulfuric acid catalyzed esterification without separation of the glycerol layer. After evaporation of the excess of methanol the glycerol layer is separated, followed by washing with water and then drying.

Previous experiments combining esterification and transesterification have been reported but in all cases first the acidic esterification was carried out (Canacki, 2003; Issariyakul, 2007).

The following reaction conditions have been used in a combination transesterification—esterification:



Reaction steps:

1. Warming up to 60°C in I
2. Neutralisation FFA, transesterification (II)
3. Acid esterification
4. Recuperation methanol (II)
5. Separation glycerol (III)
6. Washing step (II or III)

Figure 10.5. Conversion of crude palm oil (CPO) by transesterification and esterification.

Step 1: alkaline transesterification
catalyst: 0.6% (w/w) KOMe as 30% solution in MeOH
+ additional amount for neutralisation FFA
methanol: 20% (w/w)
reaction: 65 °C/90 m

Step 2: acidic esterification
Catalyst: 2% (w/w) H_2SO_4
+ additional amount to neutralize excess catalyst and soaps
methanol: 5% (w/w)
reaction: 65 °/3 hours

The oil at 60 °C is added to the reactor, followed by the methanol solution of the catalyst. The mixture is stirred for 90 min at 65 ° to 70 °C. During the reaction the glycerol is precipitated out but not separated. After the transesterification the sulfuric acid in methanol is added and stirred for another 3 hours. The methanol is recuperated by evaporation at 65 °/50 min. Water is added, and the glycerol/water layer is separated off. The biodiesel is washed with water until neutral and dried under vacuum. Thus crude palm oil (CPO) is converted into biodiesel meeting the majority of the E. U. standards (Table 10.1).

The major advantages of the two-step interesterification-esterification are:

- The soaps, which are present during the interesterification, act as emulsifiers giving rise to a higher reaction speed due to a better interaction between the polar methanol and apolar triglycerides. In one step a conversion of 90 to 95% has been reached.

TABLE 10.1. Properties of biodiesel from crude palm oil (CPO).

Analysis	Result	EN14214 limit
Ester content	96.8%	Min 96.5% (m/m)
Carbon Conradson residue	<0.001%	Max 0.3% (m/m)
Sulfated ash content	<0.01%	Max 0.02% (m/m)
Total contamination	44 mg/kg	Max 24 mg/kg
Acid value	1.7 mg/g	Max 0.5 mg KOH/g
Methanol content	0.02%	Max 0.2% (m/m)
Monoglyceride content	0.52%	Max 0.8% (m/m)
Diglyceride content	0.15%	Max 0.2% (m/m)
Triglyceride content	<0.01%	Max 0.2% (m/m)
Free glycerol	<0.01%	Max 0.02% (m/m)
Total glycerol content	0.15%	Max 0.25% (m/m)

- During the acid esterification and the acid work-up, the remaining phospholipids, proteins and pigments are removed by a degumming and bleaching reaction in this medium, and are discarded with the acidic glycerol and water layers.
- During the acid esterification, the remaining acylglycerides are converted to methylesters by acidic transesterification.
- There is a better separation of the biodiesel/glycerol and water layers in acid medium; due to the absence of soaps there is no emulsification.
- The amount of methanol is lower.
- Methanol is easily recuperated, due to evaporation in the absence of water.
- High ester yield.

The method can be used for oils containing less than 10% FFA, otherwise too much base is consumed.

The same procedure has been used for the conversion of used frying oils (Table 10.2) and crude chicken fat (Table 10.3).

10.3. VALORISATION OF REFINING SIDE STREAMS

During the refining of vegetable oils a number of side streams are formed which consist mainly of fatty acids. During neutralisation, soap stocks are formed from which fatty acids can be isolated upon acidifying. Similarly the glycerol layer obtained during the alcohol refining can be rich in fatty acids. Deodorisation, especially during physical refining, gives rise to a distillate which contains 85–90% fatty acids, as well as monoglycerides, tocopherols and sterols. These acid streams can be converted into biodiesel via acidic esterification.

TABLE 10.2. Production of biodiesel from used cooking oils (UCO).

Yield FAME	89%
FFA content	0.2%
Cloud Point	–7.5 °C
Iodine Value	122
Water content	80–200 ppm
Phosphorous content	0.2 ppm
Density	0.880 kg/l (15 °C)
OSI (110 °C)	0.35–2.4 hour

TABLE 10.3 Production of biodiesel from chicken fat.

Analysis	Test1	Test2
Cloud point (°C)	2.3	0.8
FFA (% C18:1)	0.08	0.14
Acid Value (mg KOH/g fat)	0.16	0.28
Element analyses (mg/kg)		
P	0	0
Fe	0.01	0.01
Ca	0.07	0.55
Mg	0.04	0.25
Iodine Value (Wijs)	80.0	84.1
Water content (mg/kg)	825	856
Monoglycerides (%)	0.15	0.08
Diglycerides (%)	n.d.	n.d.
Triglycerides (%)	n.d.	n.d.
Density 15 °C (g/cm^3)	0.875	0.871
Viscosity 40 °C (mm^3/s)	5.1	5.0

Test1: crude chicken fat; Test2: chicken fat olein; n.d.: not detectable.

The valorisation of these side streams is illustrated by the conversion of the deodoriser distillate into biodiesel. Deodoriser distillate is dissolved in methanol in a weight ratio 1/1 and 5% (w/w) sulfuric acid is added and heated at 75 °C for 5 hours with stirring. Under these circumstances the FFA are esterified and the remaining monoglycerides are mostly undergoing a transesterification giving FAME.

Water is added in a 20% weight ratio and the mixture is stirred for 15 minutes, after which the water layers are separated. The FAME layer is washed with water until neutral, then dried and distilled at 200–220 °C/10 mbar resulting in a biodiesel of excellent quality. From the residue, sterols can be isolated by trituration with methanol. Technologies are available to isolate tocopherols from the residue but are not performed in this study.

10.4. CONVERSION OF ANIMAL FAT FOR ELECTRICITY PRODUCTION

Waste animal fat from rendering can no longer be added in the feed-food chain. These animal fats can now be used in diesel engines to produce "green electricity" after refining. The fuel for these engines can only contain maximum 2% FFA, a low ash content, and the P-content must be lower than 10 ppm.

Animal fats (phospholipids and residues of bones and tissues) contain high amounts of FFA (up to 30%) and a P-content up to 300 ppm. efining them (Fig. 10.6) involves a degumming step at 80 °C by high shear mixing with 0.2% of a 42.5% phosphoric acid or 0.3% of a 30% citric acid solution (all w/w). The next step is an adsorption (bleaching), by either 0.3% Trysil 300, or Tonsil 550 F and 0.5% water, or 0.3% of a 30% citric acid solution. This suspension is stirred in an adsorber for 30 m at 90 °C, followed by filtration.

Afterwards the fat is dried for 1 hour at 95 °C and at 50 mbar. To remove the remaining free fatty acids, a stripping is performed in a modified deodoriser at 220 °C, with 2% steam at 2–3 mmbar for 30 m. The resulting animal fat contains less than 1% FFA and less than 10 ppm phosphor, an excellent biofuel basis for producing electicity (De Greyt W., 2006). Table 10.4 shows the composition of biofuel produced from animal fats.

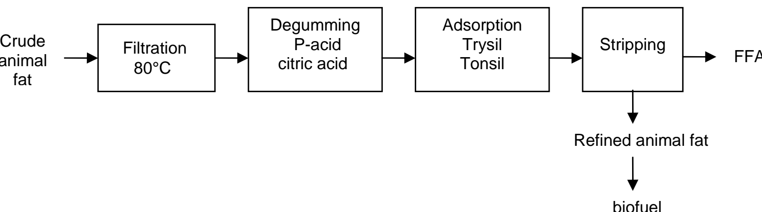


Figure 10.6. Production of biofuel for electricity from animal fat.

TABLE 10.4. Composition of biofuel from animal fat.

Parameter	Trial 1	Trial 2	Specification
Moisture (KF-ppm)	101	94	Max. 5000
FFA (% C18:1)	0.4	0.7	Max. 5
impurities (ppm)	300	233	Max. 500
ash (ppm)	224	303	Max. 500
P (ppm)	5.8	9.5	Max. 5
Fe (ppm)	0.4	0.8	
Ca (ppm)	14.3	23.7	
Mg (ppm)	1.6	2.7	

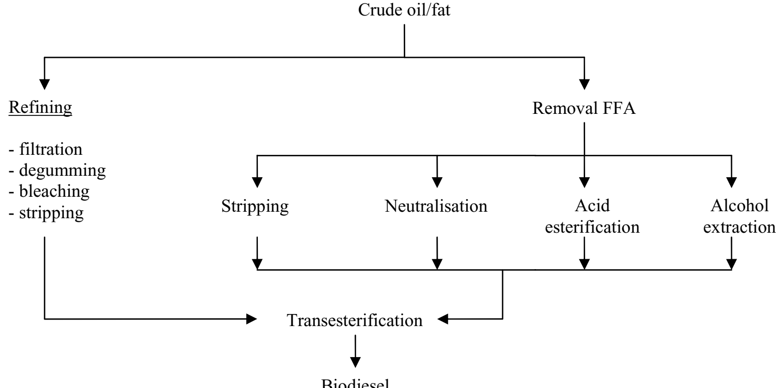


Figure 10.7. General scheme for conversion of crude lipids into biodiesel.

10.5. CONCLUSIONS

New technologies and processes are available to convert crude vegetable oils and waste oils from frying and animal fats into biodiesel, either by a pre-refining pretreatment or by a two step transesterification-esterification process (Fig. 10.7).

Animal fats can be refined and used as a biofuel for products of "green electricity."

Using the proposed technologies, waste steams which can no longer enter anymore the food chain are converted into biodiesel or biofuel.

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PART II

BIOETHANOL

CHAPTER 11

Biotechnology of Holocellulose-Degrading Enzymes

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11.1. INTRODUCTION

Plant cell walls are the source of lignocellulosic materials, also known as biomass, whose structure is chiefly represented by the physico-chemical interaction of cellulose (a linear glucose polymer), with hemicellulose (a highly

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branched heteropolymer), and lignin (a very high molecular weight and cross-linked aromatic macromolecule) (Himmel *et al.*, 2007; Howard *et al.*, 2003; Joseleau *et al.*, 1992; Meshitsuka and Isogai, 1996; Sakakibara, 1991). Cellulose, the most abundant polysaccharide on earth, is a highly ordered polymer of cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose), representing over 50% of wood mass. The length of cellulose molecules varies among different sources. Native cellulose from wood has about 10,000 glycosyl units in the cellulose chain that form fibrils (long bundles of molecules), which are stabilized by numerous strong intermolecular hydrogen bonds between hydroxyl groups of adjacent molecules (Sjöström, 1993). Cellulosic materials present crystalline domains separated by less ordered, amorphous, regions that are potential points for chemical and biochemical attacks. Approximately 4.10^{10} tons of cellulose is produced annually (Coughlan, 1985).

By contrast, lignin is a three-dimensional polyphenolic network of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenil, H) phenylpropanoid units. Derived from the corresponding *p*-hydroxycinnamyl alcohols, it gives rise to a variety of sub-units including different ether and C-C bonds. Lignin is hydrophobic and highly resistant towards chemical and biological degradation. It is located in the middle lamella, acting as cement between the plant cells; in the cell wall layers, it forms, together with hemicellulose, an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation. Lignin content and composition in terms of the H:G:S ratio vary among different plant groups: woody gymnosperms (softwoods) have the highest lignin content and their lignin is made up mostly of G units; lignin of woody angiosperms (hardwoods) consists of S and G units; that from non-woody angiosperms also contains H units. Moreover, lignin composition between the different wood tissues and cell wall layers varies. Hemicelluloses (polyoses) are the linking materials between cellulose and lignin. Wood hemicelluloses are short (degree of polymerization within 100 to 200), highly branched heteropolymers of glucose, xylose, mannose, galactose and arabinose, as well as different sorts of uronic acids. Depending on the predominant sugar type, the hemicelluloses are referred to as mannans, xylans or galactans. The C5 and C6 sugars, linked through 1,3, 1,6 and 1,4 glycosidic bonds and often acetylated, form a loose, very hydrophilic structure that acts as a glue between cellulose and lignin. Other non-structural components of plant tissues, including the compounds that are extractable with organic solvents (phenols, tannins, fats and steroids), water (sugars, starch and proteins) as well as ashes, usually represent less than 5% of the wood dry mass (Martínez *et al.*, 2005). The association between polysaccharide (cellulose and hemicellulose) and non-polysaccharide (lignin) components in the structure of plant cell walls is, in great part, responsible for its mechanical and biological resistance (Joseleau *et al.*, 1992; Pérez *et al.*, 2002).

With the exception of non-vascular plants and marine systems, cellulose and hemicellulose are always associated with lignin which plays a foremost protective role in the molecular integrity of biomass. Therefore lignin degradation is

a key step in biomass decay. In nature, fungi are able to alter lignin through the action of an extracellular enzymatic apparatus of different oxidative enzymes (oxidoreductases) such as laccases, high redox potential ligninolytic peroxidases, and oxidases. Fungi are also able to degrade cellulose and hemicellulose by a complex set of excreted hydrolytic, rather than oxidative, enzymes. A comprehensive list on the distribution of cellulases in fungi is presented elsewhere (Gosh and Gosh, 1992). Thus, an arsenal of enzymes (hydrolytic and oxidative) works synergically to perform lignocellulose degradation. These enzymes are produced by a great variety of fungi and bacteria (Pérez *et al.*, 2002). In general, fungi colonize the plant material which they degrade. Fungal growth within the plant material is mainly responsible for the ultimate disintegration of the plant, producing huge masses of residue and contributing to the major bulk of the soil. The residue is ultimately oxidized to CO_2 and H_2O. Wood decay is caused mainly by aerobic fungi responsible for the lignocellulose decay patterns known as "white," "brown," and "soft–rot" resulting from the selective action of fungi on the biomass components, *e.g.,* cellulose, hemicellulose and lignin. White rot fungi are heterogeneous and degrade all components of wood including lignin. The metabolism of the polysaccharide component provides the energy and hydrogen peroxide needed to degrade lignin. Brown rot fungi, generally Basidiomycetes, degrade cellulose and hemicellulose extensively with limited action on lignin; soft rot fungi are found among Fungi imperfecti and Ascomycetes. They soften the wood by degrading solely the polysaccharide (Ghosh and Ghosh, 1992). Actinomycetes are filamentous bacteria which are found in soil and composts where lignocellulose is decomposed. Species of the genus *Streptomyces* (*S. viridosporous* and *S. badius*, among others) are able to excrete enzymes to solubilize cellulose, hemicellulose and lignin. These bacterial enzymes, contrary to the biocatalysts produced by fungi, are more temperature stable and are able to perform in alkaline pH (Macedo *et al.*, 1999; Zerbini *et al.*, 1999; Gottschalk *et al.*, 1999; Bon *et al.*, 1999; Semêdo *et al.*, 2000; Bon and Picataggio, 2002; Bon *et al.*, 2003; Lima *et al.*, 2005). The fundamental structural role of lignin in plant tissues is only matched by the effectiveness of the microbial consortium of complex and complementary lignin degrading enzyme systems that act at nature's pace. An extensive review on the biodegradation of lignocellulosic materials by fungi and bacteria can be found elsewhere (Odier and Artaud, 1992; Gosh and Gosh, 1992).

Biomass, widely available at low cost, has been explored by humans as a favored source of energy, whereas the polyaromatic lignin structure plays a positive role as a solid fuel. Lignin, however, has represented a major encumbrance to the design of relevant industrial processes, such as are used in the pulp and paper industries, that use biomass as raw materials (Odier and Artaud, 1992). As industrial oxidases and peroxidases are not yet available at the necessary amount and price to allow its industrial use, lignin derivatives resulting from lignocellulose processing pose a significant environmental impact. Nevertheless the production of these enzymes has been studied (Odier Artaud, 1992; Ferrara *et al.*, 2002), as has their stabilization (Hernández *et al.*, 2005) and use in the paper industry (Carvalho *et al.*, 1998). Furthermore

microbial and plant peroxidases have been studied for the degradation of xenobiotics with related structure to lignin (Ferreira *et al.*, 2000; Odier and Artaud, 1992; Bon *et al.*, 2003; Leitão *et al.*, 2007).

Major biotechnological uses of the biomass carbohydrate moiety have attracted worldwide attention. Controlled cellulose degradation by cellulases may produce materials for important multifarious applications: carbohydrates that can be used in the food and beverage industries, cellulose microfibril fragments for non-caloric food additives, hyperabsorbent cellulose fibers from fragmented cellulose microfibrils which can be used in biomedical, commercial and house-hold absorbent materials. Biomass-derived glucose syrups can also be used as carbon source in industrial fermentations for the production of antibiotics, industrial enzymes, amino-acids, and bulk chemicals.

There has been a steadily growing interest worldwide in replacing oil as a source of fuel. The use of fossil fuels adds a significant amount of carbon dioxide to the atmosphere, which has been connected to global climate change due to the greenhouse effect. Furthermore cellulose, that is abundant, renewable and available throughout the world, has been considered as a safe source of energy for the production of fuel ethanol (Galbe and Zacchi, 2002; Bon and Picataggio, 2002; Bon *et al.*, 2004). Although hydrolysis to the desirable end products may be performed via chemical and/or enzymatic treatments, biomass hydrolysis to fermentable sugars must be performed via technologies that should be both environmentally friendly and economically feasible (Lynd *et al.*, 2005). The advantages of enzyme application over chemical treatment are higher conversion efficiency, the absence of substrate loss due to chemical modifications and the use of more moderate and non-corrosive physical-chemical operating conditions (lower reaction temperatures, almost neutral pH, and the use of biodegradable and nontoxic reagents), which makes the process more environmentally friendly. Although this calls for the use of enzymes, hydrolysis of cellulose is a highly complex multienzymatic process. Besides, cellulose in the lignocellulosic matrix forms complexes with other substances, which must be untangled via a choice of adequate pre-treatments, such as steam pretreatment, hydrothermolysis, or dilute (catalytic amounts) acid hydrolysis (Dekker and Wallis, 1983; Dekker, 1991; Söderström *et al.*, 2003), to render the raw cellulose digestible to cellulases. In contrast to the lignin degrading enzymes, industrial cellulases and xylanases are well known commercial products, tailor-made according to the target commercial application. The importance and interest on enzymatic biomass hydrolysis has renewed and concentrated research focus on cellulases in different areas: the search for high cellulase producing organisms, the production of hypercellulolytic mutants of organisms suitable for cellulose production, genetic engineering for the construction of high cellulose producing organisms with high specific activity, and theoretical studies on the mechanism of the action of a multienzyme system on a complex polymer.

Pre-treatment and hydrolysis of lignocellulose releases hexoses and pentoses that are efficiently, nevertheless individually, fermented to ethanol by

the yeasts *Saccharomyces, Pichia* and *Pachysolen*, respectively (Dekker, 1982). However, in order to have an efficient fermentation of lignocellulose-derived substrates, it is necessary to develop strains with a broad substrate-utilization range, capable of using hexose and pentose sugars simultaneously, presenting minimal nutrients requirement, and growing at low pH and high temperature (Hahn-Hägerdal *et al.*, 1994; Zaldivar *et al.*, 2001; Hahn-Hägerdal *et al.*, 2001). To date, the lack of a microorganism able to ferment efficiently all sugars, of six and five carbons, released by hydrolysis from lignocellulose, has been one of the main factors preventing the large scale utilization of lignocellulose to produce fuel ethanol.

The term holocellulose is used to describe the total carbohydrate content of lignocellulose (Freer *et al.*, 1998) and the material that is obtained after the removal of lignin (Zhang *et al.*, 2006). The occurrence, abundance, distribution and structure of holocellulose, the most abundant form of carbon in woody and non-woody plant tissues, is dependent on the species, tissue and growth conditions of the plant. Agricultural and forestry residues, municipal solid waste, industrial processing residues, and energy crops are all sources of holocellulose materials that can be used to obtain chemical feedstocks, fuels, foods and feeds (Filho, 1998; Galante *et al.*, 1998a, b). Depending upon the feedstock, holocellulose is composed of cellulose, hemicellulose and/or pectin, corresponding to 55% to 80% of the lignocellulose dry mass (Freer *et al.*, 1998). For economical utilization of holocellulose wastes, conversion of both cellulose and hemicellulose to useful products is required as sources of fermentable sugars (Kitpreechavanich *et al.*, 1992).

11.2. HOLOCELLULOSE-DEGRADING ENZYMES

Microorganisms able to degrade holocellulose produce a battery of hydrolytic enzymes. Most of these enzymes and microorganisms have been object of a great variety of studies, including production, purification, and characterization, besides regulation of gene expression, gene cloning, and heterologous expression (Penttilä *et al.*, 1986; Penttilä *et al.*, 1987; Penttilä *et al.*, 1988; El-Gogary *et al.*, 1989; Henrissat *et al.*, 1989; Claeyssens *et al.*, 1990; Bailey *et al.*, 1993; Davies *et al.*, 1993; El-Dorry *et al.*, 1996; Ilmén *et al.*, 1997; Hayashida *et al.*, 1998; Kubicek and Penttila, 1998; Mäntylä *et al.*, 1998; Okada *et al.*, 1998; Takashima *et al.*, 1998; Coutinho and Henrissat, 1999; Boer *et al.*, 2000; Park *et al.*, 2000).

The complexity and heterogeneity of holocellulose structures demands the synergistic action of main- and side-chain attacking enzymes with different specificities for its complete hydrolysis. The inter-chain hydrogen-bonding on cellulose crystalline, along with higher-order structures in the plant cell wall, are recognized as factors for holocellulose recalcitrance to enzymatic hydrolysis (Himmel *et al.*, 2007). Besides, xylan is thought to present a close interaction with cellulose through hydrogen-bonding (Filho, 1994).

Although extensive work has been done in the fields of cellulose and hemicellulose degradation and kinetics (Medve *et al.*, 1998; Nojiri and Kondo, 1999), the enzyme systems that facilitate holocellulose degradation to simpler constituents have not been fully characterized. Nevertheless, enzymes can be split up into main-chain- and side-chain-cleaving enzymes and exo- and endo-acting enzymes. The exo-hydrolases cleave terminal linkages and release monosaccharide units from the reducing or non-reducing end, while the endo-hydrolases attack internal bonds at random or at specific positions (Filho, 1998). Endo-enzymes affect an initial decrease in the average degree of polymerization of the substrate with formation of soluble short-chain oligosaccharides that are degraded by the exo-type enzymes (Filho, 1994). The holocellulose-degrading enzymes include endo-type (β-xylanase, EC 3.2.1.8; β-mannanase, EC 3.2.178; α-arabinanase, EC 3.2.1.99; β-galactanase, EC 3.2.1.89; β-glucanase, EC 3.2.1.4) and exo-type enzymes (β-xylosidase, EC 3.2.1.37; β-mannosidase, EC 3.2.1.25; cellobiohydrolase, EC 3.2.1.91; β-glucosidase, EC 3.2.1.21; α-galactosidase, EC 3.2.1.22). Additional enzymes such as acetyl xylan esterase (EC 3.1.1.72), α-arabinofuranosidase (EC 3.2.1.55), acetyl mannan esterase (3.1.1.6), feruloyl and p-coumaroyl esterases (3.2.1.73) and α-glucuronidase (EC 3.2.1.139) are required to remove side-chain substituents that might be attached at various points on the holocellulose structure, creating more sites for subsequent enzymic hydrolysis (Filho, 1998). The side-chain substituents on holocellulose may strongly inhibit the action of endo-enzymes, preventing the complete degradation of the polymer structure to its basic units because they lack uninterrupted sequences of similar sugars and linkages that are subject to enzyme attack (Coughlan, 1992). Some endo-enzymes need considerably long unsubstituted stretches for activity. Moreover, the presence of side-chains restricts the enzymatic hydrolysis of some holocellulose components and represents a technological barrier that retards the development of various industrial processes (Numan and Bhosle, 2006).

A mechanism for hemicellulose degradation involves an initial decrease in the average degree of polymerization of the substrate, with formation of short soluble oligosaccharides that are degraded by the side group-cleaving enzymes. In the above context, the endoxylanases cleaves the β-1,4 bonds in the xylan main-chain polysaccharide, liberating a mixture of xylo-oligosaccharides, while β-xylosidase hydrolyses the xylo-oligosaccharides from the non-reducing end to produce xylose (Iembo *et al.*, 2006). Accessory enzymes, such as α-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and p-coumaric esterase remove substituents that are attached at various points on xylan. The cellulase system is composed of endo-1,4-β-glucanases (EGs), exo-1,4-β-glucanases (cellobiohydrolases, CBHs) and β-glucosidases (Lynd *et al.*, 2002; Pérez *et al.*, 2002; Zhang *et al.*, 2006). EGs hydrolyse at random the amorphous regions of cellulose, liberating soluble cellodextrins of various lengths and creating new terminal ends on the insoluble substrate. CBHs act in a processive way from the reducing or nonreducing ends of cellulose chains, releasing glucose or cellobiose as major products (Lynd *et al.*, 2002). Only CBHs are considered to be capable of degrading

crystalline cellulose. This depolymerization step is rate-limiting for the complete degradation of cellulose. β-Glucosidases break down cellobiose to glucose. In some cases, β-glucosidases are also active on longer cellodextrins (Zhang *et al.*, 2006). As an example, the composition of the cellulase complex (with exception of the β-glucosidase) produced by *Trichoderma reesei* (recently denominated as *Hypocrea jecorina*), and the properties of the individual enzymes are shown in Table 11.1 below.

As described earlier, the extensive hydrolysis of holocellulose requires the synergistic action of a variety of endo- and exo-enzymes of different specificities, including main-chain and side-chain enzymes. According to Coughlan *et al.* (1993), the synergism is only detected when the total products formed by two or more enzymes acting together exceeds the arithmetic sum of the products formed by the action of each individual enzyme. A clear example of synergism involves the interaction between two or more different types of side-chain enzymes, or between two or more types of main-chain enzymes. In this case, the enzymatic interactions are classified as homeosynergy (Coughlan *et al.*, 1993). Heterosynergy is defined as the interaction between a side-chain and main-chain enzyme. In the case of cellulases, two classes of synergism, endo-exo synergism (the cooperation between endoglucanases and exoglucanases), and exo-exo synergism (the cooperative action between two cellobiohydrolases during hydrolysis), were described. Synergism is essential for the effective degradation of crystalline cellulose. It is highest on semicrystaline cellulose of high degree of polymerization, lower on amorphous cellulose, and non-existent on soluble cellulose substrates (Anu Nutt, 2006; Henrissat *et al.*, 1989; Nidetzky *et al.*, 1993; Samejima *et al.*, 1997).

The enzymatic break down of holocellulose main structure operates via a lysozyme type mechanism of glycosidic bond cleavage. Most of the holocellulose-degrading enzymes act via a two consecutive single displacements mechanism in which anomeric configuration is retained, while some few show single displacement reactions with the inversion of configuration (Coughlan, 1992; Coughlan *et al.*, 1993). Both mechanisms are acid-base mechanisms and involve an oxacarbonium ion intermediate and a pair of carboxylic acid at the active site (Rye and Whiters, 2000). In the case of the double displacement mechanism (S_N2-type, nucleophilic substitution), the oxacarbonium ion stabilization occurs by electrostatic interaction with the carboxylate of an asparagine or glutamate at the active site and formation of a covalent intermediate by nucleophilic attack of the asparagine or glutamate on C-1 of the incipient reducing sugar (Coughlan, 1992). In the single displacement mechanism (S_N1-type) one carboxylic acid acts as a general base and the other as a general acid (Rye and Whiters, 2000).

11.3. HOLOCELLULOSE-DEGRADING ENZYME APPLICATIONS

Research and development activity around the holocellulose-degrading enzyme began in early 1980 and has been intense in the last twenty years

(Bhat, 2000). Today, holocellulose-degrading enzymes are used in widespread industrial applications (Bhat, 2000; Zhang *et al.*, 2006), including use in animal feed, food, brewery and wine, textile, laundry, fuel and pulp and paper industries (Howard *et al.*, 2003). According to Wong and Saddler (1993), enzymes like hemicellulases and cellulases are probably more useful when selective removal of holocellulose is required for the production of certain materials that have high commercial value. It should be noted that the industrial application of the holocellulose-degrading enzymes is not only restricted to their individual components, but it can also be in combination along with other enzyme systems, such pectinases, proteases, esterases, and amylases. For example, in the detergent industry protease, cellulase, lipase, and amylase preparations are used in the production of biological washing powders.

Public concerns about the impact of the release of environmentally hazardous compounds and cost issues have subjected conventional chemical processes to considerable scrutiny. With environmental pressure, industrial enzyme application is gaining ground rapidly due to the various advantages that it offers over conventional technologies. Enzyme stability is a crucial point at the industrial level because the use of higher temperatures prevents microorganism contamination and allows higher reaction rates and increased hydrolytic efficiency (Filho, 1994). However, the enzyme production cost is still prohibitive, being the major impediment to an extensive commercialisation (Howard *et al.*, 2003; Schülein, 2000). For instance, in some cases the cellulase cost represents 50% of the total hydrolysis process cost. According to Howard *et al.* (2003), the efforts on lowering the cost of enzymes must be concentrated on screening for organisms with novel enzymes, strain improvement of existing industrial organisms, and enzyme engineering, production, and operation related factors such as choice of substrate, culturing conditions, recycling of enzymes, and redesigning of processes. Enzymes are sensitive to environmental factors such as temperature, pH, and ionic strength. Thus, when developing industrial or analytical applications, the enzyme must be stable and catalysis performed efficiently under the selected conditions (Charnock and McCleary, 2006).

According to Bhat (2000), the world market in 1995 for industrial enzymes was above 1.0 billion U. S. dollars, where 20% was attributed to cellulase, hemicellulase, and pectinase sales. This market increased to around 1.6 billion in 2000 and 2.0 billion in 2005. Presently, it is estimated at 4 billion U. S. dollars, 60% of which is for industrial enzymes (Costa *et al.*, 2007). The world demand for enzymes is expected to rise 6.5% annually, to nearly 5.1 billion U. S. dollars, in 2009, according to the market researcher Freedonia Group (www.fredoniagroup.com). In this context, hydrolases represent 75% of the industrial enzymes, and carbohydrases are the second largest group of industrial enzymes. On a worldwide level, industrial enzymes are produced in Europe (60%); the balance is from the U. S. A. and Japan (40%).

Carbohydrases will continue to be the most widely used enzymes, in part because of their extensive use in the processing of natural materials, which include the most easily available biopolymers and renewable resources. In this enzyme group, cellulases contribute with 8% of the worldwide industrial enzyme demands, being their major application in animal feed. Historically, enzyme demand has been concentrated on the more developed economies due to the high value-added nature of enzymes, and the significant technical resources needed for their development, production and application. However, the field of industrial enzymes is now experiencing major research and development initiatives, resulting in both the development of a number of new products and the improvement and optimisation of the industrial process. Countries such as China, India, South Korea, and Taiwan, which have recently emerged as industrialised manufacturing centres with strong national research and development programs, will play a much larger role in the world market going forward and offer some of the best growth opportunities. In Latin America, enzyme demand is concentrated mainly on Brazil, Chile, and Argentina (Costa *et al.*, 2007). For these more industrialised Latin America countries, the enzyme demand was 124 million U. S. dollars in 2004, and represented only 3.4% of the world market. Brazil is responsible for 60% of the Latin America enzyme demand. However, it shows a small external enzyme market that reached 147.2 million U. S. dollars in 2005, and corresponds to 3.7% of the international market (Costa *et al.*, 2007). In this case, 126.6 million U. S. dollars were from import and 20.6 U. S. dollars from export. Speciality enzymes (those for the diagnostic, pharmaceutical, research and biotechnology markets), will continue to benefit from advances in biotechnology that facilitate new application development, thereby expanding potential demand. The international market for speciality enzymes is estimated at 1.4 billion U. S. dollars, and corresponds to approximately 40% of the world enzyme market (Costa *et al.*, 2007). The world demand for speciality enzymes is predicted to achieve an annual growth rate of 7.9% and 2 billion U. S dollars in 2009. In contrast, industrial enzymes will experience more moderate growth as the maturation of key markets limits advances. In Brazil, contrary to the major world enzyme markets, the demand is concentrated mainly on industrial enzymes. Foods and beverages continue to be the largest enzyme markets, with gains in Asia/Pacific, Eastern Europe and Latin America more than offsetting weakness in developed countries. According to a report from 2002 (Freedonia (www.fredoniagroup.com), August 1, 299 pages—Pub. ID: FG802417), U. S. demand for enzymes, including industrial and speciality, was predicted to grow 7% per annum through 2006, driven by accelerating growth in the biocatalyst sector and continued strength in the pharmaceutical market. Cellulase market has been estimated in the U. S. A. to be as high as U. S. 400 million dollars per year, representing an increase of approximately 33% in the total U. S. industrial enzyme market (van Beilen and Li, 2002; Wolfson, 2005; Zhang *et al.*, 2006). The development of cellulase application as speciality enzyme has increased considerably, especially in sugar cane processing where vast amounts of renewable biomass are

available for conversion to ethanol (Gong *et al.*, 1999). Between2004–2014 an increase of approximately 100% in the use of cellulase as a speciality enzyme is expected (Costa *et al.*, 2007).

Stunning progress in the engineering of enzymes using advanced biotechnology, like directed evolution and rational design studies, continue to open new markets, with enzymes being increasingly tailored for specific applications and higher activities. These improved enzyme preparations must present different characteristics, such as higher catalytic efficiency, increased stability at elevated temperatures and at a certain pH, and a higher tolerance to end-product inhibition (Zhang *et al.*, 2006). Protein engineering of holocellulose-degrading enzymes, including mutagenesis of potential active centre residues with subsequent kinetic analysis, has been used as a tool in the study of the catalytic mechanism and improvement of some properties of industrial enzymes (Schülein, 2000). The construction and analysis of site-directed variants for structural and mechanistic analysis have been successfully carried out in cellulase engineering. In addition, analysis of the determinants of substrate specificity has led to the engineering of enzymes with modified functions (Schülein, 2000).

11.4. ENZYME PRODUCTION

There is a huge potential market for holocellulose-degrading enzymes, and over the years a number of available commercial enzyme preparations have been produced in good quantity by microorganisms from different sources. The high cost of enzymes needed for the hydrolysis of holocellulose requires the production of low cost holocellulose-degrading enzymes and the construction of an efficient holocellulolytic microorganism. Advances in the large-scale production of cellulases and hemicellulases enable efficient screening for new enzymes. Holocellulose-degrading enzymes can be produced by solid-state and submerged liquid state fermentation: for enzyme production, solid-state fermentation has some advantages over liquid-state cultivation (Considine and Coughlan, 1989; Tuohy *et al.*, 1989; Techapun *et al.*, 2003). It is less equipment-orientated (and hence more applicable in less-developed or less-sophisticated situations), aeration requires lower pressure than that needed for liquid cultivation, and vigorous agitation is not necessary. Other advantages are lower cost, improved enzyme stability, generation of a protein-enriched by-product for use as animal feed, and easier downstream processing. However, most of the enzyme production procedures have used liquid cultivation, where it is easier to control the environmental factors required for cell growth and enzyme production. For both solid- and liquid-state cultivations, the control of parameters such as moisture content, depth of culture, O_2 and CO_2 transfer, temperature, and pH under optimum limits is necessary to

achieve a maximum yield of enzyme (Considine and Coughlan, 1989; Tuohy *et al.*, 1989).

Driven by commercial demands for enzymes that can operate under certain process conditions, a number of microorganisms have been selected, in particular those of mesophilic and thermophilic origin. Many microorganisms are known to produce enzymes capable of breaking down one or more constituents of holocellulose materials. Most of the commercial enzyme preparations are mixtures of more than one of the groups of holocellulose-degrading enzymes. The composition of a mixed enzyme preparation for a certain application can be affected by the suitable choice of the microorganism and the production conditions (Linko *et al.*, 1989). Therefore, the yield and composition of enzymes produced by fermentation/growth on a specific carbon source is greatly influenced by the choice of microorganism, substrate, and cultivation system (Considine and Coughlan, 1989). The best known producers of holocellulose-degrading enzymes are strains of *Trichoderma* and *Aspergillus* species. Other sources of holocellulose-degrading enzymes include strains of *Humicola, Talaromyces, Acrophialophora, Thermoascus, Bacillus,* and *Penicillium* species.

The relationship between fungal growth conditions and cellulase production has been discussed. From theoretical assumptions it has been suggested that to be commercially successful it is necessary to produce 1100 filter paper units $L^{-1} h^{-1}$. This amount of enzyme requires a $70 g L^{-1} h^{-1}$ growth rate of the culture. Under most favourable conditions fungal cultures produce $35 g L^{-1} h^{-1}$ of cell mass. This gap could be closed if a selected organism shows any one or both of the following properties: that it has a high enhanced capacity for cellulase production, or it has the ability to produce enzymes with a high specific activity. These desirable properties may be achieved by either new strains selection and/or strain improvement (Gosh and Gosh, 1992).

11.5. IMPORTANCE OF SUBSTRATE BINDING DOMAINS IN HOLLOCELLULOSE DEGRADATION

A basic principle of biocatalytic processes is the formation of a transition state (ES) between substrate (S) and enzyme (E), signalling molecular recognition of the substrate by the enzyme and a relatively good fit of the substrate within the catalytic site of the biocatalyst. In the case of polymer degrading enzymes, where the substrate is insoluble (cellulose, xylan), the approximation between enzyme and substrate is more difficult than with soluble substrates, and the adsorption of these enzymes on their substrates is a prerequisite step for hydrolysis. The adsorption and binding of cellulases to their substrate is generally mediated by a small (30 to 240 amino acids) non-catalytic peptide called carbohydrate binding module (CBM), for cellulases more specifically denominated as cellulose-binding domain (CBD). Almost always the CBD is bound to the core protein (catalytic domain) by a smaller linker peptide

rich in prolines, serines, and threonines, and with a length of approximately 5 to 59 amino acids. Most of the CBDs are either bound to the N- or C-terminus of the catalytic domain, but also internal functional CBDs exist (Gilkes *et al.*, 1991; Kilburn *et al.*, 1993; Boraston *et al.*, 2004).

Xylanases, glucoamylases, and chitanases are other examples of enzymes which are composed of distinct domains, of which at least one is a CBM. The existence of a multidomain structure emphasizes the functional importance of the separate domains in the breakdown of insoluble polymers (Reinikainen, 1994). Boraston and co-workers (Boraston *et al.*, 2004), having recently reviewed the literature on carbohydrate binding modules, suggest that CBMs have three general roles with respect to the function of their cognate catalytic modules: *i)* a proximity effect, *ii)* a targeting function and *iii)* a disruptive function.

The binding of the CBMs to the specific polysaccharide increases the hydrolase concentration on the surface of the polymeric substrate, maintains the enzyme in proximity to the substrate and leads to a more rapid polysaccharide degradation. Proteolytic or genetical removal of the CBM from the catalytic domain has decreased the activity of the enzymes on insoluble substrates, but not on soluble polysaccharides (Boraston *et al.*, 2004). In the presence of the CBM the overall binding efficiency of a cellulase is enhanced, and, in general, a higher activity towards insoluble cellulose is observed. Many but not all cellulases contain a CBD, which is believed to play an important role in cellulose hydrolysis (Kilburn *et al.*, 1993). Most CBDs have a high affinity for crystalline cellulose although specificities have been reported. All cellulases that contain a CBD are active against crystalline cellulose, which suggests that the CBD has a functional role in the breakdown of cellulose crystals (Reinikainen, 1994).

11.5.1. CBM Structure and Mode of Interaction between CBM and Substrate

Depending on type, CBMs might bind to the surface of crystalline polysaccharide, interact with single polysaccharide chains, or bind to non-crystalline regions of the polysaccharide. With other words, these non-catalytic modules can recognize distinct regions of the same polysaccharide, and the same catalytic module can display different catalytic capacities and a different catalytic behavior when bound to different CBMs, such as it was shown for the endoglucanase CelD of *Clostridium thermocellum* and 4 CBDs of different origin (Carrard *et al.*, 2000). This binding specificity of CBMs is important for targeting the hydrolytic enzyme in a complex substrate (such as plant cell wall) to the right substrate. Thus, cellulases, xylanases, and mannanases contain CBMs that bind to cellulose, xylan, and mannan, respectively. Furthermore CBMs that apparently bind to the same polysaccharide display clear differences in specificity, and bind to different regions of—for example—non-crystalline

cellulose, which influences the ability of the enzyme to hydrolyse the polysaccharide (Boraston *et al.*, 2003).

According to structural and functional similarities a classification of CBMs in "surface-binding" CBMs (Type A), "glycan-chain-binding" CBMs (Type B), and "small-sugar-binding" CBMs (Type C) was proposed (Boraston *et al.*, 2004). Type A CBMs have a planar or platform-like binding site fitting well to the flat surfaces of cellulose or chitin crystals, but with little or no affinity for soluble carbohydrates (Nagy *et al.*, 1998). The binding location of Type A modules to *Valonia* crystalline cellulose was shown to occur on the hydrophobic face (Lehtiö *et al.*, 2003) of the cellulose and the interaction is associated with positive entropy (Creagh *et al.*, 1996).

Carbohydrate-binding sites of Type B CBMs are extended (>1.5 nm), often described as grooves or clefts, and comprise several subsites able to accommodate the individual sugar units of the polymeric ligand. In sharp contrast with the Type A CBMs, direct hydrogen bonds also play a key role in the defining the affinity and ligand specificity of Type B glycan chain binders.

The Type C CBMs were first discovered as lectins with small-sugar-binding activity and have only subsequently been included as CBMs due to their discovery in a number of glycoside hydrolases. This family of CBMs is found exclusively in xylanases. Differences between Type B and Type C CBMs can be small, but the hydrogen-bonding network between protein and ligand is more extensive in Type C CBMs than in Type B modules.

Aromatic amino acid side chains and their orientation play a crucial role in carbohydrate binding; their specific interaction with the ligand is ubiquitous to CBM carbohydrate recognition. The side chains of tryptophan, tyrosine, and, less commonly, phenylalanine, form the hydrophobic platforms in CBM-binding sites, which can be planar, twisted, or form a sandwich (Boraston *et al.*, 2004).

Based on amino acid sequence similarity, CBMs have been classified until the time of writing (April 2007) into 49 families (http://www.cazy.org/CAZY/). CBDs contain usually low contents of charged amino acids and conserved aromatic amino acids, asparagines, and glycine residues. Bacterial CBDs are approximately three times the size of the fungal CBDs (Reinikainen, 1994).

Proteins interact with carbohydrates involving charged and aromatic amino acids able to establish hydrogen bonds and hydrophobic interactions (Van der Waals forces) (Reinikainen, 1994). For *Trichoderma reesei* CBDs, chemical modification and mutagenesis studies have shown the importance of 3 conserved tyrosine residues (Claeyssens and Tomme, 1989; Reinikainen, 1994), which are arranged on the flat hydrophilic face of the wedge-shaped structure of the 36 amino acid containing peptide. It is this flat hydrophilic surface of the CBD, that is responsible for the productive cellulose-CBD interaction, whereas the rough, more hydrophobic CBD surface is not in direct contact

TABLE 11.1. Overview of data of cellulases from the white-rot/soft-rot fungus *Trichoderma reesei* (more recently denominated as *Hypocrea jecorina*).

Enzyme (older names)	Specific data	Mechanism of hydrolysis	Activity and substrates	Cinetics
Cel7A (CBH I)	up to 60% of total secreted protein 57 kDa+ pI 3.9+ CBD (C-terminal)	• from reducing ends • retention of anomeric configuration • S_N1	• Does not degrade β-glucan or substituted cellulose • No or very low EG activity • Generates reducing ends • Degrades crystalline cellulose without other enzymes • Also transglycosylation	low k_{cat}
Cel6A (CBH II)	~20% of total secreted protein Various isoenzymes (pI 5.1–6.3; pI 5.9+) (difficult to purify) 53 kDa+ pI 5.9+ CBD (N-terminal)	• From non-reducing end • Single displacement—Inversion of the anomeric carbon	• Attacks amorphous and crystalline cellulose • Active on β-glucan	
Cel7B (EG I)	5–10% of total secreted protein only one gene, but size varies between 43–58 kDa and pI between 4–6 55 kDa+ pI 4.5+ CBD (C-terminal)	Retaining glycosylase Random attack S_N1	• Activity on substituted cellulose (CMC and HEC) • Highly active on amorphous cellulose • Some activity on Avicel (semi-crystalline) • Active on barley β-glucan • Active on xylan (is an effective xylanase) • Also transglycosylation	

Cel5A (EG II; formerly EGIII)	48 kDa, 50 kDa⁺ pI 5.1; pI 5.5⁺ CBD (N-terminal)	Double displacement, retention of configuration	• Active on substituted cellulose, β-glucan and avicel	Slightly lower activity than EG I
Cel12A (EG III)	20–25 kDa; 25 kDa⁺ pI 7,5⁺ no CBD minor quantity non-glycosylated protein	Double displacement, retention of configuration	• Negligible filter paper activity • Active on CMC and HEC • Produces more than 10% of reducing sugars as glucose • Active on barley β-glucan • Erodes microfibrils • Attacks amorphous regions • Hydrolyses also β-1,3–1,4 glucan, xyloglucan and xylan	
Cel61a (EG IV)	55 kDa⁺ CBD (C-terminal)		• Very low activity if compared to Cel 7B	
Cel45A (EG V)	36 kDa⁺ pI 2.1⁺ CBD (C-terminal)		• Active on barley β-glucan • Active on HEC • Hydrolyses glucomannan (glucomannanase activity) • Lower activity on cellulose than other EGs • Main product is C_4 • Does not hydrolyse C_3, C_4 and C_5	

Source: Adapted from Reinikainen, 1994, and Nutt, 2006⁺.

C_3 cellotriose.

C_4 cellotetraose.

C_5 cellopentaose.

with cellulose. The interaction between the aromatic ring and a glucose residue arises from ring current polarization attraction involving the delocalized π-electrons in the side chain and the pyranose ring of the glucose residue. However, a perfect crystal exposes only a small area on the obtuse corner where the ring current polarization interaction is possible, thus other interactions such as hydrogen bonding must also be involved, which could also explain the pH dependence of the apparent affinities. The spacing and alignment of the tyrosine residues of the CBD of CBH I from *T. reesei* is perfect for achieving multiple interactions with the glucose residues on the cellulose crystal (Reinikainen, 1994). In other CBMs, such as those from *C. fimi* Cen A (Kilburn *et al.*, 1993; Din *et al.*, 1994b) or *P. fluorescence* xylanase XYLA (Poole *et al.*, 1993) conserved tryptophan residues are important for the interactions.

Despite of their lack in hydrolytic activity, the CBDs of the cellulases CenA and Cex from *C. fimi* have been found to be capable of disrupting cotton fibers and releasing small particles from the substrate (Kilburn *et al.*, 1993; Din *et al.*, 1994a). This disruptive effect does not seem to be a general characteristic of CBM, since it was only found with CBD from cellulose from a *Penicillium sp.* (Gao *et al.*, 2001). Anyhow, a synergism with the catalytic module and enhanced degradation capacity were reported (Din *et al.*, 1994a).

Once adsorbed to their substrate, cellulases, like other enzymes acting on polymeric substrates, are thought to work processively, *i.e.*, they perform various subsequent hydrolytic steps on the same carbohydrate chain without dissociating from the substrate. This feature seems to be only valid for exo-cellulases and cellobiohydrolases that attack the substrate from the reducing or non-reducing end of the polymeric chain. Adsorption of the CBD (36 amino acids) of CBH I, the main CBH of *T. reesei*, was reported to be fully reversible, that means, desorption of the CBD occurs easily on dilution and equilibrium is attained fast. It was also suggested that for a given CBD a compromise exists between exchange rate and the degree of partitioning of the CBD between cellulose surface and liquid phase, so that the CBD binding would not be rate limiting for the processive action of the exoglucanase (Linder and Teeri, 1996). The authors concluded that the CBD alone could not be responsible for findings of apparent irreversible binding, and suggested that the CBD increases the concentration of the catalytic domain on the substrate and makes further interactions of the second domain with the substrate possible. Despite of the reported reversibility of the adsorption of *T. reesei* CBDs (Linder and Teeri, 1996), other CBDs, especially of longer chain length, such as those of *C. fimi*, bind much stronger to the cellulosic substrate and uncomplete or very little desorption occurs, even under alkaline conditions, or in the presence of additives (Andreaus *et al.*, 2000a, 2000b; Otter *et al.*, 1984, 1989). The strong binding of the cellulases to the cellulose via CBD can lead to a population of nonproductively bound enzymes (Stahlberg *et al.*, 1991).

11.5.2. Non-productive and Non-specific Binding of Cellulases

Beside the interactions of holocellulose degrading enzymes with the carbohydrate substrate, these enzymes can also establish similar but non-specific and non-productive (non-catalytic) interactions with synthetic dye molecules or lignin components of lignocellulosic materials. Regions rich in closely located aromatic (especially Tyr, Phe) and other non-polar amino-acid residues (indigo-binding sites) on the protein surface of the core domain were reported to be responsible for hydrophobic interactions with the indigo dye (indigo-binding sites), and various dye molecules could be adsorbed onto one enzyme molecule (Campos *et al.*, 2000; Gusakov *et al.*, 2000, 2001). In general, cellulase adsorption to indigo was less specific than cellulase-cellulose adsorption (Gusakov *et al.*, 2001). These interactions were found to be critical to render soluble the indigo and its removal from dyed cotton yarns during the stone-washing of denim, a textile process used to give indigo dyed cotton fabrics used for blue jeans an aged look. Enzymes without a CBD gave the best results in this washing process, without causing redeposition of the indigo dye on the cellulosic substrate (Andreaus *et al.*, 2000a).

Higher amounts of lignin in lignocellulosic material are generally associated with reduced saccharification of biomass cellulose (Coughlan, 1992), and delignification improves biomass to sugar conversion (Baker, 1973; Schwald *et al.*, 1988). However, this could not be verified in an experimental design, where up to 40% (w/w) of noncellulosic lignocious residue was added to a cellulose-cellulose reaction mixture to investigate its impact on cellulose saccharification in a typical biomass-to-ethanol process (Meunier-Goddik and Penner, 1999). The unproductive adsorption of cellulases to lignin in lignocellulosic substrates seems to be a major reason for reduced enzyme activity and lower hydrolyses rates and sugar yields. Adsorption studies of purified *T. reesei* cellulases CBH I and EG II and its catalytic domains demonstrated that CBH I and its catalytic domain have a higher affinity for steam pretreated softwood (SPS) than EG II or its core domain. Binding to SPS was significantly decreased for both enzymes, when only the catalytic domain without the CBD was used. Both enzymes were also found to adsorb on different lignin preparations, but only the catalytic domain of EG II was able to adsorb to alkaline isolated lignin with a high affinity, whereas the CBH I core protein did not adsorb to any of the lignins tested. CBDs seem to play a major role in the non-specific and unproductive adsorption of cellulases to Lignin (Palonen *et al.*, 2004).

Lignin was also supposed to cause steric hindrance to cellulases during softwood hydrolysis (Ramos *et al.*, 1992; Mooney *et al.*, 1998, 1999) due to encapsulation of the cellulose component so that the cellulose is less accessible to the biocatalyst (Fan *et al.*, 1980).

As discussed below in more detail surfactants and proteins might reduce the negative effect of unproductive binding on hydrolysis.

An interesting approach to explain the observed substrate inhibition in cellulose degradation was presented by Väljamäe *et al.* (2001). Finding that the maximum adsorption capacity of CBH I from *T. reesei* on bacterial cellulose ($6.8\,\mu mol\,g^{-1}$) was higher than the total amount of reducing ends ($4.8 \pm 0.4\,\mu mol\,g^{-1}$), and considering that for steric reasons it was unlikely that each reducing end was carrying one enzyme molecule, they concluded that most of the CBH I molecules were anchored via the CBD in a non-productive mode without access to free chain ends. They also found that increasing the surface density of chain ends by previous treatment of the bacterial cellulose with an endoglucanase enhanced the CBH I activity and the production of reducing soluble sugars, whereas a lower chain end density could not be compensated for by increasing the substrate concentration in order to obtain a higher absolute number of chain ends. Since cellulases have been proposed to be capable of lateral diffusion (two-dimensional diffusion) on the cellulose surface (Jervis *et al.*, 1997), Väljamäe *et al.* (2001) suggested that the nonproductively bound CBH I molecules are in a "stand-by" status and might diffuse or migrate to free chain ends, where they can start their progressive action on the substrate. The probability and velocity (average time needed for migration) with which this might occur depends on the average distance between the location of the nonproductively bound cellobiohydrolases and the available chain ends, and can be augmented by the introduction of more chain ends on the same surface, *i.e.*, by increasing the surface density of chain ends. Thus, the observed apparent substrate inhibition with increasing substrate concentration was attributed to an increased average distance (*i.e.*, lateral diffusion time) between the nonproductive binding site of the CBH and the free chain ends of the cellulose. Higher endoglucanase contents in binary mixtures with CBH I increased the optimum substrate concentration, due to the increase in surface density of chain ends by the action of the endoglucanase. The described substrate inhibition was not observed with a CBDless CBH I, which supports the importance of the CBD for unproductive binding. Furthermore, the substrate inhibition depends also on the substrate and the reaction temperature. It was not observed for the more crystalline bacterial microcrystalline cellulose, which was prepared from the same bacterial cellulose by acid removal of the amorphous parts, which are supposed to be the main targets for endoglucanases and neither at higher reaction temperatures (50 °C instead of 25 °C) (Väljamäe *et al.*, 2001). The first observation implies that endoglucanases are less important for the degradation of crystalline cellulose, whereas the second was attributed to an enhancement of lateral diffusion and a larger relative contribution of cellulose degradation resulting from three-dimensional diffusion (approximation of the enzyme to the available ends directly from solution instead from the pre-adsorbed state) with an increase in temperature. These observations and conclusions elucidate quite clearly the mechanism of synergism between endo- and exo- activities of cellulases, investigated by many authors (Coughlan, 1992; Nidetzky *et al.*, 1993, 1995; Kleman-Leyer *et al.*, 1996; Cavaco-Paulo, 1998; Bhat, 2000).

11.6. DECISIVE CONTROL PARAMETERS IN THE USE OF HOLOCELLULOSE DEGRADING ENZYMES

Beside the typical control parameters in biocatalytic or enzymatic reactions (such as pH and temperature) which are intrinsic characteristics of the individual enzymes, other parameters such as the ionic strength of the reaction medium, complexing agents, surfactants, solvents, special metal ions, and mechanical agitation might have a significant and decisive impact on catalysis and product yields. Concerning the temperature dependence of cellulose hydrolysis, it is interesting that in the case of *T. reesei* cellulases (see also Table 11.1), EG II has a higher thermostability (10–11 °C) and is more active against avicel at higher temperatures than the other three main cellulases (EG I, CBH I and CBH II) from the cocktail produced by the fungus (Baker *et al.*, 1992). Since effective holocellulose degradation depends on the cooperative action and synergism of various enzymes, differences in the pH and temperature profiles of individual enzymes in a natural or synthetic mixture of biocatalysts might be of greater importance, if the process could be adjusted to exploit better the individual characteristics of the enzymes.

11.6.1. Mechanical Agitation, Shear Forces and Ultrasound

Considering the heterogeneous character of the hydrolysis of the insoluble polymer cellulose by soluble enzymes, adequate mixing is required to provide sufficient contact between substrate and enzymes and to promote heat and mass transfer within the reaction mixture, and is expected to affect the rate and extent of cellulose hydrolysis. However shear forces, such as applied by a rotary shaker (Reese, 1980) or by a six-bladed turbine impeller within a glass reactor (Gunjikar *et al.*, 2001), were reported to denature and/or inactivate cellulases. Especially the exo-1,4-β-D-glucan-4-cellobiohydrolase or avicelase (cellulase whose activity is measured on microcristalline avicel), the major component of *T. reesei* cellulases, was deactivated most rapidly, but endoglucanase and cellobiase activities are also affected. Inactivation increases with increasing shaking or agitation speed, and occurs also in the presence of substrate (Reese, 1980; Gunjikar *et al.*, 2001). The deactivation constant increases sharply when the shear stress exceeds 15 dyn/cm^2 (Asakura *et al.*, 1978). However, for the application of cellulases on cellulosic textile substrates, to improve fabric appearance by removal of fuzz fibers and pills, or to achieve the stone-washed look of denim garments, mechanical agitation is essential. Higher levels of mechanical agitation were reported to enhance endoglucanase activity, especially relative to cellobiohydrolase activity. During the treatment of cotton fabrics with *T. reesei* cellulases (total crude mixture) high mechanical agitation increased the amount of reducing end groups on cotton fibers constantly, whereas at low mechanical agitation the number of reducing end groups diminished after the initial increase. The increase in reducing fiber end groups was attributed to EG activity, whereas the reduction of the amount

of reducing fiber end groups was attributed to CBH activity (Cavaco Paulo, 1998). Mechanical agitation seems also to remove preferentially microfibrillar material and helps to break off weak, enzymatically eroded fibers. Furthermore adsorption studies showed that higher mechanical agitation increased saturation levels of EGs and reduced adsorption constants, which means enhanced desorption. The synergism between EG activity and mechanical agitation was able to transform a piece of fabric into a microfibrillar mass whereas almost pure CBH activity left the main fabric structure intact under similar treatment conditions (Cavaco Paulo *et al.*, 1996, 1998; Cavaco Paulo, 1998). It is also interesting that the agitation levels used in laboratory textile processing/washing equipments (vertical rotation) were reported to cause no significant deactivation of CMC or FPA activity during typical processing times of a maximum of 60 minutes (Azevedo *et al.*, 2002a). Some inactivation should be expected for longer reaction times. For the hydrolysis of α-cellulose, the extent of shaking primarily influenced the initial rate of hydrolysis and not the conversion yield after 72 h. Increasing substrate concentration reduced the initial rate, which was attributed to end-product inhibition and mass transfer limitations, but at the same time the conversion yields of intermittent shaking (a combination of intervals of no-mixing with short periods of high or low speed mixing) approached those from continuous high speed shaking, which might be beneficial to reduce the energy requirements of the process (Ingesson *et al.*, 2001).

The mass transfer of enzymes and reaction products might also be enhanced by ultrasonic waves. The ultrasonic cavitations produced by sonifiers were reported to increase hydrolysis of cellulosic materials with cellulases. The treatment of cotton fabrics with a whole cellulose (Cellusoft L from Novozymes) in a special reactor chamber (in which the cotton fabric is placed between two diaphragms driven by two different frequencies of 16 kHz and 20 kHz) showed an increase up to 35% in weight loss, without affecting significantly the tensile strength. Acceleration of the diffusion rate of the enzyme and the soluble reaction products, improved transport of the enzyme through the textile structure and thus a more uniform treatment, prevention of possible agglomeration of enzyme molecules, degassing and removal of entrapped air from fiber capillaries and interstices, and eventually increased specific enzyme activity due to cavitation effects that cause an increase in pressure and temperature on the microscopic level were acclaimed by the authors (Yachmenev *et al.*, 1998, 1999, 2002). Additional application of ultrasound enhanced enzymatic saccharification of waste paper in stirred tank reactors, but at a given ultrasonic intensity the effect was less pronounced in the larger reactors, since the specific ultrasonic intensity (ultrasonic intensity/volume) decreased. An adverse effect was observed with newspaper, which was attributed to the presence of lignin or other impurities that might be activated and form a rigid and closed network, which inhibits the access and adsorption of the cellulase to the cellulosic surface (Li *et al.*, 2005). Ultrasound was also reported to stimulate ethanol production during simultaneous saccharification and fermenta-

tion of mixed waste office paper. Intermittent exposure of the process to ultrasound was found to increase the production of ethanol by approximately 20%, a result that could be achieved without ultrasound only by applying the double cellulase activity (FPAse)/g substrate. Continuous exposure of the organism *Klebsiella oxytoca* P2 to ultrasonic energy resulted in decreased ethanol production and had a bacteriostatic effect (Wood *et al.*, 1997).

11.6.2. Surfactants and Other Additives

Natural or synthetic additives might increase or reduce the rate of enzyme inactivation. Proteins such as bovine plasma albumin and gelatin are effective at relative high concentrations (1mg/mL), whereas fluorosurfactants (0,001 to 0,01 mg/mL) or other surfactants and polymers (0,01 to 0,1 mg/mL; Triton X-100, saponins; cellulose ethers, polyethylene glycol, polypropylene glycol, quaternary ammonium compounds and others) were found to have a protective effect at much lower concentrations. Preliminary studies indicated that a molar ratio of 1:1 (surfactant molecule:enzyme molecule) was sufficient for maximum protection of the cellulases (Reese, 1980). Ethanol was reported to depress the adsorption of exoglucanases and inhibit saccharification (Ooshima *et al.*, 1986). Inactivation of enzymes may result from the unfolding of the protein molecules, exposing hydrophobic groups, followed by aggregation due to hydrophobic interactions, and subsequent denaturation. Interactions of the surfactant with the hydrophobic part of the protein may minimize aggregation and denaturation.

Various authors have shown that non-ionic surfactants have a beneficial effect on the hydrolysis of cellulosic and lignocellulosic substrates, whereas anionic and cationic surfactants alone interfere negatively (Castanon and Wilke, 1981; Helle *et al.*, 1993; Park *et al.*, 1992; Ooshima *et al.*, 1986; Traore and Buschle-Diller, 1999; Ueda *et al.*, 1994; Eriksson *et al.*, 2002). Increases in the amount of reducing soluble sugars and substrate conversion were reported. The effect depends on the substrate and is not observed for soluble substrates, such as carboxymethylcellulose or cellobiose. Nonionic surfactants increased the initial rate of hydrolysis of Sigmacell 100, and when they were added later in the process they were less effective (Helle *et al.*, 1993). They same authors found also that the addition of cellulose increases the critical micelle concentration of the surfactant, which indicates that the surfactant adsorbs to the substrate. Surfactants are more effective at lower enzyme loads and reduce the amount of adsorbed protein (Castanon and Wilke, 1981; Ooshima *et al.*, 1986; Helle *et al.*, 1993; Eriksson *et al.*, 2002) which can be used to increase desorption of cellulase from the cellulosic substrate (Otter *et al.*, 1989). Anyhow, the use of surfactants to enhance desorption of cellulases from textile substrates in order to recover and recycle cellulases was not successful (Azevedo *et al.*, 2002b).

One possible explanation for the effect of surfactants on cellulose hydrolysis is that surfactants adsorb to the cellulosic substrate, lower the surface

tension, improve the wettability of the substrate, and make it more accessible for the enzymes (Helle *et al.*, 1993). Surfactants influence the adsorption process on the substrate, reducing the immobilization of the enzyme on the cellulosic substrate and affect the degree of adsorption. Especially nonionic surfactants such as Tween 20 reduce the adsorption of endoglucanase activity to insoluble cellulosic substrates and enrich their concentration in the liquid phase, thus regulating the adsorption profile of exo- and endoglucanases on the cellulose surface (Ooshima *et al.*, 1986; Park *et al.*, 1992). For the same surfactant, different interactions with different enzymes should be expected. Especially on lignocellulosic substrates, the adsorption of cellulases to the lignin-rich regions seems to be unproductive and higher enzyme loads are necessary to achieve the same hydrolysis rates. In this case the adsorption of the surfactant to the hydrophobic non-cellulosic part of the substrate prevents unproductive binding of the enzymes. The EO chains of the non-ionic surfactant molecules have two effects: first, the occupation of the hydrophobic substrate sites and, second, the reduction of protein adsorption due to their volume (Eriksson *et al.*, 2002). It is interesting that BSA, when added in excess, has practically the same effect as the surfactants. Unspecific binding of BSA (Bovine Serum Albumin) to lignin decreases unproductive binding of the cellulases. Surfactants when adsorbed to crystalline cellulose might help disrupt the hydrogen bonding environment and avoid reannealing of the glycosidic bond after hydrolysis (Helle *et al.*, 1993). Park *et al.* (1992) studied the influence of non-ionic surfactants on the hydrolysis of used newspaper, and they verified an increase in conversion and free enzyme in solution with increasing HLB (hydrophilic lipophilic balance) of nonylphenylether surfactants. The non-ionic surfactant Tween 20 was also reported to improve slightly the thermal stability of cellulolytic enzymes (Eriksson *et al.*, 2002).

Although anionic surfactants can establish electrostatic interactions with positively charged amino groups of the protein, and denature and inactivate cellulases (Traore and Buschle-Diller, 1999; Ueda *et al.*, 1994), this effect seems to be reversible upon the separation of the cellulase from the surfactant (Azevedo *et al.*, 2002b). Eriksson *et al.* (2002) have also shown that the negative charge of surfactants like sodium dodecyl sulfate (SDS) can amplify the effect of non-ionic surfactants preventing unproductive binding of negatively charged enzymes (pI of Cel 7A 3,9; pH 4,8) to lignin. In mixed micelles with non-ionic surfactants SDS is totally incorporated and the denaturing effect is reduced.

For cationic surfactants both negative effects and enhancement of the hydrolytic action of cellulases were reported (Traore and Buschle-Diller, 1999; Ueda *et al.*, 1994). Ooshima *et al.* (1986) found that the cationic surfactant $RN^{+}(CH_3)_2CH_3Cl^{-}$ (R: C_{16} and C_{18}) had an accelerating effect on hydrolysis at very low concentration (0,01%) on tissue paper, but a denaturating effect at higher concentrations. Before denaturating the enzyme significantly, the surfactant was found to inhibit the reaction. Denaturation of the enzyme was attributed only to the surfactant in the liquid phase, but not to the surfactant adsorbed to the substrate. For an amphoteric surfactant no denaturing

properties were found, and an accelerating effect on cellulose hydrolysis, however less pronounced as with the nonionic Tween 20, was reported (Ooshima *et al.*, 1986).

Various chemicals were tested to stabilize cellulases during desorption from lignocellulosic substrates under alkaline conditions (Otter *et al.*, 1989). Salts such as $CaCl_2$ and KCN were found to increase slightly Avicelase activity at pH 5 and enhance desorption at pH10. $MgCl_2$ also increased Avicelase stability at high pH, whereas $MnSO_4$ reduced its activity at pH 5 and pH 10 (Otter *et al.*, 1989). The oxidation of sulfhydryl groups of cysteine residues in the active site of cellulolytic enzymes might lead to the formation of disulfide bonds or metal binding of these residues and result in inactivation. Thiol compounds such as cysteine, dithiothreitol, gluthatione, or metal chelating agents such as EDTA (ethylene diamine tetraacetate) might act as sulfhydryl protecting agents, and were found to have a stabilizing effect at pH 5 and 10, but they did not increase desorption. Polyhydric alcohols such as ethylene glycol (10% w/v), polyethylene glycol (5% w/v), glycerol (10 and 60% w/v), and other desorbents such as urea (1 and 6M), sucrose (5% w/v), and even acetone (20% w/v), had also an stabilizing effect at pH 5 (Otter *et al.*, 1989).

High concentrations of stabilizing salts such as NaCl and Na_2SO_4 were reported to increase, and chaotropic salts to decrease, the affinity of bacterial and fungal cellulases to crystalline cellulose. Denaturing agents are capable of eluting cellulases from the cellulosic substrate (Otter *et al.*, 1989).

High ionic strength, adjusted by the addition of 1M $MgCl_2$ at pH 5.0, was reported to increase significantly the adsorption of native and mutated CBH I to cellulose, whereas the addition of 1M NaCl only affected the adsorption of native CBHI with intact CBD. The activity of the CBD-less CBH I (CBH core protein) on crystalline cellulose was also increased (Reinikainen *et al.*, 1995). The improved binding was explained by reduced electrostatic repulsion between protein molecules, which possibly was masked by the salt ions.

11.7. CONCLUSIONS

It remains clear that the main carbohydrate of holocellulose is the linear polymer cellulose, but other polymers such as hemicelluloses, pectins, and lignin residues, that remain attached to holocellulosic material even after pretreatments, will have an influence on the extent of degradation. For complete or at least efficient hydrolysis (as desired for the production of biofuel such as bioethanol), a complete understanding of the degradation mechanism, the limits of hydrolysis, and the influence of process parameters, as well as the effects of substrate properties are of outstanding importance. The heterogeneous nature of the supermolecular structures of lignocellulosic matrices, variation in substrate composition, particle and pore size, and the influence of different pretreatments on the accessibility of holocellulose for different enzyme complexes, makes it difficult to understand all possible interaction that might occur and almost impossible to simulate them. No single

mechanism of total lignocellulosic or holocellulosic saccharification has been established so far (Mansfield *et al.*, 1999) and probably will not be defined in the near future due to the complex interactions. In this review some aspects were reviewed and discussed without claiming to be complete, which is impossible when we consider the amount of literature available on this topic. However, a detailed analysis of each substrate and the creation of an extensive, interconnected database providing essential information about microorganisms, enzymes, substrates, reactor systems, and process parameters might help select empirically for each case the adequate solution. Without doubt further studies on the substrate-enzyme interactions, influence on process parameters, and efforts to improve enzymes and microorganism for this purpose have to be made.

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11.9. REFERENCES

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CHAPTER 12

From Biogas Energy to Keratinase Technology

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12.1. INTRODUCTION

Anaerobic digestion is a microbial process that breaks down organic matter and converts the carbon sources into a mixture of methane and carbon dioxide, commonly known as biogas. With its 60–70% methane content, biogas is a combustible gas fuel. As a process converting animal waste into energy, anaerobic digestion is always of great interest to environmental engineers and scientists. Its application in the management of animal waste on large animal farms is technically challenging. Through a systematic study from the laboratory to the farm, a highly efficient thermophilic anaerobic digestion (TAnD) was developed. During the operation, degradation of chicken feathers in the digester was observed. Subsequently, a feather-degrading bacterium and keratinase enzyme were discovered. The gene encoding the enzyme was also iso-

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lated, sequenced and manipulated to express the keratinase, which proved to be able to hydrolyze feathers into a more digestible feather meal. When supplemented in chicken feed, the enzyme additive could improve protein digestibility and thus promote, nutritionally, animal growth. More recently, the enzyme was demonstrated to breakdown prion protein that is believed to be associated with transmissible spongiform encelphalopathies (TSE). In this paper, a brief history from biogas production to the discovery and development of keratinase technology will be reviewed. Keratinases and their applications have also been reviewed elsewhere lately (Gupta and Ramnani, 2006; Shih and Wang, 2006).

12.2. THERMOPHILIC ANAEROBIC DIGESTION (TAnD)

Animal waste is daily generated in large amounts on large animal farms, necessitating a treatment process of high efficiency. Anaerobic digestion is a bioprocess that can simultaneously breakdown organic waste and produce biogas fuel. One of the approaches to improve digestion rate is to increase the biotreatment temperature. At higher temperatures, thermophilic anaerobic bacterial culture can be selected and adapted to a higher growth rate, to breakdown the waste and to produce biogas at a higher rate. A systematic study with various operational parameters and various concentrations of poultry manure was carried out at North Carolina State University. The selection of the bacterial culture and optimal operational conditions for a high rate of biogas production was achieved (Huang and Shih, 1981; Williams and Shih, 1989). With the optimized process, the poultry manure was degraded by 50–60%, biogas produced at a volumetric rate of 4–5 v/v/d, and biogas yield at 400 l/kg volatile solids (VS). The operation operated at a concentration of 6% VS, with a short 4–5 day retention time (RT), and thermophilic temperatures of 50–60 °C. The biogas rate is 4 times higher than the operation at mesophilic temperatures (30–40 °C) (Shih, 1987a,b). Because of the short RT, digester volume can be 4-fold smaller than a mesophilic digester and more than 10 fold smaller than an ambient digester. For large amounts of manure to be processed, this advantage is significant in lowering the construction cost of a digester and reducing the digester's footprint.

Based on the operational parameters optimized in the laboratory, a prototype digester was constructed and operated on the university poultry farm in the following years (Steinsberger and Shih, 1984). The digester was a simple plug-flow design, consisting of a sausage-shaped plastic bag, an insulation system, and a hot-water heater fueled by biogas. The plastic bag was half bioreactor at the bottom and half gas collector on the top. Fresh manure from 4,000 laying hens was collected daily by a floor scraper and conveyed by an auger into the digester. Hot water from a heater fueled by biogas was added into the digester to give the desirable concentration and temperature of the digestion mixture. Daily biogas production was monitored by a gas meter and analyzed by a gas chromatograph. In three years of operation, the pilot digester

had no major problems and reached 70–80% of the biogas potential that was obtained in laboratory flasks (Steinsberger and Shih, 1984). This prototype system proved the concept of thermophilic anaerobic digestion technically feasible. Many other ecological and health benefits associated with the system were also demonstrated (Jiang *et al.*, 1987; Steinsberger *et al.*, 1987; Shih, 1987a, 1987b, 1988; Lee and Shih, 1988). In 1989, a full-scale TAnD for 2,000 hogs was demonstrated in Taiwan, and, in 1992, a full-scale TAnD for 50,000 laying hens was constructed in Beijing, China. The Chinese TAnD, made of concrete, is still in operation but close to its retirement. The simple yet efficient TAnD design was awarded with a U. S. patent in 1996.

12.3. FEATHER-DEGRADING KERATINASE

In the TAnD operation, feathers mixed with manure were completed degraded in digestion. Since feathers, like hair and finger nails, are made of a very stable protein called keratin, resistant to most proteases, the goal was to isolate the feather-degrading bacterium from the digester. After two years of work, the bacterium was isolated in pure culture, and proved to be a thermophilic strain of *Bacillus licheniformis,* which grows optimally at 50–60 °C and can live on ground feathers as the sole source of carbon, nitrogen, and energy. Since this was the first bacterium isolated from a poultry waste digester, it was named PWD-1 strain (Williams and Shih, 1989; Williams *et al.*, 1990). The degradation of feathers is demonstrated in Figure 12.1, where the feathers were immersed in test tubes of live PWD-1 culture. The feathers were gradually digested except the control, which was not inoculated with the bacteria. Subsequently, the keratinase enzyme was purified from the culture medium of the bacterium (Lin *et al.*, 1992). This enzyme is proteolytically active on all kinds of proteins tested, including collagen, elastin, casein, keratin, and plant proteins. Further studies showed that keratinase displays optimum activity at 50–60 °C and pH 7.5–8.0. With molecular mass of 33 kDa, pI at 7.25 and serine at its active site, it belongs to the family of proteases known as subtilisins. However, it works more actively on feather keratin than any other subtilisins tested. Characterizations for its stability and immobilization by chemical and biological means were also investigated (Lin *et al.*, 1996; Wang *et al.*, 2003a, 2003b).

The keratinase gene (*kerA*) was isolated and sequenced, using a technique called “chromosomal walking by PCR” (Lin *et al.*, 1995). The promoter, ribosomal binding site, open reading frame, pre-, pro- and mature regions, and termination sequence were all identified on this isolated 14 kb *kerA* sequence. Manipulations of the gene for over expression were carried out by two different strategies; one was to introduce *kerA* and a strong promoter into a plasmid, which was subsequently transformed into a protease-deficient strain of *B. subtilis.* The resulted recombinant strain hyper-produced keratinase by 4–5 fold (Lin *et al.*, 1997). The other strategy was to integrate multiple copies of *kerA* into the chromosome of *B. licheniformis* PWD-1 (Wang *et al.*, 2004). The new strain over expressed keratinase by 3–4 fold, but it transmits the trait

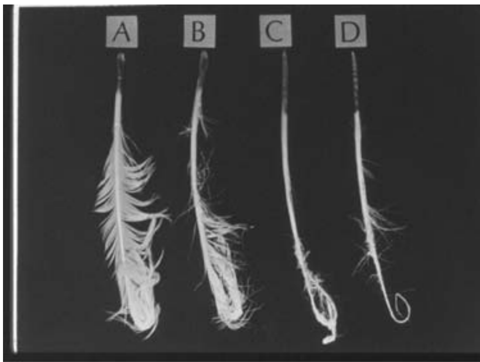

Figure 12.1. Feather degradation in the medium inoculated with *B. licheniformis* PWD-1. Source: William and Shih, 1989. A: 10 days without the inoculation. B, C, D: 3, 7, and 10 days post-inoculation.

stably over many generations. The scale-up production of keratinase was investigated in a 150-L pilot industrial fermenter, thanks to an equipment grant from the NC Biotechnology Center. Operational parameters were optimized and hundreds of grams of keratinase were produced with this facility (Wang and Shih, 1999). This quantity of keratinase made the application research possible. Six patents were awarded with this series of discoveries that included the parent strain of the bacterium, the enzyme, the gene structure, new strains of bacteria, and potential applications.

12.4. KERATINASE-BASED FEED ADDITIVE

The enzyme was first tested for its application in processing feathers. Using the PWD-1 fermentation broth as the source of keratinase to incubate feathers, the resulted product was called feather-lysate, partially hydrolyzed feathers. The highly digestible feather-lysate was able to replace soy bean meal up to 1/3 of protein in feed (Williams *et al.*, 1991). Enzymatic cooking of feathers with keratinase also improved the *in vitro* digestibility of the feather meal product. Large commercial scale of enzymatic cooking has not been fully optimized and the study is in progress.

Since keratinase is a versatile protease that digests all kinds of proteins, it is possible that supplementation of the enzyme in feed may improve the feed digestibility. In 2001 keratinase was tested as a feed additive. The first test in young chicks was positive, and reproduced by many more experiments: 0.1% of crude keratinase in a regular corn-soy diet improved the body weight gain, the feed conversion ratio (FCR), or feed efficiency in broiler chickens. Numerous experiments included the cage tests of chicks to 3–4 weeks of age (Odetellah *et al.*, 2003), pen trials on a research farm to 6 week market age (Odetellah *et al.*, 2005), and the commercial tests conducted by private com-

panies (Wang *et al.*, 2006). In general, use of the enzyme as a feed supplement in young chicks to market age could increase more than 100 g weight gain and reduce FCR by 3–6 points. The meat yield of the broilers increased when moderate to high protein diets were used. Because of the improved digestibility and utilization of dietary protein, it is possible to reduce the protein in feed by 10% when the enzyme was used. Keratinase as a feed additive opens up a huge commercial opportunity in animal and feed production. BioResource International (BRI), a biotechnology company, licensed the keratinase technology, produced the feed enzyme additive, and put the product, named Versazyme,^{TR} on the market.

12.5. PRION DEGRADATION

Prion protein is believed to be the causative agent of a group of neurodegenerative diseases, collectively called prion diseases,or transmissible spongiform encephalopathies (TSE). They include bovine spongiform encephalopathy (BSE) (commonly known as mad cow disease), scrapie in sheep, chronic wasting disease in deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans (Prusner, *et al.*, 1998). An epidemic of BSE in Europe in the late 1980s and early 1990s caused the deaths of 200,000 diseased cattle, and the preemptive slaughter of 4.5 million heads of cattle. Ingestion of diseased or contaminated beef caused nearly 200 human deaths from new variant CJD in Europe. In the U. S., only four cases of BSE have been reported. But, the shutdown of the export market for American beef has cost the beef industry hundreds of millions of dollars each year since the first case reported in December 2003.

Prion protein (PrP^C) is a normal protein that exists in the nervous system of mammals. A conformational change of the protein molecule, caused by unknown mechanism, converts the normal isoform into the pathogenic isoform (PrP^{Sc}), and subsequent aggregation into a β-amyloid plaque. PrP^{Sc} and its aggregates are infectious, insoluble, stable against digestion by most proteases, and resistant to standard sterilizing methods, such as boiling, autoclaving, and alcohol. Interestingly, β-amyloid plaque and its monomeric PrP^{Sc} are structurally similar to feather keratin and its component molecules. They are rich in β-sheet structure, tightly packed aggregates, insoluble, and resistant to proteases. Since PWD-1 keratinase is capable of degrading feather keratin, it is possible that the enzyme can also degrade β-amyloid plaque and PrP^{Sc}. This hypothesis was tested with Dr. Jan Langveld at CIDC-Lelystad, the Netherlands. Brain stems from BSE cattle were homogenized with detergent, preheated for 10 min at 115°C and incubated with the keratinase. The digested homogenates were analyzed by SDS-PAGE and Western blot. The results indicated that PrP^{Sc} was degraded to a level undetectable by the very sensitive and specific immunoblot (Langeveld *et al.*, 2003). The result of the very first experiment is given in Figure 12.2. While keratinase alone could only partially

digest PrP^{Sc}, preheating helped the enzyme complete the digestion. Proteinase K and some subtilisins were equally active as keratinase on prions when detergent and preheating were included in the process. That was the first report of enzymatic degradation of PrP^{Sc}. Consequently this discovery indicated a potential method for prion disinfection, control of prion transmission and prevention of prion diseases. Animal tests to confirm the enzymatic inactivation of PrP^{Sc} are in progress.

Because of the infectivity and danger of working with pathogenic prions, all experiments with this pathogen must be controlled in P-3 isolation lab facilities. In addition, the disease materials from sick animals are highly restricted and difficult to obtain. To continue the study the degradation of prion protein in the laboratory, we have recently developed a prion surrogate protein (PSP) from yeast (Wang *et al.*, 2005; Chen *et al.*, 2005; Shih and Wang, 2006). PSP is physico-chemically similar to the disease-causing prions, but non-pathogenic and safe to handle under standard laboratory conditions. With PSP as a marker, we are studying the conditions and optimization of the enzymatic process for prion inactivation and decontamination that mimics industrial applications (Wang *et al.*, 2007).

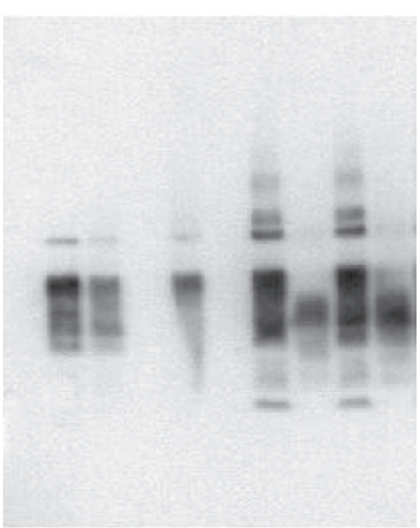

Figure 12.2. Keratinase (KE) degradation of PrP^{Sc} in BSE brain homogenates as detected by Western blot. Source: Shih and Wang, 2006. 1: Buffer only. 2: Negative brain with internal marker. 3, 5: Pre-heating, no KE digestion. 4, 6: Pre-heating and KE digestion. 7, 9: No pre-heating, no KE digestion. 8,10: No pre-heating, only KE digestion.

12.6. ACKNOWLEDGMENTS

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CHAPTER 13

Emerging Technologies in Dry Grind Ethanol Production

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13.1. INTRODUCTION

Dry grind ethanol production in the U. S. increased 300% in the last 6 years (RFA, 2007). In January 2007 there were 110 operating ethanol plants in the U. S., with another 73 under construction and 8 more under expansion. When these plants (under construction or expansion) come online, ethanol production capacity in the U. S. will reach 11.6 billion gallons/yr (RFA, 2007).

In a conventional dry grind process, ground corn is mixed with water to produce slurry. The slurry is cooked; the slurry starch liquefied, saccharified and fermented to produce ethanol. The remaining nonfermentables (germ, fiber, and protein) are recovered at the end of the dry grind process as an animal food co-product, called distiller dried grains with solubles or DDGS.

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New technologies are being implemented in dry grind ethanol plants that are less capital intensive, require less operating cost, recover multiple products, and improve the nutritional composition of the DDGS. These technologies include feedstock development, better and newer enzymes, and corn and DDGS fractionation for recovery of multiple co-products. In this paper four emerging technologies in dry grind ethanol processing will be highlighted: *i)* feedstock (corn) development, *ii)* corn wet fractionation, *iii)* raw starch hydrolyzing enzymes, and *iv)* DDGS fractionation.

13.2. CONVENTIONAL DRY GRIND PROCESS

A schematic of the dry grind process is shown in Figure 13.1. In the conventional dry grind process, corn kernels are ground using a hammermill, ground corn is mixed with water to form slurry, which is cooked at approximately 104 °C using pressurized steam to dinintegrate the crystalline structure of starch granules. Alpha-amylase is added to break down starch polymers into short chain molecules, called dextrins, to form mash. The mash is held at an elevated temperature (85 to 90 °C) for a short period of time, cooled to 32 °C, and transferred into a fermentation vessel. Glucoamylase and yeast are added for simultaneous saccharification and fermentation. In the mash, glucoamylase breaks down dextrins into mono- or di-saccharides, such as glucose and

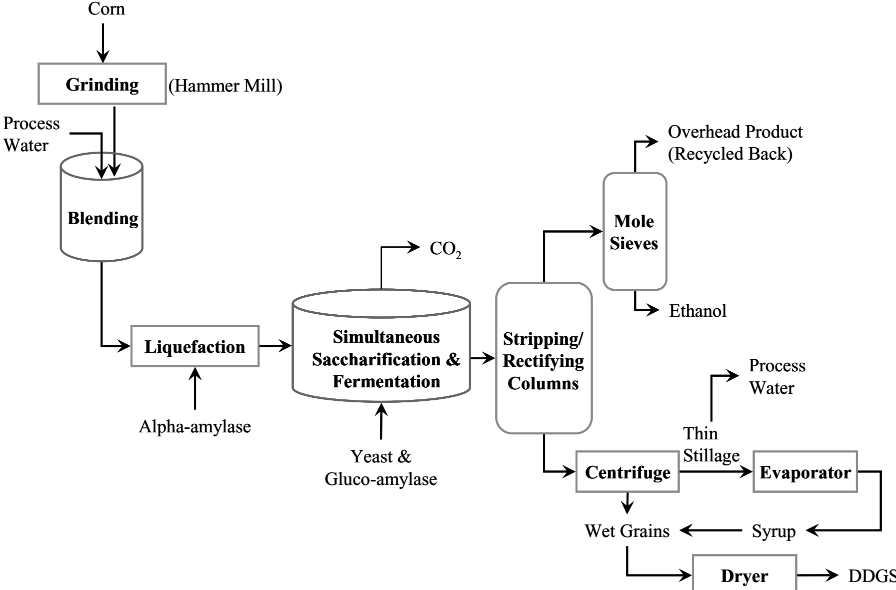

Figure 13.1. Conventional corn wet milling process.

maltose, while yeast ferment these saccharides into ethanol. At the end of fermentation, the resulting beer is transferred to a holding tank called a beer well. From the beer well, the beer is transferred to a stripper/rectifier column to remove ethanol. Overflow from the stripper/rectifier column is an ethanol and water mixture, and underflow from the column is whole stillage (nonfermentable components of corn, yeast and water). The ethanol and water mixture is processed further through molecular sieves to remove remaining water from the ethanol.

Whole stillage (WS) is centrifuged to produce thin stillage (water and soluble solids) and wet grains (suspended solids). Using an evaporator, thin stillage (TS) is concentrated into syrup and mixed with the wet grains (WG), which is dried to produce a co-product with 12% moisture content. This co-product is marketed as DDGS.

13.3. EMERGING DRY GRIND ETHANOL TECHNOLOGIES

13.3.1. Feedstock Development

In conventional yellow dent corn hybrids there is approximately 15 to 23% variability in ethanol yield (Singh and Graeber, 2005; Haefele *et al.*, 2004). Selection of hybrids could improve the amount of total ethanol produced in a dry grind corn processing plant and affect process economics. All major seed companies evaluate and short list high ethanol producing corn hybrids. Reasons for variability in ethanol yield are due, not only to starch content, but to compositional differences among hybrids. Weak or no correlation has been reported between ethanol yield and starch content (Singh and Graeber, 2005, Dien *et al.*, 2002, Zhan *et al.*, 2003); therefore, complete dry grind fermentation test is required to determine ethanol potential of corn hybrid.

In transgenic corn hybrids, an amylase corn has been developed that produces and stores alpha-amylase within the kernel (Singh *et al.*, 2006). These enzymes are activated in the presence of water at elevated temperatures (>70 °C). In conventional dry grind corn processing, exogenous alpha-amylase enzymes are added during liquefaction and are part of the operating costs in dry grind plant. With amylase corn no addition of exogenous alpha-amylase is required (Fig. 13.2). Due to high expression levels of the alpha-amylase in the amylase corn, only a small amount of amylase corn (3% of total corn per batch) is needed to replace the exogenous liquefaction enzyme currently used in the dry grind process for ethanol production (Singh *et al.*, 2006). Final ethanol concentration for 3% amylase corn treatment was similar to the control treatment (no amylase corn addition and processed with conventional exogenous alpha-amylase). Compared to the control, amylase corn treatments produced similar sugar profile of glucose, fructose, maltose, maltotriose, and DP4+ sugars. DDGS compositions from 3% amylase corn and control treatment were similar (Singh *et al.*, 2006).

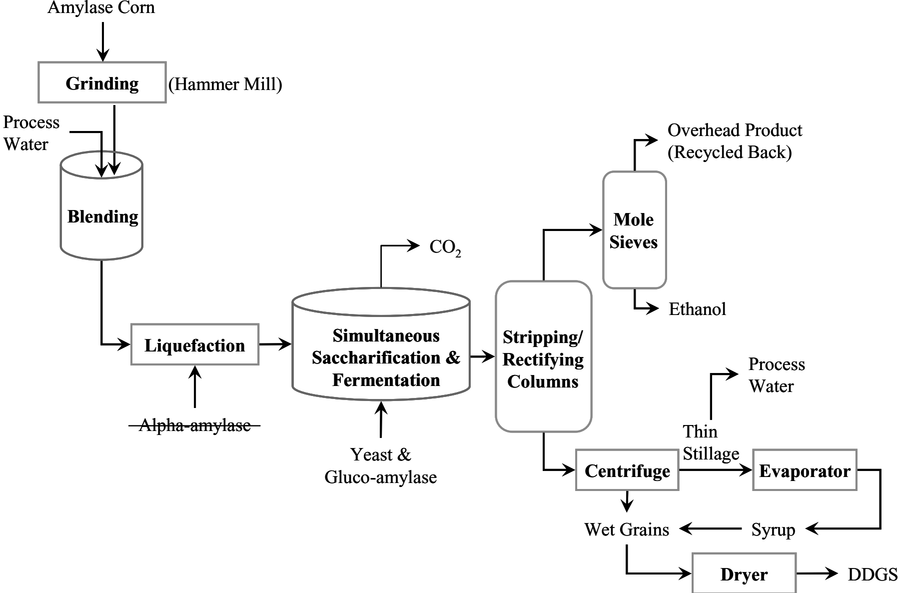

Figure 13.2. Dry grind corn process with amylase corn. Source: Singh *et al.*, 2006.

13.3.2. Corn Wet Fractionation

Corn wet fractionation processes have been development to recover germ, pericarp fiber and/or endosperm fiber as co-products besides DDGS. Corn fractionation processes reduce the amount of nonfermentables (germ and fiber) in the fermentor and improve the composition (higher protein and lower fiber content) of DDGS. Corn wet fractionation processes involve soaking corn kernels in water for a short period of time followed by milling (size reduction) and separation of components in an aqueous medium based on density difference. There are several wet fractionation processes: the quick germ process (recovery of germ as coproduct) (Singh and Eckhoff, 1996; Singh and Eckhoff, 1997), the quick germ quick fiber process (recovery of germ and pericarp fiber as co-products) (Singh *et al.*, 1999; Wahjudi *et al.*, 2000), and the enzymatic (E-Mill) dry grind process (recovery of germ, pericarp fiber and endosperm fiber as co-products) (Singh *et al.*, 2005; Wang *et al.*, 2005). The E-Mill dry grind process involves soaking of corn in water for 6 to 12 h at 55 °C, followed by coarse grind, and incubation with protease and starch degrading enzymes for 2 to 4 h (Fig. 13.3). Protease and starch degrading enzymes increase specific gravity of the slurry and aid in separation of individual corn components. Germ and pericarp fibers are recovered by floatation (hydrocylcones) (Singh and Eckhoff, 1996; Singh *et al.*, 1999; Wahjudi *et al.*, 2001). Endosperm fiber can be recovered by use of screens (200 mesh or 0.074 mm opening) either prior to (Singh *et al.*, 2005) or after fermentation (Wang *et al.*,

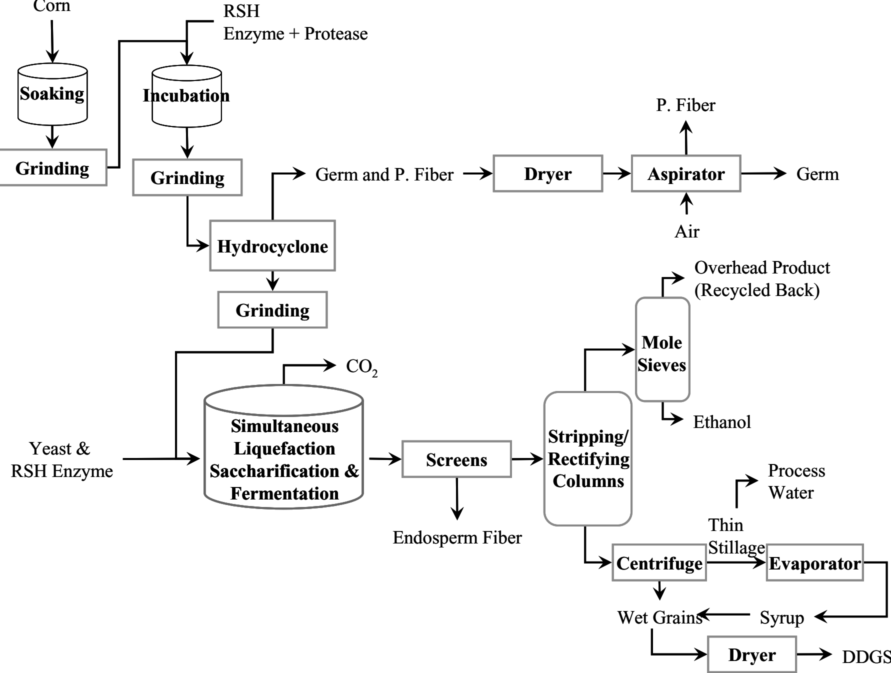

Figure 13.3. Dry grind corn process with wet corn fractionation. Source: Singh *et al.*, 2005.

2005). Recovery of endosperm fiber after fermentation reduces the loss of starch in fiber fraction and increases ethanol yield. Remaining ground corn slurry is processed for ethanol production. E-Mill process benefits dry grind ethanol production in three ways: *i)* by adding valuable co-products (corn germ, pericarp fiber, and endosperm fiber) to the process, *ii)* by increasing the plant capacity (removal of nonfermentables at front end of process), and *iii)* by reducing the amount of DDGS and improving its composition (increasing the amount of protein and reducing the amount of fiber).

13.3.3. Raw Starch Hydrolyzing Enzymes

In a dry grind plant, energy is used in jet cooking, liquefaction, distilling, dehydrating, and drying operations. Ground corn is cooked and liquefied to dextrins at ≥90 °C for 1 to 2 h using liquefaction enzymes (Kelsall and Lyons, 2003). Dextrins are hydrolyzed into fermentable sugars using saccharification enzymes during simultaneous saccharification and fermentation (SSF). A raw starch hydrolyzing (RSH) enzyme, Stargen™ 001 (Genencor International, Palo Alto, CA), has been developed. Stargen™ 001 has high raw starch hydrolyzing activity and can convert starch into dextrins at ≤48 °C, as well as hydrolyze dextrins into fermentable sugars during SSF (Fig. 13.4) (Wang *et al.*, 2007).

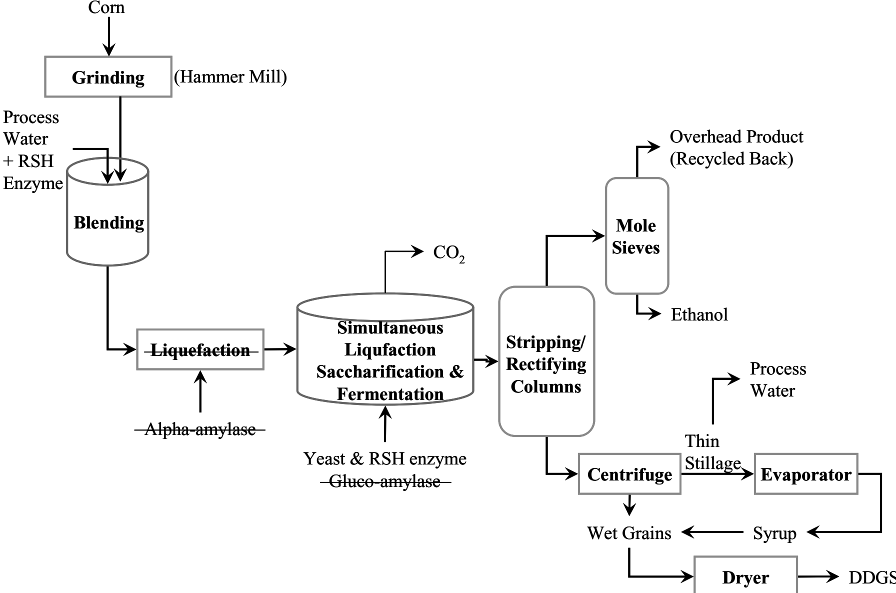

Figure 13.4. Dry grind corn process with raw starch hydrolyzing (RSH) enzymes. Source: Wang *et al.*, 2005.

Use of RSH enzymes in the dry grind process does not require high temperature cooking and liquefaction; therefore, RSH enzyme reduces energy requirements. During SSF, glucose concentrations with RSH treatment were lower compared to conventional enzyme treatments (Wang *et al.*, 2007). The final ethanol concentration, ethanol yield, ethanol conversion efficiency, and DDGS yield of the RSH process were similar to conventional dry grind process (Wang *et al.*, 2007).

13.3.4. DDGS Fractionation to Recover Fiber: Elusieve Process

The elusieve process has been developed to separate fiber from distillers dried grains with solubles (DDGS): in a dry grind ethanol plant, such a separation increases protein and fat content and reduces fiber content in the resulting DDGS. Fiber produced from the elusieve process can be used for recovery of other value added co-products. The elusieve process uses sieving and elutriation to separate fiber from DDGS (Fig. 13.5). Material carried to the top of the elutriation column is called "lighter fraction" or "fiber fraction," and material that settles at the bottom of the column is called "heavier fraction" or "enhanced DDGS." Conventional DDGS samples, obtained from dry grind corn plants, were processed using the elusieve process. By adjusting process

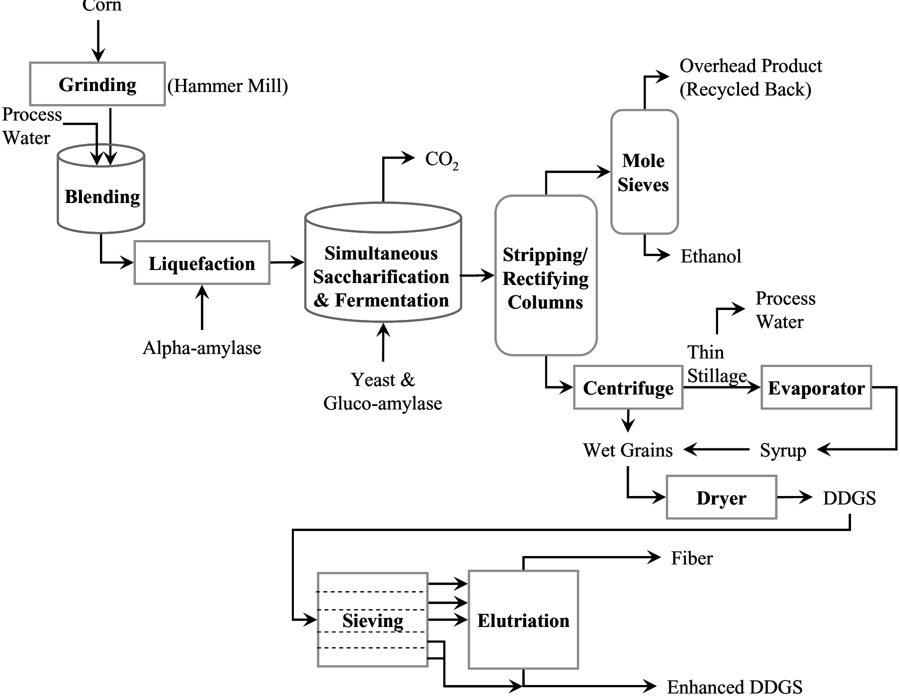

Figure 13.5. Dry grind corn process with DDGS fractionation. Source: Srinivasan *et al.*, 2006.

parameters, elusieve processing increased the protein and fat contents of enhanced DDGS from 28 to 41% and 12 to 14%, respectively, and reduced neutral detergent fiber content from 32 to 19%, compared to the original DDGS (Srinivasan *et al.*, 2005). The elusieve process is low cost solution to the reduce fiber content of conventional DDGS; payback period For a dry grind ethanol plant producing 40 million gallons per year, its payback period was estimated to be less than two years (Srinivasan *et al.*, 2006).

13.4. CONCLUSIONS

Four modified dry grind ethanol technologies (amylase corn, corn wet fractionation, raw starch hydrolysis, and DDGS fractionation) have been developed that can reduce capital and operating costs, recover multiple co-products, and/or reduce the amount of DDGS produced, as well as improve its composition. Some of these technologies have been implemented in commercial dry grind ethanol plants in the Midwestern U. S.

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CHAPTER 14

Gram-Positive Bacteria as Biocatalysts to Convert Biomass Derived Sugars into Biofuel and Chemicals

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14.1. INTRODUCTION

The cost-effective conversion of lignocellulosic biomass materials into fuels and chemicals relies not only on the efficient degradation of plant cell wall

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materials into fermentable sugars, but also on the efficiency of the microbial fermentation to convert the mixed sugars in the presence of other chemicals released from pretreatment of plant materials. Every stage of the entire process for conversion of renewable biomass materials to fuel ethanol and chemicals is critical in the overall economics. Barriers include the supply and characteristics of feedstocks, the enzymes to breakdown the recalcitrant lignocellulosic biomass (mostly plant cell wall polysaccharides) into smaller fermentable components, the fermenting microbes, and the cost-effective recovery of desired products. This paper will focus on studies to develop Gram-positive fermenting microbes for applications in the conversion process.

14.2. BIOMASS HYDROLYSATES

Lignocellulosic biomass is largely composed of cellulose (35–50%), hemicellulose (20–35%), and lignin (10–20%). The remaining fraction includes proteins, oils, and minerals (Wyman, 1999). The complete breakdown of lignocellulose biomass materials is accomplished via a combination of physical, chemical, and enzymatic pretreatments that usually generates hydrolysates containing disaccharides (cellobiose, xylobiose), and monosaccharides including five (xylose, arabinose) and six carbon (glucose, mannose, galactose) sugars. These hydrolysates can also contain organic acids (acetic acid, formic acid, ferulic acid, glucuronic acid, ρ-coumaric acids), sugar degradation products (furfural, hydroxymethyfurfural, levulinic acid,) and polyphenolics (Saha, 2003). Many of these compounds inhibit microbial fermentation and growth. The composition and proportion of sugars and other chemicals in biomass hydrolysates can vary greatly with various biomass feedstocks, which range from agriculture residues (including corn stover, wheat straw, rice straw, and sugarcane bagasse), to energy crops (including switchgrass, Miscanthus, Bermudagrass), to forest residues or waste biomass materials. Currently, no commercial microbe is available to ferment hydrolysates produced from these kinds of feedstocks. Thus, new microbes are needed for fermentative conversion of sugars originating from lignocellulose-rich feedstocks.

14.3. FERMENTATION PROCESSES

In addition to the variation among feedstocks, the requirements for fermenting microbes can differ with different pretreatment and fermentation processes. Currently, the most attractive process for biomass to biofuel conversion is simultaneous saccharification and fermentation (SSF), in which the hydrolytic enzymes and fermenting microbe(s) are mixed together and work under the same environment (Punnapayak and Emert, 1986). Use of SSF can mini-

mize product inhibition, resulting in higher ethanol yield, and lower the production cost (Wyman, 1999). In order to maximize the productivity and efficiency of the SSF process, a pretreatment step is usually needed to produce digestible cellulose and release the mixed sugars from hemicelluloses. Ideally, the SSF process also requires microbes to grow and ferment under low pH (at pH 5.0 or lower) and high temperature (up to 50 °C) conditions, to coincide with optimal activities of commercially developed fungal hydrolyzing enzymes. Otherwise, the hydrolytic enzymes must be engineered to provide favorable conditions for fermenting organisms which grow well at neutral pH and conventional temperatures (30–37 °C). To accommodate the pH and temperature requirements of both microbes and enzymes, novel enzymes and hardy microbes are needed to ensure an efficient SSF process that leads to high productivity and yield.

A number of the challenges of developing SSF processes can be overcome using the separate enzymatic hydrolysis and fermentation (SHF) approaches (Wyman, 1999). In these schemes, pretreated material is subjected to separate enzymatic hydrolysis prior to fermentation. This allows the two processes to be conducted independently, under optimal temperature and pH conditions for each. The disadvantages of SHF is that it requires additional unit operations and high enzyme loading rates (to partially overcome product inhibition), and it generates additional waste streams. As with SSF, the SHF process will require a biocatalyst capable of fermenting mixtures of hexoses and pentoses.

Meanwhile, a more aggressive consolidated bioprocessing process (CBP) strategy is being developed to combine the production of cellulases, the hydrolysis of both cellulose and hemicellulose, and fermentation in a single bioreactor (Lynd *et al.*, 2002). The CBP process could offer great economic advantage because of the significant cost reduction in process configuration, but so far, no available microbes can be directly used for CBP in lignocellulosic biomass to biofuel production. The microbe or consortium that can be used for the CBP process must be capable of producing cellulolytic enzymes, in addition to the traits required for the SSF process mentioned above. The metabolic machinery required for cellulose and hemicellulose utilization, as well as glucose and xylose co-fermentation, will all need to be included in the desired microbes by genetic engineering, or via co-culture of microbes that compliment but not compete with the carbon source and other nutrients for efficient product formation.

Therefore, the main challenge in developing robust microbes is to ensure efficient fermentations of the heterogeneous substrates released from biomass materials in the presence of inhibitors and fermentation products and under restricted pH and temperature conditions to ensure maximum product production. For the SSF process, special considerations are needed in searching for new microbes to allow optimum activities of hydrolytic enzymes. For the CBP process, pathways for producing endogenous hydrolytic enzymes should be included in new microbes.

14.4. ETHANOLOGENS

As mentioned above, the major sugar substrates derived from biomass degradation are mixtures of hexoses (C6) and pentoses (C5), predominantly glucose and xylose. Currently, the two most productive organisms, the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*, can produce significant amounts of ethanol from C6 glucose and fructose, but not from C5 xylose. Other microorganisms known to consume mixtures of C5 and C6 sugar substrates, such as *Escherichia coli, Klebsiella oxytoca*, and *Erwinia* strains, do not produce ethanol, or produce it at a low level that cannot be used commercially. Although significant advances have been made toward engineering yeast and Gram-negative bacteria that co-ferment multiple sugar substrates and produce ethanol from renewable biomass (Dien *et al.*, 2003), the current ethanologens are often inhibited by other chemicals present in the biomass hydrolysates. In most cases, an extra detoxification step is required prior to fermentation. In addition, most available ethanol producing microbes are not ethanol tolerant, and are inhibited by relatively low levels of ethanol or even killed by accumulated ethanol (Somerville, 2006).

Gram-positive bacteria possess several desirable traits: the ability to ferment multiple sugars simultaneously, to grow at lower pHs, and for some strains, to grow at a temperature range from 30 °C–50 °C (Bothast *et al.*, 1999). Therefore, this group of microbes could have potential application in converting biomass to biofuel and chemicals. In this review, we will discuss the current research progress and limitations dealing with engineering Gram-positive bacteria to be used as biocatalysts.

14.4.1. Lactic Acid Bacteria

Lactic acid bacteria (LAB) are considered attractive biocatalysts for biofuel production for several reasons. They lack cytochromes and are obligately fermentative, they are aero-tolerant or anaerobic, and they can utilize various carbohydrates and sugar derivatives via efficient transporters and catabolic pathways for energy production. LAB have GRAS (Generally Recognized As Safe) status, and many strains are already used in industrial production of various dairy products, lactic acid, nutraceuticals, bacteriocins, and other chemicals (Kleerebezemab *et al.*, 2000). In addition, LAB are found as the major bacteria contaminants in ethanol plants, and most LAB species are ethanol tolerant, capable of growing in up to 10–16% of ethanol (Alegri *et al.*, 2004; Gold *et al.*, 1992). Certain species, such as *Lactobacillus brevis* and *Lactobacillus buchneri*, are known to grow in the presence of inhibitors derived from plant materials such as wine polyphenolics or hop acids present in beer (Lonvaud-Funel, 1999; Sakamoto and Konings, 2003). Most LAB can grow and ferment in acidic environments (Alegri *et al.*, 2004; Calderon *et al.*, 2003; De Angelis *et al.*, 2001): readily satisfying the pH optimal for enzymes applied to corn stover and rice straw hydrolysis in SSF processes (Karimi *et al.*, 2006;

Ohgren *et al.*, 2006). Recent work suggests that *L. brevis* and *L. buchneri* simultaneously consume C5 and C6 sugars (Kim *et al.*, 2007; Liu *et al.*, 2008), a factor that facilitates complete consumption of all lignocellulosic-derived sugars without catabolite repression. As a result, these strains are desirable hosts for additional manipulations toward efficient conversion of biomass to fuels and chemicals.

Selected LAB species including *Lactobacillus buchneri* PTA6138 and NRRL B-30866; *L. crispatus* NRRL B-30868, 30869 and 30870; *L. reuteri* NRRL B-30867, *L. brevis* NRRL B-30865 can produce ferulate esterases which break down the cross-links between lignin and hemicellulose (Nsereko *et al.*, 2006), suggesting potential applications in lignocellulosic pretreatment and fermentation. Therefore, the direct fermentation of lignocellulosic biomass by genetically modified LAB offers potential candidates for the CBP process (Lynd *et al.*, 2002). Furthermore, LAB have relative small genomes (range from 1.7~3.3 Mb) (Makarova *et al.*, 2006) and a relatively simple metabolism. Several species have been genetically modified for production of B-vitamins, sorbitol and mannitol (Hugenholtz and Kleerebezem, 1999; Hugenholtz *et al.*, 2002). Success in cloning the pentose pathway genes of *L. brevis* and *L. pentosus* into *L. plantarum* demonstrates the feasibility for metabolic engineering of LAB to utilize lignocellulosic biomass derived sugars (Chaillou *et al.*, 1998a, 1998b, 1999). Thus, LAB are well-positioned for genetic engineering strategies aimed at establishing and improving biofuel production. As already mentioned, LAB can also potentially be modified for production of various valuable byproducts from biomass, parallel with the fermentative production of fuel ethanol, and therefore, could indirectly reduce overall cost for ethanol production.

Microbes including *S. cerevisiae* and *Z. mobilis* produce ethanol as principle fermentation products by using pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in a coupled stepwise decarboxylation and dehydrogenation reaction. However, heterofermentative LAB use a different pathway to produce ethanol via pyruvate dehydrogenase, acetaldehyde dehydrogenase, and alcohol dehydrogenase catalyzed reactions (Fig. 14.1) (Keenan and Lindsay, 1967). The genomes of LAB do not contain homologs to pyruvate decarboxylase that produce acetaldehyde directly from pyruvate via decarboxylation. Thus, it was anticipated that adding the missing pyruvate decarboxylase gene into selected recipient genomes would add new ethanol pathway to make selected LAB strains ideal ethanologens (Fig. 14.1).

Research was carried out to introduce the *pet* (Production of Ethanol) operon including the *pdc* and *adhB* genes from *Z. mobilis* (Ingram *et al.*, 1987) into *Lactobacillus casei* (Gold *et al.*, 1996) using *Bacillus subtilis* SPO2 promoter. Recombinant strains produced 189 mM ethanol from the host with the *pdc* gene alone and 142 mM ethanol from the host carrying the entire *pet* operon, suggesting the endogenous alcohol dehydrogenase in *L. casei* is enough to act with recombinant pyruvate decarboxylase for ethanol production. The *pet* operon was also introduced in other lactic acid bacteria under

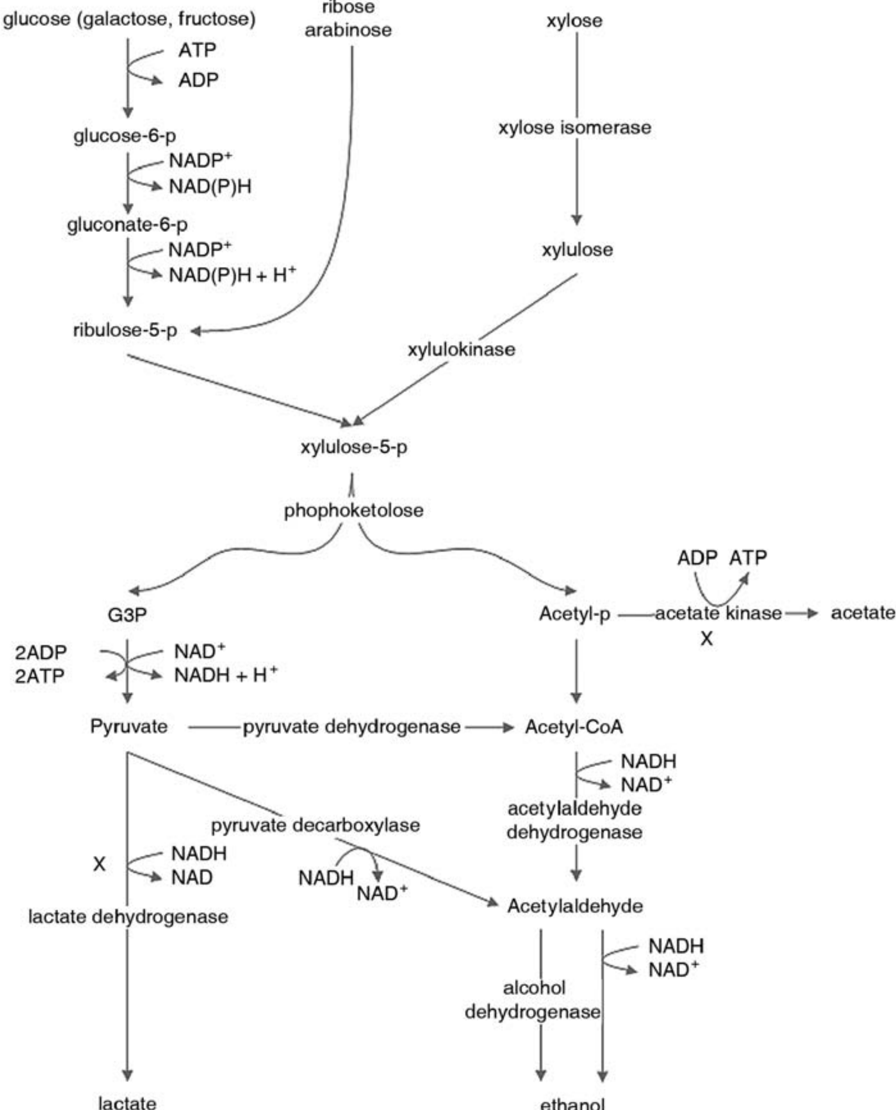

Figure 14.1. Schematic diagram of mixed sugar metabolism in heterofermentative lactic acid bacteria. Engineering for ethanol production would encompass the following strategies simultaneously or sequentially: *i)* inactivation of lactate dehydrogenase, and acetate kinase steps to prevent carbon flow to lactate and acetate respectively, *ii)* the addition of exogenous pyruvate decarboxylase and alcohol dehydrogenase, *iii)* a supplement of acetyl-CoA precursor to enhance carbon flow from acetyl-CoA to ethanol via acetylaldehyde, and *iv)* the introduction of an exogenous aldehyde dehydrogenase that converts acetate to acetaldehyde. The latter can be converted to ethanol by endogenous or exogenous alcohol dehydrogenases.

the control of a *ldh* promoter from *Streptococcus bovis* (Wyckoff *et al.*, 1997) and the CP40 promoter from *L. lactis* (Jensen and Hammer, 1998). The results showed varied levels of ethanol depending on host species examined (Nichols *et al.*, 2003). The *pdc* gene alone from *Z. mobilis* and *Zymobacter palmae* was introduced separately into *L. lactis*, resulted in increased acetaldehyde concentrations (Bongers *et al.*, 2005; Liu *et al.*, 2005), suggesting functional expressions of the *pdc* could be achieved under the control of *nisA* and other *L. lactis* specific promoters (De Ruyter *et al.*, 1996; Madsen *et al.*, 1999).

When the Gram-positive *pdc* gene from *Sarcina ventriculi* (Talarico *et al.*, 2001) was introduced in *L. plantarum* TF103, a strain in which both the *ldhL* and *ldhD* genes were inactivated, the recombinant strains produced 90–130 mM ethanol compared with 17 mM ethanol in the control strain carrying an empty pTRKH2 vector (Liu *et al.*, 2006). This *S. ventriculi pdc* was also introduced into *Bacillus megaterium* (Talarico *et al.*, 2005), which produced 20 mM ethanol from 0.5% xylose. However, the *S. ventriculi pdc* and a construct adding an *adh* gene did not increase ethanol production in host *Lactobacillus brevis* ATCC367, a strain capable of fermenting both xylose and glucose and naturally produces about 20 grams of ethanol per liter from glucose (Liu *et al.*, 2007).

These studies indicate that appropriate hosts can be engineered with selected promoters and vectors though the results for ethanol production differ among strains. In most cases, carbon flow is not significantly improved toward ethanol instead of lactate due to competition for pyruvate between lactate dehydrogenases and pyruvate decarboxylases. Obviously more research is needed in order to modify LAB for efficient ethanol production. First, a genetic knock-out system for pentose utilizing LAB strains needs to be developed. When manipulating end-product pathways, other factors such as NADH/NAD^+ ratio and energy balance will need to be considered. As discussed above, in LAB, ethanol is naturally produced via acetaldehyde catalyzed by dehydrogenase action on acetyl-CoA. If the pool of acetyl-CoA is increased, carbon flow to acetaldehyde might be enhanced. In a fermentation study using *Oenococcus oeni*, addition of pantothenate in culture media, the immediate precursor of CoASH, resulted in increased ethanol fermentation attributable to the enhanced redox-regeneration capacity (Richter *et al.*, 2003; Wagner *et al.*, 2005). Moreover, it may be possible to direct carbon flow from pyruvate to acetyl-CoA instead of lactate via pyruvate dehydrogenase complex or pyruvate oxidase depending on the target species (Wagner *et al.*, 2005). The additional acetyl-CoA generated can be converted to acetaldehyde via endogenous acetaldehyde dehydrogenase (*acdh*) and alcohol dehydrogenase (*adh*) that will amplify the endogenous ethanol production pathway. Another strategy would be to replace the endogenous lactate dehydrogenase with a heterologous pyruvate decarboxylase simultaneously. Since the only available Gram-positive PDC from *S. ventriculi* has lower affinity to pyruvate (Talarico *et al.*, 2001, 2005), a new screening search and/or genetic engineering approach is necessary to obtain a more favorable PDC with higher pyruvate affinity and

preferred Gram-positive codon usage. In addition, it might be necessary to remove the undesired pathway that leads to acetate production (Fig. 14.1) (Wolfe, 2005) or to convert acetate to acetaldehyde by aldehyde dehydrogenase (Figs. 14.1 and 14.2); however, so far no such enzyme has been identified.

More recently, a xylose utilizing strain of *Lactobacillus buchneri* NRRL B-30929 was identified from bacterial contaminants in a commercial ethanol plant (Liu *et al.*, 2008). The strain is unique in its ethanol tolerance (up to 12%), grows rapidly in presence of high concentrations of xylose and glucose (12.5% each), and is capable of metabolizing glucose and xylose simultaneously and utilizing a broad spectrum of additional energy sources including various monosaccharides (C5 and C6), disaccharides and oligosaccharides, particularly under anaerobic conditions. The major fermentative products from glucose and xylose are lactate, ethanol and acetate (Fig. 14.3). However, the organisms should ideally produce only one of these products. Since genetic

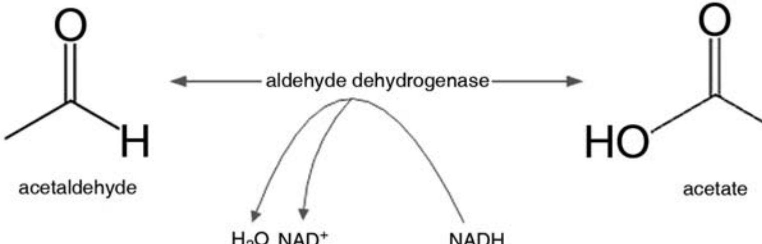

Figure 14.2. The reuse of acetate to acetaldehyde by this proposed single step enzymatic reaction, catalyzed by aldehyde dehydrogenase using NADH as the co-factor.

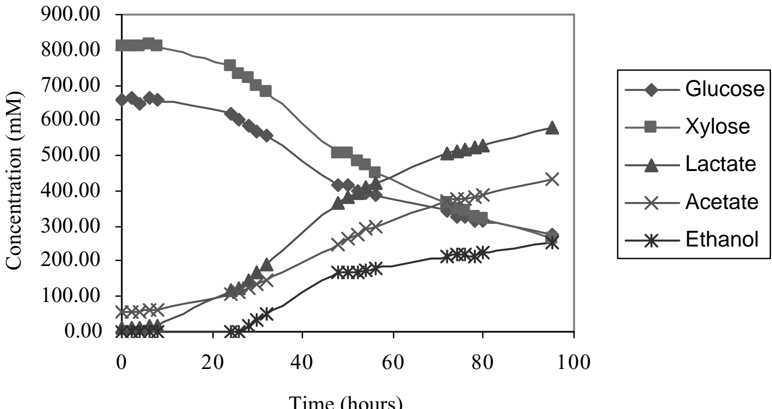

Figure 14.3. Bioreactor fermentation of mixed sugars (about 10–12.5% w/v each, glucose and xylose) by *L. buchneri* strain NRRL B-30929, with 100 rpm stirring and constant pH 5.0. Initial volume is 1000 ml; final concentrations are calculated after volume adjustment. Data represent the mean of duplicate fermentation experiments (Liu *et al.*, 2008).

tools of manipulating LAB are limited to several model strains, more research is needed for development of molecular techniques to engineer C5 and C6 co-utilizing strains such as *L. brevis, L. pentosus* and *L. buchneri.* With the sequencing of *L. brevis* genome (http://genome.ornl.gov/microbial/lbre/) finished and *L. buchneri* project ongoing (U. S. DOE, Joint Genome Institute), comparisons and analyses of these genome sequences with that of the model strain *L. plantarum*, in addition to genome-wide proteomic and metabolomic studies, will predict, guide and facilitate engineering approaches to build robust biocatalysts.

14.4.2. Other Gram-Positive Bacteria

The *Z. mobilis pet* operon has been introduced into several Gram-positive hosts, including several strains of *Bacillus subtilis* and *B. polymyxa* (Ingram and Barbosa-Alleyne, 1999). Heterologous expression and enzyme activities of pyruvate decarboxylase and alcohol dehydrogenase were detected in these hosts, but at levels too low to permit efficient ethanol fermentation. Another host that was explored for ethanol fermentation is *Corynebacterium glutamicum*, in which the *Z. mobilis pet* operon was placed under a *ldhA* promoter of the *C. glutamicum* (Inui *et al.*, 2004). The results showed a 3-fold higher ethanol production (approximately 150 mM) under oxygen-deprivation conditions in the host strain carrying *ldhA* mutation. To broaden substrate utilization, the aerobic *C. glutamicum* was engineered to include *xylA* (xylose isomerase) and *xylB* (xylulose kinase) genes from *Escherichia coli* (Kawaguchi *et al.*, 2006), with the resultant strain producing lactic acid, succinic acid and acetic acid. However, glucose-mediated regulation was still exerted on xylose consumption.

A thermophilic strain, *B. thermoglucosidasius*, was shown to produce 4–8 grams of ethanol per liter per hour with 2–4% glucose/xylose feeds. In this engineered strain, the *Z. mobilis* ethanol production genes were integrated into the host chromosome via homologous recombination while the *ldh* gene was inactivated (Javed *et al.*, 2002). This patented recombinant *B. thermoglucosidasius* strain has potential application in a SSF process since it can ferment at temperatures ranging from 40–75°C. Other advantages of using the thermophilic microbes include reduced contamination by other bacteria; fermenting at temperatures suitable for hydrolytic enzymes; and reducing ethanol inhibition via product evaporation at higher temperatures. However, the final ethanol content (174 mM) from this strain is lower than some naturally occurring lactic acid bacteria such as *L. brevis*, and *L. buchneri* (Liu *et al.*, 2007; 2008). Nevertheless, with the capability of fermenting at high temperatures, further fermentation adaptation tests of this strain using biomass hydrolysates and additional modifications to improve ethanol yield are warranted.

Another promising thermophilic Gram-positive microbe is *Thermoanaerobacterium saccharolyticum* which ferments xylan and produces ethanol, acetate, lactic acid, CO_2, and H_2. The engineered strain TD1, in which the

L-ldh gene was inactivated, was reported to produce increased acetic acid (16.4–16.8 mM) and ethanol (38.6–40.8 mM) with neglectable amounts of lactic acid (Desai *et al.*, 2004). Additional manipulations of this microbe are needed to achieve efficient simultaneous thermophilic saccarification and fermentation for cellulosic ethanol production.

14.5. LACTATE PRODUCTION BY GRAM-POSITIVE MICROBES

Biobased polymers from renewable materials have received increased attention recently. Lactate is a building block for bio-based polymers. In the United States, production of lactic acid is greater than 50,000 metric tons/yr and projected to increase exponentially to replace petroleum-based polymers. Domestic lactate is currently manufactured from corn starch using the filamentous fungus *Rhizopus oryzae* and selected species of lactic acid bacteria. The produced lactic acid can then be polymerized into polylactic acid (PLA) which has many applications (Hatti-Kaul *et al.*, 2007). However, so far, no facility is built to use biomass derived sugars for lactic acid production. More research needs to be done to develop microbes using biomass derived sugars for lactate production.

Most C5 utilizing heterofermentative *Lactobacillus* strains use phosphoketolase to split C5 into lactate and acetate (Fig. 14.1). One unique strain *Lactobacillus* MONT4, isolated from high-temperature grape musts, uses a homofermentative pathway to ferment arabinose into lactate (Picataggio *et al.*, 1998). This strain was engineered to utilize xylose by introducing the xylose isomerase and xylulokinase genes from *L. pentosus*. The recombinant strain MONT4sup pLP3537 ferments 2% xylose to lactate with a yield of 86%, but requires erythromycin to maintain the plasmid carrying the xylose utilizing genes. In addition, undesired catabolic repression by glucose was observed in fermentations with mixed sugars by the engineered strain (Picataggio *et al.*, 1998).

The majority of *Lactobacillus* species are incapable of fermenting xylose. Only a few selected strains, including *Lactobacillus casei* subsp. *rhamnosus* ATCC 10863, were reported to ferment xylose. The ATCC 10863 strain produced lactate (with a yield of 80%) and a small amount of acetate at 45 °C with optimum pH of 6.0 (Iyer *et al.*, 2000). Although the catabolic repression in growth with mixed glucose and xylose was observed, this strain showed high tolerance to inhibitors generated from acid-treated softwood while lactate yield reached 75% with 80% of hydrolysate loading (Iyer *et al.*, 2000).

When screening a large number of *Lactobacillus* strains for lactate production using hemicellulose hydrolysate, strains *L. pentosus* CHCC2355 and *L. brevis* CHCC2097 were selected and evaluated further (Garde *et al.*, 2002). Each strain produced lactate with 88% (for *L. pentosus*) and 61% of the theoretical yield (*L. brevis*), respectively, from wheat straw hydrolysate without visible inhibition. The operation of the phosphoketolase pathway in these

strains limits the theoretical yield to 0.6 g/g of xylose. Furthermore, neither strain was able to fully utilize the broad spectra of carbon sources (xylobiose, glucose, xylose, xylulose, and arabinose) released by the pretreatments. Interestingly, the production of lactate reached 95% of the theoretical maximum yield by co-inoculation of the two strains (Garde *et al.*, 2002). Another study of co-cultivation of *Enterococcus casseliflavus* and *Lactobacillus casei* resulted in complete consumption of xylose (50 g/l) and glucose (100 g/l), produced 95 g/l lactate with 96% L-lactic acid (Taniguchi *et al.*, 2004).

Theoretically, due to the operation of the phosphoketolase pathway in pentose utilizing lactic acid bacteria (Fig. 14.1), only one mole of lactate can be produced from one mole of pentose while two moles of lactate can be produced from one mole of glucose in homo-fermentative strains. Searching for the homo-lactate pathway from pentose to lactate in pentose utilizing bacteria has not been successful. Nevertheless, a unique strain of *Lactococcus lactis* IO-1 was found to produce lactate from xylose in yields that often exceeds 1.0 mol/mol (Tanaka *et al.*, 2002). The activity changes of several key enzymes, including phosphoketolase, transketolase and transaldolase under various culture conditions, indicated that *L. lactis* IO-1 metabolized xylose mainly by the pentose-phosphate/glycolytic pathway at high concentrations (50 g/l), leading to higher lactate yields. Under lower concentrations (5 g/l), xylose is primarily metabolized by the phosphoketolase pathway producing equal molar of lactate and acetate (Tanaka *et al.*, 2002). A bioinformatics study using this IO-1 strain indicated that about 70% metabolic flow from xylose goes to the pentose-phosphate/glycolytic pathway with a feeding of 20g/l xylose. This study also predicted that an increase of NADH should enhance lactate production upto 1.4 mol/mol of xylose (Ohara *et al.*, 2007). Although not mentioned in this study, one would speculate that the ATP production pathway and the carbon flow via pyruvate formate lyase (the calculated f_{19} in that paper) will need to be manipulated in order to minimize the production of acetic acid, formic acid and ethanol by this strain (Ohara *et al.*, 2007).

Recently, strains of *Leuconostoc lactis* SHO-47 and SHO-54, known to produce 6-phosphoglucanate dehydrogenase, reportedly produced D-lactate from xylooligosaccharide derived from enzymatic hydrolysis of birch wood xylan (Ohara *et al.*, 2006). These strains produced xylosidases and used the phosphoketolase pathway to metabolize xylose. Therefore, equal molar of acetic acid was also produced. The simultaneous production of the clinically useful phosphogluconate dehydrogenase and D-lactate by SHO-47 and SHO-54 could potentially reduce the production cost of both products from woody biomass materials.

Perhaps the acid-tolerant, thermophilic *Bacillus coagulans* is the only known biocatalyst that naturally produces lactic acid from xylose via the pentose phosphate pathway, not the phosphoketolase pathway (Patel *et al.*, 2006). Three strains, 17C5, P4-102B, and 36D1, can ferment both hexoses and pentoses to pure L(+)-lactic acid at 50 °C and pH 5.0, an optimal environment

for fungal cellulase during SSF processes. These strains can also grow and ferment in 50% sugar cane bagasse hemicellulose acid hydrolysate and therefore hold great potential for biomass to lactate conversion. Additional research is needed to ensure rapid bacterial growth in large scale and to maximize lactate productivity.

14.6. CONCLUSIONS

Due to limited petroleum resources and environmental concerns, the chemical industry has been pressured to shift toward a renewable bio-based industry. The production of chemicals from biomass feedstocks led to the concept of the biorefinery, analogous to the petroleum refinery. In concept, multiple products produced from fermentative microbes using biomass feedstocks will be processed into a variety of downstream product lines. The simultaneous production of biofuel and co-products via a biorefinery platform will maximize the value of lignocellulosic biomass based industry. The economic conversion of various low valued biomass feedstocks to fuel and other high valued bioproducts calls for novel and robust biocatalysts that are capable of using mixtures of substrates to produce multiple products, along with cost-effective processes for product separation and recovery. Either single microbes such as several promising Gram-positive bacteria mentioned in this chapter or a consortium of complementary microbes need to be further explored and developed for biorefinery applications.

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CHAPTER 15

Biological Hydrogen Production by Strict Anaerobic Bacteria: Fundamentals, Operational Strategies, and Limitations

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15.1. INTRODUCTION

For more than a century, fossil fuels have been extensively used to satisfy human's needs. The application of fossil fuels is very diverse—producing electricity, powering transportation activities, providing raw materials for clothes and construction. However, the world's fossil fuels inventory is rapidly declining and soon demand will outstrip production. Based on current consumption rates, fossil fuel supply will only last for a few centuries.

The environmental consequences of an extensive use of fossil fuels have already begun to surface. The excessive use of fossil fuels is one of the primary causes of global warming and acid rain, which have started to affect the earth's climate, weather conditions, vegetation and aquatic ecosystems (Hansen *et al.*, 1981). Some researchers believe that green house gas emissions have already caused a global warming. In addition, the issue of ozone depletion in the stratospheric layer is becoming critical, due to the breakage of ozone by NO_X gases emitted by the combustion of fossil fuels. An evaluation on the economic loss by worldwide environmental damage in 1990 estimated that the total environmental damage costs $2,360 billion per year or $460 per capita per year due to fossil fuels use (Barbir *et al.*, 1990). It is also believed that the environmental damage costs are even higher today.

Considering energy security and the global environment, there is a pressing need to find non-polluting and renewable energy sources. Hydrogen is a clean energy carrier, producing water as its only by-product when it burns. Hence, hydrogen could be a potential energy substitute for fossil fuels. The complete concept of hydrogen production, application, and its scientometric analysis has been developed since the middle 1980s. The concept involves hydrogen production from non-renewable and renewable energy sources, hydrogen transportation and storage, hydrogen utilization, and hydrogen safety issues (Goltsov *et al.*, 2006). With regard to the impact of hydrogen on human society, currently some researchers are evaluating the future of hydrogen economy (Winter, 2005; Milciuviene *et al.*, 2006), and are beginning to predict the development of hydrogen civilization and its culture (Ohta, 2006).

Hydrogen can be produced from both non-renewable (coal, nuclear energy) and renewable (sun, hydro, wind, biomass, tides, and so forth) energy sources. The non-renewable energy sources will be depleted eventually; and hydrogen production from non-renewable sources will release environmentally unfriendly or hazardous wastes. On the other hand, renewable energy sources for hydrogen production are unlimited, and the process of hydrogen production has little impact on the environment (Da Silva *et al.*, 2005; Sherif *et al.*, 2005; Tsai, 2005). Biological processes evolving hydrogen production are categorized as renewable energy sources and, for several decades, have been

subject to basic and applied research. In the biological process, hydrogen production is carried out by microorganisms, which can split water into hydrogen and oxygen moleculars, or they can ferment organic materials into hydrogen. Based on the metabolic pathways performed by different groups of microorganisms, biological hydrogen production processes can be classified as: *i*) biophotolysis of water using algae and cyanobacteria, *ii)* photo fermentation of organic materials by photosynthetic bacteria, and *iii)* dark fermentation of organic materials using fermentative bacteria (Hallenbeck and Benemann, 2002). A novel hybrid system of combining dark and photo fermentations to enhance hydrogen production is also being proposed (Das and Veziroglu, 2001).

Hydrogen through dark fermentation can be produced from organic wastes. Additionally, there is also a need to dispose of human-derived wastes in an environmentally friendly manner. Some of these wastes are by-products/residuals of food processing plants and agricultural entities. From the perspective of pollution prevention and resource conservation, hydrogen production from organic waste through dark fermentation offers dual benefits of waste treatment and production of renewable bioenergy.

15.2. DARK FERMENTATION

Dark fermentation is a catabolism: anaerobic bacteria convert sugars and proteins to carboxylic acids, hydrogen, carbon dioxide, and solvents. This biological chemical reaction is carried out under anaerobic condition in which the presence of oxygen is prohibited.

15.2.1. Fundamentals of Dark Fermentation

In dark fermentation, different groups of bacteria (such as *Enterobacter*, *Clostridium*, and *Bacillu*) are responsible for hydrogen production. Fang *et al.* (2002) reported that in a biohydrogen production mixed culture, approximately 70% of the population was of the genus *Clostridium* and 14% belonged to the *Bacillus* species. Another study investigated hydrogen-producing microbial populations and also showed that hydrogen production is directly correlated to *Clostridium* population in a continuous flow bioreactor (Duangmanee *et al.*, 2002a).

Clostridium is an anaerobic spore former. In response to hostile conditions (oxygen, heat, acid, base, alcohol, etc.), the physiology of *Clostridium* changes from a vegetative cell to an endospore, a stress-resistant state, with greatly reduced metabolic activity. A standard procedure to isolate *Clostridium* from mixed microbial communities requires heating to 70 °C for 10 min to kill vegetative and nonspore forming organisms (*Bergey's Manual,* 1984). In addition to the selection process of *Clostridium* by heat treatment, some species even require heat activation for endospore germination (Mead, 1992).

According to its catabolism, *Clostridium* can be classified as saccharolytic and proteolytic of fermenting bacteria. Saccharolytic clostridium ferments

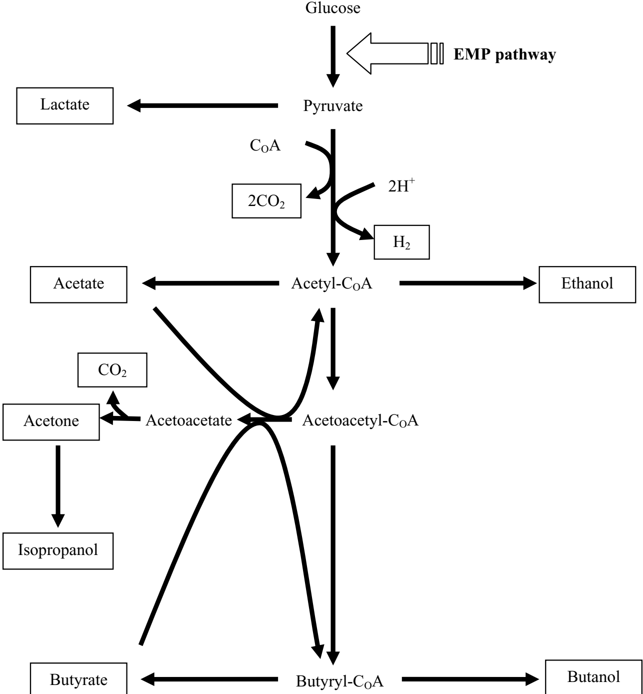

Figure 15.1. Catabolic pathway of *Clostridia* in hydrogen fermentation. Sources: Jones and Woods, 1989; Mitchell, 2001.

carbohydrates, consisting of simple sugars, disaccharides, oligosaccharides, and cellulose; whereas, proteolytic clostridium hydrolyzes protein and ferments amino acids (Ljungdahl *et al.*, 1989). However, most proteolytic clostridium can also ferment carbohydrates, and, hence, carbohydrates are very common substrates for the genus of *Clostridium*.

With regard to dark fermentation, the fermentation of *Clostridium,* utilizing simple sugars, follows the Embden-Meyerhof-Parnas (EMP) glycolytic pathway, where glucose is converted to pyruvate, an intermediate of hexose metabolism (Schroder *et al.*, 1994). Pyruvate is then oxidized by pyruvate-ferredoxin oxidoreductase to yield acetyl-CoA, carbon dioxide, and reduced ferredoxin (Hallenbeck and Benemann, 2002). The re-oxidation of reduced ferredoxin is catalyzed by hydrogenase to evolve hydrogen production. The fermentation pathway is shown in Figure 15.1.

Using glucose as a model substrate, hydrogen production is accompanied with either acetate formation (Eq. (15.1) or butyrate formation (Eq. (15.2) (Miyake, 1998). In the acetate fermentation 4 ATPs are produced; whereas, 3 ATPs are produced in the butyrate fermentation. Thus, it seems that the acetate fermentation is energetically more favorable than the butyrate fermentation. However, acetate and butyrate fermentations are commonly carried

out simultaneously (Eq. (15.3) (Wood, 1961). Butyrate fermentation is the more dominant reaction than acetate fermentation, where overall stoichiometric yields of ATP and hydrogen are 3.3 and 2.5 mol/mol glucose, respectively.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G°\text{-}184\,kJ \quad \text{[Eq. 15.1]}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \Delta G°\text{-}257\,kJ \quad \text{[Eq. 15.2]}$$

$$4C_6H_{12}O_6 + 2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2 \quad \text{[Eq. 15.3]}$$

The genus of Clostridium can convert glucose not only to hydrogen and organic acids but also to solvents. In a batch culture, *Clostridium* sp. produced hydrogen and organic acids at an exponential growth phase; whereas, the metabolism shifted to solvents production during the late growth phase (Afschar *et al.*, 1986; Brosseau *et al.*, 1986). *Clostridium acetobutylicum*, a well-known species that can produce hydrogen and organic acids, is favored solvent fermentation at a pH below 5 (Gottwald and Gottschalk, 1985). In addition, the metabolic pathway of *Clostridium pasteurianum* showed an abrupt shift from hydrogen and acid production to solvent production under iron or phosphate limiting condition, high substrate concentrations (125 g glucose/L), and the appearance of carbon monoxide (an inhibitor of hydrogenase) (Dabrock *et al.*, 1992).

15.3. OPERATING PARAMETERS AFFECTING HYDROGEN PRODUCTION

For sustainable and enhanced hydrogen production, the proliferation of hydrogen consumers, *e.g.*, methanogens, and sulfate reducers, needs to be inhibited in a mixed culture system. With regard to the process engineering, strategies such as heat treatment, pH control, hydraulic retention time (HRT), and improved reactor design could be used as selection pressures to obtain enriched culture of hydrogen-producing bacteria.

15.3.1. Inoculums and Start-Up

In the studies biological hydrogen production, populations of *Clostridium* were extracted from soil (Van Ginkel *et al.*, 2001), anaerobic digested sludge (Lay *et al.*, 2003), compost (Khanal *et al.*, 2006), and so forth. Since *Clostridium* has an inherent mechanism of shifting its morphology and physiology to endospore at an elevate temperature, the heat shock method is a common treatment to select spores forming *Clostridium*, while killing hydrogen-consuming, nonspore forming bacteria, such as methanogens, and sulfate reducing bacteria. Boiling anaerobic digested sludge has been broadly used to

preserve *Clostridium* from other microbial populations in hydrogen fermentation studies (Lay, 2000; Duangmanee *et al.*, 2002a). Meanwhile, baking compost or soil is the other heat shock method, while the inoculum is obtained from the solid samples (Fan and Chen, 2004; Khanal *et al.*, 2004; Lay *et al.*, 2005a).

In addition to heat shock treatment, acid and base treatments have been developed as alternative selection processes for the population of *Clostridium*. Chen *et al.* (2002) compared acid and base treatment reported that methanogens were effectively inhibited at both pH 3 and 10. In the later study, Cai *et al.* (2004) applied base treatment to deactivate hydrogen consumers in sewage sludge. Kawagoshi *et al.* (2005) used acid treatment to eliminate hydrogen consumers from various inocula including activated sludge, anaerobic digested sludge, refuse compost, watermelon soil, kiwi soil, and lake sediment.

15.3.2. Feedstock Characteristics

Research investigating hydrogen fermentation has been conducted on various types of feedstocks. Simple sugars (glucose, and sucrose) used for fermentative hydrogen production have been reported (Majizat *et al.*, 1997; Mizuno *et al.*, 2000; Van Ginkel *et al.*, 2001; Duangmanee *et al.*, 2002a; Lin and Jo, 2003; Khanal *et al.*, 2004). Other investigators explored the potential of hydrogen production from complex substrates, *e.g.*, food and food processing wastes (Shin *et al.*, 2004; Van Ginkel *et al.*, 2005; Wu and Lin, 2004), cellulose containing waste (Okamoto *et al.*, 2000), municipal solid wastes (Ueno *et al.*, 1995), activated sludge (Wang *et al.*, 2003), *etc.*

In addition to testing the potential of hydrogen production from different feedstocks, Lay *et al.* (2003) compared hydrogen production from synthetic carbohydrate-rich, protein-rich, and fat-rich organic solid wastes. Their batch study results indicated that hydrogen-producing bacteria could evolve much more hydrogen from carbohydrate-rich organic waste. A similar conclusion was also obtained from the investigation of converting bean curd manufacturing waste (protein-rich waste), rice and wheat bran (carbohydrate-rich waste) into hydrogen, where rice and wheat bran were more favorable for hydrogen fermentation (Noike and Mizuno, 2000). More carbohydrate-rich organic wastes, e.g., rice winery wastewater (Yu *et al.*, 2002; Yu *et al.*, 2003) and starch-manufacturing wastewater (Yokoi *et al.*, 2002; Hussy *et al.*, 2003), were also utilized for hydrogen fermentation.

15.3.3. pH

pH has been the most pivotal parameter for *Clostridium*-rich bioprocess. Hydrogen production was associated with the generation of organic acids in the first (acidogenic) phase of the anaerobic fermentation (Jones and Woods, 1989). This instinctively suggests that biohydrogen production is more favorable in an acidic range. Many investigations have evaluated the optimal pH

from various types of substrates. Batch studies using sucrose as a limiting substrate for hydrogen production suggested that the ideal initial pHs ranged from 5.5 to 5.7 (Van Ginkel *et al.*, 2001; Khanal *et al.*, 2004; Wang *et al.*, 2005). Zhang *et al.* (2003) reported that the optimal initial pH for converting starch to hydrogen was 6.0 under thermophilic conditions. Another study also showed that an initial pH of 6.0 was favorable for hydrogen production from cheese whey (Ferchichi *et al.*, 2005). Fang *et al.* (2006) found that better performing hydrogen production from rice slurry was obtained at an initial pH of 4.5. These studies concluded that an initial pH at slightly acidophilic conditions helps to enhance hydrogen production.

Apart from the initial pH in batch studies, an ideal pH of 5.5 was found to be the optimum for hydrogen production from glucose in a continuous operation (Fang and Liu, 2002). Lay (2000) consecutively controlled pH at 5.2 to optimize hydrogen production from a synthetic starch wastewater. In addition, based on the statistical contour plot analysis, Lay and his coworkers determined the optimum pH of 5.8 for a complete mixed bioreactor converting brewery wastes into hydrogen (Lay *et al.*, 2005a). When fermenting palm oil mill effluent at different pH (4.0 to 7.0), the pH of 5.0 was found to be the optimum for fermentative hydrogen production (Vijayaraghavan and Ahmad, 2006).

15.3.4. Temperature

Fermentative hydrogen production is a biochemical reaction, which is related to the activity of temperature-sensitive enzyme (hydrogenase). To enhance hydrogenase activity for increasing hydrogen production, a suitable temperature would be required. A batch test using glucose as substrate for hydrogen fermentation showed that hydrogen yield increased 72% with a temperature increase from 33 to 41 °C (2 °C increment) (Mu *et al.*, 2006). The study also found that the specific hydrogen production rate increased 86% with increasing the temperature from 33 to 39 °C, but it decreased at 41 °C. A response surface method analyzing temperature effect (20 to 45 °C) indicated that hydrogen converted from sucrose achieved the maximum yield at 35.1 °C (Wang *et al.*, 2005). Zhang and Shen (2006) found that a temperature range from 25 to 40 °C favored the conversion of sucrose to hydrogen, but hydrogen production was almost inhibited as the temperature increased to 45 °C. Wu *et al.* (2005) determined that 40 °C was the ideal temperature for the sludge immobilized by ethylene-vinyl acetate copolymer to perform hydrogen fermentation. Contrary to those results which found optimal temperatures in mesophilic range, the potential of hydrogen production from cow waste slurry at 60 °C was more than 30 folds higher than that at 37 °C (Yokoyama *et al.*, 2007).

In a continuous operation, 40 °C was determined to be the optimum for a carrier-induced granular sludge (CIGSB) reactor converting sucrose to hydrogen (Lee *et al.*, 2006). However, Yu *et al.* (2002) reported that 55 °C is the optimal temperature for hydrogen production from rice winery wastewater in

an upflow anaerobic sludge blanket (UASB) reactor. For energy saving and simplicity of reactor operation, Lin and Chang (2004) evaluated the hydrogen production under ambient temperature in a continuous stirred tank reactor (CSTR). However, their results suggested that hydrogen yield and hydrogen production rate at ambient temperature are lowered than those at 35 °C.

15.3.5. Hydraulic Retention Time (HRT)

A kinetic study of hydrogen production using sucrose as a limiting substrate was conducted. It was found that the maximum specific growth rate was 0.172 h^{-1} for hydrogen producers, which allowed them to remain in a continuously stirred tank reactor (CSTR) at a short HRT (Chen *et al.*, 2001). An investigation regarding the effect of HRT on hydrogen production from glucose indicated that the maximum hydrogen yield of 1.76 mol/mol $hexose_{consumed}$ was obtained at HRT of 6 h in a CSTR (Lin and Chang, 1999). Ueno *et al.* (1996) reported that a maximum hydrogen yield from sugary wastewater was achieved at HRT of 12 h. By treating rice winery wastewater using a CSTR, hydrogen yield of 1.32 mol/mol $hexose_{consumed}$ at HRT of 24 h was conversely decreased to 1.04 mol/mol $hexose_{consumed}$ at HRT of 4 h (Yu *et al.*, 2003). With a full factorial experimental design varying HRT from 12 to 48 h, Lay *et al.* (2005a) found that the HRT of 32 h was ideal for fermenting beer processing waste. For converting insoluble wheat starch co-product, reducing HRT from 18 to 12 h improved hydrogen yield without affecting starch removal efficiency (Hussy *et al.*, 2003)

Since varying HRT altered the organic loading rate simultaneously, there is a concern with the ambiguity between the effect of HRT and the organic loading rate on hydrogen production. Using granular acidogenic sludge, a study was conducted to examine the influence of HRT (4.6 to 28.8 h) with its corresponding sucrose concentration (4.8 to 29.8 g/L) at a constant organic loading rate of 25 g sucrose/L/d. It was reported that the maximum yield occurred at HRT of 13.7 h with a sucrose concentration of 14.3 g/L (Liu and Fang, 2002).

15.3.6. High-Rate Reactor for Fermentative Hydrogen Production

Most studies on fermentative hydrogen production using mixed culture were conducted in a conventional CSTR. The early studies have shown that short HRT was favorable for hydrogen production in a CSTR. At short HRT, methane-producing microbes could essentially be washed-out due to its low specific growth rate of 0.0167 h^{-1} (van Haandel and Lettinga, 1994). However, short HRT could reduce the efficiency of substrate utilization by the bacteria and therefore overall process efficiency as well. Methods to enhance hydrogen-producing bacteria concentration for the improvement of substrate utilization efficiency have been developed based on various reactor configurations.

These reactors were capable to decouple sludge retention time (SRT) from HRT, allowing the reactors to retain hydrogen-producing bacteria concentration at higher level.

Lately, the membrane bioreactor (MBR) has become increasingly popular for municipal wastewater treatment. In MBR, membrane modules are incorporated with bioreactors in order to retain the biomass in the reactor. Oh *et al.* (2004) used MBR for hydrogen production and reported that at a HRT of 3.3 h, the glucose consumption increased from 90 to 98%, and the conversion efficiency of glucose to hydrogen increased from 22 to 25% when SRT was increased from 3.3 to 12 h. However, with SRT increasing from 5 to 48 h with HRT held constantly at 5 h, the hydrogen conversion efficiency decreased from 37 to 18% even glucose consumption remained over 99%.

Using a UASB reactor to perform hydrogen fermentation, Gavala *et al.* (2006) reported that glucose consumption could remain more than 85% at HRT as low as 2 h. For hydrogen production using UASB from rice winery wastewater, the hydrogen yield only slightly dropped from 2.14 to 1.74 mol/mol $hexose_{consumed}$ when the HRT was decreased from 24 to 2 h, respectively (Yu *et al.*, 2002). It can be concluded that high biomass concentration in a UASB reactor guarantees not only high substrate consumption but also a stable hydrogen yield.

Immobilizing biomass in packed media or granular provides an alternative option to retain biomass within the bioreactor. With cell immobilization by acrylic latex plus silicone in a three-phase fluidized-bed bioreactor, HRT could be reduced to as short as 2 h; however, hydrogen yield of as high as 2.67 mol/mol $sucrose_{consumed}$ was still achieved (Wu *et al.*, 2003). In fixed-bed reactors using activated carbon (AC) and expanded clay (EC) as packed media for cell immobilization, Chang *et al.* (2002) reported that the optimum hydrogen production rates of 1.32 (AC) and 0.42 L/h/L (EC) were achieved at HRT of 1 and 2 h, respectively. For biomass immobilized in granular, a carried-induced granular sludge bed bioreactor could be operated at a HRT of 0.5 h with a maximal hydrogen yield of 3.03 mol/mol $sucrose_{consumed}$ (Lee *et al.*, 2004).

15.3.7. Nutrients

Biological processes require nutrient supplements for microbial metabolism and growth. For microbial multiplication, nitrogen is the most essential element for cell synthesis. As with hydrogen fermentation, nitrogen could affect hydrogen production to some extent. A study was conducted by varying NH_4HCO_3 concentrations between 560 and 11,280 mg/L (99–1,999 mg N/L) in synthetic starch wastewater (15 g starch/L). It was found that the maximum hydrogen yield and specific hydrogen production rate were at 5,640 mg/L (999 mg N/L) (Liu and Shen, 2004). Meanwhile, Lay *et al.* (2005b) suggested that NH_4^+ concentration of 537 mg/L (418 mg N/L) was beneficial for hydrogen production from food wastes. Lin and Lay (2004a) explored that, at a carbon to

nitrogen ratio (C/N) of 47 in synthetic sucrose wastewater, hydrogen yield and hydrogen production rate were found to be at their maximum of 2.4 mol/mol $hexose_{consumed}$ and 270 mmol/L/d, respectively. In addition, the origin of nitrogen sources—from inorganic portion or organic portion—plays an important role in determining the discrepancy of hydrogen production potential. Substituting peptone for NH_4Cl as a nitrogen source reduced hydrogen yield by as much as 50% (Ueno *et al.*, 2001).

Beside nitrogen, phosphor is another important element for biosynthesis. One of its chemical forms, phosphate, is commonly used for buffering. Lin and Lay (2004b) found that a Na_2HPO_4 concentration of 600 mg/L in synthetic sucrose wastewater enhanced the hydrogen production rate by 1.9 times in comparison to that in an acidogenic nutrient formulation by Endo *et al.* (1982). For converting high solid food wastes, Lay *et al.* (2005b) suggested that PO_4^{3-} of 1,331 mg/L resolved by multivariate analysis was optimal concentration for the development of hydrogen-producing bacteria.

Apart from the macronutrients (N and P), iron is the most common trace element in all the cultural media. A pure culture (*Clostridium acetobutylicum*) study revealed that lactate fermentation became the predominant under iron limitation, which was not favorable for hydrogen production (Bahl *et al.*, 1986). On the other hand, with sufficient iron concentration (1.2 to 100 mg/L), Liu and Shen (2004) reported that 10 mg Fe^{2+}/L was the optimum for hydrogen production from starch. However, the optimum iron concentrations obtained from the researches are still varied based on the different research conditions. Using sucrose as a limiting substrate, Zhang *et al.* (2005) recommended the supplementation of 589 mg Fe^{2+}/L for hydrogen production. However, Lay *et al.* (2005b) determined that 132 mg Fe^{2+}/L was suitable for mixed cultures converting food wastes to hydrogen.

In addition to iron, fermentative hydrogen production requires some other essential micronutrients. To date, diverse nutrient formulas have been used in many research studies. Nevertheless, it is doubtful that the nutrients formulas adopted in these studies were optimal to supplement the hydrogen-producing bacteria. To determine the most optimal formula, Lin and Lay (2005) designing an experiment based on Taguchi orthogonal arrays, resolving the optimum formula containing the following nutrients (mg/L): Mg^{2+} 4.8; Na^+ 393; Zn^{2+} 0.25; Fe^{2+} 1; K^+ 2.94; I^- 9.56; Co^{2+} 0.25; $NH4^+$ 16.8; Mn^{2+} 2.4; Ni^{2+} 1.23; Cu^{2+} 1.25; Mo^{6+} 0.04; and Ca^{2+} 544. This study concluded that Mg^{2+}, Na^+, Zn^{2+}, and Fe^{2+} were the important trace metals affecting hydrogen production while Mg^{2+} is the most significant one.

15.4. CHALLENGES OF THE REACTOR OPERATION

Strategies to improve hydrogen production have been attained by optimizing operating pH, temperature, reactor configurations, and seed biomass manipulation before inoculation. However, some matters still limit the hydrogen

production. Only optimizing the reactor operation cannot fully overcome the limitations: acids inhibition, hydrogen partial pressure, methanogenesis, and microbial population competition.

15.4.1. Volatile Fatty Acids (VFAs)

Fermentative hydrogen production is always accompanied by the production of acetate and butyrate. However, these acids could result in product inhibition for hydrogen-producing bacteria, the critical factor leading to deteriorating performance of biological hydrogen production. In an early study, Heyndrickx and his coworkers (1987) reported that, when adding acetate up to 18.0 g/L to *Clostridium butyricum*, no significant impact on hydrogen production was found. However, the addition of butyrate higher than 17.6 g/L began to inhibit the activity of the cell culture. Van den Heuvel *et al.* (1988) agreed with these results that only butyrate up to 17.6 g/L inhibited acidogenic bacteria growth in the mixed culture, but acetate did not inhibit the growth of bacteria. To quantify the inhibitory effect, Zheng and Yu (2005) used IC_{50} value to represent butyrate concentration causing 50% inhibition in hydrogen production rate and hydrogen yield. They reported that the IC_{50} values for hydrogen production rate and hydrogen yield were 19.39 and 20.78 g/L, respectively.

Van Ginkel and Logan (2005) compared the effect of the addition of acids from an external source with the effect of self producing acids on acid inhibition to biohydrogen production. They found that hydrogen yields were suppressed by self-produced acids. Because of the high level of self-produced VFAs during hydrogen fermentation, the possibility of VFAs inhibition was inevitable. However, based on current technology, the methodology for removing VFAs from a fermentative reactor content has not been satisfactory.

15.4.2. Hydrogen Partial Pressure

Hydrogen partial pressure in the liquid phase is the other issue burdening biological hydrogen production. Currently, several technologies have been able to reduce hydrogen partial pressure. Lamed *et al.* (1988) studied the effect of stirring on hydrogen production from cellulose and cellobiose. They found three-fold hydrogen content with less total hydrogen production in the unstirred culture broth compared to that in the stirred culture. Lay (2000) observed that increasing the agitation speed from 100 to 700 rpm in a lab-scale completely mixed reactor could double the hydrogen production rate from starch.

In addition to stirring, another approach using nitrogen gas sparging showed that hydrogen content was reduced from 50 to 7%, but hydrogen yield increased from 1.3 to 1.9 mol/mol $hexose_{consumed}$ (Hussy *et al.*, 2003). A similar experiment also carried out by Hussy *et al.* (2005) found that sparging nitrogen

increased 70% of hydrogen yield from refined sucrose. A study by Mizuno *et al.* (2000) illustrated that nitrogen gas sparging could help enhance hydrogen yield from 0.85 mol/mol $glucose_{consumed}$ to 1.43 mol/mol $glucose_{consumed}$. Rather than nitrogen gas sparging, Kim *et al.* (2006) concluded that carbon dioxide gas sparging was more effective to improve hydrogen production. Their results indicated that at the same gas sparging rate (300 mL/min), the maximum hydrogen yield of 1.68 and 0.95 mol/mol $hexose_{consumed}$ were observed by using carbon dioxide gas and nitrogen gas sparging, respectively.

Although the early investigations have shown the high efficiency of gas sparging on the improvement of hydrogen production, an evaluation of the process must be undertaken to understand whether the dilution of hydrogen stream by gas sparging is feasible, and cost-effective for a full-scale hydrogen-producing plant.

15.4.3. The Growth of Methanogens

The high specific growth rate of hydrogen-producing bacteria allows them to grow faster than their washout rate in a completely mixed reactor operating at a short HRT. Thus, the short HRT facilitates the washout of the hydrogen consumers, especially methanogens from the hydrogen-producing bioreactor. However, Lin and Jo (2003), operating an anaerobic sequencing batch reactor (ASBR), found that methane was detected even at HRT of 10 h, which was not discovered in CSTR operated at the similar HRT. ASBR is classified as a high-rate reactor, which can maintain a high level of biomass concentration. In the ASBR, long sludge retention time (SRT) could be disassociated from short HRT. Therefore, the most possible explanation for Lin and Jo's case is due to the long SRT for the potential survival of hydrogen consumers.

To prevent methanogenesis at long SRT, the well-acclimated hydrogen-producing sludge was used as an inoculum instead of thermal/chemical pre-treated anaerobic sludge. The well-acclimated hydrogen-producing sludge has already been subjected to thermal/chemical pretreatment (heat, acidification, and alkalization) and hydraulic selection in a CSTR. Recent studies have observed that no methane production was found from high-rate bioreactors seeded by the well-acclimated hydrogen-producing sludge (Wu *et al.*, 2003; Lee *et al.*, 2004, 2006).

15.4.4. Microbial Populations

The genus *Clostridia* has long been known for its contribution to the biochemical reaction of hydrogen fermentation under anaerobic condition. However, the sole *Clostridia* population growing in a continuous reactor can not be guaranteed in biohydrogen production seeded with mixed culture. Lay *et al.* (2005a) reported that not only *Clostridium thermocellum*, but also *Klebsiella pneumoniae* were predominated in the hydrogen-producing bioreactor. In addition, Duangmanee *et al.* (2002b) observed the shift of microbial popula-

tions in a continuous reactor operated for 25 days with sludge recirculation. Their results indicated that the microbial population was shifted from *Clostrdia* to *Bacillus* beginning at day 18. The genus *Bacillus* is able to form endospore and carry out the fermentative hydrogen production as well. Nevertheless, the physiology of *Bacillus* is different from *Clostridia*, which may interfere the performance of hydrogen production. Strategies to enhance hydrogen production from *Bacillus* should be re-evaluated in the continuous operation.

15.5. PRACTICAL LIMITATION

The feasibility of the technology of fermentative hydrogen production from wastewater and organic wastes has been demonstrated in many papers; however, the technology to the practical application still faces its limitation. High carbohydrate concentration and low hydrogen yield are the critical issues restricting the development of fermentative hydrogen production.

15.5.1. High Substrate Concentration/High Organic Loading Rate Requirement

Chen *et al.* (2006a), studying the kinetics of hydrogen production from three characteristics of substrates, reported the half saturation constant (K_s) of 1.4, 6.6, and 8.7 g COD/L for sucrose, nonfat dry milk (NFDM), and food waste, respectively. Other investigations conducted on the growth kinetics of hydrogen-producing bacteria from various cultures and substrates are summarized in Table 15.1. Based on K_s values, hydrogen-producing bacteria have more affinity to carbohydrate-rich substrate. A significant conclusion according to the K_s values is that high substrate concentration is essential for hydrogen fermentation. This finding explained the key choices to be made in successfully operating a hydrogen-producing reactor, *i.e.*, either high substrate concentration or high organic loading rate. One of the studies reflected that with sucrose concentrations from 11.2 to 56.0 g COD/L at HRT of 12 h, hydrogen yield decreased from 1.65 to 0.81 mol/mol $hexose_{added}$ in a CSTR at pH between 5.2 and 5.3 (Kyazze *et al.*, 2005). A similar investigation regarding the effect of sucrose concentrations (10 to 60 g COD/L) on hydrogen production was performed by Kim *et al.* (2005). However, their research reported that hydrogen yield reached its peak of 1.09 mol/mol $hexose_{added}$ (1.23 mol/mol $hexose_{consumed}$) at sucrose concentration of 30 g COD/L at HRT of 12 h with pH 5.2 in a CSTR.

Again, it is wise to note that hydrogen fermentation demands carbohydrate-rich substrate with high substrate concentration. From the perspective of environmental engineering, such characteristics of organic wastes practically only appear in some specific industrial waste streams, *e.g.*, sugar factory wastewater (Ueno *et al.*, 1996), rice winery wastewater (Yu *et al.*, 2002), starch-manufacturing waste (Yokoi *et al.*, 2002), molasses wastewater (Ren

TABLE 15.1. Summary of growth kinetics of hydrogen-producing bacteria.

Test type	Culture	Temperature (°C)	Substrate	μ_{max} (h^{-1})	R_{max} ($mL \cdot h^{-1}$)	K_S (g COD/L)	$Y_{X/S}$ ($_{g\ biomass/mol\ substrate}$)	Reference
Continuous	*Citrobacter intermedius*	37	Glucose	0.220	121.4	NA	20.6	Brosseau and Zajic (1982)
Continuous	Mixed	35	Sucrose	0.172	NA	0.068	34.2	Chen *et al.* (2001)
Continuous	Mixed	37	Glucose	0.333	NA	NA	45.0	Horiuchi *et al.* (2002)
Batch	*Enterobacter cloacae*	37	Glucose	0.568	NA	3.914	15.1	Kumar *et al.* (2000)
Batch	*Caldicellulosiruptor saccharolyticus*	70	Sucrose	0.130	67.2	0.801	NA	Van Niel *et al.* (2003)
Batch	Mixed	35	Sucrose	ND	13.9	1.446	ND	Chen *et al.* (2006a)
Batch	Mixed	35	NFDM	ND	25.6	6.616	ND	Chen *et al.* (2006a)
Batch	Mixed	35	Food waste	ND	29.9	8.692	ND	Chen *et al.* (2006a)

Source: Chen *et al.*, 2006a.
NA: Not available
ND: Not determined

et al., 2006), *etc.* In the household, food waste has received little attention as a feedstock on hydrogen fermentation (Shin *et al.*, 2004; Van Ginkel *et al.*, 2005; Chen *et al.*, 2006a). However, the carbohydrate content of food waste is varied, depending on the source of collection, and the difference of local diet. In addition, Khanal *et al.* (2006) found that the carbohydrate-rich substrate entering the reactor should remain fresh without pre-fermentation in a reservation tank. On the other hand, abundant of microbial communities existing in food waste may pre-ferment carbohydrate prior to hydrogen fermentation. Therefore, high concentration of carbohydrate would not be guaranteed in food waste. In summary, the suitable waste streams containing high carbohydrate concentration for fermentative hydrogen production are limited in the field of environmental engineering. The development of the technology of hydrogen fermentation must necessarily have a narrow scope.

15.5.2. Low Hydrogen Yield

Low hydrogen yield in dark fermentation could restrict the entire development of biological hydrogen production. In Equation 15.1, the theoretically maximum hydrogen yield of 4 mol/mol hexose is achieved with acetate production. Nevertheless, the growth of hydrogen producing microbes is not included in that equation. Therefore, the theoretic hydrogen yield in Equation 15.1 is overestimated. A stoichiometric calculation showed that the theoretically maximum hydrogen yield of 3.26 mol/mol hexose accompanied with biomass yield of 0.16 g/g hexose (0.29 mol/mol hexose) is generated on acetate fermentation pathway (Chen *et al.*, 2006b). The stoichiometric equation of hydrogen production containing biomass yield is described in Equation 15.4:

$$\begin{aligned}&(41+2Z)C_6H_{12}O_6+(9+3Z)NH_3 \rightarrow (9+3Z)C_4H_7O_2N+\\&\quad 35(1-Z)CH_3CH_2CH_2COOH+70ZCH_3COOH+\\&\quad (18-64Z)H_2O+70CO_2+70(1+Z)H_2\end{aligned} \quad \text{[Eq. 15.4]}$$

where Z = acetate/(acetate + butyrate), and 1 − Z = butyrate/(acetate + butyrate). To date, it seldom happens that hydrogen production is solely associated with acetate fermentation in a mixed culture. To the contrary, researchers have reported that butyrate fermentation is more dominant than acetate fermentation (Chang and Lin, 2004; Shin and Youn, 2005; Kyazze *et al.*, 2006). Based on Equation 15.3 with a butyrate to acetate ratio of 1.5 mol/mol, the theoretically maximum hydrogen yield in Equation 15.4 could be modified as 2.11 mol/mol hexose with biomass yield of 0.13 g/g hexose (0.23 mol/mol hexose). The influences of Z on the yields of hydrogen, biomass, acetate, and butyrate are depicted in Figure 15.2 (Chen *et al.*, 2006b).

Recent evidence from a batch study conducting on the removal of carbon dioxide from headspace suggested the possibility of acetogenesis in fermentative hydrogen production (Park *et al.*, 2005). Low hydrogen yield could be attributed to the existence of homoacetogens, mainly homoacetogenic

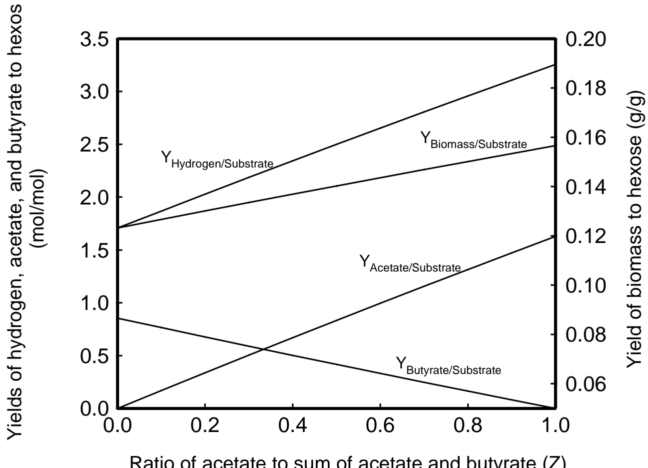

Figure 15.2. Effect of the molar ratio of acetate to the sum of acetate and butyrate on the yields of biomass, hydrogen, acetate, and butyrate to glucose. Source: Adopted from Chen *et al.*, 2006b.

clostridia, in the mixed-microbial culture. Homoacetogenic clostridia can grow heterotrophically by converting simple sugar to acetic acid without evolving hydrogen (Eq. 15.5) (Ljungdahl *et al.*, 1989). Homoacetogenic clostridia are also able to grow autotrophically with acetate production on a mixture of hydrogen and carbon dioxide as the only energy and carbon source (Eq. 15.6).

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \qquad \text{[Eq. 15.5]}$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{[Eq. 15.6]}$$

In addition, propionate can be produced by the clostridia themselves or other microbial populations competing in the mixed culture, decreasing the hydrogen yield as well (Hallenbeck, 2005). A species of saccharolytic clostridia, such as *Clostridium articum, Clostridium novyi*, or *Clostridium propionicum* in the mixed culture, convert sugar to propionate. Khanal *et al.* (2006) observed that the levels of propionate were consistently higher than acetate and butyrate, which demonstrated the existence of propionate-producing clostridia in hydrogen fermentation.

15.6. CONCLUSIONS

The potential of biological hydrogen production by strict anaerobic bacteria has generated considerable interest over the past few decades. With renewable

characteristics, biological hydrogen could be produced from organic waste streams or agriculture entities. The mechanism of biological hydrogen production under dark fermentation involves anaerobic bacteria converting sugars and proteins to carboxylic acids, hydrogen, carbon dioxide, and solvents. To maximize fermentative hydrogen production, several strategies (pH control, HRT, temperature control) have demonstrated their efficacy on biological hydrogen production. However, competitions among microbial populations, methanogenesis, and byproduct inhibition have been challenges for successful reactor operation. At the same time, the biotechnology of making hydrogen production practical still faces challenges, requiring a high carbohydrate concentration for the catabolism of hydrogen-producing bacteria. Because of this, feedstock for fermentative hydrogen production has been narrowed to specific waste streams. Lastly, fermentative hydrogen production incurs low yield, which may hinder the development of biological hydrogen production as a process to produce renewable fuel in the future.

15.7. REFERENCES

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PART III

BIOCATALYSIS (PRODUCTS FROM RENEWABLE RESOURCES)

CHAPTER 16

Some Properties of a Self-Sufficient Cytochrome P-450 Monooxygenase System from *Bacillus megaterium* Strain *ALA2*

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16.1. INTRODUCTION

Oxygenated fatty acids are common in nature (Hou, 2000). In mammals, polyunsaturated fatty acids (PUFAs) can be metabolized to prostaglandins, leukotrienes, lipoxins, and other hydroxy and epoxy fatty acids. These fatty acid metabolites are important chemical mediators of biological responses in mammals. They also *i)* can act as fungicides in agricultural applications, *ii)* are precursors of flavor ingredients such as ketones, alcohols, and aldehydes, *iii)* are feedstocks to create oxiranes, and *iv)* can be useful chiral synthons in possible drug discoveries (Kato *et al.*, 1984; Needleman *et al.*, 1986; Masui *et al.*, 1989; Hou and Forman, 2000). Plant systems also produce hydroxyl and epoxy fatty acids, which are important industrial materials. These products are used in applications including resins, waxes, nylons, plastics, cosmetics, and coatings (Hou, 2000).

Common biological fatty acid oxidation chemistries are provided by oxygenase enzymes, which include dioxygenases, monooxygenases, and electron transfer oxidase enzymes. The dioxygenases consume one molecule of O_2 per molecule of substrate with both O atoms incorporated into the substrate. The monooxygenases, which also consume one molecule of O_2 per molecule of substrate, only incorporate a single O atom into each product molecule. The electron transfer oxidases commonly reduce molecular O_2 into H_2O_2 or water as part of the reaction processes (Mason, 1957, 1965; Waterman, 2005). The middle group, the monooxygenases, are typically metal-containing mixed-function enzymes that require reducing equivalents to catalyze both the two-electron activation of one atom of dioxygen for addition to the substrate and the reduction of the second atom of dioxygen to water or H_2O_2 (Fleming *et al.*, 2003; Waterman, 2005). This monooxygenase enzyme group will be the focus of this paper.

Monooxygenases are known to hydroxylate alkanes and/or epoxidate alkenes including saturated and unsaturated fatty acids: *e.g.: Pseudomonas oleovolans* monooxygenase system (Peterson *et al.*, 1966; Abbott and Hou, 1973; May and Abbott, 1973); monooxygenase systems of methylotrophs (Hou, 1984); and *Bacillus megaterium* ATCC 14581 (Miura and Fulco, 1974, 1975; Buchanan and Fulco, 1978; Ruettinger and Fulco, 1981). The hydroxy groups in plant-derived fatty acids are usually located in the middle of the fatty acyl chain. Castor and lesquerella oils are the two major biological sources. Ricinoleic and sebacic acids, two of the castor oil derivatives, are classified by the U. S. Department of Defense as strategic and critical materials revealing the commercial importance of oxygenated fatty acid molecules. Like ricinoleic acid, lesquerella's hydroxy fatty acids also have double bonds and carboxyl functionality.

Additionally, microbial systems convert unsaturated fatty acids to monohydroxy-, dihydroxy- and trihydroxy-fatty acids (Hou, 1995, 2000). It is feasible to produce various value-added hydroxy fatty acids and derivatives for industrial applications by exploiting the unique reaction specificities of excep-

tional microbial enzymes, and if need be, targeted modification of these native enzymes. One of these, an indigenous soil bacterium collected in Alabama around 1995, designated as *ALA2* (NRRL B-21660), has revealed high activity in converting common fatty acids such as linoleic acid into an array of interesting oxygenated products via *in vivo* reactions (Hou, 1996). The oxygenase system of *ALA2* has not yet been fully studied but initial findings show it producing a number of potential value-added metabolites detailed in Figures 16.1 through 16.3, and discussions below. Strain *ALA2* was recently reclassified as *Bacillus megaterium* based on DNA analysis (Hou *et al.*, 2005). This opened a link to comparison with the well-studied *Bacillus* monooxygenase system.

The catalytically self-sufficient cytochrome P450 monooxygenase from *B. megaterium* ATCC 14581, designated as $P450_{BM\text{-}3}$ (CYP102A1), is the signature soluble enzyme of this monooxygenase class, and has been extensively studied (Miura and Fulco, 1974, 1975; Matson *et al.*, 1977; Buchanan and Fulco, 1978; Matson *et al.*, 1980; Michaels *et al.*, 1980; Matson and Fulco, 1981; Ruettinger and Fulco, 1981; Narhi and Fulco, 1986; Fulco and Ruettinger, 1987; Narhi and Fulco, 1987; Wen and Fulco, 1987; Ruettinger *et al.*, 1989; Wen *et al.*, 1989; Boddupalli *et al.*, 1990; Fulco, 1991; Boddupalli *et al.*, 1992a; Boddupalli *et al.*, 1992b; Boddupalli *et al.*, 1992c; Miles *et al.*, 1992; Peterson and Boddupalli, 1992; Ravichandran *et al.*, 1993; Smith *et al.*, 1994; Degtyarenko, 1995; Govindaraj and Poulos, 1995; Li and Poulos, 1995; Sevrioukova and Peterson, 1995; Yeom *et al.*, 1995; Capdevila *et al.*, 1996; Govindaraj and Poulos, 1996; Li and Poulos, 1996; Sevrioukova and Peterson, 1996; Sevrioukova *et al.*, 1996; Daff *et al.*, 1997; Govindaraj and Poulos, 1997; Hazzard *et al.*, 1997; Li and Poulos, 1997; Peterson *et al.*, 1997; Sevrioukova *et al.*, 1997; Yeom and Sligar, 1997; Schneider *et al.*, 1998; Noble *et al.*, 1999; Schneider *et al.*, 1999; Sevrioukova *et al.*, 1999; Cowart *et al.*, 2001; Gustafsson *et al.*, 2004; Lentz *et al.*, 2004; Warman *et al.*, 2005). Two related P450 genes, CYP102A2 and CYP102A3 from *B. subtilis*, code for soluble single-peptide monooxygenases like CYP102A1, comprise both a heme and a FAD/FMN-containing reductase domain which are covalently fused, and demonstrate a notable sequence and oxygenase reaction chemistry similarity to CYP102A1 (Gustafsson *et al.*, 2004; Lentz *et al.*, 2004). The CYP102A3 P450 monooxygenase enzyme is also involved in the hydroxylation of unsaturated, saturated, and branched-chain fatty acids (Lentz *et al.*, 2004). Recently, these three P450 enzymes were directly compared with strain *ALA2* via *in vivo B. megaterium* reactions using a palmitic acid substrate and found to possess quite similar oxidation products, thus further supporting the supposition of similarity of the strain *ALA2* monooxygenase (hydroxylation) systems to that of the more well-studied $P450_{BM\text{-}3}$ class of enzymes (Hou, 2005).

This paper will review what is known about the *in vivo* reactions involving *B. megaterium* strain *ALA2*, the initial $P450_{BM\text{-}3}$ gene analog sequence data findings, and plans for future enzymatic studies using the cloned, purified strain *ALA2*-based P450 enzyme(s).

16.2. *IN VIVO BACILLUS MEGATERIUM ALA2* REACTIONS

A number of saturated and unsaturated compounds have been used as substrates for *in vivo* reactions with the *B. megaterium* strain *ALA2*. These initial efforts are reviewed below and were biased toward characterization of novel bacteria-derived compounds of potential commercial applicability.

16.2.1. From α-Linoleic Acid (ω-6)

Strain *ALA2* is a unique microbe, which produces a variety of hydroxy fatty acids from the substrate linoleic acid (18:2 both *cis*-9,12) (Hou, 1996; Hou *et al.*, 1997; Hou *et al.*, 1998; Gardner *et al.*, 2000; Hou, 2000; Iwasaki *et al.*, 2002; Hosokawa *et al.*, 2003c; Hou, 2005; Hou *et al.*, 2005; Hou, 2006). Specifically, the compounds 12,13-epoxy-9(*Z*)-octadecenoic acid; 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12, 13-DHOA); 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA); 12,13,16-trihydroxy-9(Z)-octadecenoic acid (12,13,16-THOA); 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (THFA from the phrase "tetrahydrofuranyl fatty acid"); 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (HO-THFA); 12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid (DEOA); 7-hydroxy-12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid (7-HO-DEOA); and its isomer 16-hydroxy-12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid (16-HO-DEOA) have been isolated and characterized from reactions of linoleic acid with the strain *ALA2 Bacillus megaterium*, according to the proposed summarized chemical pathway detailed in Figure 16.1 (Hou, 2006). Similarly, *ALA2* also converts other ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) into a variety of related oxylipins.

16.2.2. From α-Linolenic Acid (ω-3)

In vivo reactions of the strain *ALA2* with an α-linolenic acid (18:3 all cis-9,12,15) substrate yielded the following novel fatty acid products: 13,16-dihydroxy-12,15-epoxy-9(*Z*)-octadecenoic acid (13,16-dihydroxy-THFA) and 7,13,16-trihydroxy-12,15-epoxy-9(*Z*)-octadecenoic acid (7,13,16-trihydroxy-THFA) (Hosokawa *et al.*, 2003b). Both products had a tetrahydrofuranyl ring in their structure analogous to the (oxidized carbon 16) right side linoleic acid pathway detailed in Figure 16.1. The 7,13,16-trihydroxy-THFA was a new chemical entity with the trihydroxy fatty acid containing a cyclic structure (Hosokawa *et al.*, 2003b). Brodowsky *et al.* (1992) reported the production of 8-hydroxy-octadecatrienoic acid, 17-hydroxy-octadecatrienoic acid and 7,8-dihydroxy-octadecatrienoic acid from α-linolenic acid by the fungus *Gaeumannomyces graminis* but no similar tetrahydrofuranyl ring-based compositions.

Note that in contrast to products from linoleic acid, bioconversion of α-linolenic acid by strain *ALA2* yielded products differing in the position of their tetrahydrofuranyl ring and hydroxy groups on the starting material

Figure 16.1. The proposed *ALA2* strain *in vivo* reaction scheme with 18:2 as the substrate. Source: Hou, 2006.

(Hosokawa *et al.*, 2003c; Hou, 2006). In addition, diepoxy bicyclic fatty acid products such as DEOA, 7-HO-DEOA, and 16-HO-DEOA were not observed with α-linolenic acid as the substrate but only with linoleic acid. These results indicate that the chemical structure of products produced by strain *ALA2* depends heavily on the type of polyunsaturated fatty acid substrate employed. This aspect of the reactions of strain *ALA2* was further investigated, again via *in vivo* reactions, using a number of related unsaturated fatty acid substrate molecules (Hosokawa *et al.*, 2003a).

16.2.3. From γ-Linolenic Acid and Arachidonic Acid (ω-6)

Strain *ALA2* converted γ-linolenic acid (18:3 all *cis*-6,9,12) to several products including: 12,17;13,17-diepoxy-6,9-octadecadienoic acid; 12,13,17-trihydroxy-6(*Z*),9(*Z*)-octadecadienoic acid; and 12-hydroxy-13,16-epoxy-6(*Z*),9(*Z*)-octadecadienoic acid as depicted in the top half of Figure 16.2 (Hosokawa *et al.*, 2003a). Strain *ALA2* also converted the substrate arachidonic acid (20:4 all *cis*-5,8,11,13) to cyclic and trihydroxyl fatty acids as reported previously (Hosokawa *et al.*, 2003a). These reactions resulted in compounds: 14,19;15,19-diepoxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid; 14-hydroxy-15,18-epoxy-5(*Z*),

γ-linolenic acid

Product 3: 12,17;13,17-diepoxy-6,9-octadecadienoic acid

Product 4: 12-hydroxy-13,16-epoxy-6,9-octadecadienoic acid

Product 5: 12,13,17-trihydroxy-6,9-octadecadienoic acid

arachidonic acid

Product 6: 14,19;15,19-epoxy-5,8,11-dieicosatrienoic acid

Product 7: 14-hydroxy-15,18-epoxy-5,8,11-eicosatrienoic acid

Product 8: 12,15,19-trihydroxy-5,8,11-eicosatrienoic acid

Figure 16.2. The *ALA2* strain *in vivo* reaction scheme with 18:3 and 20:4 ω-6 substrates. Source: Hosokawa *et al.*, 2003a.

8(*Z*),11(*Z*)-eicosatrienoic acid; and 12,15,19-trihydroxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid, which were isolated as shown in the lower portion of Figure 16.2 (Hosokawa *et al.*, 2003a). Both of these sets of compounds are analogous to metabolites derived from linoleic acid as detailed in Figure 16.1.

16.2.4. From Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) (ω-3)

Strain *ALA2* converted the substrate EPA (20:5 all *cis*-5,8,11,14,17) *in vivo* to 15,18-dihydroxy-14,17-epoxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid (Hosokawa *et al.*, 2003a). Figure 16.3 demonstrates this. The strain *ALA2* enzyme(s) hydroxylated the two double bonds at C-14 to C-15, C-17 to C-18, but did not alter the double bonds at the C-5, C-8 and C-11 positions in this highly unsaturated substrate. Analogously, strain *ALA2* also converted the substrate DHA (22:6 all *cis*-4,7,10,13,16,19) to a related product: 17,19-dihydroxy-16,18-epoxy-4(*Z*),7(*Z*),10(*Z*),13(*Z*)-docosatetraenoic acid which is also detailed in Figure

EPA

15,18-dihydroxy-14,17-epoxy-5(Z),8(Z),11(Z)-eicosatrienoic acid

DHA

17,20-dihydroxy-16,19-epoxy-4(Z),7(Z),10(Z),13(Z)-docosatetraenoic acid

Figure 16.3. The *ALA2* strain *in vivo* reaction scheme with 22:5 and 24:6 ω-3 substrates. Source: Hosokawa *et al.*, 2003a.

16.3 (Hosokawa *et al.*, 2003a). These products each contain two hydroxy groups and a tetrahydrofuranyl (THF) ring in their structures near their hydrocarbon termini.

It was reported earlier that strain *ALA2* converted α-linolenic acid to 13,16-dihydroxy-12,15-epoxy-9(*Z*)-octadecenoic acid, and 7,13,16-trihydroxy-12,15-epoxy-9(*Z*)-octadecenoic acid (Hosokawa *et al.*, 2003b). From product structures obtained from the ω-3 PUFAs EPA and DHA, it seems that strain *ALA2* places hydroxyl groups at similar positions from the omega (ω)-terminal end of the substrates and cyclizes them to related THF ring structures, despite their increased total carbon chain lengths (by 2 and 4 carbons, respectively) and numbers of double bonds (by 1 and 2 respectively).

The products obtained from the ω-6 fatty acids (linoleic acid, γ-linolenic acid, and arachidonic acid) by *in vivo* reactions with strain *ALA2* contain diepoxy bicyclic structures, tetrahydrofuranyl rings, and/or trihydroxy groups in their molecules. In contrast to these ω-6 PUFAs, substrates classified as ω-3 PUFAs (α-linolenic acid, EPA, and DHA) are only converted to hydroxyl THFAs by strain *ALA2* with no diepoxy bicyclic or trihydroxy derivatives uncovered to date. Both the hydroxyl groups and cyclic structures derived there from appear to be placed at the same positions on the substrates from the ω-carbon termini within each PUFA class, despite differences in carbon chain length and the number of double bonds in the specific PUFA substrates.

Strain *ALA2* showed the highest relative *in vivo* activity for the bioconversion linoleic acid to trihydroxy-9(*Z*)-octadecenoic acid per Table 16.1 (Hosokawa *et al.*, 2003c). The relative activities for α- and γ-linolenic acids toward trihydroxy products were lower than that of linoleic acid, though still relatively

TABLE 16.1. Relative activities of strain *ALA2* for various substrates.

Substrate	Product	Relative activity (%)
ω-3 PUFA		
α-Linolenic acid	7,13,16-Trihydroxy-12,15-epoxy-9(*Z*)-octadecadienoic acid	100
	13,16-Dihydroxy-12,15-epoxy-10(*Z*)-octadecadienoic acid	19
EPA	15,18- Dihydroxy-14,17-epoxy-5,8,11 eicosatrienoic acid	16
DHA	17,20- Dihydroxy-16,19-epoxy-4,7,10,13 docosatetraenoic acid	14
ω-6 PUFA		
Linoleic acid	Trihydroxy-9(*Z*)-octadecenoic acid	130
	12,17;13,17-Diepoxy-9(*Z*)-octadecenoic acid	19
	12-Hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid	2
γ-Linoleic acid	Trihydroxy-6,9- octadecenoic acid	76
	12,17;12,17-Diepoxy-6,9-octadecenoic acid	27
	12-Hydroxy-13,16-epoxy-6,9-octadecenoic acid	9
Arachidonic acid	Trihydroxy-5,8,11-eicosatrienoic acid	—
	14,19;15,19-Diepoxy-5,8,11-eicosatrienoic acid	11
	14-Hydroxy-15,18-epoxy-5,8,11-eicosatrienoic acid	7

Source: Hosokawa *et al.*, 2003c.
PUFA = polyunsaturated fatty acid.

high. The increase of carbon chain number and/or number of double bonds, from around 18 and three, respectively, remarkably reduced the relative *in vivo* strain *ALA2* oxidation activities. It was also of interest to determine the effect of *in vivo* reactions of strain *ALA2* using saturated fatty acid substrates.

16.2.5. Palmitic Acid Biotransformation

Recently, the strain *ALA2* was found to hydroxylate palmitic acid to mixtures of ω-1, ω-2, and ω-3 mono-hydroxy palmitic acids as detailed in Table 16.2 (Hou, 2005). This *in vivo* reaction behavior was quite analogous to two other *Bacillus* microorganisms examined in the same study: NRRL B-3712 and B-4219. The similar chemistry between the *Bacillus* microbes but differences in absolute specificity suggest likely similarities in enzyme function between the examined cultures *ALA2* (NRRL B-21660), B-3712 (wild type *B. megaterium* including CYP102A1) and B-4219 (*B. subtilis* including both CYP102A2 and CYP102A3). No secondary products were observed in these saturated fatty acid substrate reactions.

TABLE 16.2. Relative ω-1, ω-2, and ω-3 products from related *in vivo* enzymes.

	ω-1	ω-2	ω-3
Bacillus megaterium strain *ALA2*	1.00	0.93	0.35
B. megaterium strain NRRL-B-3712	0.42	1.00	0.35
B. megaterium strain NRRL-B-4219	1.00	ND	ND

Source: Hou, 2005.
ND = not detected.

16.3. $P450_{BM\text{-}3:ALA2}$ SEQUENCE INFORMATION

Given the varied and potentially valuable oxylipins derived from strain *ALA2* and detailed in Figures 16.1–16.3, it was thus of interest to isolate and clone its assumed $P450_{BM\text{-}3}$–like monooxygenase enzyme. This was targeted to assist in the thorough physical and sequence characterization and to provide purified enzyme for more specific *in vitro* substrate binding and chemical reaction studies aimed at understanding its role in controlling the chemistries detailed in Figures 16.1 through 16.3. *In vitro* comparisons with $P450_{BM\text{-}3:WT}$ and CYP102A2 and CYP102A3 are also desired. Using degenerate P450 primers, a target $P450_{BM\text{-}3}$ analog was obtained from NRRL-21660, cloned into the pGEM®-T Easy vector (Promega) and sequenced to yield a product of 3150 bp encoding a 1049 AA protein. This *ALA2*-based enzyme was found to be 94% identical to the wild-type $P450_{BM\text{-}3}$ (from B-3712) by nucleotide comparison and 97% identical by amino acid (AA) sequence (Fig. 16.4).

Further comparisons revealed about 30 AA differences between the two $P450_{BM\text{-}3:WT}$ and $P450_{BM\text{-}3:ALA2}$ enzymes which consisted of 13 major chemical changes. Two of these differences were found in the heme domain of the molecule and the other eleven were in the reductase domain of this fused molecule. It was also determined that all eight critical reductase domains of Porter were also very highly conserved in this ALA2 sequence and actually fully identical with only one minor AA substitution in region 6 (Oster *et al.*, 1991; Porter, 1991). These regions spanned conserved FMN-, FAD-PPI-, and NADPH-FAD binding areas.

A comparative BLAST search of related P450 monooxygenase heme domain structures to the measured $P450_{BM\text{-}3:ALA2}$ nucleotide sequence revealed this new composition to have its closest relative as the wild-type $P450_{BM\text{-}3}$ domain with a few other notable nearest compositions. See Figure 16.4. This new P450 monooxygenase is thus also likely classified as within the CYP102A1 isozyme.

16.4. FURTHER STUDIES ON $P450_{BM\text{-}3:ALA2}$

The *ALA2* BM-3 clone is being sub-cloned into protein expression vectors in attempts to isolate purified $P450_{BM\text{-}3:ALA2}$ protein for substrate binding

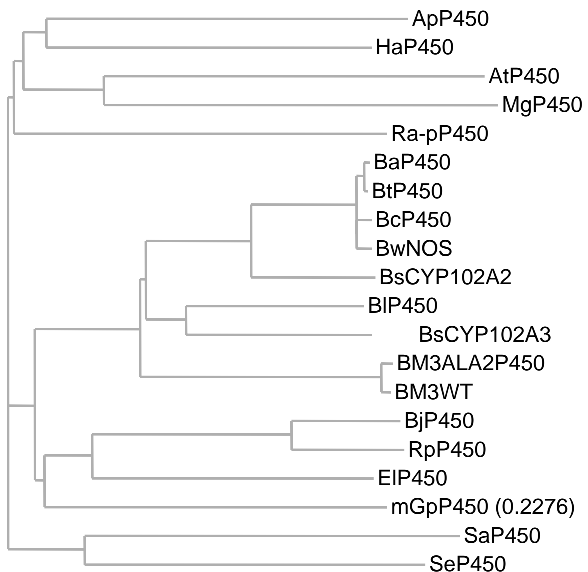

Figure 16.4. The phylogenetic tree of P450 Region of $P450_{BM\text{-}3}$ homologs. All of these are P450:P450 reductase fusion proteins. Only their P450 domain sequences are compared. Ap, *Actinosynnema pretiosum* subsp. *auranticum*; At, *Aspergillus terreus*; Ba, *Bacillus anthracis*; Bc, *Bacillus cereus*; Bj, *Bradyrhizobium japonicum*; Bl, *Bacillus licheniformis*; BM, *Bacillus megaterium*; Bs, *Bacillus subtilis*; Bt, *Bacillus thuringiensis*; BwNOS, *Bacillus weihenstephanensis* nitric oxide synthase; El, *Erythrobacter litoralis*; Ha, *Herpetosiphon aurantiacus*; Mg, *Magnaporthe grisea*; mGp, marine gamma proteobacterium; Ra-p, *Ralstonia pickettii*; Rp, *Rhodopseudomonas palustris*; Sa, *Streptomyces avermitilis*; Se, *Saccharopolyspora erythraea*.

studies, *in vitro* chemical reaction characterization, and various planned reaction kinetics studies. Our ultimate goal is to be able to control this enzyme (or related enzymes) for the preparation of larger quantities of specific molecules in the Figure 16.1 pathway (and related Figs 16.2 and 16.3) for additional applications testing. Plans and their bases are detailed below. Initial work will focus on comparative saturated and unsaturated fatty acid substrate binding studies vs. $P450_{BM\text{-}3:WT}$ to provide a fundamental understanding of how these various related substrates bind differently to the same enzyme.

16.4.1. Analysis of the Figure 16.1 Reaction Pathway for $P450_{BM\text{-}3:ALA2}$

The proposed reaction scheme for the 18:2-based products from *B. megaterium* stain *ALA2* in Figure 16.1 suggests that the first step is an epoxidation of the highest numbered double bond on the substrate. This chemistry is likely from the P450 enzyme as it is analogous to reactions the substrates 16:1

through 20:5 catalyzed by the wild-type $P450_{BM\text{-}3}$ monooxygenase enzyme (Ruettinger and Fulco, 1981; Capdevila *et al.*, 1996). The hydration of the epoxide intermediate is likely an enzymatic secondary reaction analogous to the reported 16:1 epoxide hydrolase activity involving the same reaction center as the initial oxygenase reactions (Michaels *et al.*, 1980; Ruettinger and Fulco, 1981). Notably, hydrolysis of the epoxy 18:1 compound may be catalyzed by an epoxide hydrolase. Fulco isolated such a soluble epoxide hydrolase activity from *B. megaterium* in 1980 (Michaels *et al.*, 1980). The next step of Figure 16.1 involves a non-terminal hydroxylation usually as ω-1 or ω-2. This type of reaction is considered signature $P450_{BM\text{-}3}$ monooxygenase chemistry. Thus, to this point in the pathway, there is a high likelihood, though no guarantee, that all of the reactions may be performed by a single enzyme, the $P450_{BM\text{-}3:ALA2}$ monooxygenase via sequential reactions (Boddupalli *et al.*, 1992c). This supposition will be tested with isolated pathway products used as fed substrates using *in vitro* conditions and purified $P450_{BM\text{-}3:ALA2}$ protein.

The next step on either branch of the noted reaction pathway seems to involve ring-closures by dehydration. There is a high likelihood that (an)other enzyme(s) catalyze this chemistry in the *in vivo* reactions discussed above, although it is entirely possible for the substrate to shift in the same binding pocket and undergo yet another reaction. Further, it would be logical to expect yet another enzyme to add the final mid chain hydroxyl group to the various cyclic-epoxide molecules in Figure 16.1, although again additional chemistry in the same $P450_{BM\text{-}3}$ enzyme pocket is possible. These comments are merely logical speculation and points to be experimentally tested. All related products discussed above for the ω-3 and ω-6 PUFA via strain *ALA2* likely follow similar mechanisms.

What is known regarding these kinds of secondary product issues with $P450_{BM\text{-}3}$ monooxygenase enzymes is that no secondary products have been observed from reconstituted mixtures of the independent fused enzyme domains of $P450_{BM\text{-}3}$ (Sevrioukova *et al.*, 1997). Secondary reactions only take place with native fused enzymes. This paper goes on to report that when secondary products are observed in native fused systems, the primary products typically are formed and accumulated first, then secondary products produced (Boddupalli *et al.*, 1990; Sevrioukova *et al.*, 1997) at a slower rate. Lower concentrations of substrates favor secondary product formation as do higher O_2: NADPH ratios (Boddupalli *et al.*, 1990). Substrate binding of the primary reaction products on route to secondary products may be controlled by a subtle, yet-to-be understood binding related to the FMN:FAD ratio (Sevrioukova *et al.*, 1997). Similarly, secondary product metabolites may be related to contacts with the b3 and b4 sheets at the C terminus of the BM-3 in its crystal structure (Peterson *et al.*, 1997). It has also been observed that the ω-2 and ω-3 are preferentially reacted to form secondary products over ω-1 primary ones for C16:0 (Sevrioukova *et al.*, 1997) and that this trend towards higher ω-# oxidation activity is favored for higher carbon number substrates (Boddupalli *et al.*, 1992c). Both are key for strain *ALA2* reactions. In general,

reactions of these types of enzymes and their catalytic outcome are critically dependent on:

i) the chemical properties of the acceptor C-H bonds
ii) the optimal orientation of the C-H acceptor with respect to the heme-bound reactive oxygen intermediate
iii) the substrate chemistry, and
iv) the protein structural features and NOT the oxidant chemistry (Capdevila *et al.*, 1996).

16.4.2. *ALA2* and Related Enzyme Engineering

Once this substrate focused work evolves past the specific understanding of this *ALA2* enzyme, it will likely progress to the engineering phase. At this point, we may need to take advantage of a number of interesting scale-up methods from the literature. Work has shown that expression of a co-plasmid can be incorporated into reactions to increase hydrocarbon substrate uptake (Schneider *et al.*, 1998). Targeted mutations can also be introduced to increase enzyme compatibility with organic solvents for higher productivity (Wong *et al.*, 2004). As NADPH can be expensive in the production of oxygenated species, techniques such as recycling (Maurer *et al.*, 2003, 2005) or replacing it with a Zn/Co^{III} combination (Nazor and Schwaneberg, 2006) may be considered.

16.5. CONCLUSIONS

This chapter reviewed the many interesting and related *in vivo* products derived from reactions of the *B. megaterium* strain *ALA2* and various related PUFA substrates. It showed that products obtained from the ω-6 PUFAs (linoleic acid, γ-linolenic acid, and arachidonic acid) possessed diepoxy bicyclic structures, tetrahydrofuranyl rings, and/or trihydroxy groups in their molecules, while the products from ω-3 PUFAs (α-linolenic acid, EPA, and DHA) only held hydroxyl THFAs. All of these products were sufficiently similar to have resulted from parallel oxidation mechanisms. Despite the chemical structure of results produced by strain *ALA2* depending heavily on the type of PUFA substrate employed, all of the chemistry in each PUFA class was identical when viewed from the ω terminal end of the substrates. Even the ω-6 to ω-3 reaction comparisons showed similar highest numbered double bond hydration, hydroxylations, and/or dehydration-based ring closures.

This work also reports the initial gene sequence data for a cloned strain *ALA2*-based P450 enzyme(s). As suspected from its *in vivo* chemistries, this *B. megaterium* strain included a monooxygenase enzyme of the soluble, self-

sufficient $P450_{BM-3}$ sub-class that was highly homologous with the wild type protein. Not surprisingly, this new enzyme also possessed a significant high homology in all of the expected reductase regions as well. *In vitro* reactions and substrate binding characteristics of this enzyme will be further characterized in the near future.

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CHAPTER 17

Biocatalysis-based Development of Oligosaccharides in Japan

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17.1. INTRODUCTION

During last 30 years various kinds of oligosaccharides have been developed and commercialized in Japan (Taniguchi, 2004, 2005). Table 17.1 lists the major oligosaccharides found in the Japanese market with their annual production and methods of production. Total production of these oligosaccharides amounts to 60,000 tons/year with total sales of 13 million U. S. dollars. They find their application in food or food additives claiming health beneficial functions. A government approval system of "Foods for Special Health Use," FOSHU, began in 1991, greatly stimulated the vigorous development of oli-

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TABLE 17.1. Oligosaccharide production in Japan.

Oligosaccharides (OS)	Production t/year	Method of Profuction
Trehalose	30,000	malto-oligosyl trehalose synthase malto-oligosy trehalose trehalohydrolase
Isomalto-OS	11,000	Transglucosidase
Galacto-OS	6,000	β-Galactosidase
Fructo-OS	4,000	β-Fructosidase
Lactulose	2,800	Chemical Isomerization
Lactosucrose	2,000	β-Fructosidase
Cyclodextrin	1,800	Cyclodextrin glucanotransferase
Soy OS	1,000	Extraction
Gentio-OS	1,000	β-Glucosidase
Xylo-OS	650	β-Xylosidase
Nigero-OS	300	α-Glucosidase
Raffinose	230	Extraction
Palatinose	150	Glucosyltransferase

gosaccharides in Japan. Ten of 13 oligosaccharides listed in Table 1 are produced using enzyme biocatalysis. Most of these enzymes belong to EC 3, hydrolases, but usually their hydrolysis reaction is not used. Instead, transfer reaction or condensation reaction is utilized. Enzymes used for trehalose (malto-oligosyl trehalose synthase) and palatinose (glucosyltransferase) are a unique group belonging to EC 5, isomerases. Xylo-oligosaccharide and galacto-oligosaccharides are produced from xylan and lactose, respectively.

17.2. SOURCES OF OLIGOSACCHARIDES

Figure 17.1 shows oligosaccharides derived from three major starting materials. Most of them are already commercialized, some are under development. The majority of these oligosaccharides are derived from starch, some are from sucrose, and a few from lactose. Starch is abundant, and the cheapest carbohydrate obtained annually from agricultural products. Sucrose is the most abundant, cheap carbohydrate obtained from sugar cane or sugar beet. Lactose is a by-product of the dairy industry, and therefore also inexpensive. Thus all the oligosaccharides shown in Figure 17.1 are produced from renewable and cheap sources. The exceptions are xylo- and galacto-oligosaccharides, which are produced from xylan and galactose, respectively.

The appearance in the market in mid 1980s of fructo-oligosaccharide as a sugar with a physiological function created a strong impact on both the food industry and academia, and a rush of oligosaccharide development followed this discovery. Commercial fructo-oligosaccharide is a mixture of tri- and tetrasaccharides, having additional fructose residues joined though β-2-1 linkage

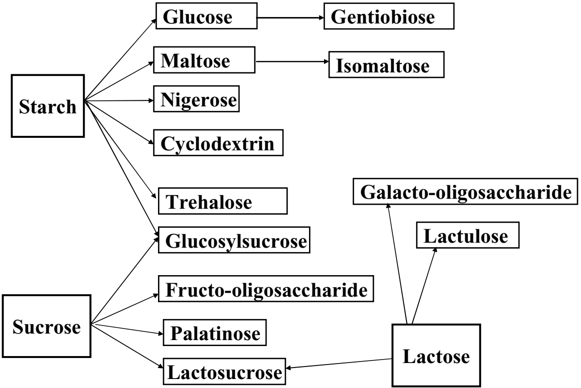

Figure 17.1. Source of oligosaccharide.

at the fructose residue of sucrose. These are produced by the action of β-fructofuranosidase from a special strain of *Aspergillus niger* (Hidaka *et al.*, 1988). At 50% sucrose this enzyme exhibited a strong transferring activity rather than hydrolysis activity.

As shown in Table 17.1, trehalose has the largest annual production, 30,000 tons/year. During the mid 1990s, Hayashibara Biochemical Laboratories Inc. discovered an enzyme system that produced trehalose directly from starch (Maruta *et al.*, 1995). The enzyme system found in *Arthrobacter* consists of two enzymes, the first one is a isomerase, EC5(glucan glucosylmutase). It catalyzes a conversion of α-1,4 linkage at the non-reducing end of malto-oligosaccharides or starch, into α-1,1 linkage to form malto-oligosyl trehalose (Nakada *et al.*, 1995a). Then, the second enzyme, a kind of hydrolase, cleaves the α-1,4 linkage next to the α-1,1 linkage, and produces trehalose and malto-oligosaccharides (Nakada *et al.*, 1995b). Large scale production of this sugar has a yield of 85% from starch.

In the following sections, I will focus on the developments carried out during last 10 years in Japan. The developments have been led mainly by research groups from Hayashibara Biochemical Laboratories Inc. and Biochemical Research laboratory, Ezaki Glico Co., Ltd. Finally I will add one example developed mainly by National Food Research Institute.

17.3. DEVELOPMENT OF CYCLIC OLIGOSACCHARIDES WITH HETEROGENOUS LINKAGES

17.3.1. Cyclic Tetrasaccharides

Very recently the Hayashibara group discovered a series of new cyclic oligosaccharides (Fig. 17.2). All of these oligosaccharides are produced from starch by special transferases found in microorgnsms. The first example is cyclic tet-

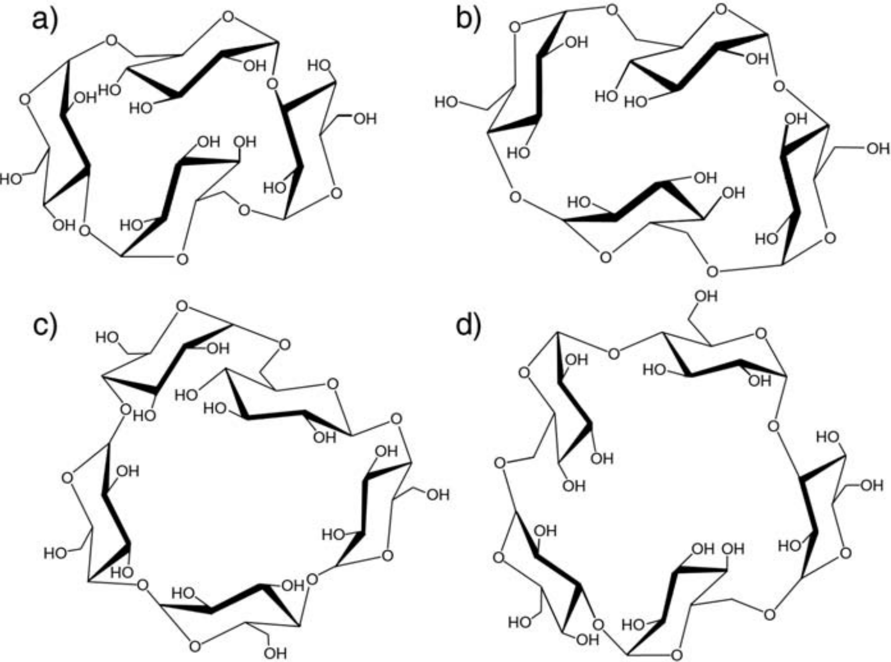

Figure 17.2. Cyclic tetra- and penta-saccharides.

1)

2)

3)

Figure 17.3. Mechanism of nigerosylnigerose formation.

rasaccharide, nigerosylnigerose (Aga *et al.*, 2002a, 2002b; Nishimoto *et al.*, 2002; Aga *et al.*, 2003) (Fig. 17.2a). *Bacillus globiformis* produces two enzymes, one being 6-glucosyltransferase (EC 2.4.1.24) and the other 3-isomaltosyltransferase (EC 2.4.1). As shown in Figure 17.3, the first enzyme transfers a glucose residue on the non-reducing end of another maltooligosaccharide forming isomaltosyl residue. Then, the second enzyme transfers this isomaltosyl residue to another isomaltosyl residue in a non-reducing end, forming α-1,3

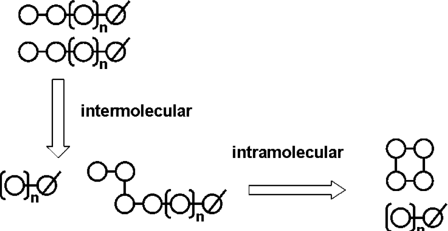

Figure 17.4. Mechanism of maltosylmaltose formation.

linkage. It further cleaves the α-1,4 linkage and transfers the reducing end of tetrasaccharide to 3-OH of its own non-reducing end glucose residue, forming cyclic nigerosylnigerose.

Figure 17.2b shows a structure of another cyclic tetrasaccharide, cyclic maltosylmaltose, again obtained from starch (Mukai *et al.*, 2005, 2006). In this case, a single enzyme from *Arthrobacter globiformis* catalyzes two step reactions. As shown in Figure 17.4, the first step is an intermolecular maltosyl transfer from non-reducing end to 6-OH of non-reducing glucose residue. Then the same enzyme catalyzes the intramolecular transfer of non-reducing end tetrasaccharide to form cyclic maltosylmaltose. These tetrasaccharides are the smallest cyclic glucans and their functions and applications to foods are being actively studied.

17.3.2. Cyclic Pentasaccharides

Further research found a cyclic pentasaccharide, isocyclomaltopentaose (Watanabe *et al.*, 2006a, 2006b, 2006c) (Fig. 17.2c). An enzyme called isocyclomaltooligosaccharide glucanotransferase (from *Bacillus circulans*) catalyzes a disproportionation reaction of maltooligosaccharides as Figure 17.5 demonstrates. When maltoheptaose or longer oligosaccharides are available, the enzyme catalyzes an intramolecular transfer, namely, cutting out maltopentaose and cyclizing it, through α-1,6 linkage. The enzyme can synthesize isocyclomaltohexaose too (Aga *et al.*, 2002a) in the similar reaction mechanism described above.

Another cyclic pentasaccharide with more complex linkages was discovered also by the Hayashibara group (Watanabe *et al.*, 2005). They found that the enzyme system synthesizing cyclic nigerosyl nigerose (Fig. 17.2a) synthesized a novel cyclic pentasaccharide as a minor product in the same reaction mixture. This cyclic pentasaccharide contains α-1,3-, α-1,6- and α-1.4- linkages as shown in Figure 17.2d.

Thus, starting from starch, its hydrolysis products such as glucose, maltose, maltooligosaccharides, and cyclodextrins have been developed. Now, a new group of cyclic glucans having heterogenous linkages in their structures are rapidly growing in Japan.

Disproportionation
α-1,4-transfer,intermolecular
(DP 2-6)
(DP>6)
Cyclization
α-1,6-transfer, intramolecular

Figure 17.5. Mechanism of isocyclomaltopentaose formation.

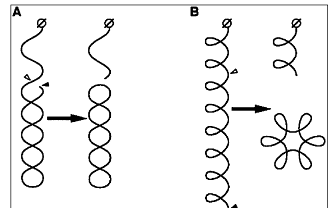

Figure 17.6. Cycloamylose formation by D-enzyme.

17.4. DEVELOPMENT OF CYCLIC POLYSACCHARIDES

17.4.1. Cycloamylose

D-enzyme (4-α-glucanotransferase, EC 2.4.1.25) has been long known to catalyze intermolecular transglycosylation reactions on maltooligosaccharides. Takaha *et al.* of Ezaki Glico found in 1996 that potato D-enzyme produces cycloamylose with a DP of 17 or higher when acted on by high molecular weight amylase (MW = 320,000) as shown in Figure 17.6. The reaction mechanism of this enzyme is thought to be close to that of cyclodxtrin glucanotransferse. Thermophillic *Thermus aquaticus* amylomaltase, having the same EC number as D-enzyme, catalyzes the same intramolecular transglycosylation reaction on amylose to form cycloamylose (Terada *et al.*, 1999). Cycloamylose is produced with high yield (more than 95%), is very soluble, and can form an inclusion complex with a wide range of compounds. They further found that a glycogen debranching enzyme from *Saccharomyces cerevisiae* also catalyzes cycloamylose synthesis (Yanase *et al.*, 2002). A glycogen debranching enzyme is unique, having both glucanotransferase and amylo-1,6-glucosidase activities on a single peptide. This enzyme can use not only amylase, but also starch as a substrate, and can synthesize cycloamylose even in the presence of glucose. Investigation on 4-glucanotransferses revealed that long-known

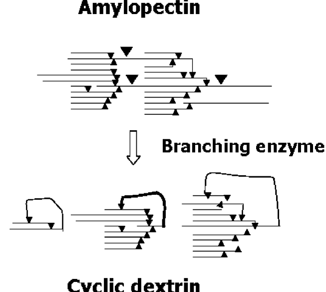

Figure 17.7. Cyclic dextrin from amylopectin by branching enzyme.

cyclodextrin glucanotransferase (EC 2.4.1.29) is not an exceptional enzyme, but just a member of 4-glucanotranferases, which commonly catalyzes cyclization reaction (Terada *et al.*, 1997). Application of cycloamylose in the fields of food and pharmaceutical is being actively explored.

17.4.2. Highly Branched Cyclic Dextrin

Another interesting discovery is a finding of highly branched cyclic dextrin. *Bacillus stearothermophillus* (Takata *et al.*, 1994) produces a very unique branching enzyme (EC 2.4.1.18) that catalyzes both intermolecular transfer to form branched dextrins and intramolecular transfer to form cyclic dextrins. When acting on amylopectin, it cleaves glucan chains only between clusters, and forms cyclic dextrins by intramolecular transfer reaction (Takata *et al.*, 1996) as shown in Figure 17.7. The cyclic dextrins thus obtained have sharp molecular weight distributions compared to those of devtrins obtained by partial hydrolysis of starch (Takata *et al.*, 1997). This finding is already released to the market underthe brand name "Cluster Dextrin."

17.5. CYCLODEXTRAN

Cyclodextrans (Fig. 17.8) are cyclic glucans consisting exclusively of α-1,6 linkage and derived from dextran by a special enzyme, cyclodextran glucanotransferase (Oguma *et al.*, 1994, 1995; Yamamoto *et al.*, 2006). Three types of cyclodextrans, consisiting of 7, 8, or 9 glucose residues, are known. *Bacillus circulans* T-3040 produces the enzyme which has both intermolecular and intramolecular transglucosylation reactions like cyclodextrin glucanotransferase but acts only on dextran. Cyclodextrans show potent inhibitor activity against mutan synthesis (Kobayashi *et al.*, 1995) and its large scale production is being actively developed.

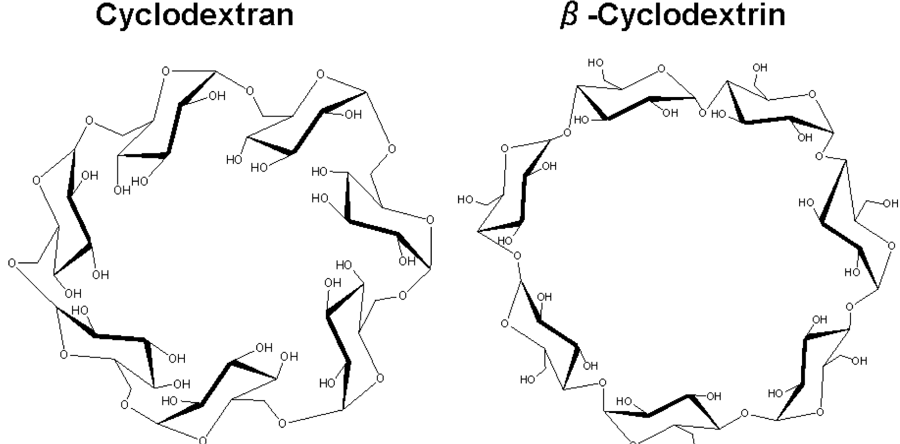

Figure 17.8. Structure of cyclodextran and cyclodextrin.

17.6. CONCLUSIONS

Starch is a high molecular weight compound, with DP of 10^5. The application of starch has been limited to use high molecular weight starch as it is, nmodified, or to hydrolyze it to low molecular weight products. The Hayashibara group developed a series of cyclic oligoglucans with heterogenous linkages. They successively discovered new microbial enzymes synthesizing these cyclic glucans starting from starch. Researchers at Ezaki Glico discovered new functions of known enzymes of α-amylase family leading to cyclic amylose and dextrins with higher molecular weight. Development of glucans with intermediate molecular sizes using biocatalysis, will be a prosperous field for future application. Among the oligosaccharides described above, cyclodextran makes a sharp contrast to other saccharides in that its starting material is not starch but dextran, which is produced from sucrose by the action of bacterial dextransucrase, and therefore, is an expensive raw material. If cyclic sugar is produced directly from sucrose or starch, its application will be greatly accelerated.

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CHAPTER 18

Biocatalysis: Synthesis of Chiral Intermediates for Drugs

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18.1. INTRODUCTION

Because chirality is a key factor in the safety and efficacy of many drug products, the production of single enantiomers of chiral intermediates has become increasingly important in the pharmaceutical industry (FDA, 1992). Single enantiomers can be produced by chemical or chemo-enzymatic synthesis. Biocatalysis has an advantage over chemical synthesis in that enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions which could cause problems with isomerization, racemization, epimerization, and rearrangement. the microbial cells and enzymes thus derived can be immobilized and reused for many cycles. In addition, enzymes can be overexpressed to make biocatalytic processes economically efficient, and enzymes with modified activity can be tailor-made. Directed evolution of biocatalysts can lead to increased enzyme activity, selectivity, and stability (Oliver *et al.*, 2002; Kazlauskas, 2005; Schmidt *et al.*, 2004; Reetz *et al.*, 2004; Pesti and DiCosimo, 2003). A number of review articles (Patel, 2004; Faber and Kroutil, 2005; Patel, 2006a; Turner, 2004; Alphand *et al.*, 2003; Robertson, and Bornscheuer, 2005; Ishige *et al.*, 2005; Kaluzna *et al.*, 2004; Fessner, 2004; Patel, 2006b) have been published on the use of enzymes in organic synthesis. This review provides examples of the use of enzymes for the synthesis of single enantiomers of key intermediates used in the preparation of drugs.

18.2. ENANTIOSELECTIVE ENZYMATIC REDUCTIVE AMINATION OF 2-(3-HYDROXY-1-ADAMANTYL)-2-OXOETHANOIC ACID

Glucagon-like peptide-1 (GLP-1) analogs and dipeptidyl peptidase IV (DPP-IV) inhibitors are two promising new approaches currently being explored for treatment of Type 2 diabetes. GLP-1, a peptide secreted by the gut in response to feeding, has the beneficial effects of increasing glucose-stimulated insulin secretion, decreasing glucagon secretion, delaying gastric emptying, and increasing the β-cell mass of pancreatic islets. GLP-1 (7–36) amide is rapidly inactivated by conversion to GLP–1 (9–36) amide by DPP-IV (Gallwitz, 2005; Nielsen, 2005; Sinclair and Drucker, 2005). To alleviate the inactivation of GLP-1, more stable analogs of GLP-1 as well as inhibitors of DPP-IV are approaches to provide improved control of blood glucose for diabetics. Saxagliptin **1** (Augeri *et al.*, 2005) (Fig. 18.1), a DPP-IV inhibitor under development by Bristol-Myers Squibb, requires (*S*)-*N*-BOC-3-hydroxyadamantylglycine **2** (Fig. 18.1) as an intermediate. We have previously prepared several unnatural amino acids (Hanson *et al.*, 1990, 1999, 2000) as intermediates for synthetic routes by reductive amination of the corresponding keto acids using L-amino acid dehydrogenases. We have investigated this approach to afford an improved synthesis of intermediate **4** using a modified form of a

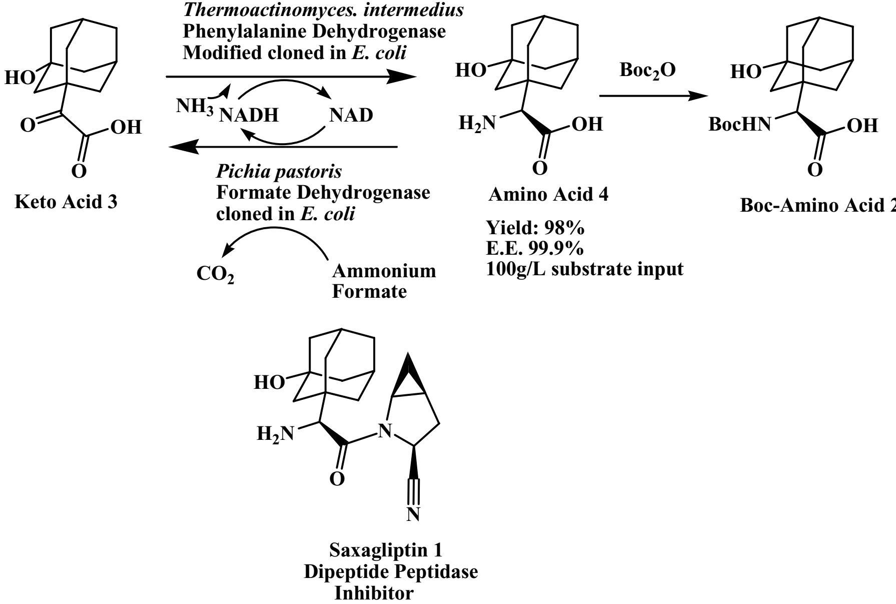

Figure 18.1. Enzymatic reductive amination of 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid **3** to prepare (*S*)-*N*-BOC-3-hydroxyadamantylglycine **2**.

recombinant phenylalanine dehydrogenase cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* or *Escherichia coli*. NAD produced during the reaction was recycled to NADH using formate dehydrogenase. The modified phenylalanine dehydrogenase contains two amino acid changes at the C-terminus and a 12 amino acid extension of the C-terminus. The modified enzyme is more effective with keto acid **3** than the wild type enzyme, but less effective with the natural substrate, phenylpyruvate. Production of multi-kg batches was originally carried out with extracts of *Pichia pastoris* expressing the modified phenylalanine dehydrogenase from *Thermoactinomyces intermedius* and endogenous formate dehydrogenase, and further scaled up using a preparation of the two enzymes expressed in *E. coli*. The amino acid **4** (Fig. 18.1) can be directly protected as its BOC derivative without isolation to afford intermediate **2**.

Using fresh cells, the reaction was evaluated under various conditions with phenylalanine dehydrogenase from *Thermoactinomyces intermedius* expressed in *Pichia pastoris* SMD 1168 (pPDH9K/10)[SC16176] (Hanson *et al.*, 2000). The enzymatic reductive amination reaction requires ammonia and NADH (Fig. 18.1). NAD produced during the reaction was recycled to NADH by the oxidation of formate to CO_2 using formate dehydrogenase (FDH) which is produced by *Pichia pastoris* during growth on methanol. Wet cells, heat-dried cells, and cell extracts were compared. *Pichia pastoris* extract was more effective than wet cells or heat-dried cells. Wet cells gave 64% yield, heat-dried

cells gave 80% yield, whereas an extract gave close to 100% yield. The best conditions using *Pichia* extract were 0.25 M keto acid, 0.5 M ammonium formate, 1 mM NAD, 1 mM dithiothreitol, pH 8, and 12.5% w/v *Pichia* extract, at 40 °C. With these conditions, a total of 1.6 kg **4** was prepared in lab batches. Yields before isolation were close to 100% with no (*R*)-enantiomer detected. The isolation (787 g ketoacid input) gave 804 g of **4** with a potency of 94.3% and a yield of 96.0% from **3**. All of the batches of **4** isolated by this procedure performed well in subsequent reactions for the synthesis of Saxagliptin (Vu *et al.*, 2004). To facilitate the BOC protection, protein was first removed by microfiltration and ultrafiltration, then ammonia was removed by boiling at pH 9.6.

Reductive amination was also conducted using cell extracts from *E. coli* strain SC16496 expressing PDHmod and cloned FDH from *Pichia pastoris*. Cells from a 15-L tank had 133 u/g FDH, 65 u/g PDH (phenylpyruvate assay), and 12.7 u/g PDH (assayed with keto acid **3**). The extract was used for conversion of 30 g **3** to **4** in close to 100% yield, and this material, after filtration for protein removal, was converted to **2** by BOC protection. Further experiments showed that the *E. coli* extract could be used at 2.5% w/v concentration instead of the 12.5% concentration used for batches with *Pichia pastoris* extract. In subsequent experiments, the substrate input was increased to 100 g/L and the reaction was carried out at pH 8.0. Cell extracts of *E. coli* strain SC16496 after polyethyleneamine treatment, clarification and concentration was used to complete the reaction in 30 hrs with >96% yield and >99.9% ee of product **4**. PDHmod and FDH expressed in *E. coli* have now been used to prepare several hundred kg of BOC-protected amino acid **2** to support the development of Saxagliptin (Hanson *et al.*, 2007).

18.3. REGIOSELECTIVE ENZYMATIC ACYLATION OF RIBAVIRIN

Ribavirin (**5**, Fig. 18.2) is an antiviral agent used in combination with α-2β interferon to treat hepatitis C (Pianko and McHutchison, 2000; Ferenci *et al.*, 2001). Although this therapy is effective against hepatitis C virus, it has several side effects (Sauleda *et al.*, 2001; Bonkovsky *et al.*, 2001). To improve the pharmacokinetic profile and reduce side effects, a ribavirin prodrug was considered. In a series of preclinical evaluations, the alanine ester of ribavirin **6** showed improved bioavalibility and reduced side effects. The synthesis of **6** required the acylation of unprotected ribavirin. The chemical acylation gave a mixture of mono-, di-, and triacylated products. An enzymatic process was developed for the regioselective acylation of ribavirin **5** with the oxime ester of L-carbobenzyloxy-alanine **7** to give the desired **8** using Novozym 435 (*Candida antarctica* lipase B or Chirazyme L-2). Chemical deprotection of **8** gave **6**. On preparative scale, the coupling of **9** with acetone oxime in the presence of di-tert-butyl dicarbonate in THF was carried out giving **7** in >96% yield. At the end of the reaction, the reaction mixture was diluted three-fold

Figure 18.2. Regioselective enzymatic acylation of ribavirin (**5**).

with THF, ribavirin was added, and the acylation was initiated by addition of the Novozyme 435. After 24 hr at 60 °C, the product **8** was isolated in 85% yield (Tamarez *et al.*, 2003).

18.4. MICROBIAL HYDROXYLATION OF EPOTHIOLONE B

Tubulin-polymerizing chemotherapeutic agents, such as taxanes, have been shown to be one of the most effective drug classes in the treatment of ovarian cancer. The clinical success of paclitaxel has stimulated research into compounds with similar modes of activity in an effort to emulate its antineoplastic efficacy while minimizing its less desirable aspects, which include non-water solubility, difficult synthesis, and emerging resistance.

The epothilones are a novel class of natural cytotoxic compounds derived from the fermentation of the myxobacterium *Sorangium cellulosum* that are non-taxane microtubule-stabilizing compounds triggering apoptosis (Gerth *et al.*, 2003; Benigni *et al.*, 2004; Goodin *et al.*, 2004). The natural product epothilone B (**10**) (Fig. 18.3) has demonstrated broad spectrum antitumor activity in vitro and in vivo, including tumors with paclitaxel resistance mediated by overexpression of P-glycoprotein or β-tubulin mutation. The role of **10** as a potential paclitaxel successor has resulted in several total syntheses of **10** and various derivatives thereof (Nicolaou *et al.*, 1998; Altmann, 2004; Boddy *et al.*, 2004). The epothilone analogs were synthesized in an effort to optimize the water solubility, in vivo metabolic stability, and antitumor efficacy of this class of antineoplasic agents (Lin *et al.*, 2003; Low *et al.*, 2005).

Figure 18.3. Microbial hydroxylation of epothiolone B (**10**) to epothilone F (**11**).

A fermentation process was developed for the production of epothilone B, and the titer of epothilone B was increased by a continuous feed of sodium propionate during fermentation. The inclusion of XAD-16 resin during fermentation to adsorb epothilone B and to carry out volume reduction made the recovery of product very simple (Benigni *et al.*, 2004). A microbial hydroxylation process was developed for the conversion of epothilone B (**10**) to epothilone F (**11**) by *Amycolatopsis orientalis* SC 15847. A bioconversion yield of 37–47% was obtained when the process was scaled up to 100–250 L. Recently, epothilone B hydroxylase and the ferredoxin gene have been cloned and expressed in *Streptomyces rimosus* from *Amycolatopsis orientalis* SC 15847 by our colleagues in Bristol-Myers Squibb. Mutants and variants thereof this cloned enzyme has been used in the hydroxylation of epothilone B to epothilone F to obtain even higher yields of product (Basch *et al.*, 2004).

18.5. BIOHYDROXYLATION 2-CYCLOPENTYLBENZOXAZOLE

(−)-Carbovir (**12)**, (Fig. 18.4) is an antiviral agent effective against HIV infection (Parker *et al.*, 1991). By employing protein and substrate engineering, the biohydroxylation of 2-cyclopentylbenzoxazole **13** to compound **14** has been demonstrated by Munzer *et al.*, to prepare a key intermediate for the synthesis of (−)-carbovir. (Muenzer *et al.*, 2005). Protecting group removal provided end product **15**.

In the initial screen, regardless of the microorganisms and fermentation conditions used, only a single diastereoisomer, (*S,S*)-**14**, could be obtained with synthetically useful diastereoselectivities. Side products were also formed during this transformation (de Raadt *et al.*, 1996). To circumvent this problem the cytochrome P450 BM-3, a medium chain (C_{12}–C_{18}) fatty acid hydroxylase from *Bacillus megaterium* engineered to accept non-natural substrates with enhanced regioselectivity, enantioselectivity, catalytic rates, and total

Figure 18.4. Biohydroxylation of 2-cyclopentylbenzoxazole **13**.

turnover, was evaluated (Urlacher *et al.*, 2004; Li *et al.*, 2001). Mutations in the active site, for example, enable the enzyme to hydroxylate alicyclic, heterocyclic, aromatic, and even polyaromatic compounds (Arnold and Otey, 2005). Biohydroxylation using the wild type enzyme expressed in *E. coli* gave very low enantioselectivity (1.5%, *S,S*) and high diastereoselectivity (87%). Mutant 139-3 also afforded (*S,S*)-**14**, but in high ee (86%) and de (96%). In dramatic contrast, mutant 1-12G was found to produce (*R,R*)-**14** in high selectivities (89% ee, 94% de) (Muenzer *et al.*, 2005).

18.6. ENZYMATIC PREPARATION OF 6-HYDROXYBUSPIRONE

Buspirone (Buspar®, **16**, Figure 5), a drug used for treatment of anxiety and depression, is thought to produce its effects by binding to the serotonin 5HT1A receptor (Fulton and Brogden, 1997; Heiser and Wilcox, 1998; Martin *et al.*, 1991). Mainly as a result of hydroxylation reactions, it is extensively converted to various metabolites (Jajoo *et al.*, 1989), and blood concentrations return to low levels a few hours after dosing. A major metabolite, 6-hydroxybuspirone (**17**), produced by the action of liver cytochrome P450 CYP3A4, is present at much higher concentrations in human blood than buspirone itself. This metabolite has anxiolytic effects in an anxiety model using rat pups, and binds to the human 5-HT1A receptor (Mayol, 2000). Although the metabolite has only

Acylase 30000

18
6-acetoxybuspirone (racemic)

(*S*)-17
(*S*)-6-hydroxybuspirone

+

Lipase A 12
or 1N HCl

(*R*)-17
(*R*)-6-hydroxybuspirone

(*R*)-18
(*R*)-6-acetoxybuspirone

Buspirone 16

Figure 18.5. Enzymatic preparation of (*S*)- and (*R*)-6-hydroxybuspirone by a resolution process.

about a third of the affinity for the human 5HT1A receptor as buspirone, it is present in human blood at 30–40 times higher concentration than buspirone following a dose of buspirone, and therefore may be be responsible for much of the effectiveness of the drug (Mayol, 2000; Yevich *et al.*, 2003a). For development of 6-hydroxybuspirone as a potential antianxiety drug, preparation and testing of the two enantiomers as well as the racemate was of interest. Both *R*- and *S*-enantiomers, isolated by chiral HPLC, were effective in tests using a rat model of anxiety (Mayol, 2000; Yevich *et al.*, 2003b). Whereas the *R*-enantiomer showed somewhat tighter binding and specificity for the 5HT1A receptor, the *S*-enantiomer had the advantage of being cleared more slowly from the blood (Yevich *et al.*, 2003c). An enzymatic process was developed for resolution of 6-acetoxybuspirone **18** (Fig. 18.5). L-Amino acid acylase from *Aspergillus melleus* (Amano acylase 30000) was used to hydrolyze racemic 6-acetoxybuspirone to (*S*)-6-hydroxybuspirone in 96% ee after 46% conversion. The remaining (*R*)-6-acetoxybuspirone with 84% ee was converted to (*R*)-6-hydroxybuspirone by acid hydrolysis. The ee of both enantiomers could be improved to >99% by crystallization as a metastable polymorph (Hanson *et al.*, 2005a). Direct hydroxylation of buspirone to (*S*)-6-hydroxbuspirone by *Streptomyces antibioticus* ATCC 14980 has also been described (Hanson *et al.*, 2005a).

19
6-Keto Buspirone

Pseudomonas putida Rec. Escherichia coli

(*S*)-17
(*S*)-6-Hydroxybuspirone

Hansenula polymorpha Rec. Escherichia coli

(*R*)-17
(*R*)-6-Hydroxybuspirone

Figure 18.6. Enzymatic preparation of (*S*)- and (*R*)-6-hydroxybuspirone by a reduction process.

In an alternate process, enantioselective microbial reduction of 6-oxobuspirone (**19**, Fig. 18.6) to either (*R*)- and (*S*)-6-hydroxybuspirone was described. About 150 microorganisms were screened for the enantioselective reduction of **19**. *Rhizopus stolonifer* SC 13898, *Rhizopus stolonifer* SC 16199, *Neurospora crassa* SC 13816, *Mucor racemosus* SC 16198, and *Pseudomonas putida* SC 13817 gave >50% reaction yields and >95% ee's of (*S*)-6-hydroxybuspirone. The yeast strains *Hansenula polymorpha* SC 13845 and *Candida maltosa* SC 16112 gave (*R*)-6-hydroxybuspirone in >60% reaction yield and >97% ee (Patel *et al.*, 2005).

The NADP-dependent (*R*)-reductase (RHBR) which catalyzes the reduction of 6-oxobuspirone to (*R*)-6-hydroxybuspirone was purified to homogeneity from cell extracts of *Hansenula polymorpha* SC 13845. The subunit molecular weight of the enzyme is 35,000 daltons based on sodium dodecyl sulfate gel electrophoresis and the molecular weight of the enzyme is 37,000 daltons as estimated by gel filtration chromatography. (*R*)-reductase from *H. polymorpha* was cloned and expressed in *Escherichia coli*. To regenerate the cofactor NADPH required for reduction we have cloned and expressed the glucose-6-phosphate dehydrogenase gene from *Saccharomyces cerevisiae* in *E. coli* (Goldberg *et al.*, 2006). The NAD-dependent (*S*)-reductase (SHBR) which catalyzes the reduction of 6-oxobuspirone to (*S*)-6-hydroxybuspirone was purified to homogeneity from cell extracts of *Pseudomonas putida* SC 16269. The subunit molecular weight of the enzyme is 25,000 daltons based on sodium dodecyl sulfate gel electrophoresis. The (*S*)-reductase from *P. putida* was cloned and expressed in *E. coli*. To regenerate the cofactor NADH required for reduction we have cloned and expressed the formate dehydrogenase gene from *Pichia pastoris* in *E. coli* (Goldberg *et al.*, 2006). Recombinant

E. coli expressing (*S*)-reductase and (*R*)-reductase catalyzed the reduction of 6-oxobuspirone to (*S*)-6-hyroxybuspirone and (*R*)-6-hyroxybuspirone, respectively, in >98% yield and >99.9% ee. (Goldberg *et al.*, 2006).

18.7. ENZYMATIC PREPARATION OF (S)-2-CHLORO-1-(3-CHLOROPHENYL)ETHANOL

The synthesis of the leading candidate compound in an anticancer program (Carboni *et al.*,2004; Beaulieu *et al.*, 2004), required (*S*)-2-chloro-1-(3-chlorophenyl)ethanol (**20)** (Fig. 18.7) as an intermediate. Other possible candidate compounds used analogs of the (*S*)-alcohol. About 100 microbial cultures were screened for reduction of the corresponding ketone **21** to the (*S*)-alcohol **20**, and *Hansenula polymorpha* SC13824 (73.8% ee) and *Rhodococcus globerulus* SC SC16305 (71.8% ee) had the highest enantioselectivity. A ketoreductase from *Hansenula polymorpha*, after purification to homogeneity, gave (*S*)-alcohol **20** with 100% ee. Amino acid sequences from the purified enzyme were used to design PCR primers for cloning the ketoreductase. The cloned ketoreductase required NADP(H), had a subunit molecular weight of 29,220 and a native molecular weight of 88,000. The cloned ketoreductase was expressed in *E. coli* together with a cloned glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae* to allow regeneration of the NADPH required by the ketoreductase. An extract of *E. coli* containing the two recombinant enzymes was used to reduce 2-chloro-1-(3-chloro-4-fluorophenyl)-ethanone (**21**) and two related ketones to the corresponding (*S*)-alcohols. Intact *E. coli* cells provided with glucose were used

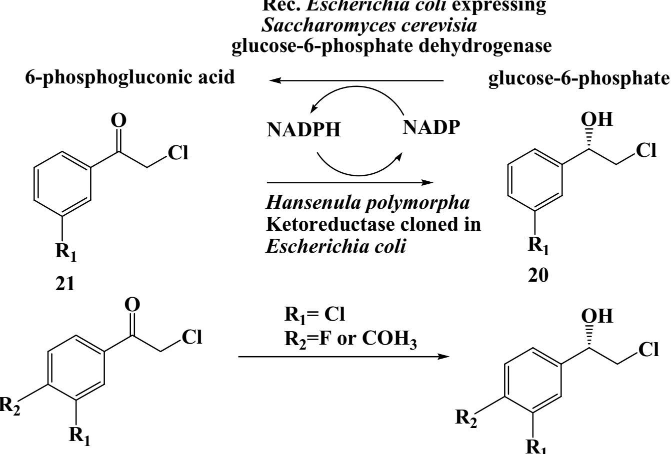

Figure 18.7. Enzymatic preparation of (S)-2-chloro-1-(3-chlorophenyl)ethanol.

to prepare (*S*)-2-chloro-1-(3-chloro-4-fluorophenyl)ethanol **20** in 89% yield with 100% ee (Hanson *et al.*, 2005b).

18.8. STEREOSPECIFIC MICROBIAL REDUCTION OF ETHYL 1-BENZYL-3-OXO-PIPERIDINE-4-CARBOXYLATE

Enantiomerically pure α-substituted-β-hydroxycarboxylates containing two chiral centers are useful building blocks in pharmaceutical synthesis. It was reported that Baker's yeast reduced the title compound **22** diastereo- and enantioselectively to *cis*-(3*R*,4*R*)-**23a** (Fig. 18.8) with de 73% and ee >95% under non-fermenting conditions (Seebach *et al.*, 1987). The de (73%) of the product was low and separation would be necessary to increase the de and ee to the level required for the synthesis of most pharmaceutical intermediates. To make the ethyl *cis*-(3*R*,4*R*)-1-benzyl-3*R*-hydroxypiperidine-4*R*-carboxylate (**23a**) in high diastereomeric and enantiomeric excess, recently we have evaluated microbial reduction of ethyl 1-benzyl-3-oxo-piperidine-4-carboxylate. The majority of evaluated microorganisms gave the ethyl *cis*-(3*R*,4*R*)-1-benzyl-3*R*-hydroxy-piperidine-4*R*-carboxylate as the major product in high diastereo- and enantioselectivities. The 3*R*,4*R*-hydroxy ester was produced in 97.4% diastereomeric excess (de) and 99.8% enantiomeric excess (ee) by *Candida parapsilosis* SC16347, while 99.5% de and 98.2% ee were obtained from reduction by *Pichia methanolica* SC16415. A few of the evaluated microorganisms gave 10–40% of the ethyl *trans*-(3*R*,4*S*)-1-benzyl-3*R*-hydroxypiperidine-4*S*-carboxylate **24a** as the minor product (Guo *et al.*, 2006a).

Figure 18.8. Enzymatic preparation of cis-(3*R*,4*R*)-1-benzyl-3*R*-hydroxypiperidine-4*R*-carboxylate **23a**.

Uridine phosphorylase & purine nucleotide phosphorylase

Figure 18.9. Enzymatic preparation of a prodrug of 9-β-D-arabinofuranosyl guanine (**25**).

18.9. ENZYMATIC PREPARATION OF A PRODRUG OF 9-β-D-ARABINOFURANOSYL GUANINE

Compound **25** (Fig. 18.9), a prodrug of 9-β-D-arabinofuranosyl guanine (**26**), was developed for the potential treatment of leukemia. Compound **24** is poorly soluble in water and its synthesis by conventional techniques is difficult. An enzymatic demethoxylation process was developed using adenosine deaminase (Mahmoudian *et al.*, 1999, 2001). Compound **25** was enzymatically prepared from 6-methoxyguanine (**27**) and ara-uracil (**28**) using uridine phosphorylase and purine nucleotide phosphorylase. Each protein was cloned and overexpressed in independent *Escherichia coli* strains. Fermentation conditions were optimized for production of both enzymes and a co-immobilized enzyme preparation was used in the biotransformation process at 200 g/L substrate input. Enzyme was recovered at the end of the reaction by filtration and reused in several cycles. A more water soluble 5′-acetate ester of compound **26** was subsequently prepared by an enzymatic acylation process using immobilized *Candida antarctica* lipase in 1,4-dioxane (100 g/L substrate) with vinyl acetate as the acyl donor (Krenitsky *et al.*, 1992).

18.10. ENZYMATIC DESYMMETRIZATION OF DIETHYL 3-[3′,4′-DICHLOROPHENYL]GLUTARATE

Tachykinins are a group of biologically active neuropeptide hormones implicated in a variety of biological processes such as pain transmission, inflammation, vasodialation, and secretion (Burcher *et al.*, 1995). The effect of tachykinins is modulated via the specific G-protein coupled receptors like NK1 and NK2. Thus non-peptide NK-receptors antagonists are potentially useful in the treatment of variety of chorinic diseases including asthma, bronchospasm, arthritis, and migraine (Veronesi *et al.*, 1999; Yuan *et al.*, 1998). The

Figure 18.10. Enzymatic desymmetrization of diethyl 3-[3′,4′-dichlorophenyletglutarate (**30**).

structure-activity relationship of several non-peptide NK1/NK2 antagonists has led to the discovery of a new class of oxime-based NK1/NK2 dual antagonists (Ting *et al.*, 2000; Reichard *et al.*, 2000) such as compound **29** (Fig. 18.10). The biological activity of **29** resides mainly in the *R,R*-diastereomer. An enzymatic process for desymmetrization of the prochiral diethyl 3-[3′,4′-dichlorophenyl]glutarate (**30**) to the corresponding (*S*)-monoester **31** has been developed using lipase B from *Candida antarctica* with either the free or the immobilized enzyme. At 100 g/L substrate input, a reaction yield of 97% and an ee of >99% were obtained for the desired (*S*)-monoester. The process was scaled up to produce 200 kg of product in 80% overall isolated yield (Homann *et al.*, 2001). DNA family shuffling was used to create a chimeric lipase B protein with improved activity towards diethyl 3-[3′,4′-dichlorophenyl]glutara te. Three homologous lipases from *Candida antarctica* ATCC 32657, *Hyphozyma* sp. CBS 648.91 and *Cryptococcus tsukubaensis* ATCC 24555 were cloned and shuffled to generate a diverse gene library, and using a high-throughput screening assay, a chimeric lipase B protein having 20-fold higher activity towards the substrate was identified (Suen *et al.*, 2004). The thermostability of the lipase was also improved by directed evolution (Zhang *et al.*, 2003).

18.11. ENZYMATIC SYNTHESIS OF INTERMEDIATES FOR PROPANOLOL AND DENOPAMINE

Propanolol (**32**, Fig. 18.11) belongs to the group of β-adrenergic blocking agents of the general structure $ArOCH_2CH(OH)CH_2NHR$, where Ar is aryl and R is alkyl. These compounds are potentially useful for the treatment of

Figure 18.11. Enzymatic synthesis of intermediates (*R*)-**35** and (*R*)-**37** for the synthesis of propanolol (**32**) and denopamine (**38**).

hypertension. β-Adrenergic receptor blocking activity mainly resides in the (*S*)-enantiomers. Synthesis of (*S*)-**32** was achieved by dynamic kinetic resolution (DKR) of (±)-**33** using Novozyme-435 in toluene at 80 °C and *p*-chlorophenyl acetate as acyl donor in the presence of ruthenium complex **34**. The (*R*)-acetate **35** was produced in ee >99% and 86% isolated yield. The enzyme was recycled and used again for another cycle without any loss of activity. Using the same procedure, racemic **36** was subjected to DKR to produce the (*R*)-acetate **37** (ee > 99%, 92%conversion and 84% isolated yield), a precursor of (*R*)-denopamine **38**, a potent orally active β_1 receptor agonist for the treatment of hypertension (Pamies and Baeckvall, 2001).

18.12. BIOCATALYTIC ASYMMETRIC DIHYDROXYLATION

Aromatic hydrocarbon dioxygenases are enzymes that catalyze the oxidation of aromatic hydrocarbons. They represent an important subset of a large family of "aromatic ring hydroxylating dioxygenases" or "Rieske non-heme iron oxygenases" as they have been variously designated, all with similar mechanism and structure (Gibson and Parales, 2000; Butler and Mason, 1997). Aromatic ring hydroxylating dioxygenases initiate the degradation of a wide range of aromatic compounds, including polycyclic aromatic hydrocarbons, nitroaromatic and chlorinated aromatic compounds, aromatic acids, and heterocyclic aromatic compounds. Toluene dioxygenase (TDO) from *Pseudomonas putida* F1 was the first characterized aromatic hydrocarbon dioxygenase.

Figure 18.12. Benzene dioxygenase, toluene dioxygenase and naphthalene dioxygenase catalyzed dihydroxylation arenes to arene cis-dihydrodiols.

Identified by Gibson and co-workers, TDO (Fig. 18.12) was shown to catalyze *cis*-dihydroxylation of benzene and toluene (Yeh *et al.*, 1977). A great deal of our understanding of the structure, function, and substrate specificity of aromatic hydrocarbon dioxygenases has come from studies of TDO and naphthalene dioxygenase (NDO) (Lee *et al.*, 2005; Karlsson *et al.*, 2003). To date, more than 100 Rieske non-heme iron oxygenases have been identified based on biological activity or nucleotide sequence identity. To date more than three hundred arene *cis*-dihydrodiols (Fig. 18.12) have been obtained using toluene dioxygenase and these have been extensively used as chiral precursors in organic synthesis (Hudlicky *et al.*, 1999; Boyd *et al.*, 2001, 2005; Boyd and Bugg, 2006). Benzene dioxygenase, toluene dioxygenase and naphthalene dioxygenase have also been reported to catalyze the dihydroxylation of alkenes and recently biocatalytic asymmetric dihydroxylation of conjugated mono- and poly-alkenes have been demonstrated to yield enantiopure cyclic *cis*-diols. The diol metabolites were obtained from monosubstituted, gem-disubstituted, cis-disubstituted, and trisubstituted alkene substrates, using whole cells of *Pseudomonas putida* strains containing toluene and naphthalene dioxygenases. Dioxygenase selection and alkene type were important factors in the preference for dioxygenase-catalyzed 1,2-dihydroxylation of conjugated alkene or arene groups, and monohydroxylation at benzylic or allylic centers. Cis-1,2-diol metabolites from arenes, cyclic alkenes, and dienes were generally observed to be enantiopure (>98% ee), while 1,2-diols from acyclic alkenes had lower enantiomeric excess values (<88% ee) (Boyd *et al.*, 2006).

18.13. ENZYMATIC SYNTHESIS OF ETHYL (3*R*,5*S*)-DIHYDROXY-6-(BENZYLOXY) HEXANOATE

The diol ethyl (3*R*,5*S*)-dihydroxy-6-(benzyloxy) hexanoate (**40a**, Fig. 18.13) is a key intermediate in synthesis of [4-[4α,6ß(E)]]-6-[4, 4-bis[4-fluorophenyl)-3-(1-methyl-1H-tetrazol-5-yl)-1,3-butadienyl]tetrahydro-4-hydroxy-2H-pyren-2-one (**41**), a potential new anticholesterol drug which acts by inhibition of HMG CoA reductase (Sit *et al.*, 1990; O'Connor *et al.*, 2005).

The enantioselective reduction of the diketoester ethyl 3,5-dioxo-6-(benzyloxy) hexanoate (**39**) to the diol ethyl (3*R*,5*S*)-dihydroxy-6-(benzyloxy)hexanoate (**40a**) has been demonstrated by *Acinetobacter calcoaceticus* SC 13876 in a yield of 85% with a diastereoselectivity of 97%. Cell extracts of *A. calcoaceticus* SC 13876 in the presence of NAD^+, glucose, and glucose dehydrogenase reduced **39** to the corresponding isomeric monohydroxy compounds **42** and **43**, which were further reduced to the compound **40a**. A reaction yield of 92% and enantiomeric purity of 98% were obtained when the reaction was carried out at 10g/L in a 1-L batch (Patel *et al.*, 1993).

Figure 18.13. Enzymatic synthesis of ethyl (3*R*,5*S*)-dihydroxy-6-(benzyloxy)hexanoate (**40a**).

Three different ketoreductases were purified to homogeneity, and their biochemical properties were compared. Reductase I only catalyzes the reduction of ethyl diketoester **39** to its monohydroxy products **42** and **43** whereas reductase II catalyzes the formation of dihydroxy products from monohydroxy substrates. A third reductase (III) was identified which catalyzes the reduction of diketoester **39** to desired *syn*-(3*R*,5*S*)-dihydroxy ester **40a** (Guo *et al.*, 2006b).

18.14. ENZYMATIC PREPARATION OF A 2,4-DIDEOXYHEXOSE DERIVATIVE

The chiral 2,4-dideoxyhexose derivative required for the HMG CoA reductase inhibitors has also been prepared using 2-deoxyribose-5-phosphate aldolase (DERA). The reactions start with a stereospecific addition of acetaldehyde (**44**) (Fig. 18.14) to a substituted acetaldehyde to form a 3-hydroxy-4-substituted butyraldehyde **45**, which reacts subsequently with another acetaldehyde to form a 2,4-dideoxyhexose derivative **46**. DERA has been expressed in *Escherichia coli* (Gijsen and Wong, 1995).

The above process has been improved and optimized. An almost 400-fold increase in volumetric productivity relative to the published enzymic reaction conditions has been achieved, resulting in a attractive process that has been run on up to 100-g scale in a single batch at a rate of 30.6 g/L/hr. The catalyst load has been improved by 10-fold as well, from 20 to 2.0 wt % DERA. These improvements were achieved by a combination of the discovery of a DERA with improved activity and reaction optimization to overcome substrate inhibition. The two stereogenic centers are set by DERA, with an ee of >99.9% and a diastereomeric excess of 96.6%. In addition, downstream chemical processes have been developed to convert the enzymic product efficiently to versatile intermediates applicable to the preparation of atorvastatin and rosuvastatin (Greenberg *et al.*, 2004).

18.15. ENZYMATIC PREPARATION OF (*R*)-4-CYANO-3-HYDROXYBUTYRATE

An enzymatic process has been developed for the preparation of 4-halo-3-hydroxybutyric acid derivatives by ketoreductase-catalyzed conversion of 4-

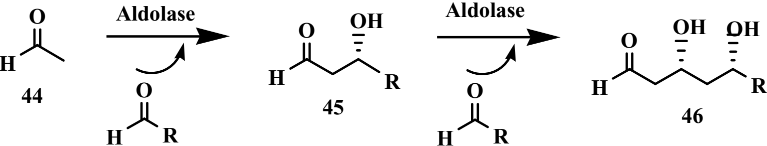

Figure 18.14. Enzymatic preparation of a 2,4-dideoxyhexose derivative **46**.

Ketoreductase cloned in *E. coli*
Halohydrin dehalogenase cloned in *E. coli*
Sodium Cyanide

48
Ethyl-4-chloro acetoacetate

49
4-Chloro-3-hydroxy-butyric acid ethyl ester

47
(R)-4-Cyano-3-hydroxybutyrate

Figure 18.15. Enzymatic preparation of (*R*)-4-cyano-3-hydroxybutyrate (**47**).

Nitrilase

50

51

Figure 18.16. Enzymatic desymmetrization of prochiral 3-hydroxyglutaronitrile (**50**).

halo-3-ketobutyric acid derivatives (Davis *et al.*, 2004a). The genes encoding halohydrin dehydrogenase from *Agrobacterium tumefaciens*, ketoreductase from *Candida magnoliae*, glucose dehydrogenase from *Bacillus subtilis*, and formate dehydrogenase from *Candida boidinii* were separately cloned into *Escherichia coli* BL21. Each enzyme was then produced by fermentation, isolated, and characterized. Then ethyl (*R*)-4-cyano-3-hydroxybutyrate (**47**) (Fig. 18.15) was prepared from ethyl 4-chloroacetoacetate (**48**) by the following procedure: Ethyl 4-chloroacetoacetate **48** was incubated at pH 7.0 with ketoreductase, glucose dehydrogenase, and NADP for 40 hr to produce ethyl (*S*)-chloro-3-hydroxybutyrate (**49**). The ethyl (*S*)-chloro-3-hydroxybutyrate **49** was extracted with ethyl acetate, dried, filtered and concentrated to yield ~ 97% pure ester. The dried ethyl (*S*)-chloro-3-hydroxybutyrate (**49**) was dissolved in phosphate buffer and mixed with halohydrin dehalogenase and sodium cyanide at pH 8.0. After 57 hr, essentially pure (*R*)-4-cyano-3-hydroxybutyrate (**47**), an intermediate used in many HMG-CoA reductase inhibitors syntheses, was recovered (Davis *et al.*, 2004b).

18.16. ENZYMATIC SYNTHESIS OF CHIRAL CARBOXYLIC ACIDS

Enzymatic desymmetrization of prochiral 3-hydroxyglutaronitrile (**50**) using a nitrilase (DeSantis *et al.*, 2002, 2003a, 2003b) has been demonstrated (Fig. 18.16). Following esterification of the resulting (*R*)-3-hydroxy-4-cyanobutyric acid (**51**), an intermediate useful for the manufacture of the cholesterol-lowering drug Lipitor (atorvastatin calcium) was produced. Nitrilases were identified in genomic libraries created by extraction of DNA directly from environmental samples and were expressed in *E. coli*. The resulting library was screened for highly enantioselective (*R*)-specific nitrilases (Robertson *et al.*, 2002). (*R*)-3-Hydroxy-4-cyanobutyric acid was produced using a 100-mM

initial nitrile concentration in 98% yield and 94.5% ee. The enantioselectivity of this wild-type nitrilase decreased with increasing nitrile concentration, where only 87.8% ee was obtained at 2.25 M substrate concentration. Mutagenesis of the nitrilase using a technique that combinatorially saturated each amino acid in the protein to each of the other 19 amino acids resulted in an improved variant (Ala190His) that was expressed in *E. coli*. This variant nitrilase gave an enantiomeric excess of 98.5% at 3 M substrate concentration with a volumetric productivity of 619 g/L/day. Nitrilases from this library have been used to produce a range of (*R*)-mandelic acid derivatives and analogs, and (*S*)-phenyllactic acid, with high yields and enantioselectivities (DeSantis, *et al.*, 2002). A nitrilase from this library has been expressed in *Pseudomonas fluorescens* (Squires and Talbot, 2004).

18.17. ENZYMATIC RESOLUTION OF α-METHYL-1,3-BENZODIOXOLE-5-ETHANOL

(−)-Talampanel (**52**, Fig. 18.17) has been identified as an orally active compound with potential antiepileptic, neuroprotectant and skeletal muscle relaxant activities (Tarnawa *et al.*, 1993; Vizi *et al.*, 1996). An efficient enantioselective reduction of 3,4-methylenedioxyphenylacetone (**53**) has been developed using

Figure 18.17. Enantioselective microbial reduction of 3,4-methylenedioxyphenylacetone (**53**) and enzymatic resolution of α-methyl-1,3-benzodioxole-5-ethanol (**55**).

an NAD(P)H-dependent dehydrogenase from *Zygosaccharomyces rouxii*. The product (S)-3,4-methylenedioxyphenylisopropanol, a key chiral intermediate (**54**) was obtained in >95% yield and >99.9% ee Since the substrate and product were both toxic to cells, polymeric hydrophobic resins were used to both supply substrate and remove the product from the reaction mixture as it formed. This approach allowed the reaction concentration to be increased from 6 to 40 g/L. The reaction was scaled up to 300 L by utilizing a commercially available agitated filter as the reactor which allowed the reaction, product isolation, and resin recycle to be accomplished within a single piece of equipment with overall productivity of 75 g/L/day (Vicenzi *et al.*, 1997).

In an alternate process, enantioselective enzymatic acylation of racemic α-methyl-1,3-benzodioxole-5-ethanol (**55**, Fig. 17) was developed using Amano lipase PS-30 (lipase from *Pseudomonas cepacia*) with vinyl acetate as acylating agent in n-hexane:benzene (2:1). This process gave (+)-**56** in 54% yield with 80% ee and (−)-**57** in 46% yield with 96% ee After separation of alcohol (+)-**56** from acetate (−)-**57** by methanolysis in the presence of K_2CO_3, the acetate was converted to alcohol (−)-**56** in 95% yield with 96% ee Mitsunobu inversion of (−)-**56** provided (+)-**56** in 94% yield with 96% ee The conversion of (*S*)-alcohol **56** to (−)-talampanel was carried out in 54% overall yield (Easwar and Argade, 2003).

18.18. ENANTIOSELECTIVE ENZYMATIC REDUCTION OF N,N-DIMETHYL-3-KETO-3-(2-THIENYL)-1-PROPANAMINE

Although considerable progress has been made in improving the tolerability of antidepressant drugs, the classical tricyclic antidepressants (TCA) are still a standard for efficacy. The selective serotonin reuptake inhibitors (SSRI) are much better tolerated than the TCAs, but their antidepressant efficacy is, at best, equivalent and probably inferior to the TCA, clomipramine, in many situations. The introduction of the SSRIs naturally focused both fundamental and clinical research effort on the role of serotonin (5-HT) in the pharmacogenesis and pharmacotherapy of depression. More recently the probable role of noradrenaline (NA) has been "rediscovered" and increasingly both 5-HT and NA dysfunctions are seen as fundamental to depressive illness. The therapeutic importance of this has been underlined by studies showing the increased antidepressant efficacy obtained when selective serotonergic drugs have been used in conjunction with selective noradrenergic drugs. The development of the new class of serotonin and noradrenaline reuptake inhibitors (SNRI) was a logical extension of these ideas. Compounds of this class, which currently comprises venlafaxine, milnacipran and duloxetine, act to inhibit the reuptake of both monoamines with no direct actions at postsynaptic receptors and are very effective antidepressant drugs (Montgomery, 2006; Delgado, 2006; Blier, 2006). (*S*)-Duloxetine [(*S*)-N-methyl-3-(1-naphthalenyloxy)-3-(2-thienyl)-1-propanamine] is regarded as a useful chiral drug which is desired in the single

Figure 18.18. Microbial reduction of N.N-dimethyl-3-keto-3-(2-thienyl)-1-propanamine (**58**).

enantiomeric form for the treatment of psychiatric and metabolic disorders (Robertson *et al.*,1990).

Candida tropicalis PBR-2, a yeast strain isolated from soil, is capable of carrying out the enantioselective reduction of N,N-dimethyl-3-keto-3-(2-thienyl)-1-propanamine **58** to (*S*)-N,N-dimethyl-3-hydroxy-3-(2-thienyl)-1-propanamine **59** (Fig. 18.18), a key intermediate in the synthesis of the chiral drug (S)-Duloxetine (Soni and Banerjee, 2005). The organism produced the enantiopure (*S*)-alcohol with a good yield (>80%) and almost absolute enantioselectivity, with an ee >99%. Parameters of the bioreduction reaction were optimized and the optimal temperature and pH for the reduction were found to be 30 °C and 7.0, respectively. The optimized substrate and the resting cell concentration were 1 g/l and 250 g/l, respectively. The preparative-scale reaction using resting cells of *C. tropicalis* yielded the (*S*)-alcohol at 84–88% conversion and ee >99%.

18.19. DYNAMIC KINETIC RESOLUTION (DKR)

The deracemization of a number of pharmaceutically valuable building blocks has been carried out by biocatalytic processes. This includes epoxides, alcohols, amines and acids. DKR involves the combination of an enantioselective transformation with an *in situ* racemisation process such that, in principle, both enantiomers of the starting material can be converted to the product in high yield and ee. The racemization step can be catalysed either enzymatically by racemases, or non-enzymatically by transition metals.

A robust and scaleable technology has been developed for the preparation of optically active chiral amines by deracemization of racemic mixtures. The approach employs the simultaneous use of a highly selective oxidase biocatalyst and a chemical reducing agent or catalyst, and can be used for the preparation of a wide range of optically pure amines in yields often approaching 100%. An example is the conversion of racemic α-methyl benzylamine (**60**) (Fig. 18.19) *R*-α-methyl benzylamine (R-**60**) by amine oxidase from *Aspergillus niger*. The key advantages of the technology lie in the coordinated action of proven industrial catalysts and efficient methods of genetic screening to adapt the approach for the preparation of valuable industrial catalysts and efficient methods of genetic screening to adapt the approach for the

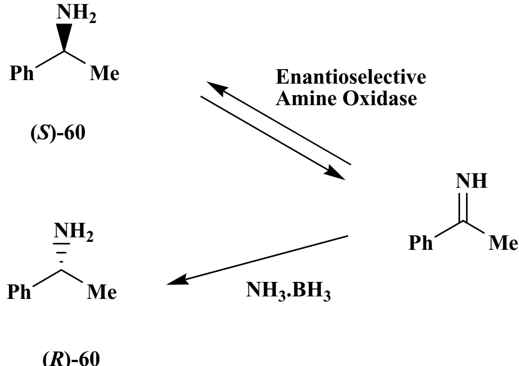

Figure 18.19. Dynamic kinetic resolution of α-methyl benzylamine (**60**).

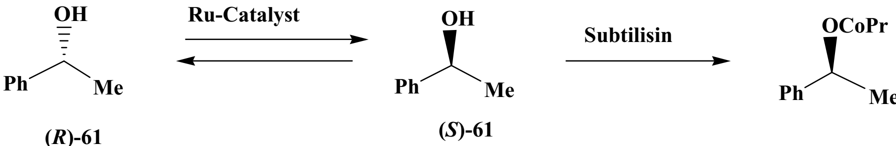

Figure 18.20. Dynamic kinetic resolution of secondary alcohols **61**.

preparation of valuable industrial targets (Turner *et al.*, 2004; Carr *et al.*, 2005; Roff *et al.*, 2004).

The DKR of secondary alcohols (Fig. 18.20) has been demonstrated by combining enantioselective lipases with transition-metal-based racemisation catalysts by the Bäckvall and Kim groups (Kim *et al.*, 2003; Pàmies and Bäckvall, 2003a). Kim has recently shown that (*S*)- as well as (*R*)-configured alcohols **61** can be prepared by the use of a commercially available (*S*)-selective subtilisin from *Bacillus licheniformis* as the enantioselective acylating catalyst (Kim *et al.*, 2003). The reactions were carried out using THF as a solvent, trifluoroethyl butyrate as an acyl donor, and an aminocyclopentadienylruthenium complex as the racemising catalyst. Pre-treating the subtilisin with a non-ionic surfactant significantly improved the activity of the biocatalyst about. 4000-fold under the reaction conditions. Backvall also reported the DKR of both α-and β-hydroxyalkane phosphonates using either *Candida antarctica* lipase B or *Pseudomonas cepacia* lipase using an alternative ruthenium complex with *p*-chlorophenoxy acetate as acyl donor (Pàmies and Bäckvall, 2003a, 2003b).

The development of a large-scale process for the DKR of alcohols using various lipases in combination with a range of ruthenium catalysts has been demonstrated. The reactions can be carried at concentrations up to 1 M with lower catalyst loadings (Pamies, and Backvall, 2003b). The process for the preparation of (*R*)-3,5-bis-trifluoromethyl-phenylethan-1-ol using [$RuCl_2$(*p*-

Figure 18.21. Deracemization of benzoin (**62**) by *Rhizopus oryzae* ATCC 9363.

cymene)]$_2$ as the racemization catalyst in combination with *Candida antacrtica* lipase B as the acylating catalyst has also been demonstratred (Verzijl *et al.*, 2001; Broxterman *et al.*, 2003). Deracemization of racemic secondary alcohols by two different alcohol dehydrogenases with complementary enantiospecificity has been well described (Allan and Carnell, 2001; Azerad and Buisson, 2000; Kroutil and Faber, 1998; Carnell, 1999). Comasseto *et al.* have shown that *Aspergillus terreus* CCT 3320 and *A. terreus* CCT 4083 are able to deracemise *ortho*- and *meta*-fluorophenyl-1-ethanol in good yields and high ee (Comasseto *et al.*, 2003). The deracemization of racemic benzoin (**62**, Fig. 18.21) *Rhizopus oryzae* ATCC 9363 has been demonstrated. The pH of the medium was used to control the absolute configuration of the enantiomer produced. Thus, at pH 7.5–8.5 the (*R*)-enantiomer was obtained in 73–76% yield and 97% ee, whereas at pH 4–5 the (*S*)-enantiomer was produced in 71% yield and 85% ee (Demir *et al.*, 2002). Deracemization of the racemic α-hydroxy ester ethyl 2-hydroxy-4-phenylbutanoate to yield the (*S*)-enatiomer in 85–90% yield and >99% ee using whole cells of *Candida parapsilosis* has been demonstrated (Kato *et al.*, 2002; Kato *et al.*, 2003).

The kinetic resolution of 2,2-disubstituted epoxides **63** (Fig. 18.22) by epoxide hydrolase has been demonstrated with excellent enantio- and regioselectivity yielding the corresponding (*S*)-diol and (*R*)-epoxide. Subsequently in the second step, the remaining (*R*)-epoxide was transformed by acid catalysis with *inversion* of configuration under carefully controlled reaction conditions to yield the corresponding (*S*)-diol in virtually enantiopure form and in high chemical yields (>90%). This methodology proved to be highly flexible and was also applicable to styrene oxide substrates (Paedragosa-Moreau *et al.*, 1997; Orru *et al.*, 1999).

The deracemization of *rac*-1-methyl-1,2-epoxycyclohexane (**64)** (Fig. 18.23) has been demonstrated by cells of *Corynebacterium* sp C12 and *Methylobacterium* sp. (Archer *et al.*, 1996; Ueberbacher *et al.*, 2005). A one pot combination of *Corynebacterium* C12 epoxide hydrolase and acid catalysed ring opening converted *rac*-1-methyl-1,2-epoxycyclohexane to (1-*S*, 2-*S*)-1-methylcyclohexane-1,2-diol. Alternatively, instead of the chemical conversion of the unreacted oxirane, the formed diol can be converted into the remaining epoxide (Monfort *et al.*, 2004).

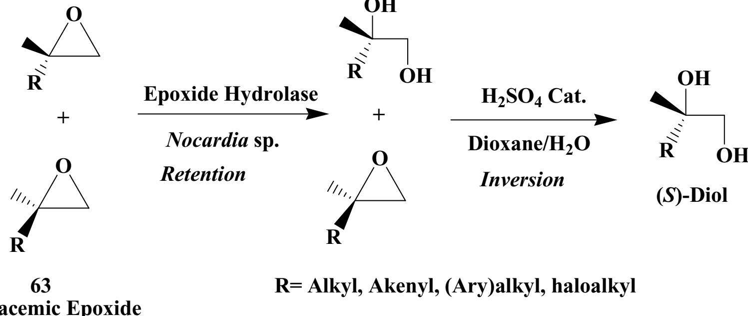

Figure 18.22. Dynamic kinetic resolution of 2,2-disubstituted epoxides **63** by epoxide hydrolase.

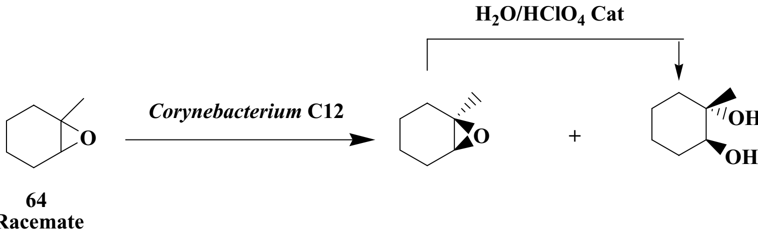

Figure 18.23. Deracemization of *rac*-1-methyl-1,2-epoxycyclohexane (**64**) by cells of *Corynebacterium* sp C12.

S-1-2′,3′-Dihydrobenzo[b]furan-4′-yl)ethane-1,2-diol (**65**, Fig. 18.24) is a potential precursor of *melatonin receptor agonist* (Catt *et al.*, 1999). The dynamic kinetic resolution of the racemic diol **65** to the (*S*)-enantiomer **65** has been demonstrated (Goswami *et al.*, 1999). *Candida boidinii* SC 13822, *Candida boidinii* SC 16115, and *Pseudomonas methanolica* SC 13860 transformed the racemic diol **65** in 3–4 days to *S*-diol **65** in >70% yield and 90–100% ee. A new compound was formed during these biotransformations and was identified as the hydroxy ketone **66** by LC-MS. The area of the HPLC peak for hydroxy ketone **66** first increased with time, reached a maximum, and then decreased, as expected for the proposed dynamic kinetic resolution.

An efficient kinetic resolution of racemic γ-hydroxy amides **67** (Fig. 18.25) was developed by *Pseudomas cepacia* lipase (PS-C)-catalyzed transesterification to yield the corresponding chiral acetate **68**. The enzyme PS-C tolerates both variation in the chain length and different functionalities giving good to high enantioselectivity (*E* values to >250). The combination of enzymatic kinetic resolution with a ruthenium-catalyzed racemization led to a dynamic kinetic resolution. The use of 2,4-dimethyl-3-pentanol as a hydrogen source to suppress ketone formation in the dynamic kinetic resolution yields the corresponding acetates in good yield and good to high enantioselectivity (ee’s to

Figure 18.24. Microbial dynamic kinetic resolution of the racemic diol **65** to (*S*)-diol.

Figure 18.25. Dynamic kinetic resolution of racemic γ-hydroxy amides **67**.

98%). The synthetic utility of this procedure was illustrated by the practical synthesis of the versatile intermediate γ-lactone (*R*)-5-methyltetrahydrofuran-2-one (Fransson *et al.*, 2005). Chiral γ-lactones are important structural synthons for the synthesis of natural products and biologically active compounds (Benincori *et al.*, 2004).

18.20. CONCLUSIONS

The production of single enantiomers of drug intermediates is increasingly important in the pharmaceutical industry. Biocatalysis provides organic chemists an alternate opportunity to prepare pharmaceutically important chiral compounds. The advantages of biocatalysis over chemical catalysis are that enzyme-catalyzed reactions are stereoselective and regioselective, and can be carried out at ambient temperature and atmospheric pressure providing an environmentally friendly system. The selective examples presented in this

review demonstrate the interest of process scientists using biotransformations in the preparation of chiral compounds. Different types of biocatalytic reactions are capable of generating a wide variety of chiral compounds. The use of hydrolytic enzymes such as lipases, esterases, proteases, dehalogenases, acylases, amidases, nitrilases, lyases, epoxide hydrolases, decarboxylases, and hydantoinases for the resolution of variety of racemic compounds, and in the asymmetric synthesis of enantiomerically enriched chiral compounds, has been widely demonstrated. Dehydrogenases and aminotransferases have been successfully used along with cofactors, and cofactor regenerating enzymes, for the synthesis of chiral alcohols, aminoalcohols, amino acids, and amines. Aldolases and decarboxylases have been effectively used in asymmetric synthesis by aldol condensation and acyloin condensation reactions. Monoxygenases have been used in enantioselective and regioselective hydroxylation, epoxidation, and Baeyer-Villiger reactions. Dioxygenases have been used in the chemo-enzymatic synthesis of chiral diols. The development of methods for the preparation of chiral compounds in 100% chemical and optical yields from racemates is one of the current challenges in asymmetric synthesis. Several approaches, such as re-racemization and repeated resolution or dynamic resolution, or on the transformation of enantiomers *via* enantioconvergent pathways, usually achieved by combination of chemo- and/or biocatalysts in sequential reactions or by a single biocatalyst, have been described. Stereoinversion *via* an oxidation-reduction sequence has also been demonstrated. In the course of the last decade, progress in protein chemistry, molecular cloning, random and site-directed mutagenesis, directed evolution of biocatalysts, and fermentation technology has opened up unlimited access to a variety of enzymes and microbial cultures as tools in organic synthesis.

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CHAPTER 19

Screening of Novel Microbial Enzymes and Their Application to Chiral Compound Production

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19.1. INTRODUCTION

The need for chiral (optically active) drugs is increasing due to the efficacy of these drugs, and market pressure for safe chemical compounds. Chiral starting materials are also increasingly needed in the pharmaceutical and agrochemical fields (Breuer *et al.*, 2004; Patel, 2000). The market for chiral compounds like chiral intermediates and pharmaceuticals is called the "chiral industry," and thus "chiral technology" means the various technologies involved in the chiral industry. In particular, the production technology for chiral compounds, except those easily obtained from natural resources such as L-amino acids, is one of the most important technologies in the chiral industry. To date, chiral compounds have been produced primarily through organic synthesis involving the optical resolution of racemic substrates. Recently, several artificial asymmetric catalysts, such as BINAP, have also been developed and used for the practical synthesis (Mikami *et al.*, 2000; Noyori and Ohkuma, 2001). On the other hand, biocatalysts are recognized as important catalysts for chiral technology because they catalyze chemical reactions specifically at three levels, *i.e., chemo-, regio-*, and *stereo*-selectivity. Furthermore, the fact that biocatalysts work under mild conditions is an attractive property, and is becoming more and more important as environmental restrictions make greater demands on the chemical process industry.

Generally, there are two ways to produce chiral compounds by using stereospecific biocatalysts. One is the optical resolution of racemic substrates or their derivatives, which is essentially the same as conventional chemical production processes. The other is the direct synthesis of chiral compounds from prochiral substrates. The advantage of the former method is that the biocatalysts are easily applicable to the corresponding conventional chemical resolution method, since essentially the same starting material, a racemic compound, is used. The most striking advantage of the latter is that the theoretical yield of a chiral compound is 100%, although optical resolution yields a maximum yield 50% of the amount of the starting material.

Here, we describe several successful examples of the development of "chiral technologies" using biocatalytic methods. These developments were based on extensive screening of microorganisms, and the consequent discovery of novel enzymes and their functions.

19.2. ASYMMETRIC REDUCTION OF PROCHIRAL CARBONYL COMPOUNDS FOR THE PRODUCTION OF CHIRAL ALCOHOLS: D-PANTOYL LACTONE PRODUCTION BY ASYMMETRIC REDUCTION

Chiral alcohols are some of the most important chiral building blocks for the production of pharmaceuticals. The creation of chiral alcohols through the asymmetric reduction of prochiral carbonyl compounds using biocatalysts, such as microbial cells and commercially available oxidoreductases, has been

thoroughly studied (Csuk and Glänzer, 2000; D'Arrigo *et al.*, 2000; Grunwald *et al.*, 1985; Kula and Kragl, 2000; Mori, 2000; Patel *et al.*, 1992; Wong *et al.*, 1985). In particular, many attempts at the asymmetric reduction of carbonyl compounds using baker's yeast as a catalyst have been made (Chin-Joe *et al.*, 2001; Csuk and Glänzer, 1991; D'Arrigo *et al.*, 1997; Kometani *et al.*, 1993; Nakamura *et al.*, 1991; Shieh *et al.*, 1985; Ward and Young, 1990; Zhou *et al.*, 1983). Most of the enzymes catalyzing the asymmetric reduction of carbonyl compounds require a stoichiometric amount of an expensive cofactor, NADH or NADPH, as the substrate for the reactions, therefore a sufficient cofactor supply system is also necessary. When the whole cells of naturally-occurring microorganisms such as baker's yeast are used as catalysts for asymmetric reduction, carbohydrates (glucose etc.) are usually added as an energy source to the reaction mixture for the regeneration of endogeneously present-cofactor NAD(P)H, in the cells (Kataoka *et al.*, 1992a).

The development of a novel production system for D-pantoyl lactone (which is a lactone compound carrying a chiral hydroxy group and a chiral intermediate for the commercial production of D-pantothenate) by microbial asymmetric reduction has been undertaken. D-pantothenate is mainly used in various pharmaceutical products and as an animal feed additive, the current world production of calcium pantothenate being about 6,000 tons per year. Conventional commercial production of D-pantoyl lactone has depended exclusively on chemical synthesis involving optical resolution of a chemically synthesized racemic pantoyl lactone, which is the most troublesome step of the pantothenate synthesis process.

Microbial reduction activity toward ketopantoyl lactone, a prochiral carbonyl form of D-pantoyl lactone, was screened, and many microorganisms were found to convert added ketopantoyl lactone to pantoyl lactone (Fig. 19.1a). However, the ratios of D- and L-isomers of the formed pantoyl lactone varied randomly, and their stereospecificities showed almost no relation to the genus or source. For example, among nine strains of *Rhodotorula glutinis* which produced pantoyl lactone with greater than 90% molar yields, five gave racemic mixtures, two the L-isomer, and the remaining two gave the D-isomer with more than 70% ee (Shimizu *et al.*, 1984). Preparative-scale stereospecific reduction was carried out with washed cells of one of the potent strains found on the screening, *Candida parapsilosis*, as a catalyst and glucose as an energy source for the reduction. About 90 g/l of D-pantoyl lactone was produced with a molar yield of nearly 100% and an optical purity of 94% ee (Hata *et al.*, 1987).

19.3. DISCOVERY OF NOVEL LACTONASES PROMISING FOR ENZYMATIC RESOLUTION OF RACEMIC PANTOYL LACTONE

In the course of the above-mentioned screening, we found another kind of novel conversion; stereospecific ester-hydrolysis of the lactone ring of

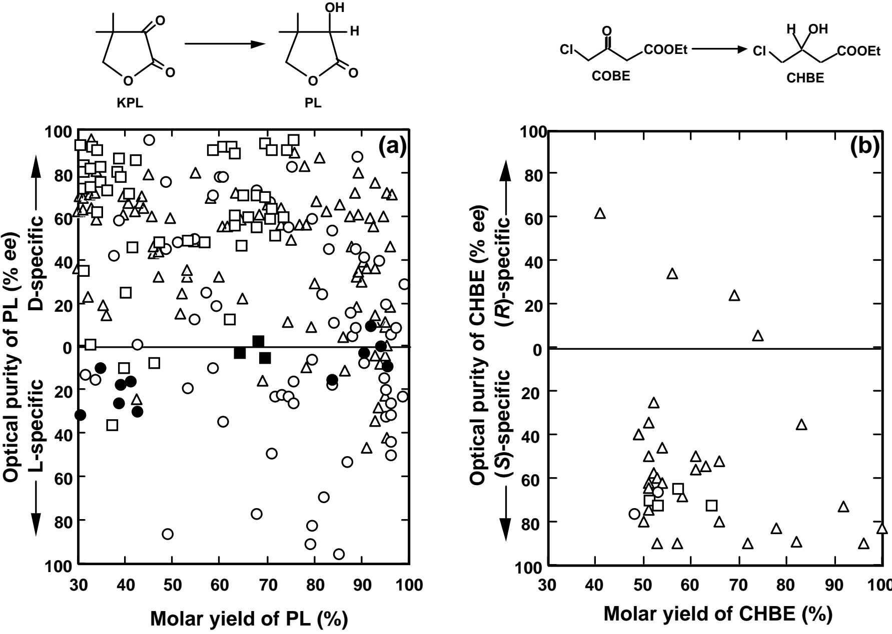

Figure 19.1. Diversity of microbial reduction of ketopantoyl lactone (a) and COBE (b). △, yeasts; ○, molds; □, bacteria; ■, actinomycetes; ●, basidiomycetes; PL, pantoyl lactone; KPL, ketopantoyl lactone.

F. oxysporum

Pantoyl lactone H_2O D-Pantoic acid + L-Pantoyl lactone

Figure 19.2. Enantioselective hydrolysis of D-panotyl lactone in a racemic mixture by *F. oxysporum*.

D-pantoyl lactone (Fig. 19.2). This type of hydrolysis reaction is catalyzed by many filamentous fungi such as *Fusarium oxysporum*. Since the reaction seemed to be a promising one for the enzymatic resolution of racemic pantoyl lactone, the enzyme responsible for this hydrolysis was characterized in some detail.

19.3.1. A Novel Lactonase from *Fusarium oxysporum*

An enzyme isolated from *F. oxysporum* catalyzes the hydrolysis of various aldonate and aromatic lactones (Table 19.1) (Shimizu *et al.*, 1992). The hydrolysis of aldonate lactones proceeds stereospecifically through recognition of

TABLE 19.1. Properties of microbial lactonases.

	Fusarium oxysporum	*Agrobacterium tumefaciens*	*Acinetobacter calcoaceticus*
Native M_r	125,000	62,000	55,000
Subunit M_r (SDS-PAGE)	63,000	26,500	30,000
Number of subunits	2	2	2
Substrate specificity [K_m (mM)/V_{max} (μmol/min/mg)]			
Hydrolysis			
D-Pantoyl lactone	123/653	no activity	no activity
L-Pantoyl lactone	no activity	3.59/13.7	no activity
D-Galactono-γ-lactone	23/1,300	no activity	no activity
L-Mannono-γ-lactone	3.6/1,440	no activity	no activity
3,4-Dihydrocoumarin	6.3/2,800	2,560/7.74 × 10^4	0.801/4,760
Homogentisic-acid lactone	2.5/257	4.35/1.83 × 10^4	0.560/0.963
2-Coumaranone	8.7/701	455/1.52 × 10^4	0.761/8.04
3-Isochromanone	4.4/6.88	1.43/2.37	no activity
ε-Caprolactone	ND	ND	no activity
Lactonization			
D-Pantoic acid	9.1/103	no activity	no activity
D-Galactonic acid	52.6/216	no activity	no activity
Optimum pH (hydrolysis)	7–7.5	8.5	7.0
Optimum temperature (hydrolysis)	50 °C	45 °C	30 °C
pH stability (30 °C, 30 min)	5.0–10	>5.0	>5.0
Thermal stability[a]	<50 °C (pH 7.4, 20 min)	<50 °C (pH 7.4, 20 min)	<75 °C (pH 7.0, 30 min)
Inhibitors	chelating reagents, L-pantoyl lactone, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}	chelating reagents, Fe^{2+}, Pb^{2+}, Sn^{2+}, Al^{3+}, Fe^{3+}	diisopropylphosphofluoridate, phenylmethanesulfonyl fluoride, Hg^{2+}, Fe^{3+}
References	Shimizu *et al.*, 1992	Kataoka *et al.*, 2000b	Kataoka *et al.*, 2000a

[a]Enzymes retained 100% activity under the conditions with the indicated pHs or temperatures.
ND, not determined.

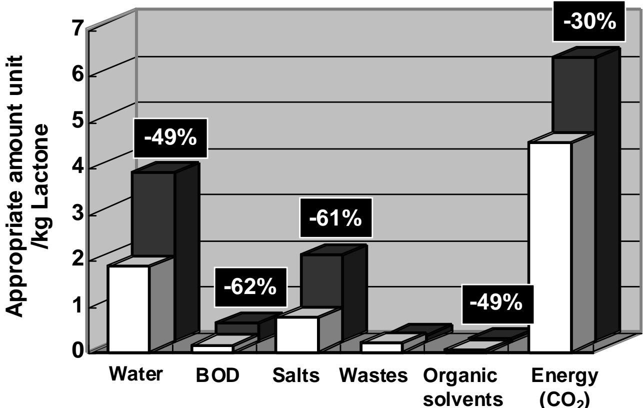

Figure 19.3. Comparison of environmental items between biocatalytic and chemical resolution. Dark bars, chemical; light bars, biocatalytic. The numbers are the reduction percentages for individual items.

the configuration at the 2-position carbon; only lactones carrying a downward hydroxy group, according to Haworth's projection, are hydrolyzed at this position. D-pantoyl lactone has similar structural properties to substrate aldonate lactones. When mycelia of *F. oxysporum* were incubated with 700 g/l of racemic pantoyl lactone, only the D-isomer was hydrolyzed, the L-isomer remaining intact (molar converion, 46%; optical purity, 96% ee) (Kataoka *et al.*, 1995a, 1995b). Thus, the practical use of this lactonase for the enzymatic resolution of pantoyl lactone was examined.

Fungal mycelia containing the enzyme were immobilized in a calcium alginate gel. When the gel was incubated in 300 g/l of racemic pantoyl lactone for 21 h at 30 °C with automatic pH control (pH 7.0), more than 40% of the substrate was hydrolyzed with high optical purity (90% ee). After 180 reaction cycles (*i.e.*, 180 days), the gel retained about 70% of its initial activity (Sakamoto *et al.*, 2005). The addition of Ca (90 mM) to the reaction mixture was essential for maintaining the physical strength of the calcium alginate gel. Since Ca is also necessary for both the enzyme activity and stability (Shimizu *et al.*, 1992), its addition enhanced not only the physical strength of the gel but also the stability of the enzyme, and consequently prolonged the life-time of the catalyst. This process has been used since 1999 for the commercial production of D-pantoyl lactone (ca 3,000 tons per year as calcium D-pantothenate) by Daiichi Fine Chemicals (Japan), and has been shown to be highly satisfactory, not only from an economic aspect, but also an environmental one (Fig. 19.3).

19.3.2. Microbial Lactonases from Other Microorganisms

Lactonase belongs to the esterase family, and can act on the intramolecular ester bonds of lactones. Like already-known esterases such as lipases,

lactonase has the potential for practical use thus the screening of novel lactonases from other microbial sources was performed (Bommarius and Polizzi, 2006; Kataoka *et al.*, 2007).

Lactonase from *Agrobacterium tumefaciens* shows opposite stereospecificity to *Fusarium* lactonase, that is, L-pantoyl lactone is specifically hydrolyzed by the *Agrobacterium* lactonase (Table 19.1) (Kataoka *et al.*, 2000b). In principle, the L-isomer-specific lactonase of *Agrobacterium* also can be applied to the enzymatic resolution of racemic pantoyl lactone (Kesseler *et al.*, 2002), as well as *Fusarium* lactonase. However, a D-isomer-specific lactonase is more advantageous than an L-isomer-specific one for practical purposes. With use of a D-isomer-specific lactonase, D-pantoic acid (easily converted to D-pantoyl lactone) of high optical purity could be constantly obtained regardless of the hydrolysis yield, while the use of an L-isomer-specific lactonase, the optical purity of the remaining D-isomer might be low, except when the L-isomer is hydrolyzed completely. Therefore, control of the optical purity of the product, D-pantoyl lactone, in practical production with a D-isomer-specific lactonase, is easier than that with an L-isomer-specific one.

One example of the diversity and practical potential of lactonases is 3,4-dihydrocoumarin hydrolase (DHase) from *Acinetobacter calcoaceticus* (Table 19.1) (Honda *et al.*, 2003; Kataoka *et al.*, 2000a). This enzyme was found on screening for aromatic lactone-hydrolyzing enzymes. Although the DHase is highly specific toward 3,4-dihydrocoumarin, several other aromatic lactones, such as 2-coumaranon and homogentisic acid lactone, and linear esters are also hydrolyzed. The specificity towards linear esters is very characteristic: *i)* the enzyme is specific toward methyl esters; *ii)* it recognizes the configuration at the 2-position; and *iii)* it hydrolyzes diesters to monoesters. Recombinant *E. coli* cells overexpressing this DHase gene were shown to be useful for the stereoselective hydrolysis of methyl β-acetylthioisobutyrate and regioselective hydrolysis of cetraxate methyl ester (Fig. 19.4) (Honda *et al.*, 2002).

19.4. BIOREDUCTION SYSTEM FOR LARGE-SCALE PRODUCTION OF CHIRAL ALCOHOLS

The microbial process for D-pantoyl lactone production has been industrialized by using the novel fungal lactonase described above. However, D-pantoyl lactone production through microbial asymmetric reduction of ketopantoyl lactone is not effective for industrial use; judging from the screening results for ketopantoyl lactone reduction, we found diversity of microorganisms as to stereospecificity and reducing ability. The asymmetric reduction of carbonyl compounds is still an attractive process for the production of chiral alcohols, since the theoretical yield of the chiral products is 100%. Thus, the development of an efficient bioreduction system for the production of other useful chiral alcohols has been performed (Kataoka *et al.*, 2003).

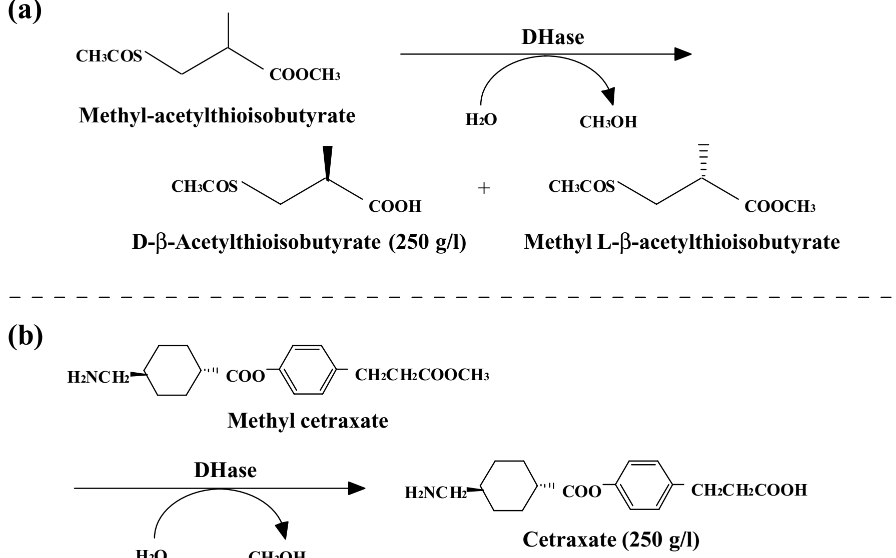

Figure 19.4. Enantioselective hydrolysis of methyl β-acetylthioisobutyrate (a) and regioselective hydrolysis of methyl cetraxate (b) catalyzed by DHase. The numbers in parentheses are the maximum yields of individual products in reactions with *E. coli* transformant cells overexpressing the DHase gene. Source: Honda *et al.*, 2002.

19.4.1. Bottlenecks for Bioreduction of Prochiral Carbonyl Compounds by Microbial Cells for Industrial Use: Production of (*R*)-4-Chloro-3-Hydroxybutanoate Ethyl Ester

(*R*)-4-Chloro-3-hydroxybutanoate ethyl ester (CHBE) is also an important chiral building block for the synthesis of pharmaceuticals or other materials. Screening for microorganisms catalyzing the reduction of the prochiral carbonyl compound 4-chloro-3-oxobutanoate ethyl ester (COBE) was performed as well as ketopantoyl lactone reduction (Fig. 19.1b). Only a few strains, such as *Sporobolomyces salmonicolor*, were found to produce the (*R*)-enantiomer predominantly (<60% ee) with high molar conversion (<70%). In contrast, many strains, such as *Candida magnoliae*, showing more than 50% conversion almost specifically yielded the (*S*)-enantiomer (80–95% ee) (Wada *et al.*, 1998). Stereospecific production of (*R*)-CHBE using whole cells of potent strains, such as *S. salmonicolor*, did not give sufficient molar or optical yield results.

What are the bottelnecks for bioreduction? The drawbacks of a bioreduction process involving whole cells of microoganisms can be summarized: *i)* Microbial strains possessing both carbonyl reductase activity and cofactor (NAD(P)H)-regenerating activity are necessary to obtain a high molar yield, because a stoichiometric amount of cofactor is required for substrate reduc-

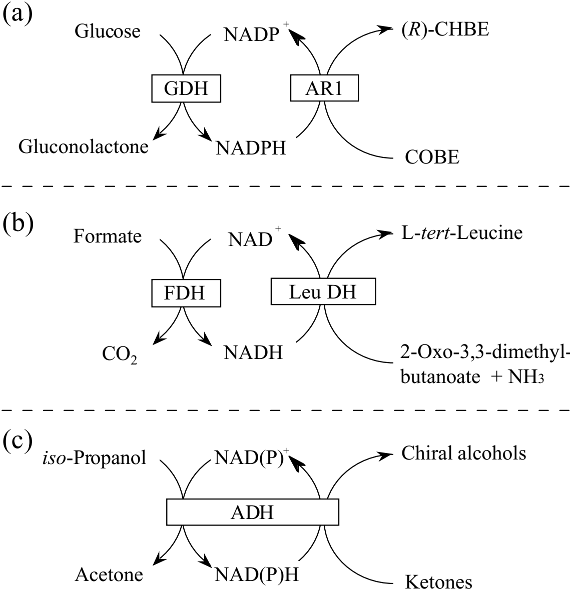

Figure 19.5. Cofactor regeneration systems for NAD(P)H-dependent enzyme reactions. The enzyme-coupled one involving GDH (a), that involving FDH (b), and the substrate-coupled one (c). AR1, aldehyde reductase from *S. salmonicolor*; Leu DH, leucine dehydrogenase; ADH, *sec*-alcohol dehydrogenase.

tion. And *ii)* microbial strains carrying only one potential carbonyl reductase showing high stereospecificity, or multiple carbonyl reductases producing only single enantiomer, are also necessary to obtain a high optical yield.

To satisfy these requirements, isolated, immobilized or commercially available enzymes have been used. For example, a crude extract of *S. salmonicolor*, which produces (*R*)-CHBE predominantly, was heated, precipitated with acetone, and used as a catalyst together with commercially available glucose dehydrogenase (GDH) as a cofactor regenerator (Fig. 19.5a), 33.1 g/l of (*R*)-CHBE being produced from COBE (molar yield, 74%; optical purity, 85% ee) (Shimizu *et al.*, 1990b). Furthermore, by using an *n*-butyl acetate-water two phase system, in which substrate and product inhibition, and inactivation of the enzymes were avoided, 77 g/l of (*R*)-CHBE (86% ee) was produced from COBE with a molar yield of 95.4% (Shimizu *et al.*, 1990a).

Enzymatic synthesis of L-*tert*-leucine is another example of the use of isolated enzymes (Bommarius *et al.*, 1995). An NADH-dependent leucine dehydrogenase was used as a catalyst for the reductive amination of the corresponding keto acid together with formate dehydrogenase (FDH) and formate as a cofactor regenerator (Fig. 19.5b; Shaked and Whitesides, 1980; Wichmann *et al.*, 1981). Furthermore, a unique membrane reactor system involving FDH and PEG-modified-NAD for continuous NADH regeneration

has been developed (Kula and Wandrey, 1987; Wückmann *et al.*, 1981), and applied to L-*tert*-leucine production (Wandrey 2004).

In addition to the enzyme-coupled cofactor regeneration system involving two oxidoreductases, *i.e.,* an enzyme converting the substrate to the product (*e.g.*, carbonyl reductase and leucine dehydrogenase) and one regenerating cofactors (*e.g.*, GDH and FDH), a substrate-coupled one—in which a single oxidoreductase performs both reduction of the substrate and regeneration of the cofactors—has also been developed (Buchholz and Gröger, 2006; Kroutil *et al.*, 2004). *sec*-Alcohol dehydrogenases are typical examples of a substrate-coupled cofactor regeneration system. An enzyme catalyzes the asymmetric reduction of carbonyl compounds together with the self-regeneration of NAD(P)H *via* enzymatic dehydrogenation of *iso*-propanol (Fig. 19.5c). Alcohol dehydrogenases from *Lactobacillus brevis* (Wolberg *et al.*, 2000), *Pseudomonas fluorescens* (Hildebrandt *et al.*, 2001), *Leifsonia* sp. (Inoue *et al.*, 2005), *Rhodococcus ruber* (Stampfer *et al.*, 2002), *etc.* have been applied to the asymmetric reduction of chiral alcohols, using a substrate-coupled cofactor regeneration system involving *iso*-propanol.

19.4.2. Development of a Novel Bioreduction System

A more efficient and simple bioreduction system, which avoids the problems with the system involving naturally-occurring microorganisms or cell-free systems described above, has been required. A novel bioreduction system, in which *Esherichia coli* transformant cells coexpressing the genes of an NAD(P)H-dependent carbonyl reductase and GDH, as a cofactor regenerator, were used as the catalyst, has been constructed (Kataoka *et al.*, 2003). The production of chiral CHBEs is a typical successful example with this bioreduction system.

An aldehyde reductase (AR1) of *Sporobolomyces salmonicolor* (Kataoka *et al.*, 1992b; Kita *et al.*, 1996; Yamada *et al.*, 1990) and a carbonyl reductase (S1) of *Candida magnoliae* (Wada *et al.*, 1998, 1999a; Yasohara *et al.*, 2000) were found to catalyze NADPH-dependent stereospecific reduction of COBE to (*R*)- and (*S*)-CHBE, respectively. These yeast enzymes have been successfully applied to the *E. coli* coexpression system for practical production (Kataoka *et al.*, 1999; Kizaki *et al.*, 2001). (*R*)- or (*S*)-CHBE (300–350 g/l) of high optical purity (92–100% ee) was stoichiometrically obtained on incubation of the transformant cells in a reaction mixture containing COBE, glucose and a catalytic amount of $NADP^+$. Very high turnover numbers of $NADP^+$ (13,500 and 35,000 mol CHBE/mol, respectively) have been reported. Based on these results, the Kaneka Corporation (Japan) started commercial production of (*S*)-CHBE in 2001.

This bioreduction system is applicable to the production of many other useful chiral alcohols by replacing the carbonyl reductase gene with that of another appropriate enzyme for carbonyl reduction (Fig. 19.6). A good library of microbial carbonyl reductases with different substrate and stereospecifici-

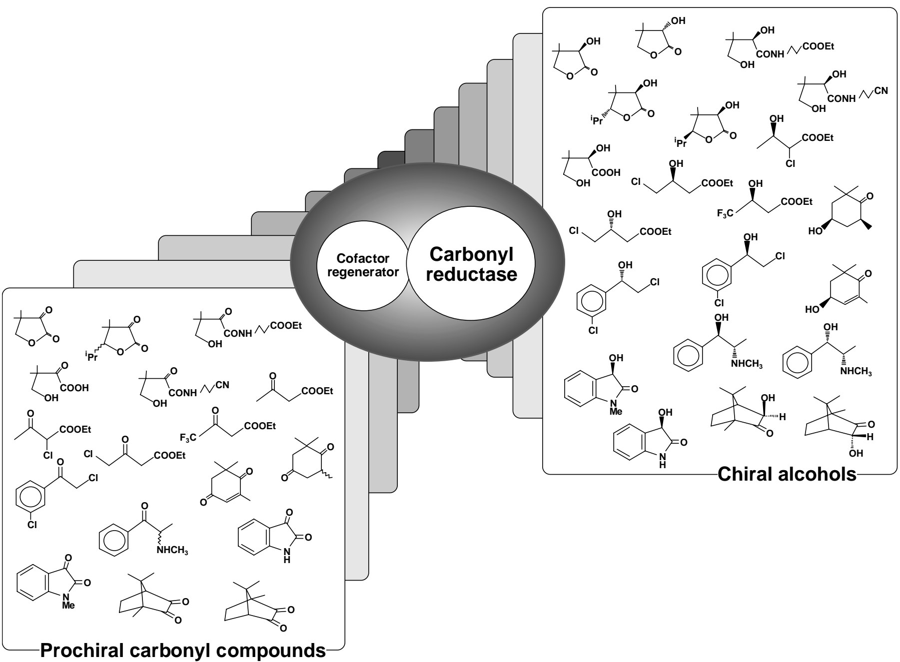

Figure 19.6. Multi-purpose bioreduction system, involving recombinant microorganisms coexpressing carbonyl reductase and cofactor regenerator genes.

ties has been made (Kataoka *et al.*, 2003). There is still the possibility, by more extensive screening, of finding novel carbonyl reductases possessing functions different from those of known enzymes.

Whole cell reactions involving and *E. coli* transformant overexpressing appropriate enzyme gene(s) have also been use for cofactor regeneration systems other than the enzyme-coupled one involving GDH, *i.e.,* that involving FDH and the substrate-coupled one involving *sec*-alcohol dehydrogenase (Buchholz and Gröger, 2006, Gröger *et al.*, 2006; Honda *et al.*, 2006; Kroutil *et al.*, 2004).

19.4.3. Application of the Bioreduction System to Other Cofactor-Dependent Enzyme Reactions: Discovery of New Functions of Old Yellow Enzymes

A bioreduction system might be applied to many NAD(P)H-dependent enzyme reactions other than carbonyl reduction. Recently, two novel old yellow enzymes (OYEs) catalyzing the asymmetric hydrogenation of C=C bonds were found and applied to a bioreduction system for the production of double chiral compounds.

Microorganisms were screened for the reduction of ketoisophorone (KIP; 3,5,5-trimethyl-2-cyclohexene-1,4-dione), and *Candida macedoniensis* was found to produce (6*R*)-levodione (2,2,6-trimethylcyclohexane-1,4-dione) through asymmetric hydrogenation of the C=C bond of KIP (Kataoka *et al.*, 2002). Enzymological and genetic analyses of the enzyme involved in this reaction identified it as an OYE family protein (Kataoka *et al.,* 2002, 2004). OYE (NADPH dehydrogenase; EC 1.6.99.1) is the first-reported flavoprotein found in a brewer's bottom yeast by Warburg and Christian (1932, 1933), and catalyzes the hydrogenation of the C=C bonds of α,β-unsaturated carbonyl compounds such as 2-cyclohexen-1-one (Vaz *et al.*, 1995).

The asymmetric reduction of KIP to (6*R*)-levodione was successfully performed with *E. coli* transformant cells, in which both the *Candida* OYE (CYE) and GDH genes were coexpressed. The (6*R*)-levodione formed amounted to 96.6 g/l (627 mM), the molar yield being 95.4% (Kataoka *et al.*, 2004). The resultant (6*R*)-levodione was stoichiometrically and stereospecifically converted, by using *E. coli* transformant cells coexpressing the levodione reductase (LVR) gene from *Corynebacterium aquaticum* (Wada *et al.*, 1999b; Yoshizumi *et al.*, 2001) and the GDH gene, to the double chiral compound (4*R*,6*R*)-actinol (4-hydroxy-2,2,6-trimethylcyclohexanone) for the commercial production of zeaxanthin and xanthoxin (Leuenberger *et al.*, 1976).

The simultaneous sequential conversion of KIP to (4*R*,6*R*)-actinol *via* (6*R*)-levodione with both OYEs (including CYE) and LVR has been performed (Wada *et al.*, 2003). However, the main product of the reaction was (4*S*)-phorenol (4-hydroxy-2,6,6-trimethyl-2-cyclohexanone), suggesting that the OYEs can catalyze the asymmetric hydrogenation of only KIP, while LVR can catalyze the reduction of both (6*R*)-levodione and KIP (Fig. 19.7a). Thus, (4*R*,6*R*)-actinol production with OYEs and LVR must be performed through two separate reactions.

Microorganisms were screened again as to the reduction of (4*S*)-phorenol, and *Torulopsis* sp. was found to produce (4*R*,6*R*)-actinol through asymmetric hydrogenation of (4*S*)-phorenol. The enzyme involved in this reaction was also identified as an OYE family protein, but *Torulopsis* OYE (TYE) catalyzes the hydrogenation of both KIP and (4*S*)-phorenol (Fig. 19.7b). Using cells of a single *E. coli* transformant coexpressing the TYE, LVR and GDH genes as the catalyst, 10 g/l of KIP was almost stoichiometrically and stereospecifically converted to (4*R*,6*R*)-actinol through simultaneous sequential conversion (unpublished data).

OYEs are powerful and excellent biocatalysts for the reduction of the C=C bonds, especially asymmetric ones, of α,β-unsaturated compounds (Bommarius and Polizzi, 2006; Chaparro-Riggers *et al.*, 2007; Hall *et al.*, 2006, 2007, 2008a, 2008b; Müller *et al.,* 2006, 2007a, 2008b; Stuermer *et al.*, 2007; Swiderska and Stewart, 2006a, 2006b; Williams and Bruce, 2002). A mechanism for OYE reactions producing chiral products was also proposed based on X-ray crystallographic and reaction experimental data (Kohli and Massey, 1998; Swiderska and Stewart, 2006a, 2006b; Vaz *et al.*, 1995).

(a)

(b)

Figure 19.7. (4*R*,6*R*)-Actinol production from KIP with the combination of CYE and LVR (a), and that of TYE and LVR (b).

19.5. CONCLUSIONS

Our first research target was the microbial asymmetric reduction of ketopantoyl lactone for the stereospecific production of D-pantoyl lactone. Contrary to our expectation, the industrial production of D-pantoyl lactone had become possible through the discovery of a novel microbial lactonase. For the microbial asymmetric reduction of prochiral carbonyl compounds, extensive screening provided information on the microbial diversity of reducing ability and stereospecificity, and consequently disclosed a novel bioreduction system constructed by means of gene technology, and applicable to industrial use. Furthermore, through extension of the bioreduction system for NAD(P)H-dependent reactions other than carbonyl reduction, a novel function, the asymmetric C=C bonds hydrogenation of microbial OYEs was also discovered.

Most scientists first assume a story line of experimental results, and then carry out experiments expecting the results will support the assumed plot. As shown in this chapter, however, they often obtain results different from those first assumed ones. Nature exhibits much more diversity than we suppose, and gives us results we did not expect. In that case, you had better readily accept

the results as they are, and expect further new developments due to those unexpected results. If you are unable to ignore the first assumption, new development of research might be missed. This might be true for not only the screening of microorganisms but also all experimental work and research.

19.6. ACKNOWLEDGMENTS

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CHAPTER 20

Hydrogenation Technologies for the Production of High Quantity of Biobeneficiary Conjugated Fatty Acids

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20.1. INTRODUCTION

Conjugated linoleic acids are a group of natural linoleic acids with a conjugated double bond. It has been reported that conjugated linoleic acids can prevent or cure cancer (Ha *et al.*, 1987; Liew *et al.*, 1995; Ip *et al.*, 1996), atherosclerosis (Lee *et al.*, 1994) and Type II diabetes (Houseknecht *et al.*, 1998). Conjugated linoleic acids may regulate cytokines production to strengthen muscle and bone activity in humans (Cook *et al.*, 1993). The fat partitioning activity of conjugated linoleic acids reduces fat syntheses in mice, and abdominal circumferences in humans (Park *et al.*, 1999; Rieserus *et al.*, 2001). Conju-

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gated linoleic acids are abundant in dairy products and meats from ruminant animals (Ha *et al.*, 1989; Chin *et al.*, 1992; Shantha *et al.*, 1992; Kim and Liu, 2002; Boylstone and Beitz, 2002). Ha *et al.* (1989) reported high levels of conjugated linoleic acids in cheeses and milk. Chin *et al.* (1992) reported that milk, butter, cheese, yogurt or meats from ruminants have 3–8 mg conjugated linoleic acids/g fat. Vegetable oils contained very low levels of CLA, ranging from 0.1 mg conjugated linoleic acids/g coconut oil to 0.7 mg conjugated linoleic acids/g safflower oil (Chin *et al.*, 1992). Banni *et al.* (1994) carried out a series of HPLC/UV/MS analyses to characterize the fatty acids with conjugated dienes in a partially hydrogenated oil (a mixture of partially hydrogenated soybean oil and palm oil)and concluded that it contained a small quantity of conjugated linoleic acid isomers (4.24 mg total CLA/g oil). In our laboratories, however, we found that a large quantity of conjugated linoleic acids could be formed during hydrogenation of vegetable oils, and their formation was greatly affected by the reaction conditions. The review for the hydrogenation for low trans and high conjugated linoleic fatty acid is also available in the literature (Jang *et al.*, 2005). In this paper we review CLA isomeric identification and distribution, and the processing effects on their formation in vegetable oils during hydrogenations.

20.2. CLA FORMATION DURING SELECTIVE AND NONSELECTIVE TYPE HYDROGENATIONS

In our laboratories, spectroscopic scanning at near 233 nm was carried out to check conjugated diene formation during hydrogenation and it was found that the absorbance near 233 nm of the samples increased sharply during hydrogenation (Fig. 20.1). The results suggested that the hydrogenation greatly induced the conjugated diene formation in vegetable oils. Further ^{1}H NMR analysis also showed the clear evidence for the formation of conjugated dines in soybean oil during hydrogenation (Figs. 20.2 and 20.3). Based on these findings, Jung and Ha (1999) studied CLA formation during selective and non-selective hydrogenation processes. The authors identified the individual CLA by the combined results from GC-MS, equivalent chain length (ECL), GC retention time, and GC peak elution order comparison with previously reported works (Ha *et al.*, 1989; Lavillonniere *et al.*, 1998). The authors used the polyethylene glycol type capillary column (Supelcowax 10, 60 m × 0.25 mm, 0.25 um film thickness, Supelco Inc., Bellefonte, PA) for the gas chromatographic analysis. Jung and Ha (1999) concluded that a large quantity of CLA could be formed during hydrogenation. The total CLA contents in vegetable oil sharply increased during the initial stage of hydrogenation. After reaching maximum level, the CLA content in the vegetable decreased. The authors reported that selective hydrogenation was much more favorable for the formation of CLA than non-selective (Jung and Ha, 1999). The maximum level of conjugated linoleic acid in hydrogenated soybean oil obtained from the hydrogenation at

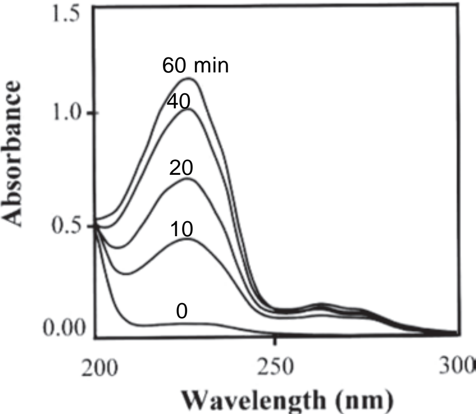

Figure 20.1. Partial UV spectrum of soybean oil and hydrogenated soybean oil obtained during 10, 20,40, 60 minutes of selective hydrogenation under the conditions (230 °C, 300 rpm, 0.5 kg/cm^2).

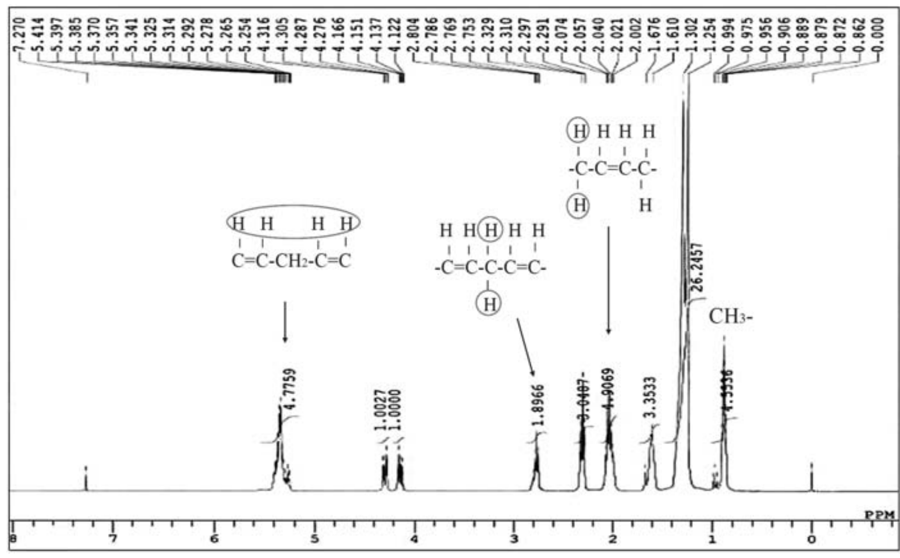

Figure 20.2. ^{1}H nuclear magnetic resonance (NMR) spectrum of soybean oil.

the temperature of 215 °C and hydrogen pressure of 0.5 kg/cm^2 was 98 mg/g oil, which was one of the highest ever reported in foods (Jung and Ha, 1999). However, the authors reported that maximum contents of conjugated linoleic acids in hydrogenated soybean oil obtained by conventional hydrogenation with nonselective nickel catalyst at the temperature of 215 °C and hydrogen pressure of 2.5 kg/cm^2 was 9.06 mg/g oil.

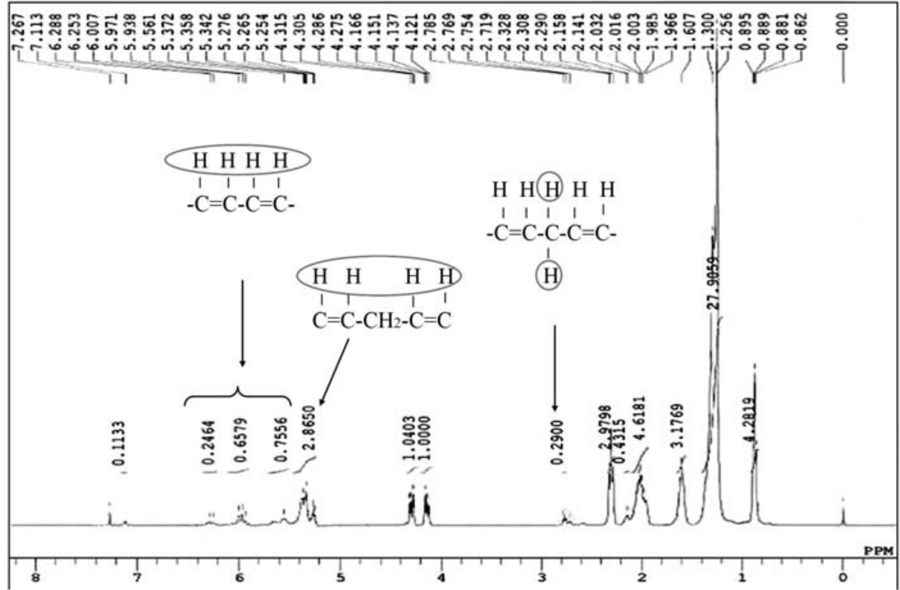

Figure 20.3. ^{1}H nuclear magnetic resonance (NMR) spectrum of selectively hydrogenated soybean oil obtained after 60 min selective hydrogenation.

20.3. IDENTIFICATION OF CLA ISOMERS IN HYDROGENATED VEGETABLE OILS

Jung and Jung (2002) separated conjugated linoleic acids by a silver-ion (Ag^+) impregnated high pressure liquid chromatography (HPLC) after the methylation of fatty acids in hydrogenated soybean oil (Fig. 20.4). In the silver ion impregnated HPLC chromatogram, the CLA isomers of hydrogenated soybean oil were separated into three groups (*trans-trans; cis-trans; cis-cis*). The authors collected the individual isomeric peaks by the repeated running of the HPLC. Then the isolated CLA isomers were converted into their 4,4-dimethyloxazoline (DMOX) derivatives. The authors carried out the gas chromatography-mass spectrometry of CLA isomer DMOX derivatives for the identifications of their chemical structures. By interpreting the mass spectra of the DMOX derivatives of conjugated linoleic acid isomers isolated by silver ion-impregnated HPLC, 20 different CLA isomers present in hydrogenated soybean oil were identified. Figure 20.5 shows the GC-MS spectrum for *c*9 *t*11 CLA isomer obtained from hydrogenated soybean oil. The methylesters of individual CLA isomers collected from Ag^+-HPLC were also injected into the gas chromatograph equipped with a long highly polar capillary column (cyanopropyl type, Suppelco 9908, 100 m × 0.22 mm, 0.20 um thickness, Supelco Inc.) for the identification of the CLA isomer peaks shown in the gas chromato-

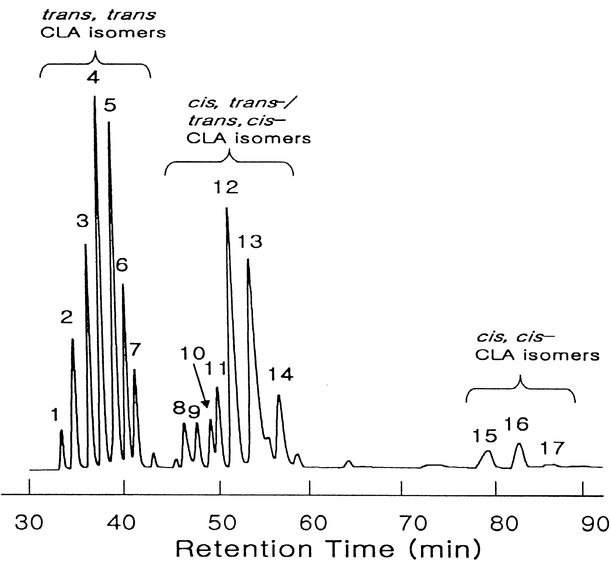

Figure 20.4. Silver-ion impregnated HPLC chromatogram of methylated conjugated fatty acids of hydrogenated soybean oil. *trans* 13, *trans* 15-(peak 1), *trans* 12, *trans* 14-(peak 2), *trans* 11, *trans* 13-(peak 3), *trans* 10, *trans* 12-(peak 4), *trans* 9, *trans* 11-(peak 5), *trans* 8, *trans* 10-(peak 6), *trans* 7, *trans* 9-(peak 7)/*trans* 12, *cis* 14-(peak 8); *cis* 12, *trans* 14-(peak 9); *trans* 11, *cis* 13-(peak 10) *cis* 11, *trans* 13-(peak 11); *cis* 10, *trans* 12-/*trans* 10, *cis* 12-(peak 12); *cis* 9, *trans*11-/*trans* 9, *cis* 11-(peak 13); *trans* 8, *cis* 10-/*cis* 8, *trans* 10-(peak 14), *cis* 10, *cis*12-(peak 15); *cis* 9, *cis* 11-(peak 16), *cis* 8, *cis*10-(peak 17). Source: Jung and Jung, 2002.

gram. The gas chromatogram and identification of methyl esters of conjugated linoleic acids in hydrogenated soybean oil are shown in Figure 20.6.

20.4. EFFECTS OF HYDROGENATION CONDITIONS ON THE CLA FORMATION

Reaction conditions greatly affect the types of fatty acids produced in vegetable oils after hydrogenation reaction. Jung *et al.* (2001) studied the effects of temperature and agitation rate on the CLA formation in soybean oil during hydrogen process with selective Ni catalyst. The authors reported that reaction temperature and agitation rate greatly affected the quantities of total CLA and the time required to reach the maximum quantity of CLA in soybean oil during hydrogenation. The authors reported that as the hydrogenation temperature increased, the maximum quantity of CLA in soybean oil increased, but the time to reach the maximum CLA content decreased. By increasing the hydrogenation temperature from 170 to 210 °C, the maximum quantity of CLA obtained was about 2.6 times higher (Fig. 20.7). As the agitation rate decreased,

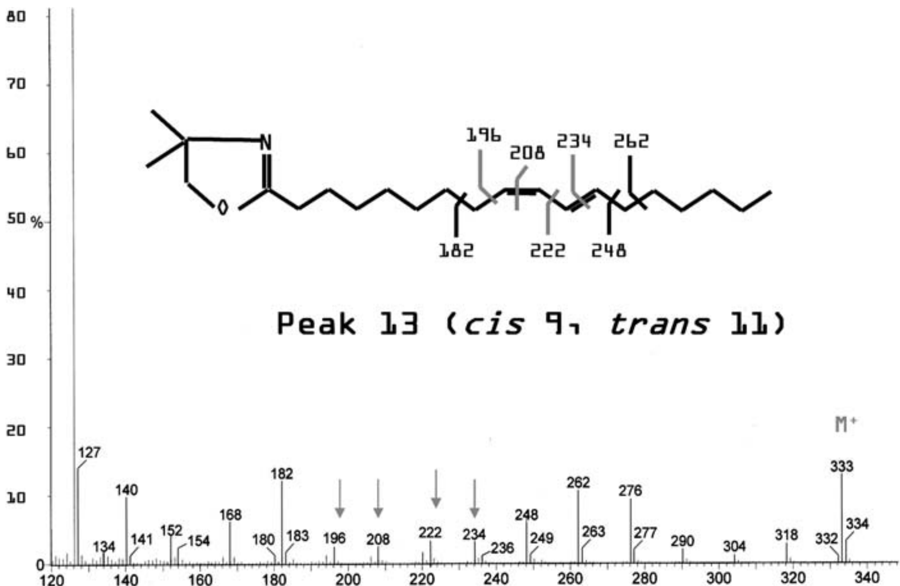

Figure 20.5. GC-MS spectrum for cis 9 trans 11-conjugated linoleic acid obtained from hydrogenated soybean oil.

the CLA formation in soybean oil increased, as did the time to reach the maximum CLA content: The maximum CLA contents in soybean oil obtained from hydrogenation at 210 °C with agitation rates of 300, 500, and 700 rpm were 162.8, 108.6 and 66.5 mg total CLA/g oil, respectively (Fig. 20.8). The results showed that by decreasing the agitation rate from 700 to 300 rpm, the quantity of CLA obtained was 2.5 times higher. The authors further reported that the CLA isomeric distribution in the hydrogenated soybean oil was also affected by the reaction temperature and agitation rate during hydrogenation. Jung and others (2002) also studied the effects of catalyst types, catalyst amount, hydrogen pressure, and different oils on the quality and quantity of CLA of hydrogenated soybean oil. They reported that selective type nickel catalyst increased CLA in soybean oil much more efficiently than non-selective ones. The catalyst amount greatly affected the formation of CLA in soybean oil during hydrogenation. As the catalyst content increased, so did the conjugated linoleic acids content of hydrogenated soybean oil. By increasing the amount of catalyst from 0.05 to 0.3%, the quantity of CLA obtained was about 1.9 times higher (Fig. 20.9). Hydrogen pressure also influenced the maximum quantity of CLA formed during hydrogenation. As the hydrogen pressure increased, the conjugated linoleic acids content in soybean oil decreased. By decreasing hydrogen pressure from 2.5 to 0.25 kg/cm^2, the quantity of CLA obtained was about 1.3 times higher (Fig. 20.10). Jung *et al.* (2002) concluded that the gas-liquid mass transfer limitation conditions of low hydro-

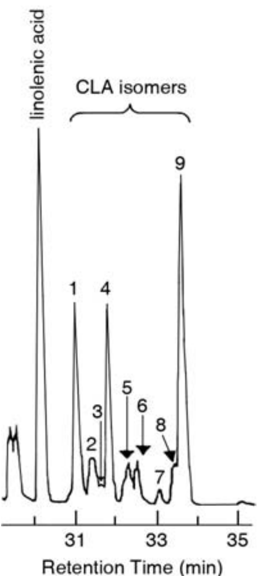

Figure 20.6. Gas chromatogram of fatty acid methyl esters of hydrogenated soybean oil. *trans* 7, *cis* 9-/*cis* 9, *trans* 11-/*trans* 8, *cis* 10-(peak 1); *cis* 10, *trans* 12-(peak 2); *trans* 9, *cis* 11-/*cis* 11, *trans* 13-(peak 3); *cis* 12, *trans* 14-/*trans* 10, *cis* 12-(peak 4); *trans* 11, *cis*13-/*cis* 9, *cis* 11-(peak 5); *trans* 12, *cis* 14-/*cis* 10, *cis* 12/*cis* 11, *cis*13-(peak 6); *trans* 12, *trans* 14-(peak 7); *trans* 11, *trans*13-(peak 8); and *trans* 10, *trans*12-/*trans* 9, *trans* 11-/*trans* 8, *trans*10-/*trans* 7, *trans* 9-conjugated linoleic acids (peak 9).

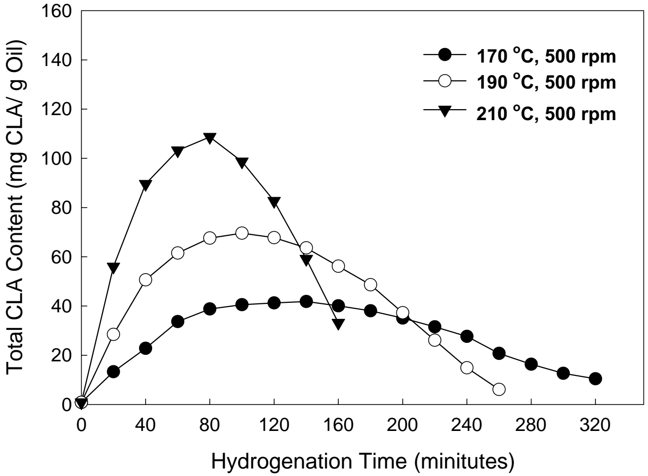

Figure 20.7. Effects of hydrogenation temperatures on the conjugated linoleic acids of soybean oil with selective type nickel catalyst, hydrogen pressure 0.5 kg/cm^2, and agitation rate 500 rpm. Source: Jung *et al.*, 2001.

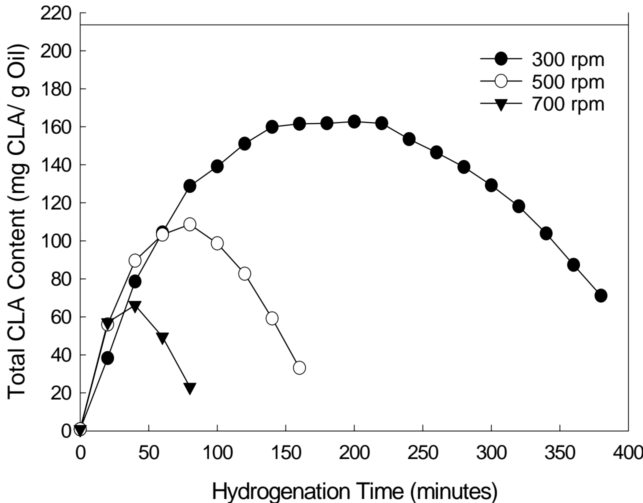

Figure 20.8. Effects of stirring rate on the conjugated linoleic acids of soybean oil with selective type nickel catalyst, hydrogen pressure 0.5 kg/cm^2, and temperature 210 °C. Source: Adopted from Jung *et al.*, 2001.

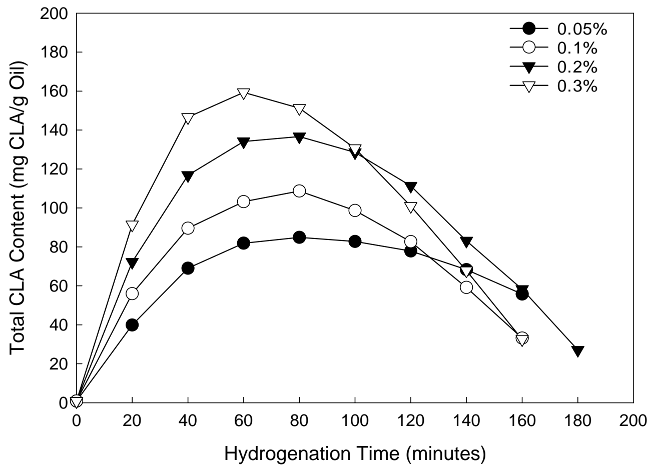

Figure 20.9. Effects of catalyst amount on the conjugated linoleic acids of soybean oil with selective type nickel catalyst, hydrogen pressure 0.5 kg/cm^2, and agitation rate 500 rpm. Source: Jung *et al.*, 2002.

gen pressure, low stirring rate, high catalyst content, and high reaction temperature favored the production of conjugated linoleic acids in vegetable oils during hydrogenation. They also reported that the maximal quantity of CLA and the distribution of their isomers were not significantly different with the

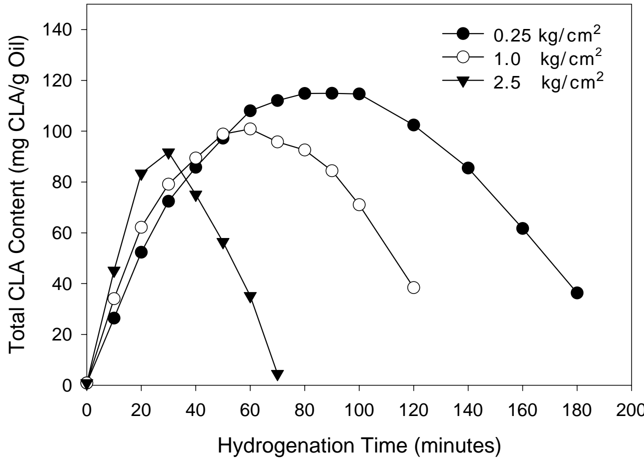

Figure 20.10. Effects of hydrogen pressure on the conjugated linoleic acids of soybean oil with 0.1% selective type nickel catalyst, temperature 210 °C, and agitation rate 500 rpm. Source: Jung *et al.*, 2002.

different oils (corn oil, cottonseed oil, soybean oil) tested when these vegetable oils were hydrogenated under the same reaction conditions. The authors reported, however, that the time to reach the maximal quantity of CLA was different with different oils:. fastest in soybean oil, followed by cottonseed oil and corn oil. The contents of conjugated linoleic acids in hydrogenated corn oil, cottonseed oil, or soybean oil under the tested hydrogenation condition reached to as high as 250 mg/g oils. These oils also contained relatively high quantity (about 8–10%) of trans $C_{18:1}$ isomers, which are associated with an increased risk of cardiovascular disease. But Jung and others (2002) reported that mild hydrogenation (10 minutes) with selective type nickel catalyst at low hydrogen pressure (0.25 kg/cm^2) and high temperature (230 °C) produced oils containing high amount of CLA (48.16 mg CLA/g oil), which was about eight times greater than levels in major dietary sources for CLA such as dairy products and ruminant meat. The authors reported that obtained oil contained only 3.5% trans fatty acids. Choi *et al.* (2004) selectively hydrogenated to obtain the hydrogenated soybean oil containing 21% CLA, and studied the effects of the hydrogenated soybean oil with high CLA on body composition, adipose tissue, and organ weights, and plasma lipid profiles in Male Sparague Dawley rats during 6 week feeding experiments with a purified diet containing 0–5% the hydrogenated soybean oil. The authors reported that the hydrogenated soybean oil supplementation did not significantly affect the growth performance. The weights of inguinal, epididymal, and retropeitoneal, but not mesenteric, adipose tissue were significantly influenced by the dietary hydrogenated soybean oil supplementation. Plasma triglycerides and total cholesterol decreased significantly with the supplementation of dietary hydrogenated

soybean oil with high CLA. The authors reported that 5% dietary supplementation of the hydrogenated soybean oil with high CLA induced the reductions of 23.3% body fat, 7.1% plasma triglycerides, and 44.3% total cholesterol. The results suggested that hydrogenated vegetable oils containing high CLA would be a useful fat source with body-fat reducing and anti-atherosclerosis properties.

20.5. EFFECTS OF SULFUR ADDITION ON THE CLA FORMATION DURING HYDROGENATION

Selective type nickel catalyst produced a much higher quantity of CLA than non-selective in hydrogenated vegetable oils (Jung and Ha, 1999; Jung *et al.*, 2002). Ju and Jung (2003) hypothesized that the poisoning effect of sulfur in the selective Ni catalysis might be responsible for the promotion of CLA formation during hydrogenation. The qualitative and quantitative effects of sulfur addition on the formation of conjugated linoleic acids in vegetable oils during hydrogenation with nonselective type Ni catalyst showed that sulfur addition greatly increased the conjugated linoleic acids of hydrogenated soybean oil. As the amount of sulfur increased to a certain level, the conjugated linoleic acids in soybean oil increased greatly during hydrogenation. However, the higher sulfur above the optimum level decreased the conjugated linoleic acids formation. Ju and Jung (2003) reported that during hydrogenation with 0.15% nickel, the maximum contents of CLA formed with 0, 30, 60, 90, 120 and 150 ppm sulfur additions were 38.4, 129.4, 177.6, 196.7, 188.4 and 165.3 mg of CLA/g oil, respectively (Fig. 20.11). The optimal sulfur level for the promotion of conjugated linoleic acids formation changed with the amount of nickel used. However, interestingly enough, the highest activity for the production of conjugated linoleic acid was shown at the optimal ratio of sulfur to nickel, 0.06 : 1, regardless of the nickel amount used. At the optimal ratio of sulfur to nickel, the higher the nickel content was, the higher the conjugated linoleic acids content in the hydrogenated soybean oil was. The increase of nickel from 0.05 to 0.15% at the optimal sulfur to nickel ratio increased the conjugated linoleic acids content by 50%. The conjugated linoleic acids compositions were greatly affected by both sulfur addition and amounts of nickel.

20.6. CATALYTIC TRANSFER HYDROGENATION WITH ALCOHOLS FOR THE CLA FORMATION

Ju *et al.* (2003) studied the effects of catalytic transfer hydrogenation (with alcohol as a hydrogen source) on the formation of conjugated linoleic acids. Classical hydrogenation techniques use molecular hydrogen gas, but the catalytic transfer hydrogenation uses alcohols or organic acids as donors of atomic hydrogen to the substrate (such as double bonds in triglycerides) (Mondal and

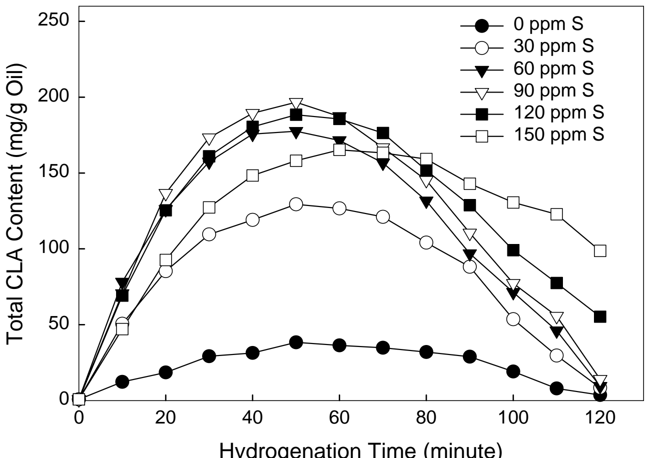

Figure 20.11. Effects of different level of sulfur addition on the quantity of total CLA formed in soybean oil during hydrogenation with 0.15% nickel catalyst. The hydrogenation was performed under the condition of hydrogen pressure 0.5 kg/cm^2, reaction temperature 220 °C, and agitation rate 500 rpm. Source: Ju and Jung, 2003.

Lalvani, 2000; Naglic *et al.*, 1998; Basu and Chakrabarty, 1966; Chakrabarty *et al.*, 1972; Arkad *et al.*, 1987; Tagawa *et al.*, 1978). One of the major advantages of catalytic transfer hydrogenation is to use safe hydrogen donors as an alternative for dangerous gaseous hydrogen. The generalized Equation 20.1 represents this process.

$$DH + A \xrightarrow{\text{catalyst}} D + AH \qquad \text{[Eq. 20.1]}$$

where D, H and A represent hydrogen donor, hydrogen, and hydrogen acceptor, respectively.

Ju *et al.* (2003) studied methanol, ethanol, propanol, and butanol as a hydrogen donors, monitoring their activity on the production of CLA during the catalytic transfer hydrogenation process. The authors found that butanol and propanol showed the highest activity for the formation of CLA, followed by ethanol, and methanol. The alcohol content added to the reaction system greatly affected the quantity of CLA: as it increased from 0 to 2.5%, CLA formation increased greatly. However, the CLA formation decreased above the 2.5% ethanol. The authors reported that the CLA contents in the hydrogenated soybean oil with 0, 0.5, 1.0, 2.5, 5.0, 10.0, and 15.0% ethanol for 150 min were 0.8, 69.6, 91.2, 120.4, 108.7, 87.3, and 61.2 mg/g oil (Fig. 20.12). Hydrogenation time and alcohol content also greatly influenced the isomeric distribution of conjugated linoleic acids in soybean oils. The higher the alcohol contents and the longer the hydrogenation time induced the greater distribution of *trans trans* CLA isomers in the oil. The compositions of the CLA isomers

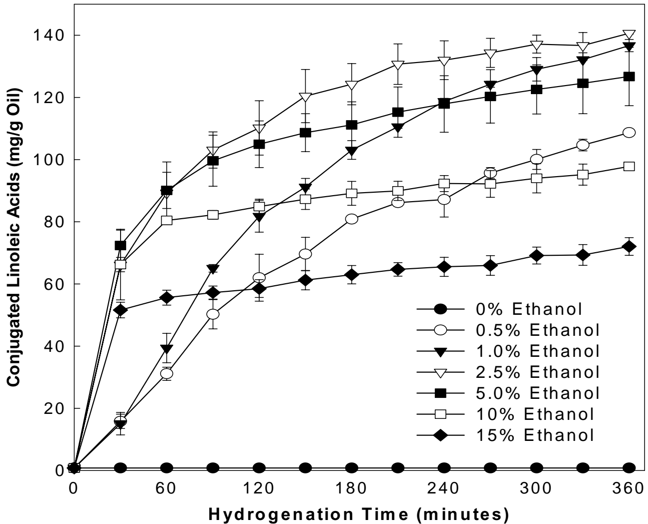

Figure 20.12. Effects of alcohol amounts on the quantity of total CLA formed in soybean oil during hydrogenation process. The hydrogenation was performed with 0.1% catalyst and different amount (0–15%) of ethanol under 210 °C of reactor temperature and 500 rpm of agitation rate. Source: Ju *et al.*, 2003.

(7t,9c-/9c,11t-/8t,10c-/12c,14t-/10t,12c-CLA isomers; peaks 1 and 4 in GC chromatogram) in soybean oil obtained after 60 min and 360 min hydrogenation with 0.5% ethanol were 85.1% and 47.8%, respectively. The percentage of *trans, trans* CLA isomers (peak 8 + peak 9) in soybean oils obtained after 360 min hydrogenation with 0.5, 1.0, 2.5, 5, 10 and 15% ethanol were 24.8, 33.74, 43.93, 46.6, 47.7, and 47.8%, respectively. The favorable reaction times and conditions for formation of each isomer were different. For example, the contents of *cis* 9, *trans* 11-CLA and *trans* 10, *cis* 12-CLA were highest in the hydrogenated soybean oils obtained after 360 min and 210 min hydrogenations with 1.0% ethanol, respectively. The results clearly suggested that the CLA isomer profiles in soybean oil could be manipulated by controlling the alcohol amount and hydrogenation time. Ju *et al.* (2003) claimed that catalytic transfer hydrogenation with alcohol has a distinct advantage over classical hydrogenation with molecular hydrogen gas in producing hydrogenated soybean oil with low trans fatty acid but still high conjugated linoleic acid.

20.7. CONCLUSIONS

Health concerns about trans fatty acids formed by hydrogenation have lead to the use of interesterification, fractionation, or blending of saturated and polyunsaturated oils as an alternate method to hydrogenation. It was found

that CLA, known as beneficiary biofunctional components, could be formed during hydrogenation. Reaction conditions such as catalyst type and amounts, hydrogen pressure, agitation rate, and temperature greatly affected the total quantity of CLA, isomeric distribution, and time required to reach their maximal quantity during hydrogenation. Hydrogenation with selective type Ni catalyst under the mass transfer limitation conditions of high catalyst content, low stirring rate, high temperature, and low hydrogen pressure favored the CLA formation, resulting in their high presence (as high as 240 mg/g oil) in vegetable oils. Short time hydrogenation (10 minutes) with a selective type nickel catalyst at low hydrogen pressure (0.25 kg/cm^2) and high temperature (230 °C) produced oils a containing high amount of CLA (48.16 mg CLA/g oil) and a low level of trans fatty acids (3.5%). The CLA amount of 48.16 mg per g oil was about eight times greater than levels in major dietary sources for CLA such as dairy products and ruminant meat. The hydrogenated vegetable oils with high quantity conjugated linoleic acid might provide the possibility for the utilization of hydrogenated oils as health-promoting food ingredients.

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CHAPTER 21

Production of Mannitol by Lactic Acid Bacteria: A Review

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21.1. INTRODUCTION

Mannitol, a naturally occurring polyol, is widely used in the food, pharmaceutical, medicine, and chemical industries (Saha, 2003). At $3.32/lb in the U. S. and with a global market of 30 million lbs/year, it is currently produced industrially by high pressure hydrogenation of fructose/glucose mixtures in aqueous solution at high temperature (120–160 °C) with Raney nickel as catalyst (Makkee *et al.*, 1985). Typically, the hydrogenation of a 50/50 fructose/glucose mixture results in an approximately 25/75 mixture of mannitol and sorbitol ($ 0.73/lb). This means that about half of the fructose is converted to mannitol

[†] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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and half to sorbitol. The glucose is hydrogenated exclusively to sorbitol. As a consequence, the commercial production of mannitol is always accompanied by the production of sorbitol, thus resulting in an inefficient process (Soetaert *et al.*, 1995). Moreover, it is relatively difficult to separate sorbitol and mannitol, which results in even higher production costs and decreased yields (Johnson, 1976).

Some microorganisms can specifically produce mannitol from glucose or fructose without making a sorbitol byproduct (Smiley *et al.*, 1967; Song *et al.*, 2002; Wisselink *et al.*, 2002; Saha, 2003). Mannitol, at 180 g/L, can be easily recovered from the fermentation broth by cooling crystallization. Thus, research efforts have been directed toward production of mannitol by fermentation and enzymatic means (Vandamme and Soetaert, 1995). In this paper, the authors review the production of mannitol by lactic acid bacteria.

21.2. MANNITOL PRODUCTION BY HOMOFERMENTATIVE LACTIC ACID BACTERIA

Some homofermentative LAB such as *Streptococcus mutans* and *Lactobacillus leichmanii* produce small amounts of mannitol from glucose (Loesche and Kornman, 1976; Chalfan *et al.*, 1975). The pathway for glucose metabolism and mannitol production by homofermentative LAB is shown in Figure 21.1. Forain *et al.* (1996) reported that a strain of *L. plantarum* deficient in both L- and D-lactate dehydrogenase (LDH) produces mannitol as an end-product of glucose catabolism. LAB uses several strategies for regeneration of NAD^+ during metabolism of sugars. Hols *et al.* (1999) showed that disruption of the *ldh* gene in *Lactococcus lactis* strain NZ20076 leads to the conversion of acetate into ethanol as a rescue pathway for NAD^+ regeneration. Neves *et al.* (2000) reported that a LDH-deficient (LDH^d) mutant of *Lactococcus lactis* transiently accumulates intracellular mannitol, which was formed from fructose-6-phosphate by the combined action of mannitol-1-phosphate dehydrogenase and phosphatase. They showed that the formation of mannitol-1-phosphate (M-1-P) by the LDH^d strain during glucose catabolism is a consequence of impairment in NADH oxidation caused by a highly reduced LDH activity, the transient formation of M-1-P serving as a regeneration pathway for NAD^+ regeneration. Gaspar *et al.* (2004) described the construction of *Lactococcus lactis* strains able to form mannitol as an end product of glucose metabolism, using a food-grade LDH-deficient strain as genetic basis for knocking out the gene *mtlA* or *mtlF*. Non-growing cells of the double mutants (Δ*ldh*/Δ*mtlA*) and (Δ*ldh* Δ*mtlF*) produced mannitol, ethanol, 2,3-butanediol, and lactate as major end products from glucose, with approximately one-third of the carbon being successfully channeled to the production of mannitol.

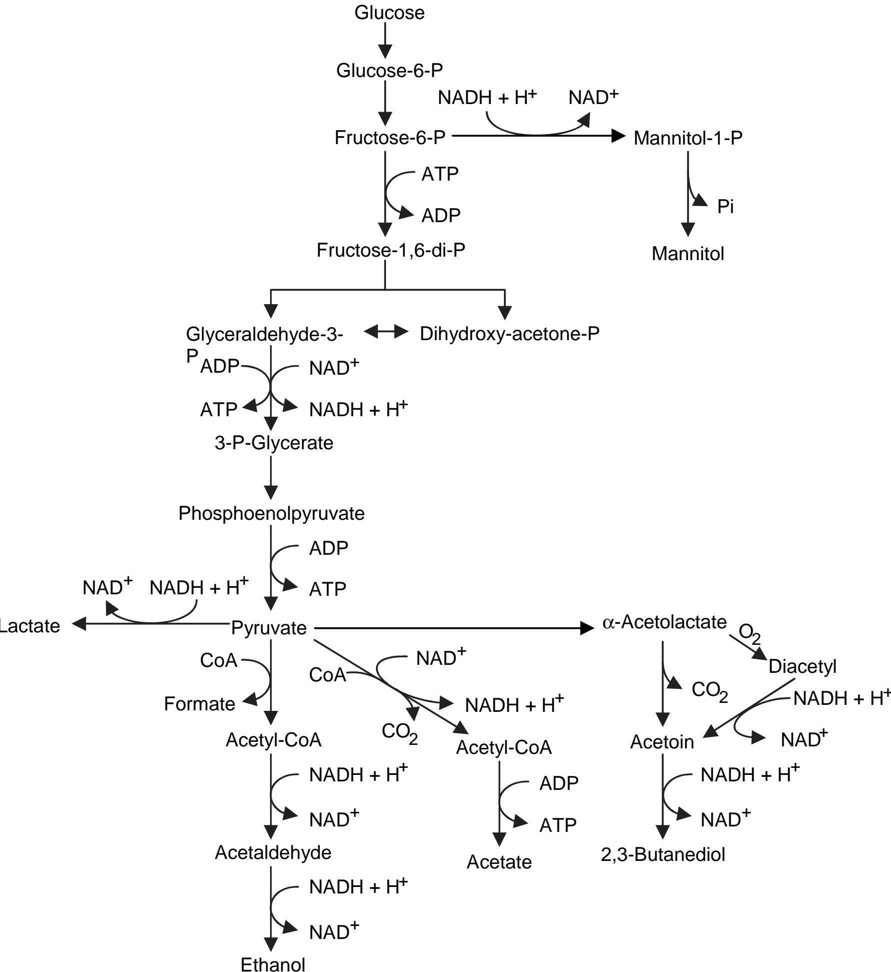

Figure 21.1. Pathway of glucose metabolism by homofermentative lactic acid bacteria.

21.3. MANNITOL PRODUCTION BY HETEROFERMENTATIVE LACTIC ACID BACTERIA

Several heterofermentative LAB belonging to the genera *Lactobacillus, Leuconostoc,* and *Oenococcus* can produce mannitol from fructose effectively (Saha, 2003). In addition to mannitol, these bacteria may produce lactic acid, acetic acid, carbon dioxide, and ethanol. The process is based on the ability of the LAB to use fructose as an electron acceptor and reduce it to mannitol with the participation of the enzyme mannitol 2-dehydrogenase (EC 1.1.1.38).

Saha and Nakamura (2003) reported that nine strains of heterofermentative LAB (*L brevis* NRRL B-1836, *L. buchneri* NRRL B-1860, *L. cellobiosus* NRRL B-1840, *L. fermentum* NRRL B-1915, *L. intermedius* B-3693, *Leu. amelilibiosum* NRRL B-742, *Leu. citrovorum* NRRL B-1147, *Leu. mesenteroides subsp. dextranicum* NRRL B-1120 and *Leu. paramesenteroides* B-3471) produce mannitol from fructose. The strain *L. intermedius* B-3693 produced 198 g of mannitol from 300 g fructose per L in pH-controlled (pH 5.0) fermentation at 37 °C. Small white needle-like crystals of mannitol appeared upon refrigeration of the cell-free fermentation broth at 4 °C. The time of maximum mannitol production varied greatly from 15 h at 150 g fructose to 136 h at 300 g fructose concentration per L. The bacterium converted fructose to mannitol from the early growth stage. One-third of fructose can be replaced with other substrates such as glucose, maltose, starch plus glucoamylase (simultaneous saccharification and fermentation, SSF), mannose, and galactose. Two-thirds of fructose can also be replaced by sucrose. The bacterium co-utilized fructose and glucose (2:1) simultaneously and produced very similar quantities of mannitol, lactic acid, and acetic acid in comparison with fructose only. The glucose was converted to lactic and acetic acids, and fructose was converted into mannitol. Application of fed-batch fermentation by feeding equal amounts of substrate and medium four times decreased the maximum mannitol production time from fructose (300 g/L) from 136 h to 92 h. The yields of mannitol, lactic acid, and acetic acid were 202, 53, and 39 g/L, respectively. With glucose (150 g/L) as the carbon source, the bacterium produced D- and L-lactic acids in equal ratios (total, 70 g/L) and ethanol (38 g/L) but no acetic acid. Saha (2004) purified mannitol dehydrogenase from *L. intermedius* NRRL B-3693 and showed that the purified enzyme can convert fructose to mannitol completely in the presence of NADPH.

Saha (2006a) studied the production of mannitol by *L. intermedius* NRRL B-3693 using molasses as an inexpensive carbon source. The bacterium produced mannitol (104 g/L) from molasses and fructose syrups (1:1; total sugars, 150 g/L; fructose:glucose, 4:1) in 16 h. Several kinds of inexpensive organic and inorganic nitrogen sources and corn steep liquor were evaluated for their potential to replace more expensive nitrogen sources derived from Bacto-peptone and yeast extract. Soy peptone D (5 g/L) and corn steep liquor (50 g/L) were found to be suitable substitutes for Bacto-peptone (5 g/L) and Bacto-yeast extract (5 g/L), respectively. The bacterium produced 105 g mannitol per L from the molasses and fructose syrup (1:1, total sugars, 150 g/L; fructose:glucose, 4:1) in 22 h using a combination of soy peptone D (5 g/L) and corn steep liquor (50 g/L). The effects of four salt nutrients (ammonium citrate, sodium phosphate, magnesium sulfate, and manganese sulfate) on the production of mannitol by *L. intermedius* NRRL B-3693 in a simplified medium containing 300 g fructose, 5 g soy peptone, and 50 g corn steep liquor per L in pH-controlled fermentation at 5.0 at 37 °C were evaluated using a fractional factorial design (Saha, 2006b). Only manganese sulfate was found to be essential for mannitol production. Added manganese sulfate concentration of

33 mg/L supported maximum mannitol production. The bacterium produced 200 g mannitol, 62 g lactic acid, and 40 g acetic acid from 300 g fructose per L in 67 h. Racine and Saha (2006) improved the fermentation process further for the production of mannitol by *L. intermedius* NRRL B-3693. A fed-batch protocol overcame limitations caused by high substrate concentrations. The fed-batch process resulted in the accumulation of 176 g mannitol from 184 g fructose and 92 g glucose per L of final fermentation broth in 30 h with a volumetric productivity of 5.9 g/L·h. Further increases in volumetric productivity of mannitol were obtained in a continuous cell-recycle fermentation process that reached more than 40 g/L·h. This is the first report of such a high volumetric productivity of mannitol by a heterofermentative lactic acid bacterium.

Saha (2006c) investigated the production of mannitol by *L. intermedius* NRRL B-3693 using inulin as a substrate at pH 5.0 and 37 °C. Inulin is a polyfructan, consisting of linear β-2,1-linked polyfructose chains terminated at the reducing end by a glucose residue (Vandamme and Derycke, 1983). This polymer is a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, and dahlia. These inulin sources have recently received attention as a potential feedstock for fuel ethanol production (Ohta *et al.*, 1993). Endoinulinases (2,1-β-D-fructan fructoanhydrolase, EC 3.2.1.7) are specific for inulin and hydrolyze the internal β-2,1-fructofuranosidic linkages to yield inulotriose, inulotetraose, and inulopentaose as the main products. Exoinulinases (β-D-fructan fructohydrolase, EC 3.2.1.80) split terminal fructose units from inulin. The synergistic action of endo- and exo-inulinases on inulin allows its efficient hydrolysis to fructose. The bacterium produced 106 g mannitol from dilute acid hydrolyzate (pH 2.0, 121 °C, 15 min) of 150 g inulin per L in 34 h. It also produced mannitol from inulin by simultaneous saccharification and fermentation (SSF) at pH 5.0 and 37 °C using inulinase (8 U/g inulin). The *L. intermedius* B-3693 strain produced 207 g mannitol from 300 g inulin per L in 72 h by SSF. The fermentation time decreased from 72 h to 62 h using a mixture of fructose and inulin (1:1; total, 300 g/L). When fructose and inulin mixture (3:5, total 400 g/L) was used as substrate, the bacterium produced 228 g mannitol per L from both inulin and fructose with a yield of 0.57 g per g substrate after 110 h of SSF. This is the highest concentration of mannitol produced by a heterofermentative LAB thus far reported in the literature.

Martinez *et al.* (1963) reported that *L. brevis* fermented 1 mol fructose to 0.67 mol mannitol and 0.33 mol lactate and 0.33 mol acetate. Soetaert *et al.* (1995) reported a fed batch fermentation method with automatic feeding strategy for very fast and rapid production of mannitol and D-lactic acid from fructose or glucose/fructose mixture (1:2) by using *Leu. pseudomesenteroides*. The maximal volumetric productivity of mannitol was 11.1 g/L·h with a final concentration of 150 g/L in 24 h and a conversion efficiency of 94%. By using a special mutant strain, quantitative conversion and a further concentration increase up to 185 g mannitol per L could be obtained. Grobben *et al.* (2001)

reported the spontaneous formation of a mannitol-producing variant of *Leu. pseudomesenteroides* grown in the presence of fructose. The mannitol producing variant differed from the mannitol-negative original strain in two physiological aspects: the presence of mannitol dehydrogenase activity and the simultaneous utilization of fructose and glucose. The presence of mannitol dehydrogenase is clearly a prerequisite for mannitol production.

Yun *et al.* (1996a) reported about 4–5 g of mannitol accumulation per kg during fermentation of kimchi, a Korean pickled vegetable. Yun and Kim (1998) isolated two different LAB, *Lactobacillus* sp. Y-107 and *Leuconostoc* sp. Y-002 during the fermentation of kimchi. These two strains utilized fructose and sucrose as substrates for mannitol formation. Under optimal conditions, the maximum mannitol produced by *Lactobacillus* sp. Y-107 (at 35 °C, initial pH 8.0, anaerobic, 100 g fructose/L, 120 h) and *Leuconostoc* sp. Y-002 (at 35 °C, initial pH 6.0, anaerobic, 50 g fructose/L, 25 h) were 73 and 26 g from 100 g fructose per L with yields of 86 and 65% based on fructose consumed, respectively. The volumetric productivities of mannitol by both strains were less than 1.0 g/L · h. Neither isolate produced other polyols such as glycerol and sorbitol as by-products. These two bacterial strains were not able to utilize high concentrations of sugars above 100 g/L due to low osmotolerance of the isolates. Yun *et al.* (1996b) obtained a mannitol yield of 70 g from 100 g fructose within 80 h at 28 °C using *Lactobacillus* sp. KY-107.

Erten (1998) studied the utilization of fructose (5 mmol/L) as an electron acceptor in two *Leu. mesenteroides* strains under anaerobic conditions. These strains produced 0.26 mol mannitol, 0.65–0.67 mol lactic acid, 0.37–0.57 mol ethanol and 0.26–0.27 mol acetic acid per mol of fructose at 25 °C. Fermentation of a mixture of fructose and glucose (1 : 1) resulted in the production of the same metabolic end-products. Korakli *et al.* (2000) reported that mannitol is produced by sourdough *Lactobacilli* from fructose with concomitant formation of acetate. They obtained a 100% yield of mannitol from fructose by *L. sanfranciscensis* (isolated from sourdough) grown in a fed-batch culture containing fructose-glucose mixture with a volumetric productivity of 0.5 g/L · h and a final mannitol concentration of 60 g/L. The turnover of fructose was at its optimum at a concentration of glucose/fructose mixture ranging between 130–140 g/L in batch fermentation. Higher concentrations of substrate were found to be growth inhibitory for *L. sanfranciscensis*. After adaptation of the cells in sucrose, the bacterium produced mannitol to only 65% yield in relation to the fructose content of sucrose. It also synthesized complex polysaccharides when grown on sucrose. *L. pontis* isolated from sourdough produced mannitol, lactic acid, and ethanol from fructose (Hammes *et al.*, 1996). An unidentified *Lactobacillus* sp., named B001, produced mannitol from fructose with a volumetric productivity of 6.4 g/L · h (Itoh *et al.*, 1992). Salou *et al.* (1994) reported that *O. oenos* converted 83 mol% of fructose to mannitol when grown in a medium containing fructose and glucose (1 : 1) with volumetric productivity of about 0.2 g/L · h. Most LAB are able to consume glucose and fructose simultaneously. Pimentel *et al.* (1994) studied growth and metabolism of sugars and

acids by *Leu. oenos* under different conditions of temperature and pH. The bacterium produced mannitol from fructose. The addition of acids, particularly, citrate, significantly repressed mannitol formation.

Von Weymarn *et al.* (2002a) studied mannitol production by eight heterofermentative LAB (*L. brevis* ATCC-8287, *L. buchneri* TKK-1051, *L. fermentum* NRRL B-1932, *L. sanfranciscensis* E-93491, *Lactobacillus* sp. (B001) BP-3158, *Leu. mesenteroides* ATCC-9135, *Leu. pseudomesenteroides* ATCC-12291, and *O. oeni* E-9762). They found that the ability to produce mannitol from fructose varied markedly among these heterofermentative LAB species. The effects of growth temperature, pH, and nitrogen flushing on mannitol production by four selected strains were studied in batch bioreactor cultivation. Using *L. fermentum* and with fructose (20 g/L) and glucose (10 g/L) as carbon source, mannitol yields from fructose were 86, 89 and 94 mol% at 25, 30 and 35 °C, respectively. Mannitol yields, but not the volumetric mannitol productivities, were improved with constant nitrogen gas flushing of the growth medium. Applying the most promising strain (*L. fermentum*), high average and maximum mannitol productivities (7.6 and 16.0 g/L-h, respectively) were achieved. Von Weymarn *et al.* (2002b) then compared the ability to produce mannitol from fructose by ten heterofermentative bacteria (the eight from above, *Leu. mesenteroides* ATCC-8086 and ATCC 8293) in resting state. They achieved high mannitol productivity (26.2 g/L·h) and mannitol yield (97 mol%) in high cell density membrane cell-recycle culture using the best strain, *Leu. mesenteriodes* ATCC 9135. A stable high-level production of mannitol was maintained for 14 successive bioconversion batches using the same initial cell biomass. Von Weymarn *et al.* (2002b) also reported that increasing the initial fructose concentration from 100 to 120 and 140 g/L resulted in decreased mannitol productivities due to both substrate and end-product inhibition of the key enzyme mannitol dehydrogenase in *Leu. mesenteriodes* ATCC 9135. Von Weymarn *et al.* (2003) scaled up the mannitol production by *Leu. mesenteroides* ATCC-9135. On a 2-L laboratory scale, high mannitol yields from fructose (93–97%) and volumetric mannitol productivities (>20 g/L-h) were achieved. In the pilot plant scale (100 L), the production levels of mannitol were similar to those in the laboratory. Also, high purity mannitol crystals were obtained at similar yield levels. Ojamo *et al.* (2000) achieved a volumetric mannitol productivity of about 20 g/L·h using high cell density fermentation of *Leu. pseudomesenteroides* ATCC 12291.

Several heterofermentative LAB produce mannitol in large amounts, using fructose as an electron acceptor. Mannitol produced by heterofermentative bacteria is derived from the hexose phosphate pathway (Soetaert *et al.*, 1999; Wisselink *et al.*, 2002). The process makes use of the capability of the bacterium to utilize fructose as an alternative electron acceptor, thereby reducing it to mannitol with the enzyme mannitol dehydrogenase. In this process, the reducing equivalents are generated by conversion of one-third fructose to lactic acid and acetic acid. The enzyme reaction proceeds according to (theoretical) Equation 21.1:

$$3\ \text{Fructose} = 2\ \text{Mannitol} + \text{Lactic acid} + \text{Acetic acid} + CO_2 \quad \text{[Eq. 21.1]}$$

The net ATP gain is 2 mol of ATP per mol of fructose fermented. For fructose and glucose (2:1) co-fermentation, the equation becomes:

$$2\ \text{Fructose} + \text{Glucose} = 2\ \text{Mannitol} + \text{Lactic acid} + \text{Acetic acid} + CO_2 \quad \text{[Eq. 21.2]}$$

For sucrose and fructose (1:1) co-fermentation, the equation is

$$\text{Sucrose} + \text{Fructose} = 2\ \text{Fructose} + \text{Glucose} = 2\ \text{Mannitol} + \text{Lactic acid} + \text{Acetic acid} + CO_2 \quad \text{[Eq. 21.3]}$$

Busse *et al.* (1961) and Erten (1998) found a lower mannitol yield from fructose in *Leu. mesenteroides*. In these cases, the enzyme reaction proceeds by the following equation:

$$3\ \text{Fructose} = 1\ \text{Mannitol} + 2\ \text{Lactic acid} + 0.5\ \text{Acetic acid} + 1.5\ \text{Ethanol} + CO_2 \quad \text{[Eq. 21.4]}$$

The net gain is 1.25 mol of ATP per mol of fructose fermented. The typical pathway for mannitol production by a heterofermentative LAB from glucose and fructose mixture (1:2) is shown in Figure 21.2.

21.4. MANNITOL PRODUCTION BY RECOMBINANT MICROORGANISMS

Kaup *et al.* (2004) constructed an efficient *Escherichia coli* strain for mannitol formation from fructose in a whole cell biotransformation. The strain expressed NAD^+-dependent mannitol dehydrogenase from *Leu. pseudomesenteroides* ATCC 12291 (Hahn *et al.*, 2003) for the reduction of fructose to mannitol, NAD^+-dependent formate dehydrogenase from *Mycobacterium vaccae* N10 (Galkin *et al.*, 1995) for NADH regeneration, and the glucose facilitator from *Zymomonas mobilis* (Weisser *et al.*, 1995; Parker *et al.*, 1995) for the uptake of fructose without concomitant phosphorylation. The strain produced 0.362 M mannitol from 0.5 M fructose within 8 h with a yield of 84 mol% and a specific mannitol productivity of more than 4 g per g cell dry weight per h. Kaup *et al.* (2005) reported that supplementation of this recombinant strain with extracellular glucose isomerase resulted in the formation of 0.8 M mannitol from 1 M glucose. They have coexpressed the *xylA* gene of *E. coli* in this recombinant *E. coli* strain which formed 0.42 M mannitol from 1 M glucose. Sasaki *et al.* (2005) cloned a gene encoding MDH from *L. reuteri* and expressed in *E. coli*.

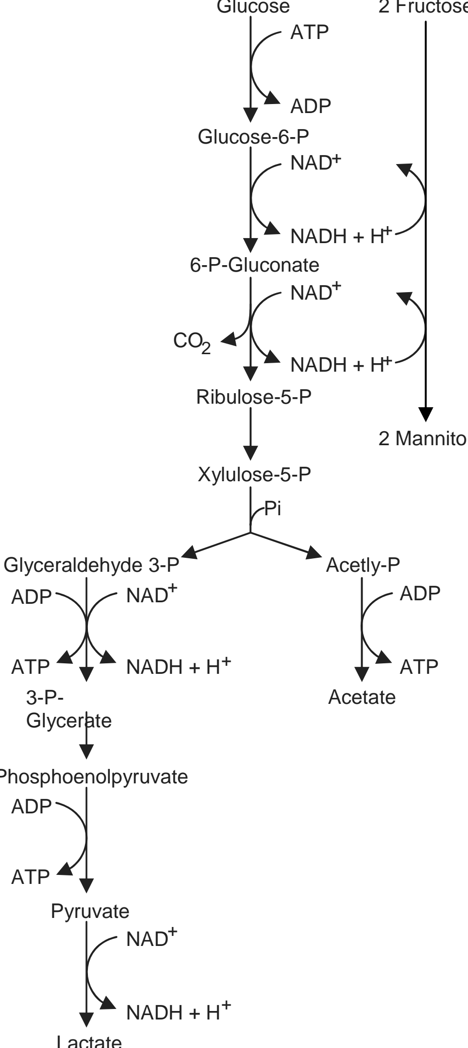

Figure 21.2. Pathway of glucose and fructose (1:2) metabolism by heterofermentative lactic acid bacteria.

The purified recombinant enzyme works optimally at 37 °C and pH 5.4 for conversion of fructose to mannitol.

Aarnikunnas *et al.* (2002) used metabolic engineering of *L. fermentum* for the production of mannitol and pure L-lactic acid or pyruvate. The authors first developed genetic tools to modify *L. fermentum* and then proceeded to inactivate first *ldhD* gene and then *ldhL* gene to create a bacterium that could produce mannitol and either pure L-lactic acid or pyruvic acid in a single process. In bioreactor cultivations, the single mutant strain constructed by

inactivation of the *ldhD* gene produced mannitol and L-lactic acid. The double mutant strain created by inactivating the *ldhL* gene produced mannitol and pyruvate. In addition, the mutant produced 2,3-butanediol and the volumetric productivity of mannitol was decreased. Helanto *et al.* (2005) described the construction and characterization of a random mutant of *Leu. pseudomesenteroides* that is unable to grow on fructose, and the positive effects of the mutation on mannitol production. They have performed the inactivation of its fructokinase activity with random mutagenesis and screening the mutants unable to grow on fructose. The fructose uptake of the mutant was unaltered and the mutant converted fructose to mannitol when grown in a medium containing both glucose and fructose. The yield of mannitol from fructose was improved from 74 to 86 mol%. A fructokinase-negative mutant could enable higher pH to be used in the mannitol production process without lowering the yield (Helanto *et al.*, 2005). Wisselink *et al.* (2004) cloned and overexpressed *mtlD* from *L. plantarum* in *Lactococcus lactis* in different genetic backgrounds (a wild type strain, a lactate dehydrogenase (LDH)-deficient strain, and a strain with reduced phosphofructokinase activity). Small amounts (<1%) of mannitol were formed by growing cells of *mtlD*-overexpressing LDH-deficient and phosphofructokinase reduced strains. The resting cells of the LDH-deficient transformant converted 25% of glucose to mannitol. They concluded that the *mtlD* overexpressing LDH-deficient *Lactococcus lactis* strain seemed to be the most promising strain for mannitol production. Liu *et al.* (2005) have cloned and characterized the *mtlK* gene encoding MDH from *L. brevis*. Genetically engineered *L. plantarum* TF103 carrying *mtlK* gene of *L. brevis* indicated increased mannitol production from glucose.

Costenoble *et al.* (2003) demonstrated that mannitol is produced under anaerobic conditions by a glycerol-defective mutant of *Saccharomyces cerevisiae* expressing the *mtlD* gene from *E. coli* coding for NADH-dependent mannitol 1-phosphate dehydrogenase. Improving efflux of the formed mannitol seems to be necessary for obtaining anaerobic growth and sustained mannitol production.

21.5. CONCLUSIONS

Heterofermentative LAB have the capability to utilize high concentrations of fructose such that the mannitol concentration in the fermentation broth could reach more than 180 g/L, which is enough to be separated from the cell-free fermentation broth by cooling crystallization. Lactic and acetic acids can be recovered by electrodialysis (Soetaert *et al.*, 1995). The enzyme mannitol dehydrogenase responsible for catalyzing the conversion of fructose to mannitol requires NADPH (NADH) as cofactor. Thus, it is possible to develop a one-pot enzymatic process for production of mannitol from fructose if a cost-effective cofactor regeneration system can be developed (Saha, 2004). The heterofermentative LAB cells can be immobilized in a suitable support, and

the immobilized cells can be used in a bioreactor to produce mannitol from fructose continuously. The production of acetic acid and D-lactic acid by some LAB can be blocked (inactivation of acetate kinase and D-LDH) by mutagenesis (Aarnikunnas *et al.*, 2002). In that case, the fermentation broth should contain only mannitol and L-lactic acid. Since both are value added-products, the fermentative production of mannitol will become more attractive.

21.6. REFERENCES

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CHAPTER 22

Evaluation of the Physiological Function of Docosahexaenoic Acid in Diet-induced Lipodystrophy Model Mice

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22.1. INTRODUCTION

Recent advances in molecular and cell biology have shown that adipose tissue not only stores excess energy in the form of fat, but also secretes physiologically active substances called adipocytokines (Matsuzawa *et al.*, 1999). In obesity, adipocytes (cells of adipose tissues) are increased and enlarged, and they secrete excessive amounts of inflammatory adipocytokines, such as tumor necrosis factor-alpha (Hotamisligil *et al.*, 1993) and monocyte chemoattractant protein-1 (Sartipy and Loskutoff, 2003). This induces type-2 diabetes, such as

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hyperinsulinemia and fatty liver (Wang *et al.*, 2005; Kanda *et al.*, 2006). On the other hand, a deficiency of adipocytes—the symptom is known as lipodystrophy in humans—also induces type-2 diabetes due to a paucity of normally-functioning adipocytekines, such as leptin (Ahima *et al.*, 2000) and adiponectin (Arita *et al.*, 1999; Hotta *et al.*, 2001). Lipodystrophies are rare acquired and genetic disorders, characterized by the complete or partial absence of body fat with a line of metabolic disorders (Yu and Ginsberg, 2005; Simha and Garg, 2006).

To understand the pathophysiology of lipodystrophy and evaluate the efficacy and safety of clinical treatments, several transgenic mouse models mimicing the features of lipodystrophy, such as aP2-SREBP-1c mouse (Shimomura *et al.*, 1998, 1999a, 1999b; Horton *et al.*, 2003) and A-ZIP/F1 mouse (Moitra *et al.*, 1998; Reitman and Gavrilova, 2000; Gavrilova *et al.*, 2000), have been established. Feeding conjugated linoleic acid (CLA) (a group of positional and geometric isomers of linoleic acid), with a low-fat diet also induces lipodystrophy, characterized by an increase in hepatic lipid content concomitant and a decrease in body fat mass in mice (Clement *et al.*, 2002; Pariza, 2004). Lipodystrophy may occur in mice because they are very sensitive to the CLA-induced reduction in body fat (Ohashi *et al.*, 2004; Poirier *et al.*, 2005; Purushotham *et al.*, 2007). We previously reported that the short-term feeding of CLA decreased adipose tissue weight and hepatic lipid levels, without inducing adverse effects in mice (Wang *et al.*, 2005). Tsuboyama-Kasaoka *et al.* also reported that increasing the amount of fat in a CLA-supplemented diet substantially reduces the lipodystrophy effect, and continuous leptin infusion reverses hyperinsulinemia in CLA-fed mice (2000, 2003). These results indicate that dietary CLA induces fatty liver and hyperinsulinemia through the drastic reduction of adipocytekine levels, due to a paucity of adipose tissue but not through the direct induction of hepatic lipid synthesis and insulin resistance (Fig. 22.1). Because the pathogenesis of these metabolic abnormalities in CLA-fed mice is similar to that in human lipodystrophy, we expect

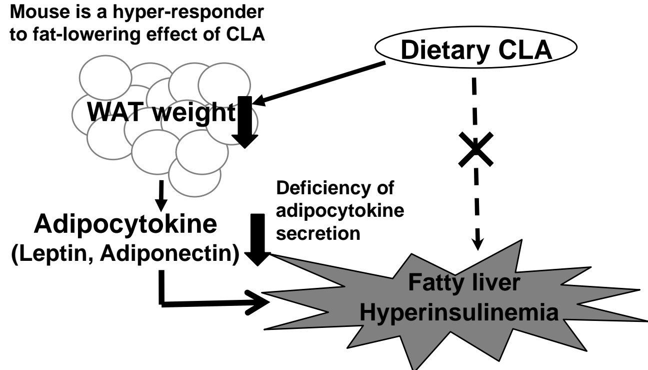

Figure 22.1. Possible mechanism of the development of lipodystrophy by dietary CLA in mice.

the use of CLA-fed mice might serve as a diet-induced lipodystrophy model (Nagao *et al.*, 2008).

Docosahexaenoic acid (DHA) is a long-chain highly unsaturated fatty acid that is abundant in fish oil and is a precursor of several eicosanoids. DHA has a lipid-lowering effect through the suppression of lipogenic gene expression in the liver of rodents (Ikeda *et al.*, 1998; Park and Harris, 2003; Buckley *et al.*, 2004). In this paper, we introduce the evaluation of the physiological function of DHA in diet-induced lipodystrophy model mice.

22.2. EFFECT OF DHA ON GROWTH PARAMETERS IN DIET-INDUCED LIPODYSTROPHY MODEL MICE

Male C57BL/6N mice were assigned to three groups, with dietary fats composed of 6% high-linoleic safflower oil (HL-SAF) in the control diet, a mixture of 4% HL-SAF + 2% CLA in the lipodystrophy model control diet, and a mixture of 3.5% HL-SAF + 2% CLA + 0.5% DHA in the lipodystrophy model plus DHA diet.

Figure 22.2 shows liver and white adipose tissue (WAT) weights of C57BL/6N mice after 4 weeks of consuming these diets. Although there was no significant difference in final body weight or food intake among groups, CLA-containing lipodystrophy model diets significantly increased the liver weight of mice, as has been reported elsewhere (Clement *et al.*, 2002; Pariza, 2004). However, the supplementation of DHA to the lipodystorphy model diet alleviated, but not significantly, hepatomegaly in diet-induced lipodystrophy

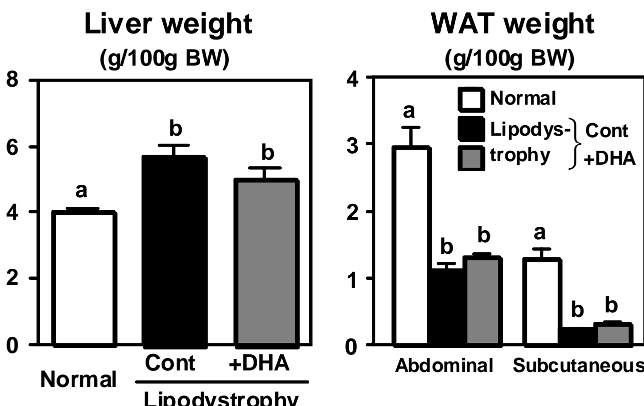

Figure 22.2. Effect of DHA on liver weights and white adipose tissue (WAT) weights in diet-induced lipodystrophy model mice.
Mice were fed diets containing either 6% HL-SAF (normal), 4 HL-SAF + 2% CLA (lipodystrophy control), or 3.5% HL-SAF + 2% CLA + 0.5% DHA (lipodystrophy + DHA) for 4 weeks. Values are expressed as mean ± standard error for six mice. Different letters show significant differences at $p < 0.05$.

model mice. Weights of west subcutaneous and abdominal (perirenal, epididymal, and omental). WATs were significantly decreased in lipodystorphy model mice, and there was no significant effect of DHA feeding on WAT weights in this model.

22.3. EFFECT OF DHA ON HEPATIC TRIGLYCERIDE METABOLISM IN DIET-INDUCED LIPODYSTROPHY MODEL MICE

Figure 22.3 shows hepatic triglyceride levels and serum alanine aminotransferase (ALT) levels of C57BL/6N mice after 4 weeks of feeding. Although the difference was not significant, the hepatic triglyceride level in mice fed the lipodystrophy model diet was 2.2-fold that in the mice fed a control diet. However, supplementation of DHA to the lipodystrophy model diet resulted in a 34% attenuation of triglyceride accumulation in the liver. The activities of ALT, one of the hepatic injury markers, in the serum of lipodystrophy model mice were markedly increased because of the development of hepatic steatosis. Supplementation of DHA to the lipodystrophy model diet, however, resulted in a 27% decrease of ALT levels consistent with the attenuation of hepatic triglyceride accumulation.

Takahashi *et al.* (2003) previously demonstrated that CLA increases the activity and mRNA levels of hepatic lipogenic enzymes; they suggested that enhanced lipogenesis is a principal mechanism of CLA-induced hepatic steatosis in mice. In this study, suppression of the activity and mRNA expression

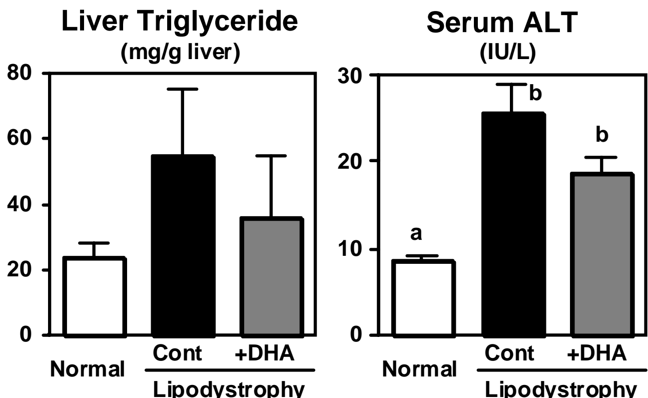

Figure 22.3. Effect of DHA on hepatic triglyceride levels and serum alanine aminotransferase (ALT) levels in diet-induced lipodystrophy model mice.
Mice were fed diets containing either 6% HL-SAF (normal), 4 HL-SAF + 2% CLA (lipodystrophy control), or 3.5% HL-SAF + 2% CLA + 0.5% DHA (lipodystrophy + DHA) for 4 weeks. Values are expressed as mean ± standard error for six mice. Different letters show significant differences at $p < 0.05$.

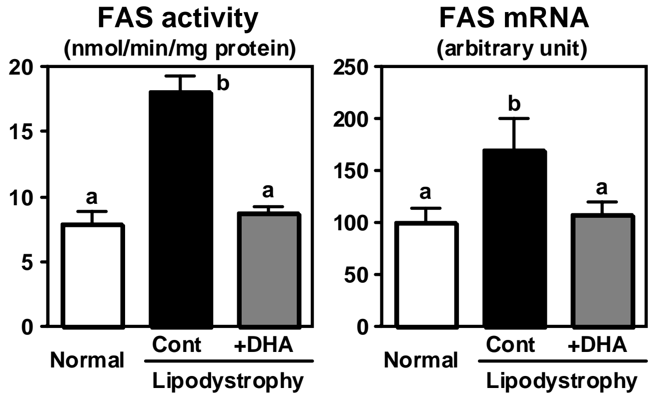

Figure 22.4. Effect of DHA on activities and mRNA levels of fatty acid synthase (FAS) in the liver of diet-induced lipodystrophy model mice.
Mice were fed diets containing either 6% HL-SAF (normal), 4 HL-SAF + 2% CLA (lipodystrophy control), or 3.5% HL-SAF + 2% CLA + 0.5% DHA (lipodystrophy + DHA) for 4 weeks. Values are expressed as mean ± standard error for six mice. Different letters show significant differences at $p < 0.05$.

of fatty acid synthase (FAS) induced decreases in liver weight and hepatic triglyceride content in mice fed a DHA plus lipodystrophy model diet (Fig. 22.4). The gene expression of FAS is regulated by sterol-regulatory element binding protein-1 (SREBP-1), a lipogenic transcriptional factor (Horton *et al.*, 2002). Previous studies have shown that DHA has a suppressive effect on SREBP-1 mRNA expression (Fujiwara *et al.*, 2003; Kim *et al.*, 1999; Nakatani *et al.*, 2002), which suggests that this fatty acid can decrease fatty acid synthesis through the transcriptional suppression of SREBP-1 signaling. Therefore hepatic steatosis induced by the lipodystrophy model diet was attenuated by DHA supplementation through the transcriptional suppression of fatty acid synthesis.

22.4. EFFECT OF DHA ON CHOLESTEROL METABOLISM IN DIET-INDUCED LIPODYSTROPHY MODEL MICE

Figure 22.5 shows cholesterol levels in the liver and serum of C57BL/6N mice after 4 weeks of feeding. Hepatic cholesterol accumulation and hypercholesterolemia were induced by the lipodystrophy model diet, and they were markedly attenuated by DHA supplementation.

HMG-CoA reductase, a rate-limiting enzyme of cholesterol synthesis, and ACAT-1, a rate-limiting enzyme of cholesterol esterification, relate to hepatic cholesterol storage. Levels of mRNA of those enzymes were also increased by the lipodystrophy model diet during the onset of hepatic steatosis, but DHA supplementation attenuated this (Fig. 22.6).

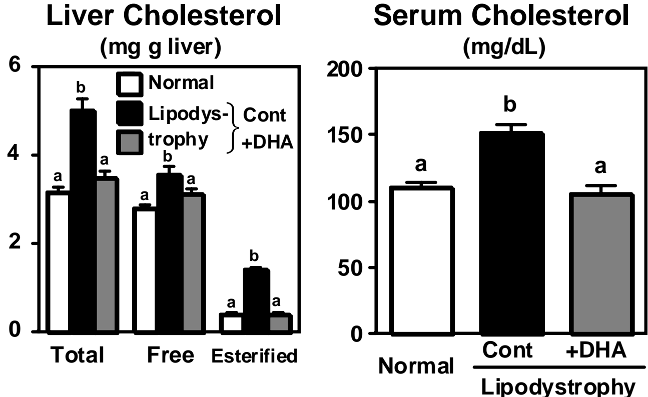

Figure 22.5. Effect of DHA on cholesterol levels in the liver and serum of diet-induced lipodystrophy model mice.
Mice were fed diets containing either 6% HL-SAF (normal), 4 HL-SAF + 2% CLA (lipodystrophy control), or 3.5% HL-SAF + 2% CLA + 0.5% DHA (lipodystrophy + DHA) for 4 weeks. Values are expressed as mean ± standard error for six mice. Different letters show significant differences at $p < 0.05$.

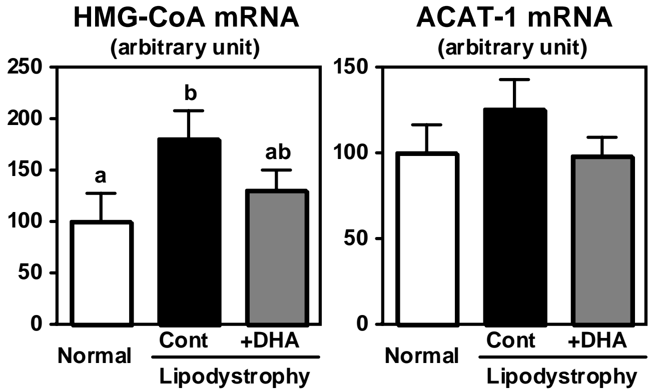

Figure 22.6. Effect of DHA on levels of hepatic mRNA related to cholesterol metabolism in diet-induced lipodystrophy model mice.
Mice were fed diets containing either 6% HL-SAF (normal), 4 HL-SAF + 2% CLA (lipodystrophy control), or 3.5% HL-SAF + 2% CLA + 0.5% DHA (lipodystrophy + DHA) for 4 weeks. Values are expressed as mean ± standard error for six mice. Different letters show significant differences at $p < 0.05$. HMG, 3-hydroxy-3-methylglutaryl-CoA reductase, ACAT1, acyl-CoA:cholesterol acyltransferase-1.

22.5. EFFECT OF DHA ON ADIPOCYTEKINE LEVELS IN DIET-INDUCED LIPODYSTROPHY MODEL MICE

As shown in Figure 22.7, serum levels of adiponectin and leptin were drastically decreased in mice fed the CLA-containing lipodystrophy model diet, as previously reported (Ohashi *et al.*, 2000; Poirier *et al.*, 2005; Purushotham *et al.*, 2007). Adiponectin and leptin are both abundantly secreted from adipose tissue and have several physiological functions, including the regulation of insulin sensitivity in humans and animals. Therefore it has been reported that the deficiency of adipocytokine secretion induced by a paucity of adipose tissue would be cause of lipodystrophy, which is characterized by a severe insulin resistance, and which leads to hyperinsulinemia and hepatic steatosis (Shimomura *et al.*, 1999c; Yamauchi *et al.*, 2001). Thus we hypothesized that the DHA-mediated alleviation of hepatic steatosis observed in this study was accompanied by a parallel increase in adipocytokine levels. However, a DHA supplementation to the lipodystrophy model diet did not affect adipocytokine levels, compared with those seen with a CLA-containing lipodystrophy model diet. Additionally, serum insulin levels were not changed by DHA supplementation to the CLA-containing lipodystrophy model diet (Fig. 22.7). Ide previously demonstrated that different amounts (1.5, 3, and 6%) of fish oil added to CLA-containing diets dose-dependently downregulated lipogenic parameters and decreased hepatic triglyceride levels in mice (Ide, 2005). In that study, however, CLA-induced hypoleptinemia, hypoadionectinemia, and hyperinsulinemia were alleviated only by the high-dose (6%) fish oil supplementation. These results suggest that in this study, hepatic steatosis induced by the lipodystrophy model diet was attenuated by DHA supplementation through the

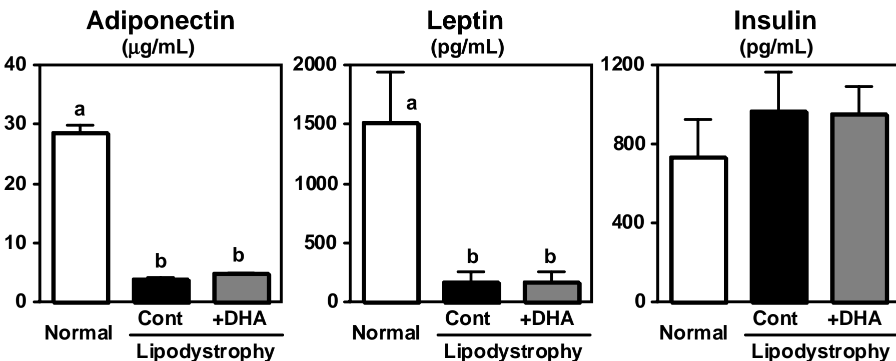

Figure 22.7. Effect of DHA on serum levels of adipocytokines and insulin in diet-induced lipodystrophy model mice.
Mice were fed diets containing either 6% HL-SAF (normal), 4 HL-SAF + 2% CLA (lipodystrophy control), or 3.5% HL-SAF + 2% CLA + 0.5% DHA (lipodystrophy + DHA) for 4 weeks. Values are expressed as mean ± standard error for six mice. Different letters show significant differences at $p < 0.05$.

suppression of hepatic lipogenesis, rather than through a regulation of adipocytokine production and insulin sensitivity in mice.

22.6. CONCLUSIONS

The present study explored the effect of DHA on the pathophysiology of diet-induced lipodystrophy in mice. A four-week diet of CLA-containing lipodystrophy showed hepatic lipid accumulation concomitant with a decrease in adipose tissue weight in mice. However, 0.5% supplementation of DHA to the diet could alleviate hepatic steatosis without affecting the lipoatrophic effect of CLA. The CLA-containing lipodystrophy model diet promoted fatty acid synthesis in the liver, but DHA supplementation significantly attenuated the increase in enzyme activity and mRNA expression induced by the diet. On the other hand, serum adipocytokines were drastically decreased by the feeding of the lipodystrophy model diet, and DHA supplementation did not affect those levels. These results indicate that DHA can attenuate hepatic steatosis through the reduction of hepatic fatty acid synthesis without affecting adipocytokine production in diet-induced lipodystrophy model mice (Yanagita *et al.*, 2005).

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CHAPTER 23

Conversion of Fishery By-products and Waste into Value-added Products: Ongoing Activity in Hokkaido, Japan

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23.1. INTRODUCTION

Local globalization, holding a clue for developing the technology of utilizing fishery by-products and wastes might provide a model developing a global

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technology fishery by-productand waste utilization. Hokkaido Island in northern Japan is one of the largest habitats for shellfish, salmon, and squid. Approximately four hundred thousand tons of scallops per year are landed in Hokkaido. But half of their body weight is occupied by shells. Among the four hundred thousand tons, only about eighty thousand tons are adduct muscle, suitable as food. Internal organs and mantles are discarded. Half of the tissues are also discarded in salmon. And, in late run chum salmon, whole bodies are often discarded after artificial fertilization.

In this paper, written from the useful biomaterial source point of view, the utilization of fishery by-products and wastes on Hokkaido Island are introduced.

23.2. UTILIZING THE SCALLOP SHELL

Scallop shells are used in part as under drain-material, and soil, feed, and sometimes as food additives. Plastic, chalk (Yoshida *et al.*, 2006), asphalt, and road sign paint additives (Yamagishi *et al.*, 2006) are expected to be industrialized shortly.

Smashed scallop shell with water was found to be very useful in removing asbestos: the scallop shell powders have a very high aspect ratio and micro stick shape itself makes plastics or chalks tough. The hot use of scallop shells is its application to an expendable bathythermograph, an XBT, which is a device utilized by a moving ship for obtaining a record of temperature as a function of depth. Instead of measuring temperature with a thermistor within an expendable, weighed casing, scallop shell powder impregnated collagen is substituted for the casing. Demands for XBT or XCTD (a kind of XBT which measures salinity) is predicted to grow drastically in the next decade, because the law of the sea has made it requisite for nations to keep basic oceanographic data. Thus, the development of an environmental friendly XBT is becoming more and more important. Tetsumura and his coworkers (2006) developed an

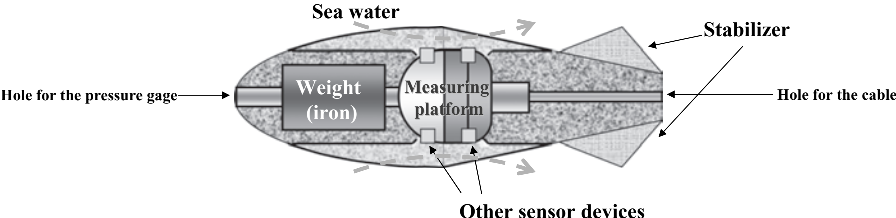

Figure 23.1. Environment friendly XBT* casing produced from scallop shell and collagen (Tetsumura *et al.*, 2006).
*A device for obtaining a record of temperature as a function of depth from a moving ship.

environment friendly XBT casing with fine wire, as illustrated in Figure 23.1. As mentioned, the casing is made of scallop shell powder impregnated collagen, the paired fine wires are made of zein-coated copper. Neither the iron for sinker nor the copper materials is toxic. The platform of the censer circuit is expected to be changed into naturally decomposable resins.

Another notable scallop shell application is for fluorescent materials. Scallop shell and a well-known fluorescent calcite have identical structures and components. Shimono *et al.* (2006) irradiated a roasted scallop shell with ultraviolet, and it gleamed, suggesting that scallop shells could be used as an environmental friendly fluorescent material for audio visual displays or fluorescent lamps.

23.3. UTILIZING SCALLOP DIGESTIVE TRACK ENZYMES

Shellfish eat algae. For this reason, the digestive track of a shellfish is rich in alginate lyases, cellulases, and beta 1,3-glucanases. Digestive juice in the digestive track maybe useful in degrading plant source by-products as well as used paper. The enzymes of shellfish which live in cold seas are generally active even under low temperature (Ojima *et al.*, 2003), soheating equipment for those enzymatic reactions would not be necessary. Another benefit of using low temperature enzymes: spoiling can be prevented by keeping those enzymatic reactions under low temperature. The digestive juices of abalone and scallop rich in those enzymes may degrade crystalline cellulose in plant source by-products into glucose. Through fermentation, esterification, and polymerization processes, environmental friendly plastics and films could be produced from the obtained glucose.

23.4. UTILIZING THE SCALLOP MANTLE

Scallop mantles can be easily separated by machine, as shown in Figure 23.2 (Takahashi, 2005). Other than ccallop mantle processed into fishing bait, or snacks for a drinker, consumption has been very limited. For this reason, purchasing a processing machine might be unprofitable. To fully utilize this kind of machine, a process shown in Figure 23.3 might first, extract native collagen with an acid and pepsin treatment. Then boil the residue to get gelatin. Trypsin can be added to the residue to carry out hydrolysis, and collagen peptides will be obtained. The remaining residue may be applicable to pet foods. Figure 23.4 (Shen *et al.*, 2005) is an example of utilizing collagen peptides which, obtained from scallop mantle, show excellent results in restoring hair damage. As seen in Figure 23.4, the splintery surface is surprisingly restored when rinsed with scallop mantle collagen. It was even more effective than the collagen peptide of bovine.

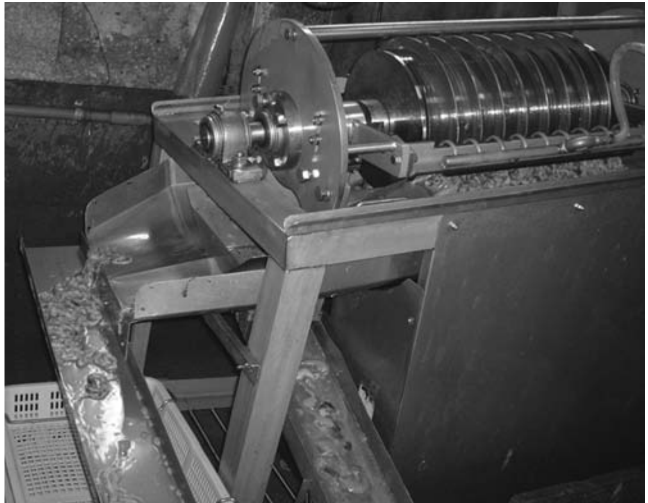

Figure 23.2. Scallop mantle separating machine (Takahashi, 2005).

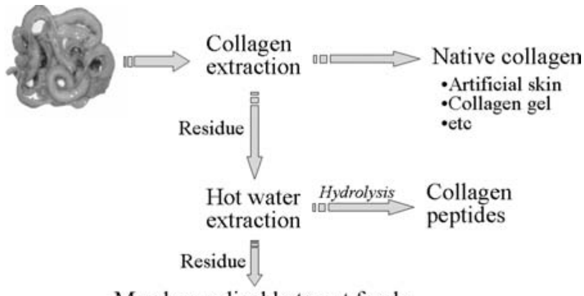

Figure 23.3. Schematic diagram to fully utilize scallop mantle by extracting collagen in a native form, then converting to gelatin and collagen peptides, and finally residues processed into a pet food.

23.5. UTILIZING SQUID INK

The percentage of the weight of the ink sack against the squid's whole body weight is around only 0.3%, but squid ink is extremely useful, being the only edible black ink available, and extremely valuable for printing directly on foods. The crucial point in producing squid ink for ink jet printers is to keep the individual ink particles separate. Lumps such as shown in Figure 23.5 (Ueno *et al.*, 2005) must be separated into individual ink particles, and this has been successfully accompliched by applying a proteolytic enzyme in combination with ultra filtration. First, a screen filtration is done, and then proteolytic enzyme is added to the filtrate, degrading the impurities between

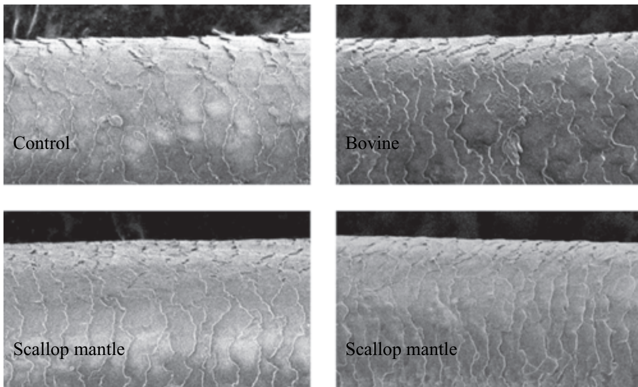

Figure 23.4. Damaged hair surface after rinsing with collagen peptide (Shen *et al.*, 2005).

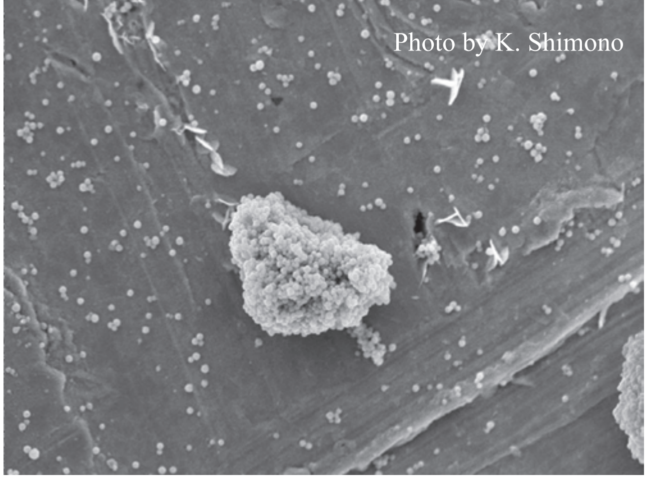

Figure 23.5. Aggregates of squid ink particles (Ueno *et al.*, 2005).

the individual ink particles. These degraded impurities are removed by a suction filtration. Finally, tangent flow hollow fiber filtration removes any impurities.

23.6. UTILIZING β-CHITIN IN SQUID

β-chitin can be extracted from the squid pen, which looks like a spine in the trunk. Squid β-chitin has a physical strength comparable to that of the conventional α-chitin. As seen in Table 23.1 (Takahashi, 2004), braking strain and

TABLE 23.1. Comparison of properties of chitin sheets between squid and crab.

	Stiffness	Burst strength ($kP \cdot m^2/g$)	Braking strain (km)	Expansibility (g/m^2)
Squid chitin sheet	12	6.9	6.9	21.9
Crab chitin sheet	66	3.8	7.1	21.9

Source: Takai and Shimizu, 1995.

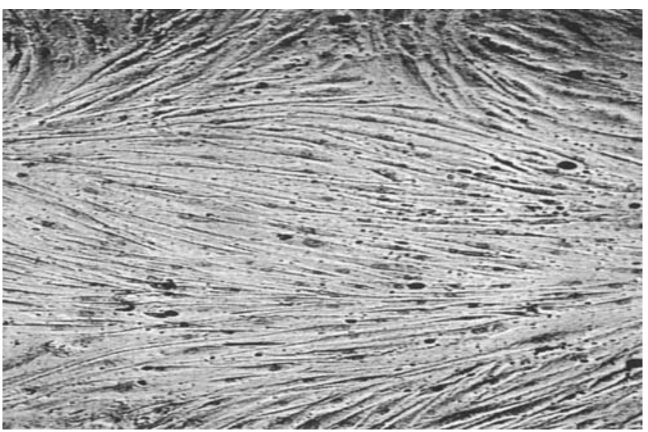

Figure 23.6. Proliferation of fibroblast on salmon skin collagen laminated squid β-chitin sheet (Takai, 1996).

expansibility are comparable between the two chitins. But the squid chitin sheet is softer than that of crab, although the burst strength is superior in squid chitin sheet. As shown in Figure 23.6 (Takai, 1996), a favorable cell growth of fibroblast on a squid β-chitin sheet laminated with salmon skin collagen was confirmed after 8 days. This complex might be developed as an artificial skin for badly burnt person.

23.7. UTILIZING SALMON-ORIGINATED BY-PRODUCTS

Fish scales, including those of salmon align their collagen fibers similar to those found in the cornea. And salmon also have a structure similar to the so called plywood-like structure of the cornea (Fig. 23.7) (Takagi, 2005). But cornea has only a limited ability of regeneration, and so implantation is the only way of restoring the cornea. On the other hand, fish scale has high regeneration ability and might be a good model to study in fabricating materials for corneal regeneration.

Salmon skin or cartilage collagen has been used as food additives, in tablets or powders. Carefully prepared salmon skin collagen is also used as gel for cell growth, since it yelds higher performance than porcine collagen.

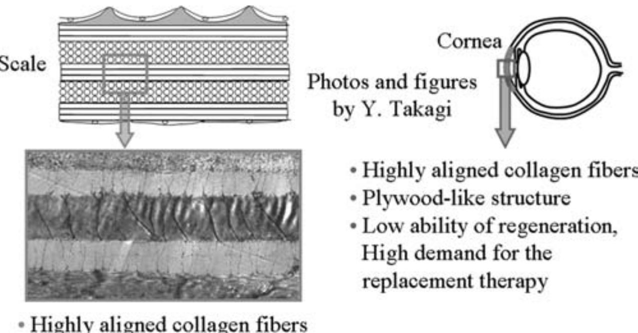

Figure 23.7. Fish scales: a unique model of cornea regeneration (Takagi, 2005).

TABLE 23.2. Antibacterial activity of alginate film with and without salmon testis DNA.

	Ag impregnated alginate film	DNA bounded Ag impregnated alginate film
Ag(μg)	4.21*	21.7*
E. coli	89*	226*
S. aureus	387*	708*

DNA as a carrier of silver ion (Ag) in relation to the Ag impregnation.
Source: Kitamura *et al.*, 1997.
*Inhibition area (mm^2) of cell growth.

Salmon testes have been used as a source of protamine, a naturally occurring food preservative. Other than protamin, DNA from testes has been used as DNA supplements and drinks, as filter impregnates for carcinogen (dioxine) trapping, as an additive for artificial skin, and as a binder, holding silver ion on cloth for medical uses. As shown in Table 23.2 (Kitamura *et al.*, 1997), DNA helps to hold more silver ion on alginate film, suppressing the bacterial growth of *E. coli* or *S. aureus.* This is evident in the larger inhibition area in Table 23.2 (Kitamura *et al.*, 1997), on the DNA added silver ion impregnated alginate film patch contacted portion.

23.8. UTILIZING FISH MEAT DEBRIS, UNUTILIZED FISH MEAT, AND UP STREAM SALMON MEAT BY APPLYING MAILLARD REACTION WITH SACCHARIDES

Fish meat debris by-produced on fish processing lines, unutilized fish meat, and upstream salmon meat are important sources of proteins, but have

practically no commercial value. Sato and coworkers (Sato *et al.*, 2003, 2005) found that those proteins can be converted into useful protein sources by applying the Maillard reaction. The easiest way to do so is by mixing the meat with sugar or glucose. But there are many other methods to produce varieties of glycosylated protein by using alginate oligosaccharides: they can be prepared by applying marine bacteria on brown algae, which causes a spot-wounded disease of *Laminaria*. The glycosylated protein thus obtained can be utilized for beverages (seafood drink), as a functional protein supply for people who cannot masticate foods, as novel textural retort foods, and as nutritional emulsifiers.

23.9. SUPPRESSION OF CANCER USING CHITOSAN-COATED MARINE PHOSPHOLIPID LIPOSOMES

Chitosan is a by-product of crab processing and has been applied in the food, agricultural, and cosmetic industries due to its high availability, high biocompatibility, biodegradability, and ease of chemical modification. Recently scientists have identified its potential application in the pharmaceutical sciences (Singla and Chawla, 2000; Aspden *et al.*, 1995; Schipper *et al.*, 1996). Its lack of toxicity and transmucosal penetration enhancer properties means it can be applied in several drug delivery systems (Paul and Sharma, 2000). It may induce very little cellular toxicity (Berscht *et al.*, 1995; Dornish *et al.*, 1997) and is naturally biodegradable because it is metabolized by lysozymes both *in vitro* and *in vivo* (Onishi and Machida, 1999). It is currently attracting much attention as a possible mediator of transepithelial drug delivery, due to its ability to mediate increased transepithelial drug permeation both *in vitro* (Dodane *et al.*, 1999; Smith *et al.*, 2004) and *in vivo* (Illum *et al.*, 2002; Degim *et al.*, 2003). Furthermore, the residence time of drug carrier systems in the gastrointestinal tract is an important factor controlling the bioavailability of drugs of high molecular weight. Recently, the mucoadhesive function of chitosan has received much attention for prolonging the residence time of dosage forms at the absorption site (Gupta *et al.*, 1990). It has been reported that the stability of chitosan-coated liposomes in stimulated gastric fluid was significantly higher as compared to uncoated liposomes, and it has been proposed that chitosan-coated liposomes might be utilized as a bioadhesive intestinal delivery system (Filipović-Grčic *et al.*, 2001). Thus, a chitosan-coated marine phospholipid may be beneficial in promoting the antitumor activity of the marine phospholipid or the chitosan itself by prolonged retention on intestinal wall. And in fact, this was true for myeloma sp2 tumor bearing mice. Daily oral administration of chitosan-coated liposomes suppressed tumor growth as shown in Figures 23.8 and 23.9. Groups served by Chitosan alone or squid phospholipid alone showed slight tumor suppression through out the experimental term. There was a significant cancer tumor suppression effect when both chitosan and squid phospholipid were administrated simultaneously,

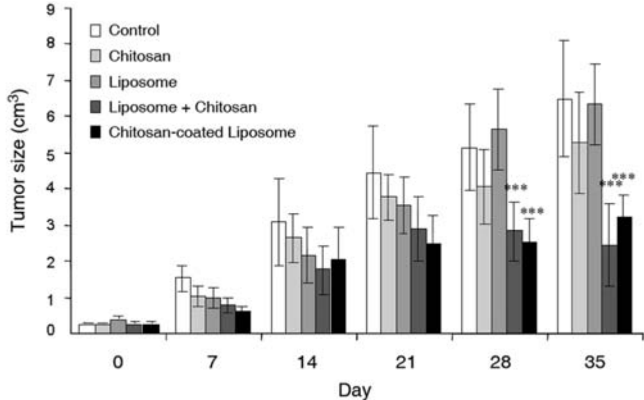

Figure 23.8. Effect of squid meal phospholipid liposome and chitosan on tumor sizes of myeloma sp2 tumors bearing BALB/c mice.
Myeloma sp2 tumor cells (1.5×10^5 cells/mice) were implanted into mice. Twenty days after implantation of the cells, liposomes (1.0 mg/ml), chitosan (5.0 mg/ml), chitosan (5.0 mg/ml)-liposomes (1.0 mg/ml) mixture and chitosan-coated (5.0 mg/ml) liposomes (1.0 mg/ml) were administered for 35 days. The results are the means ± SD (n = 12). Asterisks indicate significant difference as compared to control and liposome (***$P < 0.001$).

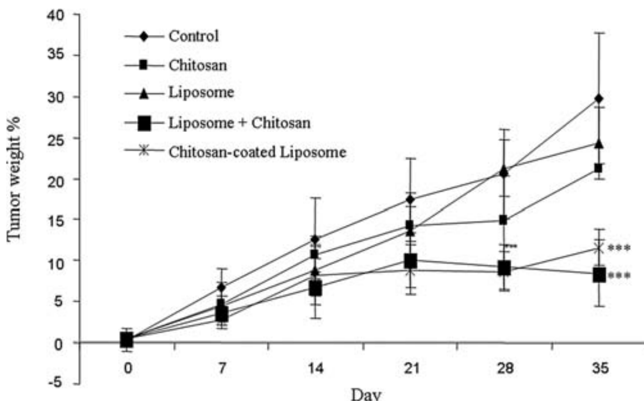

Figure 23.9. Effect of squid meal phospholipid liposome and chitosan on tumor weight (%) against body weight of the myeloma sp2 bearing BALB/c mice.
Twenty days after implantation of the cells, liposomes (1.0 mg/ml), chitosan (5.0 mg/ml), chitosan (5.0 mg/ml)-liposomes (1.0 mg/ml) mixture and chitosan-coated (5.0 mg/ml) liposomes (1.0 mg/ml) were administered for 35 days. The results are the means ± SD (n = 12). Asterisks indicate significant difference as compared to control and liposome (***$P < 0.001$).

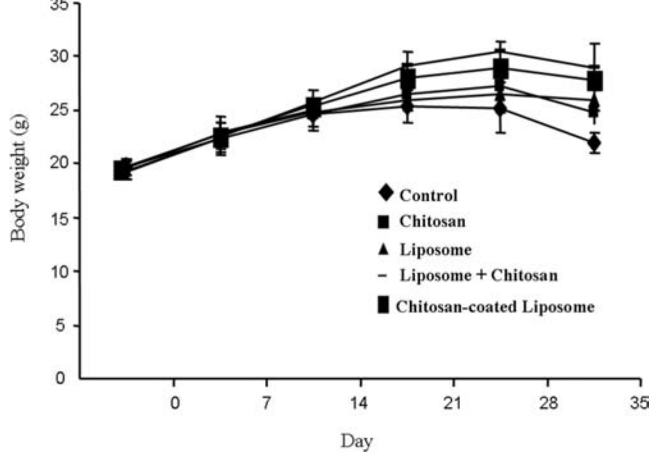

Figure 23.10. Changes in body weight of the myeloma sp2 bearing BALB/c mice during the intake of squid meal phospholipid liposome and chitosan.

both in a simple mixture form, and in a chitosan-coated liposome form. A notable feature, illustrated in Figure 23.10, was that cachexia, serious weight loss due the progression of cancer, was effectively suppressed. The mucoadhesive properties of chitosan-coated liposomes were expected to increase the probability of absorption in the intestinal tract by increasing the residence time. However, the tumor suppression effect of chitosan-coated liposomes, and the simple mixture of the chitosan and the marine phospholipid liposomes, were not clear, perhaps due to the preparation of the chitosan coated liposomes. The simple mixture of chitosan and liposomes would gradually allow liposomes to be chitosan coated. Thus should make it difficult to discriminate the function of the chitosan coated and the chitosan simple mixture. At any rate, it should be said that a longer residence time of the marine phospholipid liposomes lead to prolong therapeutic effect.

23.10. PERSPECTIVES

When extracting chondroitin sulfate from salmon head cartilage, collagen inevitably accompanies it. In this extraction process, from a pharmaceutical point of view, collagen is considered as an impurity. This is a kind of contradiction because if we administrate chondroitin sulfate and collagen simultaneously, the result will be beneficial and effective. Most marine bioresources contain many health beneficial compounds even in the same tissues. For this reason, we should design sequential extraction processes of those useful compounds from the most value added to the poorly valued compounds. For example, the first step for pharmaceutical use should be the mild extraction of compounds. And the final step should extract materials for fertilizers.

However, the useful compounds obtained through those processes still have to compete with prices for corresponding compounds from conventional sources. The technology to utilize fishery by-products or wastes should concentrate on "combining" or "converting." The environmentally friendly XBT or XCTD, artificial skin, chitosan coated marine phospholipid liposome are examples of combining technologies; fluorescent materials, corneal regeneration, and squid ink are converting technologies. Glycosylation may correspond to both. How to make those converting and combining technologies practically viable is no doubt the key to developing the technology of changing fishery by-products or wastes into value-added products.

23.11. ACKNOWLEDGMENTS

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CHAPTER 24

Chemoenzymatic Synthesis of Enantiopure Triacylglycerols

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24.1. INTRODUCTION

Structured triacylglycerols (TAG), saturated medium chain fatty acids (MCFA) at the outer positions and long-chain biologically active polyunsaturated fatty acids (PUFA) at the mid-position of the glycerol backbone, have gained the increased attention of scientists as dietary and health supplements (Miura *et al.*, 1999; Fomuso and Akoh, 1998; Christensen *et al.*, 1995). Recently, we described a highly efficient synthesis of two types of structured lipids by a two-step chemoenzymatic process. First, we examined a range of structured MLM (medium-long-medium) type TAGs comprising a pure saturated MCFA at the terminal positions and pure EPA or DHA at the mid-position (Halldorsson *et al.*, 2003; Haraldsson, 2005; Haraldsson, 2007). Then we studied similarly structured ether lipids (EL) of the 1-O-alkylglycerol type (Haraldsson, 2007).

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Figure 24.1. Chemical structure of MLM' type asymmetrically structured TAGs.

Lipases, owing to their regioselectivity, are ideally suited as biocatalysts for preparing structured TAGs and ELs of the type described (Haraldsson, 2007; Haraldsson and Hjaltason, 2001). By acting preferably or exclusively at the primary alcoholic end-positions of the glycerol backbone lipase may be employed to introduce fatty acids of certain type or composition at these positions by esterification or transesterification. An immobilized *Candida antarctica* lipase (CAL)—displayed excellent regioselectivity toward the end-positions of glycerol and 1-O-alkylglycerols at 0–4 °C using vinyl esters as acylating agents. The n-3 fatty acids were introduced into the remaining mid-position highly efficiently using EDCI as a chemical coupling agent.

A more recent work describes a chemoenzymatic approach to the synthesis of a novel type of structured TAGs. This is an enantiopure MLM' (medium-long-medium') type asymmetrically structured TAG possessing two different types of MCFA ($MCFA_1$ and $MCFA_2$) at the end-positions, and a bioactive PUFA such as EPA or DHA at the *sn*-2 position of the glycerol backbone. Figure 24.1 shows the structure of such asymmetrically structured TAG along with its optical antipode displaying the opposite configuration. This is based on a six-step chemoenzymatic process starting with optically pure solketal involving two lipase steps.

In this chapter we summarize our synthesis of positionally labeled symmetrically structured TAGs of the MLM-type constituting pure homogeneous MCFA and EPA or DHA by a chemoenzymatic approach. The synthesis of similarly structured enantiopure ELs of the 1-O-alkyl-*sn*-glycerol type will also be briefly discussed. The main emphasis will be put on describing the synthesis of asymmetrically structured MLM' type TAGs.

24.2. CHEMOENZYMATIC SYNTHESIS OF MLM-TYPE STRUCTURED TAG

The two-step chemoenzymatic approach starting from glycerol to synthesize the symmetric MLM-type structured TAGs is illustrated in the scheme in Figure 24.2. In the first step lipase regioselectivity was exploited to synthesize symmetric 1,3-diacylglycerols (DAGs) of the MCFA. This was followed by chemical introduction of the PUFA into the mid-position.

1a-d **2a-d** **3a-d**

MCFA = C_5H_{11} (**a**), C_7H_{15} (**b**), C_9H_{19} (**c**), $C_{11}H_{23}$ (**d**)

2: PUFA = EPA
3: PUFA = DHA

Figure 24.2. Synthesis of positionally labeled, symmetrically structured TAGs of the MLM-type constituting MCFA at the end-positions, and EPA or DHA at the mid-position, by a chemoenzymatic approach based on immobilized *Candida antarctica* lipase (CAL).

The immobilized *Candida antarctica* lipase (CAL) displayed superb performance in terms of the regiocontrol and yields of the enzymatic step (Halldorsson *et al.*, 2003; Haraldsson, 2007). This is based on a rapid, irreversible transesterification of glycerol using 1.25-fold stoichiometric amount of vinyl esters of the MCFA in dichloromethane or chloroform at 0–4 °C. Excellent yields (90–92%) were obtained as based on pure material after recrystallization.

The lipase acted exclusively on the glycerol end-positions. The progress of the reaction was monitored by TLC on silica and high-resolution ^{1}H NMR spectroscopy. Only 1-monoacylglycerol (MAG) intermediate was detected in small quantities during the reaction. It took the reaction only 3–5 hours to proceed to completion resulting in quantitative conversion into the desired 1,3-DAGs **1a–1d** (for C_6, C_8, C_{10} and C_{12}, respectively, in accordance with the scheme in Fig. 24.2). Only traces of the 1-MAG intermediate remained, and there were no signs of the undesired 1,2-DAG and 2-MAG regioisomers and TAG present, and thereby neither indications of any acyl-migration side reactions nor the lipase acting at the mid-position.

The subsequent chemical coupling reaction to introduce EPA and DHA into the mid-position of the 1,3-DAG adducts (see Fig. 24.2) was performed at room temperature in dichloromethane. EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride; 1.2 equivalents) was used as a coupling agent in the presence of DMAP (dimethylaminopyridine; 30–50% as based on mol) with an exact stoichiometric amount of EPA or DHA as based on the 1,3-DAG adduct. The reactions were completed in 12–15 hours. Chemically and regioisomerically pure structured TAGs were afforded as colourless and slightly yellowish oils, respectively, for the EPA (**2a–2d**, respectively, for C_6, C_8, C_{10} and C_{12}) and DHA (**3a–3d**, respectively, for C_6, C_8, C_{10} and C_{12}) adducts. The products were all obtained in excellent yields (90–95%), based on isolated and purified material by a chromatography treatment on silica gel. No sign of any acyl-migration side-reaction was observed during the coupling

reaction. All compounds were fully characterized, and their regioisomeric and chemical purity established by traditional modern organic chemistry methods.

High-resolution ^{1}H and ^{13}C NMR spectroscopy played a crucial role in monitoring the regioselectivity control in these reactions and to evaluate the purity of the compounds (Halldorsson *et al.*, 2003; Haraldsson, 2005). Similarly, ^{13}C NMR spectroscopy was also of high use to monitor the regiocontrol of the reactions (Halldorsson *et al.*, 2003; Haraldsson *et al.*, 2000; Halldorsson *et al.*, 2001).

24.3. CHEMOENZYMATIC SYNTHESIS OF ALM TYPE STRUCTURED ETHER LIPIDS

Nonpolar 1-O-alkyl-2,3-diacyl-*sn*-glycerol type ether lipids are major constituents of liver oils of various species of elasmobranch fish including shark and dogfish (Mangold and Palthauf, 1983; Kayama and Mankura, 1998). Shark liver oil has been long used as a therapeutic and preventive agent. Various beneficial effects on human health have been attributed to the 1-O-alkyl-*sn*-glycerols in it (Benveniste and Vargaftig, 1983; Mangold and Weber, 1987), which, it is claimed, prevent radiation sickness from cancer X-ray therapy, stimulate the allergic system as well as immune control, offer beneficial effects against asthma, psoriasis and arthritis, and accelerate the removal of heavy metals from the body (Brohult *et al.*, 1970; Pugliese *et al.*, 1998).

The three most prevalent fatty alcohols present in the 1-O-alkyl moiety of the ether lipids are $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, corresponding to chimyl **4**, batyl **5** and selachyl **6** alcohols, respectively, the last one being the most abundant. As implied by the *sn*-terminology their natural absolute configuration is S (see Fig. 24.3).

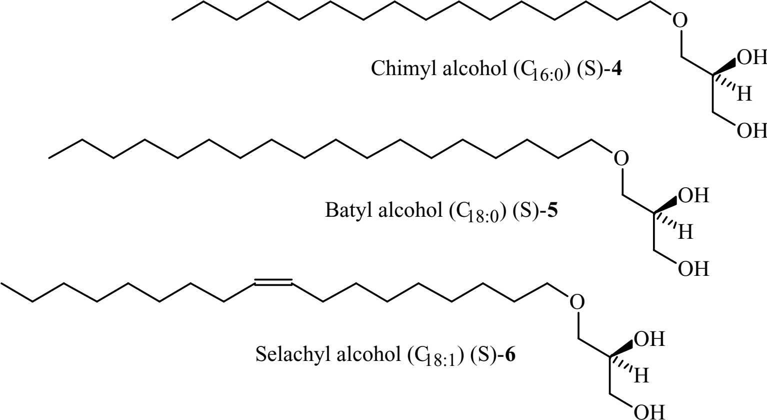

Figure 24.3. Chemical structure of chimyl (**4**), batyl (**5**) and selachyl (**6**) alcohols, the three most prevalent 1-O-alkyl-*sn*-glycerols in shark liver oil.

To combine the beneficial effects of the MLM-type structured TAGs, ether lipids of the 1-O-alkylglycerol type and the long chain n-3 PUFA a chemoenzymatic synthesis of similarly structured ALM type (alkyl-long-medium) ether lipids possessing pure EPA or DHA at the mid-position, and a pure MCFA at the end-position was designed (Haraldsson, 2007). The two-step chemoenzymatic approach is schematically illustrated in Figure 24.4 starting from optically pure chimyl, batyl and selachyl alcohols (S)-**4**, (S)-**5** and (S)-**6**, respectively. The starting materials were made in two steps in excellent yields using optically pure (R)-solketal (isopropylideneglycerol) as a chiral precursor (Haraldsson, 2007; Halldorsson *et al.*, 2004). The ether moiety was introduced by alkylation using the corresponding alkyl bromides with grounded potassium hydroxide as a base in the presence of tetrabutylammonium bromide as a phase-transfer catalyst and a subsequent deprotection of the isopropylidene moiety under acidic aqueous conditions in THF (Halldorsson *et al.*, 2004).

As in the corresponding structured TAG synthesis, the immobilized *Candida antarctica* lipase preserved outstanding regioselectivity toward the *sn*-3 position of the 1-O-alkyl-*sn*-glycerols **4–6** at 0–4 °C using vinyl esters of the MCFA (**a**, **b**, **c** and **d** for C_6, C_8, C_{10} and C_{12}, respectively) as acylating agents. This resulted in the 1-O-alkyl-3-acyl-*sn*-glycerol adducts that were obtained optically pure of high chemical and regioisomeric purity in excellent yields (90–98%) for the chimyl, batyl, and selachyl adducts (R)-**4a–4d**, (R)-**5a–5d** and (R)-**6a–6d**, respectively. The yields are based on purified material after recrystallization from hexane or petroleum ether.

The monoacylation reactions were performed under similar conditions as described for glycerol in dichloromethane, but more solvent was needed this

(S)-**4**: R = $C_{16}H_{33}$
(S)-**5**: R = $C_{18}H_{37}$
(S)-**6**: R = $C_{18}H_{35}$

CAL

(R)-**4a-d**
(R)-**5a-d**
(R)-**6a-d**

EDCI
DMAP

(R)-**7a-d** (R)-**10a-d**
(R)-**8a-d** (R)-**11a-d**
(R)-**9a-d** (R)-**12a-d**

MCFA = C_5H_{11} (**a**), C_7H_{15} (**b**), C_9H_{19} (**c**), $C_{11}H_{23}$ (**d**)

7: PUFA = EPA; R = $C_{16}H_{33}$ **10**: PUFA = DHA; R = $C_{16}H_{33}$
8: PUFA = EPA; R = $C_{18}H_{37}$ **11**: PUFA = DHA; R = $C_{18}H_{37}$
9: PUFA = EPA; R = $C_{18}H_{35}$ **12**: PUFA = DHA; R = $C_{18}H_{35}$

Figure 24.4. Synthesis of enantiomerically pure, positionally labeled, structured ether lipids of the ALM type by chemoenzymatic approach, starting from enantiomerically pure chimyl (S-**4**), batyl (S-**5**) and selachyl (S-**6**) alcohols.

time to dissolve the 1-O-alkylglycerols (Haraldsson, 2007). There were no indications of any acyl-migration products nor of the lipase acting at the mid-position. 1-O-Alkylglycerols are less prone to undergo acyl-migration than the corresponding 1-MAGs and 1,3-DAGs (Haraldsson and Thorarensen, 1994).

Pure EPA and DHA were introduced to the remaining *sn*-2 position highly efficiently using EDCI as a chemical coupling agent in the presence of DMAP. All products, the chimyl adducts (R)-**7a–7d** and (R)-**10a–10d**, the batyl adducts (R)-**8a–8d** and (R)-**11a–11d,** and the selachyl adducts (R)-**9a–9d** and (R)-**12a–12d**, respectively, for EPA and DHA, in accordance with the reaction scheme in Figure 24.4 were isolated in excellent yields (90–96%) and fully characterized by traditional organic chemistry synthesis methods. The yields are based on purified material after silica gel column chromatography. As before, high-resolution ^{1}H and ^{13}C NMR spectroscopy was used to monitor the regioselectivity and regiocontrol of these reactions in a similar manner as described for the corresponding chemoenzymatic synthesis of the MLM-type structured TAGs.

24.4. SYNTHESIS DESIGN OF ENANTIOPURE MLM’ TYPE ASYMMETRICALLY STRUCTURED TAG

Our primary goal was to design a viable synthesis of asymmetrically labeled MLM’ type structured lipids and to use that approach to synthesize a selected variety of such enantiopure MLM’ type TAGs. We were interested in a set of compounds representing all variations of medium chain (C_6, C_8, C_{10} and C_{12}) saturated fatty acids fulfilling the requirement of chirality, *i.e.*, M is not equal to M’, for both EPA and DHA located at the *sn*-2 position. However, our interest was not necessarily confined to the MCFA, since we were also interested in a whole range of C_2–C_{18} saturated fatty acids including short chain (C_2 and C_4) and longer chain (C_{14}, C_{16} and C_{18}) fatty acids.

Such compounds may be used for various purposes including screening for biological effects of individual fatty acids, possibly related to their location in stereochemical positions of the glycerol backbone. They may also be used as chiral substrates to investigate lipase enantioselectivity towards TAGs, at which time they may find application as standards, fine chemicals, drug supplements, drug carriers, or even as drugs. The methodology may also be utilized to introduce isotopically labelled fatty acids into labelled positions of TAG.

The following essential requirements had to be fulfilled in the synthesis: Pure organic single compounds, the final TAG products possessing three different pure fatty acids in each and every position of the glycerol moiety (ABC type structured TAG). Also, strict regio- and enantiopurity is required with the chemical purity, enantiopurity and regiopurity fully established for all isolated compounds.

Figure 24.5. A fully enzymatic, "ideal" three-step synthetic route to the synthesis of enantiopure MLM' type asymmetrically structured TAGs.

The work described herein is limited to the synthesis of all adducts accommodating C_{18} fixed at the *sn*-1 position with all variations of C_2–C_{16} at the *sn*-3 position as well as the corresponding optical antipodes (C_{18} at the *sn*-3 position with all variations of C_2–C_{16} at the *sn*-1 position). This means 16 asymmetric 1,3-DAG adducts. All products have been made with EPA located at the sn-2 position for all these adducts and they fully characterized. Similar synthesis of the corresponding similarly structured DHA analogous compounds is now under way.

An "ideal" synthetic approach could involve an all enzymatic three-step process starting from prochiral glycerol (Fig. 24.5). In the first step a lipase ("lipase 1") acts highly regio- and enantioselectively at the *sn*-1 (or *sn*-3) position of glycerol to afford an enantiopure 1-O-acyl-*sn*-glycerol adduct 1-MAG (S) (or the *sn*-3 enantiomer (R) depending on the enantiopreference of the lipase). A second lipase ("lipase 2") subsequently acts highly regioselectively or exclusively at the non-accommodated *sn*-3 position to introduce a different fatty acyl moiety at that position to afford the asymmetric 1,3-DAG (S or R configuration depending on the two different acyl groups). No enantioselectivity is needed for this lipase. In the third step a third lipase ("lipase 3") introduces a pure PUFA (EPA or DHA) to the *sn*-2 position to accomplish the desired MLM' type asymmetrically structured TAG product.

Despite its simplicity this route is not easily accessible. Lipases can act enantioselectively on glycerol derivatives, but their enantioselectivity is usually rather low. And, there is no such report for an efficient enough direct acylation of non-substituted glycerol. Lipases of the two type, on the other hand, are known, one of the most efficient being CAL as has been demonstrated in our previously described synthesis of structured MLM-type TAGs and ALM type ether lipids (Halldorsson *et al.*, 2003; Haraldsson, 2005, 2007). The third type of lipase is certainly not available when PUFAs such as EPA or DHA are involved.

Lipases act enantioselectively on TAG molecules, but usually the enantiopreference between the *sn*-1 and *sn*-3 positions is only moderate or relatively low (Rogalska *et al.*, 1990; Villeneuve *et al.*, 1995; Villeneuve *et al.*, 1996; Lang and Dijkstra, 1998; Rogalska *et al.*, 1993; Lang *et al.*, 1993; Uzawa *et al.*, 1990, 1993). Based on such lipase enantioselectivity there have been attempts to prepare asymmetric AAB and ABC type structured TAGs, respectively from AAA and ABA type TAGs, using immobilized *Rhizomucor miehei* lipase in an acidolysis reaction (Chandler *et al.*, 1998; Iwasaki and Yamane, 2000). Although moderate enantioselectivity was obtained in these attempts, enantiopurities were nowhere close to our requirements. However, excellent enantioselectivity (>99% enantiomeric excess or ee) was obtained in a recently reported enantioselective ethanolysis of homogeneous TAG using the immobilized *Rhizomucor miehei* lipase (Piyatheerawong *et al.*, 2006). That lipase displayed a strong enantiopreference for the *sn*-1 position of the TAGs. The performance of the lipase was observed to depend largely on the chain length of the fatty acids and the best results were obtained for the C_8 and C_{10} adducts. For the C_8 adduct a glyceride mixture consisting of 61% optically pure *sn*-2,3-DAG, 21% 2-MAG and 18% unreacted TAG was obtained in nearly 90% recovery after only 20 min reaction. Apparently, no effort was made to purify the optically pure 2,3-DAG constituent, but according to these data yields over 50% may be expected. Some glycerol was also formed by total ethanolysis. All this clearly demonstrates the complexity involved in such processes involving a potential mixture of the desired optically pure *sn*-2,3-DAG, 2-MAG, unreacted TAG and glycerol, and that a compromise is needed to optimize the yields of the desired adduct.

There are no reports thus far of an enantioselective direct acylation on non-substituted prochiral glycerol involving fatty acids and lipase to discriminate between the *sn*-1 and *sn*-3 positions (Halldorsson *et al.*, 2003; Haraldsson *et al.*, 1995). However, there is a report on a direct acylation of glycerol using various commercially available lipases and vinyl benzoate in 1,4-dioxane as a solvent (Kato *et al.*, 2000). The best results were obtained with an immobilized *Candida antarctica* lipase displaying enantiopreference for the *sn*-3 position, and offering 54% ee and 94% yield on a multi-gram scale. The optical activity was brought up to 95% by a single recrystallization.

There are, however, numerous reports on asymmetric transformations involving lipase and variously substituted glycerol derivatives. These include acylation of prochiral 2-O-benzylglycerol (Wang and Wong, 1988), kinetic resolution of racemic 1-O-benzylglycerol (Guanti *et al.*, 2004), 1-O-alkylglycerols (Halldorsson *et al.*, 2004; Ransac *et al.*, 1990) and a whole variety of 1-O-arylglycerols (Theil *et al.*, 1995). In the case of many of the above 1-O-monosubstituted glycerol derivatives, a sequential acylation was required to obtain satisfactory enantioselection with the first acylation taking place at the preferred primary alcoholic end-position with very low enantioselectivity, but a much higher enantioselectivity in the second acylation taking place at the secondary mid-position.

Regarding "lipase 3," a lipase acting directly on the mid-position is not a straightforward issue either. There are no reports on such direct introduction of EPA or DHA to the mid-position of 1,3-DAG by lipase and, in fact, there are surprisingly few reports of lipases acylating the mid-position of 1,3-DAG involving fatty acids. There is a recent report from Bornscheuer and coworkers on esterification of 1,3-dilaurin and 1,3-dicaprylin with oleic acid and its vinyl ester using various lipases (Wongsakul *et al.*, 2004). Conditions requiring 60 °C caused significant acyl-migration resulting in losses of regiocontrol. It is clear from our previous studies that lipases strongly prefer acting on the terminal positions as compared to the mid-position. In our previous synthesis of homogeneous TAGs comprised of pure EPA and DHA by direct esterification of glycerol with free acids there was convincing evidence that acylation at the mid-position took place by an intramolecular acyl-migration from an occupied end-position to the mid-position rather than the lipase acting directly on that position (Haraldsson *et al.*, 1995). This reluctance of a lipase to act directly on the mid-position is also supported by our more recent synthesis of the MLM-type structured TAGs involving the use of vinyl esters (Halldorsson *et al.*, 2003). Both cases involve CAL that is claimed to be a non-regioselective lipase. Furthermore, preliminary attempts using various lipases to introduce simple saturated fatty acids such as MCFA to the mid-position of 1,3-DAG have failed (Sigmarsdottir, U. and Haraldsson, G.G., unpublished results). The reactions were conducted at room temperature and no reactions were observed. It is possible that higher temperature is needed, making it difficult to avoid acyl-migration.

There is little doubt that lipases claimed to be non-regioselective display activity towards the secondary alcoholic mid-position of TAGs, but this may well be different when it comes to re-esterification of that position with fatty acids or their derivatives. Interestingly enough, there is a recent report on conversion of a carboxylesterase into a triacylglycerol lipase by random mutation (Reyes-Duarto *et al.*, 2005). The hydrolytic enzyme was observed to display preference for the *sn*-2 position of triacylglycerols in an ethanolysis reaction.

Therefore it was evident that a protection-deprotection based approach would be required for our intended task. One approach is proposed in Figure 24.6. That four-step chemoenzymatic approach is based on the use of a chiral enantiomerically pure solketal as a starting material. A MCFA ester group was easily introduced to the *sn*-1 position to afford the protected MAG adduct using CAL and MCFA vinyl esters. The idea was then to remove the isopropylidene protective moiety using mild acidic aqueous conditions, followed by introduction of a different acyl group to the *sn*-3 position by the same lipase, and a subsequent chemical coupling to introduce the PUFA (EPA or DHA) into the *sn*-2 position. As had been anticipated the problem here was the deprotection step, since the use of acidic condition resulted in an acyl migration of the MCFA group in the resulting 1-MAG to the corresponding 2-MAG. This resulted in a mixture of approximately 90% 1-MAG

Figure 24.6. A proposed four-step chemoenzymatic route to the synthesis of enantiopure MLM' type asymmetrically structured TAGs.

and 10% 2-MAG, which is close to a reported equilibrium (Compton *et al.*, 2007).

24.5. CHEMOENZYMATIC SYNTHESIS OF ENANTIOPURE MLM' TYPE ASYMMETRICALLY STRUCTURED TAG

Consequently, a more extensive multi-step protection-deprotection approach was needed as is illustrated in Figure 24.7. This is the route that we successfully followed, based on a six-step chemoenzymatic process starting from optically pure solketal. The first four steps were needed for sorting out the enantiocontrol of the synthesis involving a benzyl ether protection of the free hydroxyl group, deprotection of the isopropylidene moiety, a highly regioselective introduction of a pure MCFA to the primary hydroxyl group of the resulting 1-O-benzyl-*sn*-glycerol by CAL, and a catalytic hydrogenolysis of the benzyl protective group to afford a regioisomerically pure 1-MAG. The remaining part of the synthesis was rather straightforward by subsequent introduction of a second different MCFA (or a short or long chain saturated fatty acid) exclusively to the vacant primary hydroxyl group by CAL, providing an asymmetric regioisomerically pure 1,3-DAG that was finally acylated at the mid position by pure EPA or DHA by the previously described EDCI promoted coupling reaction.

In order to demonstrate that the route proposed in Figure 24.7 was a viable approach to the synthesis of such asymmetrically structured MLM' type TAGs we kept the *sn*-1 position fixed with stearic acid (by no means a MCFA, but providing crystalline intermediate adducts while sorting out the chemistry) and then introduced the whole range of C_2–C_{16} saturated fatty acids to the *sn*-3 position. EPA was subsequently introduced to the *sn*-2 position. The main challenges when bringing about such synthesis are enantiocontrol, regiocontrol, and regiopurity by avoiding acyl-migration. This requires a support by

$MCFA_1 = C_{17}H_{37}$

$MCFA_2 = CH_3$ (**a**), C_3H_7 (**b**), C_5H_{11} (**c**), C_7H_{15} (**d**), C_9H_{19} (**e**), $C_{11}H_{23}$ (**f**), $C_{13}H_{27}$ (**g**), $C_{15}H_{31}$ (**h**)

Figure 24.7. A six-step chemoenzymatic route to the synthesis of enantiopure MLM' type asymmetrically structured TAGs.

firm analytical methods, and full purification and characterization of all intermediate adducts and final products by traditional synthetic organic chemistry methods including high-resolution ^{1}H and ^{13}C NMR and IR spectroscopy, as well as high-resolution accurate mass spectrometry analyses (Halldorsson *et al.*, 2003).

A full enantiocontrol was secured by the use of commercially available enantiopure solketal possessing correct stereochemistry as a starting material. Regioselective lipase was used to control the regiochemistry. Regiopurity was maintained by the mild conditions offered by the lipase acting at low temperature. The good success is believed to relate predominantly to the enzyme displaying a superb regioselectivity and elimination of any acyl-migration side-reaction (Halldorsson *et al.*, 2003; Haraldsson, 2007). The acyl-migration is interrelated to various important factors including temperature, apparently the most crucial single parameter, fast and efficient reaction, support material of enzyme, type of reaction, acyl donor and reaction conditions. The temperature was maintained low enough to keep the acyl-migration and lipase regioselectivity completely under control with the lipase still acting fast enough, since the acyl-migration process is clearly time-dependent. Activated vinyl esters offered a fast and irreversible reaction (Halldorsson *et al.*, 2003).

In the first step sodium hydride was used as a base in THF to introduce a benzyl protective group to the *sn*-3 position of the glycerol moiety by a Williamson ether synthesis using benzyl bromide. The benzylated solketal adduct was not isolated but introduced to deprotection of the isopropylidene

protective moiety using acidic aqueous THF at room temperature. The resulting 3-O-benzyl-*sn*-glycerol adduct (R)-**13** was isolated by kugelrohr distillation *in vacuo* and fully characterized. The overall two-step yield was 87% as based on purified material after distillation.

The benzylated glycerol adduct (R)-**13** was subsequently acylated with the $MCFA_1$ (C_{18}) moiety using the immobilized *Candida antarctica* lipase (CAL) and the vinyl ester at room temperature in dichloromethane. In this step we were "playing on our homegrounds" and clearly benefiting from our previous experience with the highly regioselective acylation of the structurally analogous 1-O-alkyl-*sn*-glycerols described earlier. The benzyl ether intermediate adduct was not isolated but introduced to Pd/C promoted catalytic hydrogenolysis deprotection of the benzyl protective group in THF/hexane. As had been anticipated no acyl migration took place during this reaction. The overall two-step yield of the enantiopure 1-MAG (S)-**14** was 81% after crystallization from hexane. Table 24.1 shows the yields and specific rotation of the intermediate adducts (R)-**13** and (S)-**14**.

It is of interest to compare the specific optical rotation of the enantiopure 1-MAG (S)-**14** in Table 24.1 to the corresponding values obtained for the enantiopure 1-O-alkyl-*sn*-glycerols, especially the value for batyl alcohol (S)-**5** possessing an alkyl group of the same chain length as the acyl group in the 1-MAG adduct. Both the sign of rotation and the numeric values are comparable as can be noticed from Table 24.2 showing yields and specific optical rotation for some of the relevant batyl alcohol adducts from our previous synthesis described in section 24.3 of this paper. This comparison, however, warrants a special comment since the specific rotation of the 1-O-

TABLE 24.1. Yields and specific rotation of intermediate adducts (R)-13 and (S)-14.

Compound	$MCFA_1$	Yields (%)	$[\alpha]_D^{20}$
(R)-**13**	—	87	+5.52[1)]
(S)-**14**	$-C_{17}H_{35}$	81	+2.43[2)]

[1)]c = 20 in chloroform; [2)]c = 6 in THF.

TABLE 24.2. Yields and specific rotation of batyl alcohol (S)-5 ($-C_{18}H_{37}$ alkyl group at *sn*-1), its monoacyl adduct (R)-5c ($MCFA_1 = -C_9H_{19}$ at *sn*-3), and diacyl ALM type structured ether lipid final product (R)-8c ($MCFA_1 = -C_9H_{19}$ at *sn*-3; EPA at sn-2).

Compound	$MCFA_1$	PUFA	Yields (%)	$[\alpha]_D^{20}$
(S)-**5**	—	—	80[1)]	+2.2[2)]
(R)-**5c**	$-C_9H_{19}$	—	90	–2.9[3)]
(R)-**8c**	$-C_9H_{19}$	EPA	93	–3.4[4)]

[1)]Overall in two steps; [2)]c = 0.8 in chloroform; [3)]c = 2.0 in benzene; [4)]c = 5.2 in chloroform.

alkylglycerols has been observed to be clearly dependent on concentration (Baer and Fischer, 1941). This may be related to aggregation properties of the 1-O-alkylglycerols and it will certainly be of interest to study the enantiopure 1-MAG further in that respect.

In the second enzymatic step CAL was used again, but this time to acylate exclusively at the *sn*-3 position of the 1-MAG with the range of C_2–C_{16} fatty acids using their vinyl esters in dichloromethane at 0–4 °C. Here we were also benefiting from our previous experience in synthesizing the symmetric 1,3-DAGs from glycerol using CAL and vinyl esters of MCFA involving 1-MAG as intermediates. Only one hour was needed to complete the reaction, conducted at 0–4 °C as before. This is in good agreement with our previous observation that only traces of 1-MAGs were present during the two-step process suggesting that 1-MAG, once formed, reacts much faster than glycerol with the MCFA vinyl esters under the reaction condition used. The enantiopure asymmetric 1,3-DAG adducts (S)-**15a–15h** were all purified by crystallization from hexane. Their yields ranging from 83–92% after purification are shown in Table 24.3 along with their specific rotation.

In the final step chemical coupling with EDCI and DMAP was used to introduce pure EPA into the *sn*-2 position of adducts (S)-**15a–15h** to afford the asymmetrically structured MLM' type TAG final products (S)-**16a–16h** in high to excellent yields (78–92%) after purification using silica gel chromatography. This can be noticed from Table 24.4 also showing the specific rotation for each product. The reaction was conducted in dichloromethane at room temperature for 12–15 hours. Stoichiometric amount of EPA was used, 20% molar excess of EDCI and 0.4 equivalents of DMAP. As previously observed, no acyl migration took place during this reaction.

Specific optical rotation values are extremely low for this type of compound, both the 1,3-DAG adducts and the TAG products. For the asymmetric 1,3-DAG (S)-**15a–15h** the values range from –0.18 to +0.08 and for the corresponding asymmetric EPA TAG adducts (S)-**16a–16h** the range is –0.13 to +0.16. This is significantly lower than for the corresponding chimyl, batyl, and

TABLE 24.3. Yields and specific rotation of 1,3-DAG adducts (S)-15a–15h ($MCFA_1$ = $-C_{17}H_{35}$ at *sn*-1).

Compound	$MCFA_2$	PUFA	Yields (%)	$[\alpha]_D^{20}$[1)]
(S)-**15a**	$-CH_3$	—	83	–0.18
(S)-**15b**	$-C_3H_7$	—	84	–0.04
(S)-**15c**	$-C_5H_{11}$	—	89	+0.08
(S)-**15d**	$-C_7H_{15}$	—	85	+0.06
(S)-**15e**	$-C_9H_{19}$	—	87	+0.02
(S)-**15f**	$-C_{11}H_{23}$	—	92	+0.01
(S)-**15g**	$-C_{13}H_{27}$	—	88	+0.01
(S)-**15h**	$-C_{15}H_{31}$	—	91	+0.01

[1)] c = 10 in dichloromethane.

TABLE 24.4. Yields and specific rotation of TAG products (S)-16a–16h ($MCFA_1$ = $—C_{17}H_{35}$ at *sn*-1).

Compound	$MCFA_2$	PUFA	Yields (%)	$[\alpha]_D^{20}$ [1]
(S)-**16a**	$-CH_3$	EPA	78	–0.13
(S)-**16b**	$-C_3H_7$	EPA	90	–0.07
(S)-**16c**	$-C_5H_{11}$	EPA	84	+0.03
(S)-**16d**	$-C_7H_{15}$	EPA	88	+0.08
(S)-**16e**	$-C_9H_{19}$	EPA	91	+0.16
(S)-**16f**	$-C_{11}H_{23}$	EPA	89	+0.09
(S)-**16g**	$-C_{13}H_{27}$	EPA	92	+0.06
(S)-**16h**	$-C_{15}H_{31}$	EPA	82	+0.02

[1] c = 10 in dichloromethane.

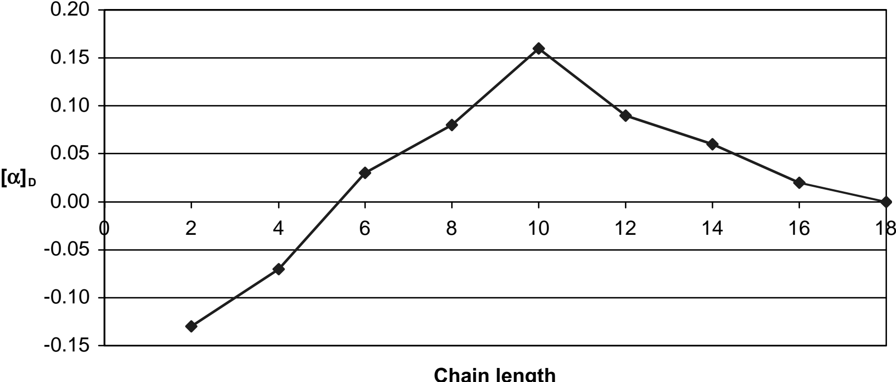

Figure 24.8. Graphic presentation of the variation in specific optical rotation values for enantiopure MLM' type asymmetrically structured TAG products **16a–16h**, when varying the chain length of the saturated fatty acid located at the *sn*-3 position of the glycerol backbone.

selachyl ether lipid adducts, where the specific rotation values roughly range between –2 to –4 as can be noticed for some comparable batyl alcohols deriviatives in Table 24.2. Despite the low specific rotation values it is of great interest to examine the behavior of these values when the $MCFA_2$ counterpart is varied from C_2 to C_{16} keeping the $MCFA_1$ acyl moiety fixed as C_{18}. This is graphically presented for the final products **16a–16h** in Figure 24.8. It is of interest to notice that for the shortest C_2 and C_4 chains the specific rotation values are negative, but from there onwards they turn positive. Maximum value was obtained for the C_{10} adduct, but from there onwards the values dropped towards zero. This is quite a striking behavior and similar observations were made for the intermediate 1,3-DAG adducts **15a–15h** as may be noticed from Table 24.3.

Finally, all corresponding optical antipodes of enantiopure TAG of the MLM' type described above have also been successfully synthesized using optically pure solketal of opposite configuration (R) as a chiral precursor. Otherwise the methodology was identical and very similar results were obtained with reverse signs of the specific rotation values as was anticipated.

24.6. ACKNOWLEDGMENTS

The University of Iceland Research Fund provided financial support, Novozyme AS in Denmark the lipase, and Olav Thorstad of Pronova Biocare ASA in Norway provided pure EPA and DHA. Björn Kristinsson efficiently synthesized the enantiopure MLM' type structured TAGs; and Carlos D. Magnusson provided valuable information. We would also like to acknowledge and thank the organizers of the 2006 IBBS at Taichung University in Taiwan for their invitation to participate in the symposium, and for their great hospitality during our stay in Taiwan.

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CHAPTER 25

Biosynthesis of Castor Oil Studied by the Regiospecific Analysis of Castor Triacylglycerols by ESI-MS

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25.1. INTRODUCTION

The presence of the hydroxy group on ricinoleate (12-hydroxy oleate) underlies many industrial uses, such as the manufacture of lithium grease, plastics, paints, coatings, and cosmetics. Castor oil contains 90% of its fatty acids (FA) as ricinoleate (Achaya *et al.*, 1964) and is the only commercial source of ricinoleate. Since the castor bean contains the toxin ricin and potent allergens, it

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is hazardous to grow, harvest, and process, and producing ricinoleate from a transgenic oilseed lacking these toxic components would be desirable. The cDNA for oleoyl-12-hydroxylase (the enzyme catalyzing the hydroxylation of oleate to ricinoleate) has been cloned from castor and expressed in tobacco, resulting in accumulation of low levels of ricinoleate in seed lipid (van de Loo *et al.*, 1995). The later expression of this enzyme in a transgenic *Arabidopsis thaliana* plant was improved, but still resulted in low levels of hydroxy FA (Broun and Somerville, 1997) compared to castor oil (20% *vs.* 90% ricinoleate in castor oil). To develop a transgenic plant that produces seed oil containing high level of ricinoleate, it is important to know the biosynthetic pathway of castor oil, and to identify the key enzymatic steps that drive ricinoleate into triacylglycerol (TAG) in the castor bean.

We have identified and quantified 16 molecular species of acylglycerols (AG) in castor oil by C_{18} HPLC and evaporative light scattering detector (ELSD) (Lin *et al.*, 2003). The content of triricinolein (RRR) in castor oil was 71%. The contents of diricinoleoyl-acyl-glycerols (RRAc) containing non-ricinoleoyl chains are as follows: diricinoleoyl-oleoyl-glycerol (RRO, 7.2%), diricinoleoyl-linoleoyl-glycerol (RRL, 6.6%), diricinoleoyl-linolenoyl-glycerol (RRLn, 0.15%), diricinoleoyl-stearoyl-glycerol (RRS, 1.1%), diricinoleoyl-palmitoyl-glycerol (RRP, 1.6%) and diricinoleoyl-lesqueroloyl-glycerol (RRL, 0.7%). The identity of these molecular species of AG in castor oil has been confirmed recently by LC-ESI-MS (Lin *et al.*, 2006). Knowing the stereospecific locations of the non-ricinoleoyl chains in these RRAc will help to interpret the biosynthetic pathway and predict the physical properties of castor oil relevant to industrial uses. The regiospecific characterization of triacylglycerols (TAG) as lithiated adducts by collisionally activated dissociation tandem mass spectrometry (CAD-MS^2) has been reported (Hsu and Turk, 1999). We used a similar technique, MS^3, to determine the regiospecific location of non-ricinoleoyl acyl groups (Ac) in RRAc.

25.2. BIOSYNTHESIS OF CASTOR OIL

Triricinolein (RRR) constitutes about 70% of TAG in castor oil (Lin *et al.*, 2003). Recently, a new tetraacylglycerol, (12-ricinoleoylricinoleoyl)diricinoleoylglycerol (RRRR), in castor oil was identified and its content was about 0.5% (Lin *et al.*, 2006). These two AG contain ricinoleate only without other FA. The biosynthetic pathway of RRR and RRRR in the castor bean has been established and the key enzymatic steps driving ricinoleate into RRR and RRRR have been identified (Lin *et al.*, 1998, 2000, 2002, 2006) as shown in Figure 25.1. Oleate of oleoyl-CoA is incorporated into 2-oleoyl-phosphatidylcholine (2-oleoyl-PC) by lysophosphatidylcholine-acyltransferase and then hydroxylated to 2-ricinoleoyl-PC by 12-oleoyl-hydroxylase in castor microsomes (Fig. 25.1) (Lin *et al.*, 1998). 2-Oleoyl-PC is also desaturated to 2-linoleoyl-PC and then to 2-linolenoyl-PC (Lin *et al.*, 1998) by desaturases.

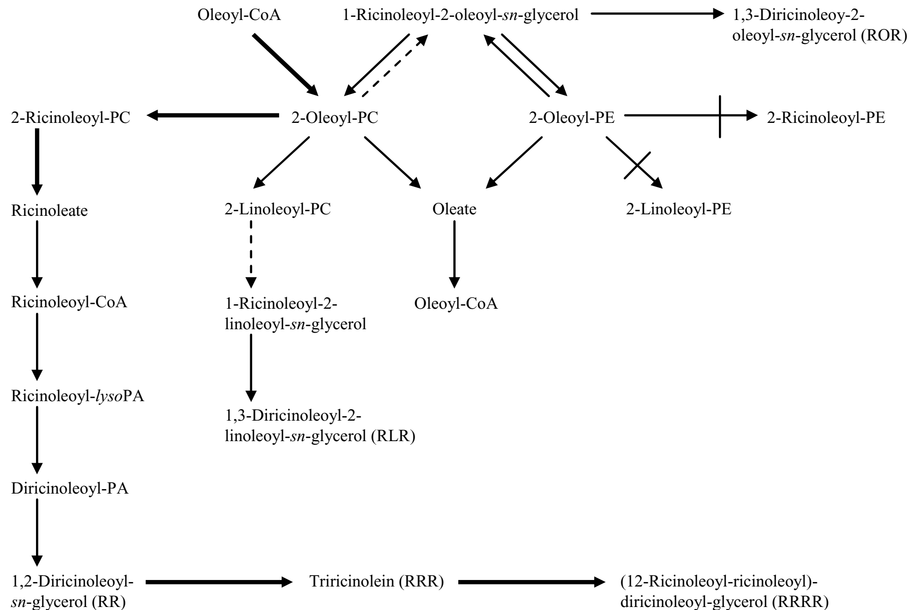

Figure 25.1. Proposed biosynthetic pathway of castor oil. Heavy arrows show the key enzyme steps driving ricinoleate into acylglycerols. Two arrows with solid bars show a complete block. Two dashed arrows show the phospholipase C hydrolysis which can be targeted to block the incorporation of non-hydroxyl fatty acids into triacylglycerols to increase presumably the content of ricinoleate in transgenic seed oils.

1-Acyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (2-oleoyl-2-PE) can not be used as the immediate substrate for the hydroxylase and desaturase (Lin *et al.*, 2000). However, the conversion of 2-oleoyl-PC to 2-oleoyl-PE and *vice versa* has been demonstrated (Lin *et al.*, 1998, 2000). In addition to oleoyl-12-hydroxylase, we have also identified phospholipase A_2 and 1,2-diacyl-*sn*-glycerol-acyltransferases in the pathway as the key enzymatic steps that drive ricinoleate into TAG (Lin *et al.*, 1998). The final step in RRR biosynthesis can be carried out by acyl-CoA:diacylglycerol acyltransferase (DGAT) and/or phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.*, 2000).

Various fatty acids can be incorporated at different rates into PC by lysophosphatidylcholine-acyltransferase (Lin *et al.*, 2002). The 2-nonricinoleoyl-PC are converted to 1,3-diricinoleoyl-2-nonricinoleoyl-*sn*-glycerol (RAcR) through reactions catalyzed by phospholipase C and then diacylglycerol acyltransferase (DGAT). Among these RAcR, only ROR and RLR, the most abundant RAcR in castor oil, are shown in Figure 25.1. PC can be hydrolyzed by phospholipase A_2 to release fatty acids, which are then activated to become acyl-CoA. Ricinoleoyl-CoA, linoleoyl-CoA, linolenoyl-CoA, and lesqueroloyl-CoA must come from the pathway involving PC where hydroxylation and desaturation occur. Oleoyl-CoA, stearoyl-CoA, and palmitoyl-CoA

can be available from the plastid. Acylation on glycerol backbone by acyl-CoA can be at *sn*-1, *sn*-2 and *sn*-3 by different acylation steps on the pathway. This biosynthetic information can be used to develop transgenic plants that produce seed oil containing RRR and RRRR without toxicity. Regiospecific and stereospecific identification of acyl chains in the molecules of RRAc in castor oil can be used in determining the acylation steps in its biosynthetic pathway.

25.3. SEPARATION OF VARIOUS DIRICINOLEOYLACYLGLYCEROLS IN CASTOR OIL

The HPLC separation of the various molecular species of RRAc in castor oil was the same as previously reported (Lin *et al.*, 1997; Lin and McKeon, 2003). The fractionation was performed using a C_{18} column (250 × 4.6 mm, 5μ). One mg of castor oil was chromatographed with a linear gradient from 100% methanol to 100% 2-propanol in 40 min, at a 1 mL/min flow rate, and detected at 205 nm. The chromatograms showing the fractionation of RRLs, RRLn, RRL, RRP, RRO and RRS in castor oil have been published (Lin *et al.*, 2003, 2006). One-half min fractions were collected and analogous fractions were pooled. The six RRAc in elution order and their fractions collected for MS analysis were as follows: RRLs (fraction #21), RRLn (#26, 27), RRL (#29, 30), RRP (#32), RRO (#32, 33) and RRS (#36, 37). The regioisomers can not be separated by C_{18} HPLC.

25.4. MS^2 SPECTRUM OF DIRICINOLEOYLOLEOYLFGLYCEROL (ROR)

The MS^2 spectrum of $[RRO + Li]^+$ at *m/z* 923.7 (Fig. 25.2) shows the precursor ion and the fragment ions of $[RRO + Li - \underline{R}COOH]^+$ at *m/z* 625.5 and $[RRO + Li - \underline{O}COOH]^+$ at *m/z* 641.5, reflecting the neutral losses of ricinoleic acid ($\underline{R}$COOH) and oleic acid ($\underline{O}$COOH) respectively. Ricinoleate, $[\underline{R}COOH + Li]^+$ at *m/z* 305.2, was detected slightly (Fig. 25.2). Oleate, $[\underline{O}COOH + Li]^+$ at *m/z* 289.3, was not detected. The structures of these fragment ions have been proposed to contain a 1,3-dioxolane ring (five-membered ring) with the two carbon atoms on the ring from the glycerol backbone (Hsu and Turk, 1999). Unlike the previous report (Hsu and Turk, 1999), the neutral loss of the lithium salts of these fatty acids from $[RRO + Li]^+$ were not observed as $[RRO + Li - (\underline{R}COOLi)]^+$ and $[RRO + Li - (\underline{O}OOLi)]^+$. The other non-lithiated adducts, e.g., $\underline{R}CO^+$, $[\underline{R}CO^+ - 18]^+$, $\underline{O}CO^+$, $[\underline{O}CO^+ - 18]^+$, shown earlier (Hsu and Turk, 1999) were also not observed here, yielding a much simpler spectrum (Fig. 25.2). The difference in spectra might be due to the different methods used. The earlier report (Hsu and Turk, 1999) used a tandem sector quadrupole instrument with "in-source" CAD, whereas we used an ion-trap instrument.

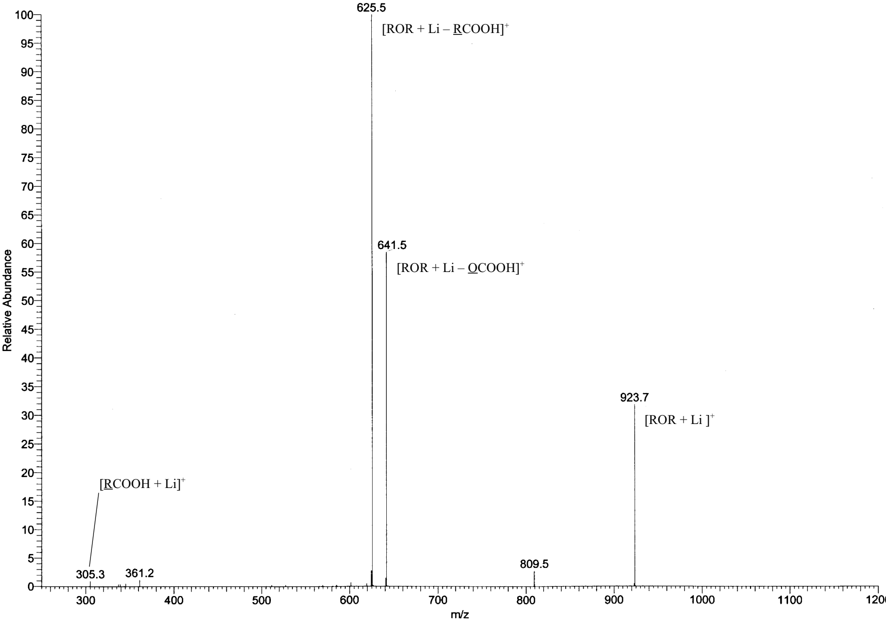

Figure 25.2. Ion trap mass spectrum of ESI-MS^2 of [ROR + Li]$^+$ ion at *m/z* 923.7. Both R and R̲COOH are ricinoleic acid. Both O and O̲COOH are oleic acid. ROR is diricinoleoy-oleoyl-glycerol (non-stereospecific) from castor oil including RRO and ORR if any.

25.5. MS^3 SPECTRUM OF [ROR + LI – R̲COOH]$^+$

Figure 25.3 shows the MS^3 spectrum of [ROR + Li – R̲COOH]$^+$ at *m/z* 625.5. Regiospecific ions were derived from the loss of α,β-unsaturated fatty acids specific at the *sn*-2 position (Hsu and Turk 1999) and were the two ions of [ROR + Li – R̲COOH – O̲′CH=CHCOOH]$^+$ at *m/z* 345.2 and [RRO and/or ORR + Li – R̲COOH – R̲′CH=CHCOOH]$^+$ at *m/z* 329.3. The abundance of the latter ion was very low (Fig. 25.3). It seems that the oleoyl moiety of ROR (non-stereospecific) in castor oil was mostly at the *sn*-2 position according to the relative abundances of m/z 345.2 and 329.3. However, most of *m/z* 345.2 was [ROR + Li – R̲COOH – R̲″CH=C=O]$^+$, from the loss of ketene, not [ROR + Li – R̲COOH – O̲′CH=CHCOOH]$^+$. The ratio of these two ions at *m/z* 345.2 and identification of the ion resulting from the loss of ketene will be given in a latter section. The fragmentation pathway of the loss of α,β-unsaturated fatty acids specific at *sn*-2 position has been proposed previously as shown in Figure 25.4A (Hsu and Turk, 1999).

Figure 25.3 also shows ricinoleate, [R̲COOH + Li]$^+$ at *m/z* 305.3, and oleate, [O̲COOH + Li]$^+$ at *m/z* 289.2, to less extent. A major ion [ROR + Li – R̲COOH – $C_7H_{14}O$]$^+$ at *m/z* 511.4 was formed from the cleavage between C-11

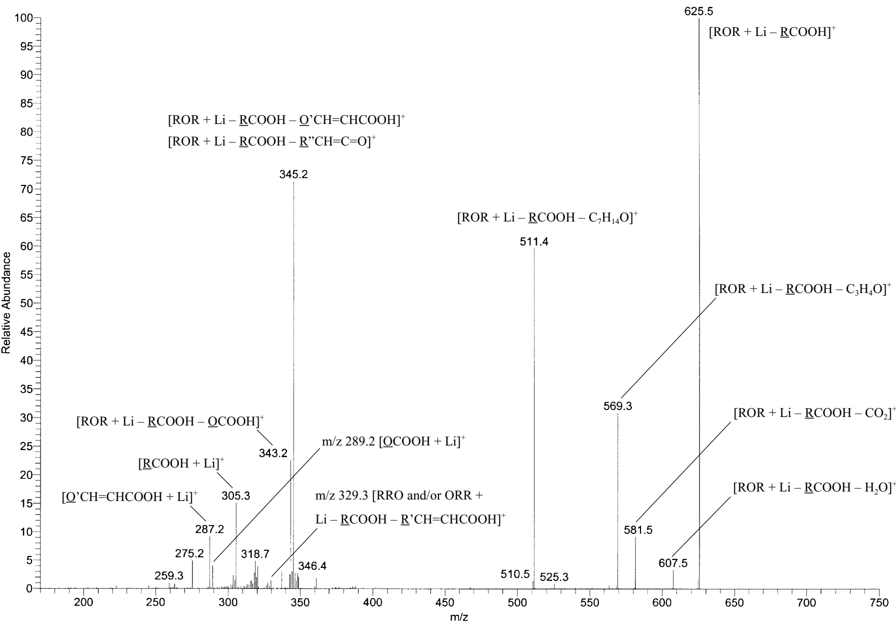

Figure 25.3. Ion trap mass spectrum of ESI-MS^3 of [ROR + Li – ROOH]$^+$ at *m/z* 625.5. For abbreviations, see Figure 25.2. O′CH=CHCOOH is α,β-unsaturated oleic acid from the *sn*-2 position. R′CH=CHCOOH is α,β-unsaturated ricinoleic acid from the *sn*-2 position. $C_7H_{14}O$ is the loss from the cleavage between C-11 and C-12 of ricinoleate chain. C_3H_4O is the loss of glycerol backbone to form acid anhydride of two fatty acids. R″CH=C=O is a ketene from ricinoleate at the *sn*-1,3 position.

and C-12 of the ricinoleoyl chain next to the hydroxyl group. The hydrogen atom of the hydroxyl group migrated to this lithium adduct during fragmentation. A major ion at *m/z* 569.3 (Fig. 25.3) may be the acid anhydride of ricinoleate and oleate, [RCOOCOO + Li]$^+$, and is the same as [ROR + Li – RCOOH – C_3H_4O]$^+$. The proposed fragmentation pathway for [ROR + Li – RCOOH – C_3H_4O]$^+$ is shown in Figure 25.4B, with the intermediate containing a 1,3-dioxolane five-membered ring with the two carbon atoms of the ring originating from the glycerol backbone. C_3H_4O is the same as CH_2=CHCHO from the glycerol backbone of the TAG.

25.6. MS^3 SPECTRUM OF [ROR + Li – OCOOH]$^+$

Figure 25.5 shows the MS^3 spectrum of [ROR + Li – OCOOH]$^+$ at *m/z* 641.5. The relative abundance of the ion at *m/z* 345.2, [RRO and/or ORR + Li – OCOOH – R′CH=CHCOOH]$^+$, resulting from the loss of α,β-unsaturated

Figure 25.4. Proposed fragmentation pathways of triacylglycerol lithium adducts by ESI-MS^3. R = fatty acid. Subscripts after R represent the stereospecific locations. (A) Loss of fatty acid at *sn*-3, then the loss of α,β-unsaturated fatty acid at *sn*-2 (Hsu and Turk, 1999). (B) Loss of fatty acid at *sn*-3, then the loss of C_3H_4O from glycerol backbone to form acid anhydride. (C) Loss of fatty acid at *sn*-2, then the loss of C_3H_4O from glycerol backbone to form acid anhydride.

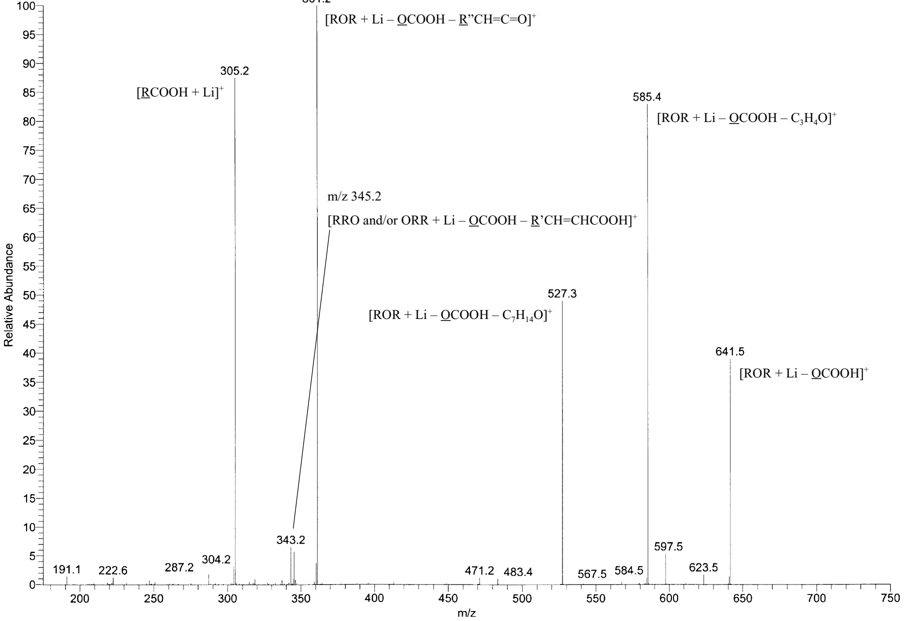

Figure 25.5. Ion trap mass spectrum of ESI-MS^3 of [ROR + Li – OCOOH]$^+$ at *m/z* 641.5. For abbreviations, see Figures 25.2 and 25.3.

ricinoleic acid specific at *sn*-2 position, was low compared to those of the five major ions. However, this ion was still significant, thus RRO and/or ORR are present in castor oil. Ricinoleate, [RCOOH + Li]$^+$ at *m/z* 305.3, was one of the major ions. As expected, oleate, [OCOOH + Li]$^+$ at *m/z* 289.3, was not present. [ROR + Li – OCOOH – R″CH=C=O]$^+$ at *m/z* 361.2 occurred as a major ion (Fig. 25.5) while [RRO and/or ORR + Li – RCOOH – O″CH=C=O]$^+$ at *m/z* 361.2 was a minor ion (Fig. 25.3). These results indicate that loss of R″CH=C=O and O″CH=C=O were regiospecific and bound to the *sn*-1 and/or *sn*-3 positions (Hsu and Turk, 1999), and that the content of RRO and/or ORR in castor oil was very low.

The ion at *m/z* 527.3 (Fig. 25.5), [ROR + Li – OCOOH – $C_7H_{14}O$]$^+$, was from the cleavage between C-11 and C-12 adjacent to the hydroxyl group of ricinoleoyl chain. The major ion at *m/z* 585.4 might be the acid anhydride of ricinoleic acid, [RCOOCOR + Li]$^+$, and was the same as [ROR + Li – OCOOH – C_3H_4O]$^+$. The loss of C_3H_4O, *m/z* 56, was also shown in Figure 25.3 at *m/z* 569.4. The proposed fragmentation pathway for [ROR + Li – OCOOH – C_3H_4O]$^+$ is shown as Figure 25.4C.

25.7. MS^3 SPECTRA OF [RLR + Li – RCOOH]$^+$ AND [RLnR + Li – RCOOH]$^+$

Figure 25.6 shows the MS^3 spectrum of [RLR + Li – RCOOH]$^+$ at *m/z* 623.5. Figure 25.7 shows the MS^3 spectrum of [RLnR + Li – RCOOH]$^+$ at *m/z* 621.5. The fragmentation pattern shown in these two MS^3 spectra is similar to that given in Figure 25.3. Figure 25.7 shows the three different ions, [RLnR + Li – RCOOH – R″CH=C=O]$^+$ at *m/z* 341.2, [RLnR + Li – RCOOH – LnCOOH]$^+$ at *m/z* 343.2 and [RLnR + Li – RCOOH – Ln′CH=CHCOOH]$^+$ at *m/z* 345.3 and these three *m/z* values are different. Figure 25.6 shows both of the ions [RLR + Li – RCOOH – R″CH=C=O]$^+$ and [RLR + Li – RCOOH – LCOOH]$^+$ at *m/z* 343.2. These two ions are not isotopic with the ion used for the regiospecific identification, [RLR + Li – RCOOH – L′CH=CHCOOH]$^+$ at *m/z* 345.3.

In Figure 25.3, most of the ion at *m/z* 345.2 was from [ROR + Li – RCOOH – R″CH=C=O]$^+$, not [ROR + Li – RCOOH – O′CH=CHCOOH]$^+$ because of Figure 25.7 showed that the abundance of [RLnR + Li – RCOOH – R″CH=C=O]$^+$ was almost four times of that of [RLnR + Li – RCOOH – Ln′CH=CHCOOH]$^+$. Minor ions from the loss of the ketene from lithiated TAGs were detected (Hsu and Turk, 1999), however, in this report the loss of ricinoleate ketene at *sn*-1,3 positions were major ions. All of the major ions from the loss of ketene in Figures 25.3, 25.5, 25.6, 25.7 were from the loss of ricinoleate ketene. The hydroxyl group on ricinoleate and the methods used made the difference. Neutral loss of ketene at the *sn*-3 position was proposed as [RCO + 74]$^+$ (Cheng *et al.*, 1998) on the CAD spectra of ESI-produced [M + NH_4]$^+$.

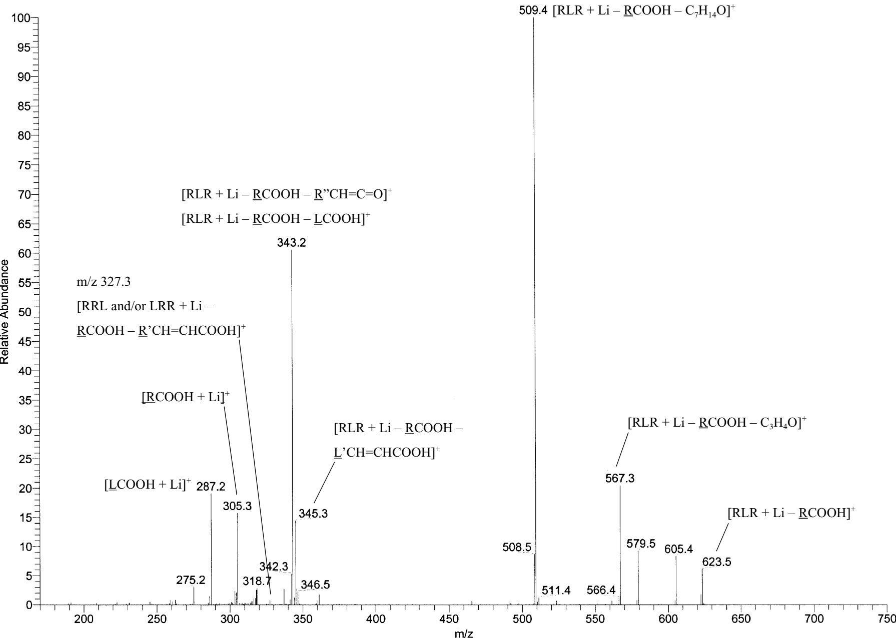

Figure 25.6. Ion trap mass spectrum of ESI-MS^3 of [RLR + Li – RCOOH]$^+$ at *m/z* 623.5. For abbreviations, see Figures 25.2 and 25.3. L′CH=CHCOOH is α,β-unsaturated linoleic acid from the *sn*-2 position. Both L and LCOOH are linoleic acid. RLR here is diricinoleoy-linoleoyl-glycerol (non-stereospecific) from castor oil included RRL and LRR if any.

25.8. MS^3 SPECTRA OF [RSR + Li – RCOOH]$^+$, [RPR + Li – RCOOH]$^+$ AND [RLsR + Li – RCOOH]$^+$

The MS^3 spectra of [RSR + Li – RCOOH]$^+$ at *m/z* 627.5, [RPR + Li – RCOOH]$^+$ at *m/z* 599.5 and [RLsR + Li – RCOOH]$^+$ at *m/z* 669.5 were also obtained. These spectra were similar to those shown in Figures 25.3, 25.6, and 25.7. The ions due to the loss of α,β-unsaturated fatty acids specific at the *sn*-2 position, [RSR + Li – RCOOH – S′CH=CHCOOH]$^+$ at *m/z* 345.3, [RPR + Li – RCOOH – P′CH=CHCOOH]$^+$ at *m/z* 345.3 and [RLsR + Li – RCOOH – Ls′CH=CHCOOH]$^+$ at *m/z* 345.3 were different from the ions of [RSR + Li – RCOOH – R″CH=C=O]$^+$ at *m/z* 347.3, [RPR + Li – RCOOH – R″CH–=C=O]$^+$ at *m/z* 319.2 and [RLsR + Li – RCOOH – R″CH=C=O]$^+$ at *m/z* 389.3. Therefore, the relative abundance of the ions resulting from the loss of the α,β-unsaturated fatty acids specific at the *sn*-2 position can be estimated directly.

25.9. QUANTIFICATION OF THE REGIOISOMERS

The contents of RAcR among RRAc, RAcR and AcRR combined were estimated by the comparison of relative abundances of the two ions, [RAcR + Li

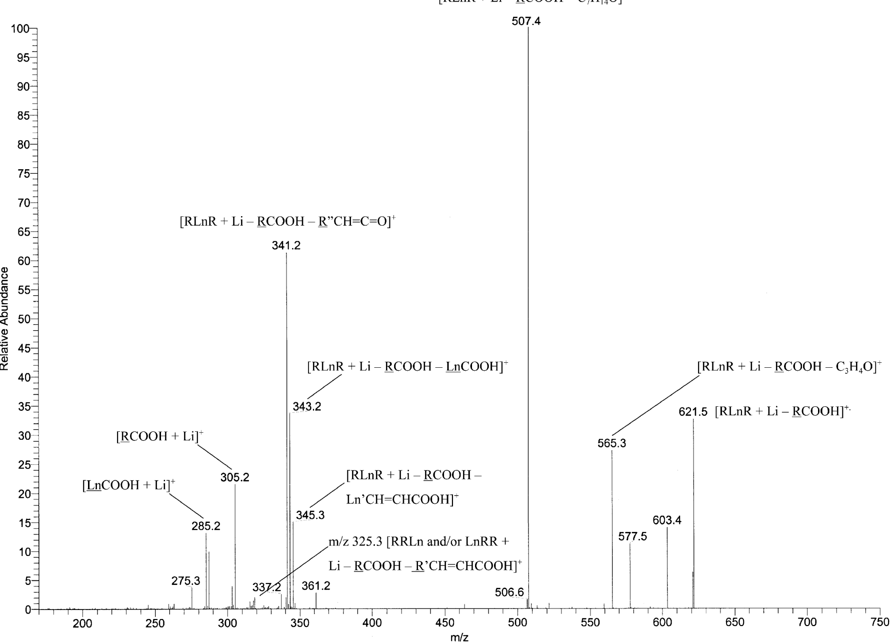

Figure 25.7. Ion trap mass spectrum of ESI-MS^3 of [RLnR + Li – RCOOH]$^+$ at *m/z* 623.5. For abbreviations, see Figures 25.2 and 25.3. Ln′CH=CHCOOH is α,β-unsaturated linolenic acid from the *sn*-2 position. Both Ln and LnCOOH are linolenic acid. RLnR here is diricinoleoy-linolenoyl-glycerol (non-stereospecific) from castor oil included RRLn and LnRR if any.

– RCOOH – Ac′CH=CHCOOH]$^+$ and [RRAc and/or AcRR + Li – RCOOH – R′CH=CHCOOH]$^+$ as shown in Figures 25.3, 25.6 and 25.7. Among the twelve ions used from the six RRAc in castor oil, only the ion [ROR + Li – RCOOH – O′CH=CHCOOH]$^+$ at *m/z* 345.2 in Figure 25.3 was mixed with the ion from the loss of ketene, [ROR + Li – RCOOH – R″CH=C=O]$^+$ at *m/z* 345.2. The relative abundance of [ROR + Li – RCOOH – O′CH=CHCOOH]$^+$ was estimated from the ratios of the three ions, [RLnR + Li – RCOOH – R″CH=C=O]$^+$ at *m/z* 341.2, [RLnR + Li – RCOOH – LnCOOH]$^+$ at *m/z* 343.2 and [RLnR + Li – RCOOH – Ln′CH=CHCOOH]$^+$ at *m/z* 345.3, in Figure 25.7. The relative abundance of [ROR + Li – RCOOH – O′CH=CHCOOH]$^+$ was then compared with that of [ROR + Li – RCOOH – R′CH=CHCOOH]$^+$ at m/z 325.3 to get the content of ROR. When the ratio of the abundances of [RLnR + Li – RCOOH – LnCOOH]$^+$ at *m/z* 343.2 and [RLnR + Li – RCOOH – Ln′CH=CHCOOH]$^+$ at *m/z* 345.2 in Figure 25.7 was used, the content of ROR among the three isomers (ROR, RRO, ORR) combined was 88%. When the ratio of the abundances of [RLnR + Li – RCOOH – R″CH=C=O]$^+$ at *m/z* 341.2 and [RLnR + Li – RCOOH –

Ln′CH=CHCOOH]$^+$ at m/z 345.3 in Figure 25.7 was used, the content of ROR among the three isomers (ROR, RRO, ORR) combined was 91%. We used the latter, avoiding the use of the third ion for calculation.

From the relative abundances of the two ions derived from the loss of α,β-unsaturated fatty acids specific at the *sn*-2 position for each of the other RRAc in castor oil, we estimated the content of the 1,3-diricinoleoyl-2-acyl-*sn*-glycerol species among the three isomers as follows: RLR (95%), RLnR (96%), RSR (96%), RPR (78%), and RLsR (31%). These values fall consistently within ±3% in repeated experiments. Both MS^3 and CAD-MS^2 were used to measure the content of each 1,3-diricinoleoyl-2-acyl-*sn*-glycerol among the three possible stereoisomers with equivalent results (data not shown).

Since ESI-MS^3 of lithium adducts detects ions from the loss of α,β-unsaturated fatty acid specific at the *sn*-2 position, the very minor regioisomers can be identified such as [RRO and/or ORR + Li − RCOOH − R′CH=CHCOOH]$^+$ at m/z 329.3 (Fig. 25.3), [RRO and/or ORR + Li − OCOOH − R′CH=CHCOOH]$^+$ at m/z 345.2 (Figure 25.5), [RRL and/or LRR + Li − RCOOH − R′CH=CHCOOH]$^+$ at m/z 327.3 (Fig. 25.6) and [RRLn and/or LnRR + Li − RCOOH − R′CH=CHCOOH]$^+$ at m/z 325.3 (Fig. 25.7). We assume a linear relation of the ion response and the amount of isomer as reported earlier (Jakab *et al.*, 2003).

25.10. CONCLUSIONS

The contents of the five RAcR containing non-hydroxyl acyl chains at the *sn*-2 position were high. This indicated that phospholipase A_2 hydrolysis of PC containing non-hydroxyl fatty acid at the *sn*-2 position is either blocked or partially blocked *in vivo* and these non-hydroxyl fatty acids stay mostly at the *sn*-2 position of triacylglycerol in castor oil. It had been shown previously *in vitro* that ricinoleate was released specifically from PC by phospholipase A_2 in castor microsomes (Bafor *et al.*, 1991; Stahl *et al.*, 1995). The lesqueroloyl moiety on the *sn*-2 position of diricinoleoyl-lesqueroloyl-glycerol represents about one third of the three possible isomers, suggesting that 2-lesqueroloyl-PC, like 2-ricinoleoyl-PC, can be efficiently hydrolyzed by phospholipase A_2. In addition, lysophosphatidic acid acyltransferase (acylation step on *sn*-2) may incorporate more non-hydroxyl fatty acids into castor oil than the acylation steps of lysophosphatidic acid acyltransferase (*sn*-1) and diacylglycerol acyltransferase (*sn*-3) combined.

In castor oil, non-hydroxyl fatty acids are mostly at the *sn*-2 position, while in seed oils lacking hydroxy fatty acid, unsaturated fatty acids are mostly at the *sn*-2 position. The incorporation of non-hydroxyl fatty acids into triacylglycerols, e.g. ROR, RLR, includes the enzymatic steps catalyzed by phospholipase C and DGAT. Down-regulation of DGAT will block the incorporation of both hydroxy and non-hydroxyl fatty acids into triacylglycerols, potentially impacting oil yield. Therefore, phospholipase C can be the target to block the

incorporation of non-hydroxyl fatty acids into triacylglycerols to increase presumably the content of ricinoleate in transgenic seed oils (Fig. 25.1).

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CHAPTER 26

Composition, Functionality and Potential Applications of Seaweed Lipids

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26.1. INTRODUCTION

Seaweeds are a group of macroscopic marine algae that form the basic biomass in the intertidal zone. Some authors even brand seaweeds as sea vegetables (Wong and Cheung, 2000). They form a part of the staple diet in the Far East and Hawaiian Islands, Japan, Korea and China, apart from being used as delicacies in some of the western world where they are principally used as sources of phycocolloids, thickening and gelling agents for various industrial applications including foods. Seaweeds have been used since ancient times as food, fodder fertilizer, and as sources of medicinal drugs. Today seaweeds are the raw material for industrial production of agar, carrageenan, and alginates, but they continue to be widely consumed as food in Asian countries. They are nutritionally valuable, in fresh and dried forms, as ingredients in a wide variety of prepared foods (Wong and Cheung, 2000). With high essential amino acids content and relatively high levels of unsaturated fatty acid, the quality of protein and lipid in seaweed is acceptable compared to other diet vegetables. Although seaweeds have been thoroughly explored for their hydrocolloids, their low-content lipids are often ignored. Of late, seaweeds have been explored for some of the bioactives and nutraceuticals present in them. It has been recently reported that the seaweeds contain some interesting polyenes, and contain some of the typical carotenoids (*e.g.*, fucoxanthin in brown seaweeds) that have been associated with anti-carcinogenic, anti-oxidative and anti-hypertensive properties. This paper will consider seaweed lipids for their beneficial biomolecules, and review seaweed lipids, their extraction, analysis and applications.

26.2. SEAWEED LIPIDS

26.2.1. Seaweed Lipid/Carotenoid Extraction, Analysis of Total Lipid, Lipid Classes and Fatty Acids

Seaweed lipids have been analysed both qualitatively and quantitatively, by gas chromatography (GC) and high performance liquid chromatography (HPLC). Most methods for the extraction of seaweed lipids use combinations of chloro-

form and methanol (Bhaskar *et al.*, 2004a, 2004b; Sanchez-Machado *et al.*, 2004a, 2004b) or methanol alone. Separation of lipid classes from total lipids is usually accomplished by open column chromatography using silica gel by successive elution with chloroform (1:10, w/v of lipid), acetone-methanol (9:1, w/v of lipid) and methanol (1:10, w/v of lipid) to get neutral- (NL), glycol- (GL) and phospho- (PL) lipids respectively (Bhaskar *et al.*, 2004a, 2004b). Thin layer chromatography (TLC) on silica gel plates with chloroform-methanol-acetone-acetic acid (70:14:24:0.4, v/v) was used as a mobile phase has also been reported as another method for separation of lipids into different classes, *viz.*, triacylglycerols (TAG), glycolipids (GL) and phospholipids (PL) (Zornitsa *et al.*, 2002). Quantitative and qualitative evaluation of fatty acids associated with seaweed lipids is achieved either by HPLC or by GC after converting the individual fatty acids into their methyl esters. Most of the methods followed by researchers for analyzing the fatty acid composition are based on GC methods. Various trans-esterification agents used for preparation of fatty acid methyl esters (FAME) from seaweed lipids include methanolic boron triflouride (Floreto and Teshima, 1998; Van Pelt *et al.*, 1999), sodium methoxide (Van Pelt *et al.*, 1999; Bhaskar *et al.*, 2004a, 2004b, 2004c; Bhaskar and Miyashita, 2005) and boron trichloride (Israel *et al.*, 1992). Fused silica capillary columns (with or without temperature gradient) and helium as the carrier gas appear to be the preferred choice for seaweed fatty acid analysis by several researchers (Israel *et al.*, 1992; Herbreteau *et al.*, 1997; Floreto and Teshima, 1998; Van Pelt *et al.*, 1999; Khotimchenko and Vaskovsky, 2000; Zornitsa, *et al.*, 2002; Sanchez-Machado *et al.*, 2004a; Bhaskar *et al.*, 2004a, 2004b, 2004c; Bhaskar and Miyashita, 2005).

Extraction of total carotenoids has been reported by several authors using different solvents or solvent combinations. Most researchers use methanol and/or acetone as the preferred solvents to recover total carotenoids. General HPLC protocols followed for identification of various seaweed carotenoids, fucoxanthin in particular, are presented in Table 26.1.

26.2.2. Recovery of Lipid Compounds From Seaweeds

Several methods have been proposed to produce polyunsaturated fatty acid (PUFA) concentrates particularly high in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Most PUFA enrichment methods are based upon a combination of techniques such as saponification, solvent extraction, urea fractionation, molecular distillation, fractionation distillation, liquid chromatography, and super critical carbondioxide extraction. Current evidence suggests that the physiological effects of omega-3 fatty acids are such that the annual world supply of fish oils will be grossly inadequate as a source of these materials, and alternative sources will be needed (Belarbi *et al.*, 2000).

Robles *et al.* (1998) have reviewed the various techniques for concentrating and purifying marine algal PUFA by urea fractionation and liquid chromatography. The urea fractionation technique is most frequently employed to obtain concentrates of PUFAs with four or more double bonds for several reasons *viz., i)* it allows handling of large quantities of material in simple equipment,

TABLE 26.1. HPLC protocols used for separation of seaweed related carotenoids.

Carotenoids examined & Source	Method details (Column, Mobile phase)	Reference
Fucoxanthin & its esters separation; Chinese clam	Reverse Phase, C18 column Chloroform : Acetonitrile (3:7 v/v); Isocratic	Maoka *et al.*, 2007
Fucoxanthin and other carotenoids; Dinoflagellate	Silica Column n-hexane (with 0.1% v/v Methanol) and acetone at 25:6 (v/v); isocratic	Bjornland *et al.*, 2003
Various carotenoids; microalgae & cyanobacteria	Reverse Phase, C18 column Binary solvent system (Solvent A : Methanol and 28mM Amm. Acetate 70:30 v/v; Solvent B : Methanol); Gradient	Frassanito *et al.*, 2005
Various carotenoids; brown and red seaweeds	Reverse Phase, C18 column Methanol and Water (9;1 v/v); isocratic Normal phase, Silica gel column Tetrahydrofuran and hexane (35:65 v/v); isocratic	Maoka *et al.*, 2002
Purified fucoxanthin; Brown seaweed	Normal phase, silica gel column Chloroform and Acetone (9:1); isocratic	Yan *et al.*, 1999
Fucoxanthin and its metabolites Brown seaweeds	Reverse phase, C 18 column Methanol and water (95:5 v/v); isocratic	Mori *et al.*, 2003
Fucoxanthin and its metabolites; Egg yolk	Normal phase, Silica gel column Acetone : hexane; gradient Normal phase, Silica gel column Hexane, Dichlormethane, 2-Propanol and Ethyl-diisopropylamine (90.9:7:3:0.1 v/v/v/v); isocratic Reverse phase, C18 column Methanol and Water (67:33 v/v); isocratic Bonded nitrile column Hexane, Isopropyl acetate, Acetone, Methanol (76:17:7:0.1 v/v/v/v); isocratic	Strand *et al.*, 1998
Algal carotenoids; Brown seaweeds	Bonded Nitrile column Hexane, Isopropyl acetate, acetone, Methanol (75.9:17:7:0.1 v/v/v/v); Isocratic Normal Phase; Silica column Hexane, Isopropyl acetate, acetone, Methanol (75.9:17:7:0.1 v/v/v/v); Isocratic	Haugan and Liaeen-Jensen, 1994

ii) biocompatible solvents such as ethanol can be used, *iii)* it employs milder conditions (*e.g.*, room temperature), *iv)* the separation is more efficient than with other methods, such as fractional crystallization or selective solvent extraction, and *v)* it is cost effective. The major drawback of the urea method as a PUFA purification process is the low PUFA recovery, as some PUFAs, such as EPA, form a very high percentage of urea compounds.

The HPLC-based fatty acid separations use silver nitrate adsorption chromatography or reverse-phase partition chromatography. The silver nitrate method is suitable for separating PUFAs, but not for separating saturated, mono or di-unsaturated fatty esters (Robles *et al.*, 1998). Reverse phase chromatographic separations are largely based on the sorption of hydrophobic moieties of the soluble molecules from a polar solvent or apolar solvent. The separation of fatty acids depends on the chain length and on the degree and type of unsaturation.

An integrated process for highly purified PUFAs from microalgae has been developed at the University of Almeria (Molina Grima *et al.*, 1996). The process basically employs solvent extraction, phase separation, urea adduction of fatty acids, and chromatographic separation of PUFA rich fraction; the process is summarized in Figure 26.1. The same is applicable to recovery of PUFA rich lipid fractions from seaweeds as well.

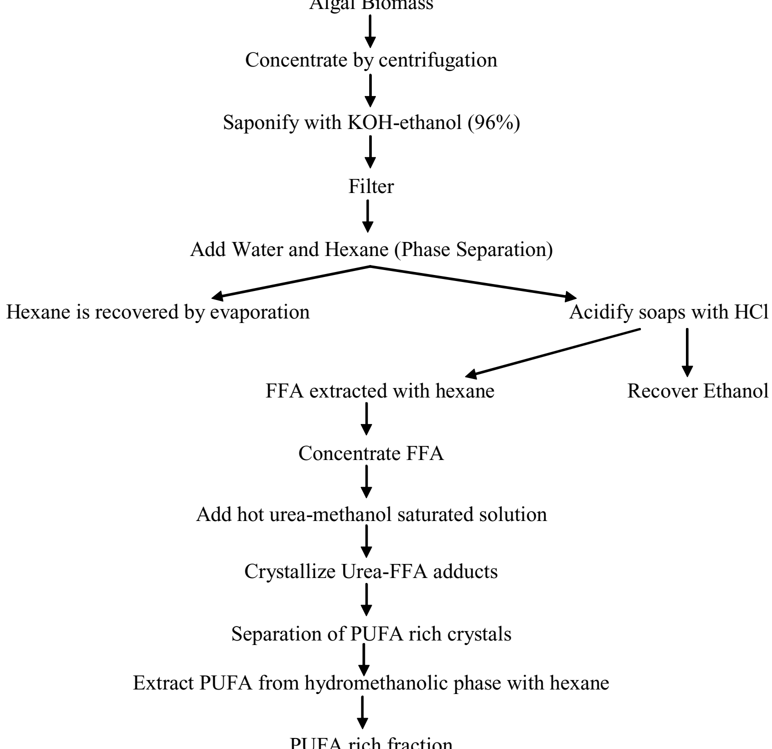

Figure 26.1. Scheme for recovery of PUFA from microalgae.

26.2.3. Qualitative and Quantitative Composition of Lipids in Seaweeds

Although the marine animals have been exploited thoroughly for food and other byproducts, their plant counter parts have not been exploited for food to the same extent, barring some seaweeds like *Porphyra* (nori) and *Laminaria* (konbu). Unlike terrestrial counter parts, marine plants have not been looked upon as important substrates for further processing of their lipids. In this section, seaweed lipids have been classified for convenience into different categories, *viz.*, general fatty acids, conjugated fatty acids, carotenoids (fucoxanthin), and sterols (fucosterol).

Seaweed lipids account for ~7% of dry algal matter, and mainly exist in three classes: neutral lipids (NL), glycolipids (GL), and phospholipids (PL). The composition of different lipid classes in selected seaweeds is summarized in Table 26.2. The lipid content of seaweed varies with species, geographical location, season, temperature, salinity, light intensity, and type of species, and/or a combination of these factors. Seasonal variation, the effect of growth conditions, and the compositional characterization of genus have all been studied for their influence on fatty acid composition of seaweeds with special reference to aquaculture (Sanchez-Machado *et al.*, 2004a, 2004b). Tropical species have significantly less lipid (<1%) than cold water species (~1.6%). Glycolipids form the major lipid class in all the seaweeds, followed by NL and PL (Bhaskar *et al.*, 2004c).

26.2.4. Fatty Acid Composition

The fatty acids of seaweeds generally have linear chains, an even number of carbon atoms and one or more double bonds (Khotimchenko, 1991). In particular seaweeds can be source of essential fatty acids including both omega-3 (n-3) and omega-6 (n-6) fatty acids. The red and brown algae are particularly rich in fatty acids with 20 carbon atoms, *viz.*, EPA ($C_{20:5}$) and arachidonic acid

TABLE 26.2 Composition of different lipid classes (as % of total lipids) in seaweeds.

Seaweeds	Total lipids	Neutral lipids	Glycolipids	Phospholipids
Padina tetrastomatica	1.83 ± 0.11	38.85 ± 0.92	44.40 ± 1.13	16.75 ± 0.21
Sargassum marginatum#	0.90 ± 0.17	37.8 ± 1.53	47.9 ± 2.12	14.3 ± 0.98
S. thunbergii#	1.60 ± 0.12	21.9 ± 0.96	62.1 ± 1.35	16.0 ± 1.56
S. confusum	1.90 ± 0.28	20.7 ± 1.88	60.4 ± 3.30	18.9 ± 0.62
Acanthophora spicifera *	0.9 ± 0.15	26.3 ± 1.18	63.0 ± 2.10	10.9 ± 0.98
*Gracilleria folifera**	0.8 ± 0.09	18.1 ± 0.56	71.7 ± 2.98	10.2 ± 1.05
*G. edulis**	0.6 ± 0.11	20.6 ± 1.26	71.7 ± 3.02	7.7 ± 1.03

**Source*: Bhaskar *et al.*, 2004a
#*Source*: Bhaskar *et al.*, 2004c.

(AA; $C_{20:4}$). The green algae show interesting levels of α-linolenic acid (ALA; $C_{18:3}$). Fatty acid composition of different seaweeds from across the globe is summarized in Table 26.3. The ratio of n-6 to n-3 fatty acids is very important as a nutraceutical for human intake, as both of these compete for the same enzyme to synthesize prostaglandins derived from both n-3 and n-6 families. Several seaweeds have a balanced ratio of n-3 and n-6 fatty acids (Bhaskar *et al.*, 2004b). Seaweeds belonging to the same genus, from different parts of the world, generally have similar fatty acid profile, although the collection site influences the actual PUFA content. Several researchers have opined that the differences in the fatty acid composition of algae depend on environmental factors (Bhaskar *et al.*, 2004b). For instance, it is well established that algae accumulate PUFAs when there is decrease in the environmental temperature (Kayama *et al.*, 1985; Khotimchenko, 1991). This in turn influences the comparisons between algae from different parts of the world (Bhaskar *et al.*, 2004b). PUFA content of several brown, red, and green seaweeds is also shown in Table 26.3. Attempts to use fatty acid composition as an aid in taxonomical conclusions of higher plants have been reviewed thoroughly. Some researchers have also found that distribution of fatty acids in marine plants is closely linked with taxonomic position (Khotimchemko and Svetashev, 1987; Kayama *et al.*, 1985; Bhaskar *et al.*, 2004a). For instance, a C20 non-methylene interrupted (NMI) fatty acid is an emerging characteristic of the genus *Sargassum* (Bhaskar *et al.*, 2004b) while conjugated fatty acids are present in a specific group of red seaweeds, namely ceramiales (Burgess *et al.*, 1991; Wise *et al.*, 1994; Bhaskar *et al.*, 2004a). Besides fatty acids, an unsaponifiable fraction of seaweeds contain carotenoids such as β-carotene, lutein, and violaxanthin (red and green seaweeds), fucoxanthin (brown seaweeds), tocopherols, sterols (mainly fucosterol in brown seaweeds), and terpenoids (Jensen, 1969).

26.2.5. Conjugated Fatty Acids

Aquatic plants possess conjugated fatty acids, with carbon chain lengths varying from 16 to 22 carbon atoms, as natural constituents in their lipids; both trienes and tetraenes occur in aquatic plant lipids. Though many workers have studied the fatty acid composition of seaweeds from different regions of the world, not much information is available on the occurrence of these conjugated PUFAs in the seaweeds. In all there are few reports on the occurrence of these conjugated polyenes, *viz.* trienes in *Ptilota* (Lopez and Gerwick, 1987; Wise *et al.*, 1994), tetraenes in *Bosiella orbingiana, Lithothamnion coralliodes* (Hamberg, 1992), and *Anadyomene stellata* (Mikhailova *et al.*, 1995). Investigation of conjugated polyenes from the seaweed *Ptilota filicina* resulted in the definition of a polyenoic fatty acid isomerase (PFI) (Wise *et al.*, 1994); work on the enzyme from *L. corallioides* explained the formation of tetraenes by that enzyme (Hamberg, 1992). PFI has been characterized and functionally expressed by DNA cloning (Zheng *et al.*, 2002). Bhaskar *et al.* (2004a) investigated for the presence of conjugated polyenoic fatty acids in seaweeds from

TABLE 26.3. Fatty acid composition (% total fatty acid content) of different macro algae.

	Species of Macro-algae (as numbeed in the list below)																
Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
12:0	0.57	0.11	0.15	0.17	0.12	—	—	—	—	—	—	—	—	—	—	—	—
14:0	6.74	6.95	2.47	2.91	6.18	2.75	3.44	2.13	3.44	3.59	4.0	3.6	5.2	4.6	3.5	4.2	3.3
15:0	0.96	0.60	0.45	0.49	0.45	0.22	0.23	0.25	0.98	0.40	0.3	0.2	0.3	0.4	0.3	0.5	0.5
16:0	40.27	43.47	84.60	81.28	43.76	24.12	21.05	22.08	0.87	0.68	23.5	22.4	33.5	37.7	28.8	27.2	26.9
18:0			1.24	1.51	0.84	0.54	0.47	0.71	0.65	0.59	0.7	0.8	0.9	0.9	0.9	1.2	1.0
19:0	1.43	0.70	—	—	0.62	0.63	0.68	—	3.97	3.30	—	—	—	—	—	—	—
20:0	0.23	1.35	—	—	0.25	0.26	0.28	0.20	0.21	0.29	—	0.3	0.2	0.3	—	—	—
16:1n-7	—	0.19	0.38	0.47	8.28	3.26	3.15	3.29	4.83	3.25	4.0	6.1	5.4	6.3	3.7	3.3	3.4
18:1n-9	1.43	7.59	0.71	1.0	9.50	6.79	6.13	8.31	11.15	6.83	8.3	7.2	8.9	10.2	9.9	11.1	10.6
18:1n-7	10.21	11.72	0.69	0.81	0.66	0.39	0.23	—	—	—	0.2	0.3	—	—	0.7	0.5	0.6
18:1n-5			—	—	0.71	0.38	0.16	—	—	—	—	—	—	—	—	—	—
16:2n-4	2.18	0.63	—	—	1.09	0.41	0.53	—	0.03	0.14	0.3	0.7	0.4	0.3	0.3	0.4	0.5
16:3n-4	0.20	0.35	—	—	0.70	0.36	0.30	1.41	0.34	0.64	—	—	—	—	—	—	—
18:2n-6	0.24	0.64	0.16	0.21	4.90	5.15	7.73	—	3.97	3.30	4.8	9.8	7.2	4.9	5.6	5.4	4.6
18:3n-3	0.38	0.58	—	0.10	2.14	11.08	7.54	4.81	0.64	0.27	0.5	7.2	6.2	6.3	7.9	6.7	8.9
20:2n-6	1.37	4.54	0.12	0.27	0.27	0.27	0.38	0.46	0.56	0.23	0.3	—	0.1	0.2	0.2	0.3	0.8
20:3n-6	0.79	1.37	0.23	0.57	0.84	0.63	2.35	0.97	0.90	0.46	0.6	3.6	0.7	0.9	1.0	1.0	1.0
20:4n-6	0.20	0.15	0.67	—	7.76	11.04	20.34	13.82	11.20	15.79	12.4	1.1	13.5	12.2	15.2	14.4	14.2
20:5n-3	0.48	0.76	—	0.50	1.52	13.76	0.10	11.63	4.82	9.65	14.4	3.8	3.8	3.2	4.6	4.3	4.5
22:1n-9	10.19	3.23	—	—	—	—	—	0.89	—	0.53	1.6	1.0	0.5	0.7	0.9	0.1	0.3
C18 PUFA		0.45	—	—	—	—	—	24.84	15.01	19.92	19.7	25.9	22.0	16.9	20.8	18.1	21.5
C20 PUFA	6.18	—	—	—	—	—	—	27.76	18.20	26.96	28.5	27.1	19.0	17.4	22.4	22.6	22.8

Source: Khotimchenko, 1991; Xiang-chun *et al.*, 1995; Bhaskar *et al.*, 2004a; Bhaskar *et al.*, 2004c; Bhaskar and Miyashita, 2005; *1- Acanthophora spicifera 2- Padina tertrastomatica 3- Gracillaria Edulis 4- Gracillaria folifera 5- Sargassum marginatum 6- S. thunbergii 7- S. confusum 8- S. muticum 9- Sargassum. sp 10-S. fusiformis 11-S. miyabei 12-S. pallidum 13-S. herklotsii 14- S. baccularia 15- S. microcystum 16- S. turbinariodes 17- S. cristaefolum*

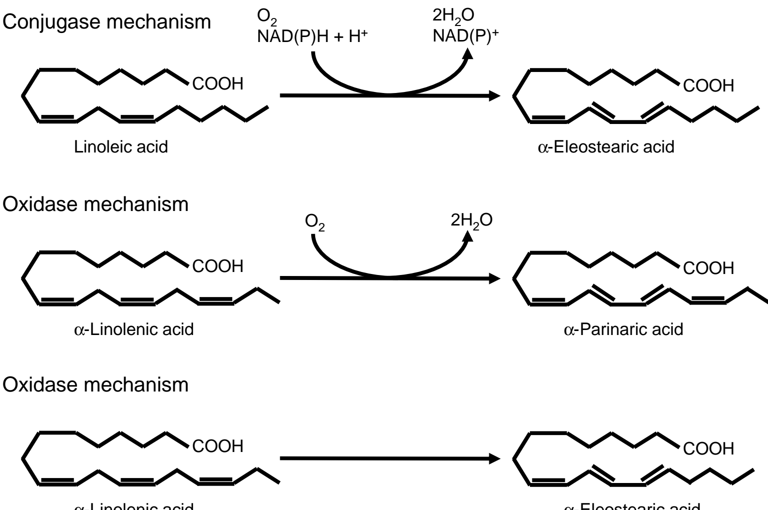

Figure 26.2. Summary of mechanisms involved in the formation of conjugated fatty acids.

Indian origin. Analysis of lipid classes of these seaweeds revealed that *Acanthophora spicifera* (Ceramiales, Rhodophyta) had significantly higher amounts of conjugated eicosapentaenoic acid (CEPA) and conjugated arachidonic acid (CAA) in all lipid classes except glycolipids. It has been reported that conjugated PUFAs such as CEPA, CAA, and conjugated DHA (CDHA) prepared by alkali isomerization had profound cytotoxic effects against human cancer cell lines (Matsumoto *et al.*, 2001). Various enzymes in aquatic plants are thought to be responsible for the formation of conjugated trienes/tetraenes endogenously, and can be grouped into three main categories: conjugases, oxidases, and isomerases. The general schemes by which conjugated fatty acids are formed is presented in Figure 26.2. For more information on conjugated fatty acids and their physiological effects, readers may refer to the comprehensive review on the subject by Bhaskar *et al.* (2006a).

26.2.6. Seaweed Carotenoids

The major carotenoids in seaweeds include β-carotene, lutein, violoxanthin, neoxanthin, and zeaxanthin in green seaweeds (chlorophytes); α- & β-carotene, lutein, and zeaxanthin in red seaweeds (rhodophytes); and, fucoxanthin, β-carotene, and violoxanthin in brown seaweeds (phaeophytes). Fucoxanthin, apart from being the most characteristic pigment of brown seaweeds, is also one of the most abundant carotenoids in nature (Matsuno, 2001; Hosokawa *et al.*, 2004).

Figure 26.3. Fucoxanthin, fucoxanthinol and related compounds.

Fucoxanthin is xanthophyll that contains two epoxy groups. It is the most abundant of all carotenoids, accounting for >10% of estimated total natural production of carotenoids (Matsuno, 2001). Several geometrical isomers of fucoxanthin also exist in nature, and in alcohol form as fucoxanthinol along with other reported metabolites (Mori *et al.*, 2003). The structure of fucoxanthin, its metabolites, and fucoxanthinol are closely related (Fig. 26.3). Both fucoxanthin and fucoxanthinol occur in brown seaweeds (Czeczuga and Taylor, 1987). Fucoxanthin, when present in thallus of seaweeds, is quite stable in the presence of organic ingredients; apart from surviving the drying process and storage at ambient temperature, although, fucoxanthin in pure form is susceptible to oxidation and/or decomposition. Fucoxanthin content in seaweeds exhibits seasonal variation and also varies depending on the life cycle of the seaweeds.

26.2.7. Seaweed Sterols

Sterols are an important family of lipids, present in the majority of eukaryotic cells. Plant cells typically contain a mixture of sterols, such as β-sitosterol, stigmasterol, and 24-methylenecholesterol (Nabil and Cosson, 1996). Cholesterol is the most abundant of the free sterols found in sea water. Sterol content in chlorophyta is similar to higher plants, and also it contains large amount of cholesterol. *Chondrus crispus* (Irish moss), one of the red seaweeds harvested in quantity, has cholesterol as its major sterol. Fucosterol is the major sterol in brown algae. Rhodophyta species contain primarily cholesterol and to some extent desmosterol. Fucosterol, the dominant sterol in pheophyta, is apparently the major sterol of every species (Table 26.4). The brown seaweeds contain mainly fucosterol and fucosterol derivatives; red seaweeds mainly

TABLE 26.4. Major sterol contents (μg/g dry weight) of the different processed seaweed samples.

Seaweeds	Fucosterol	Desmosterol
Canned		
Himanthalia elongate	2320	—
Saccorhiza polyschides	1133	—
Dried		
Himanthalia elongate	1706	—
Undaria pinnatifida	1136	—
Laminaria ochroleuca	662	—
Porpyra sp	—	337
Palmaria sp	—	186

Source: Sanchez-Machado *et al.*, 2004b.

contain cholesterol and cholesterol derivatives; and green seaweeds contain mainly ergosterol and 24-ethylcholesterol (Govindan *et al.*, 1993; Nabil and Cosson, 1996; Hamdy and Dawes, 1988). Sheu *et al.* (1999) isolated nine types of sterols from the marine brown alga *Turbinaria conoides*. The structures (Fig. 26.4) of the compounds were also established by spectral analysis. Sanchez-Machado *et al.* (2004b) quantified sterols by a reproducible HPLC method in edible seaweeds. The predominant sterol was fucosterol in brown seaweeds (83–97% of total sterol content) and desmosterol in red seaweeds (87–93% of total sterol content).

26.3. FUNCTIONALITY AND PHYSIOLOGICAL EFFECTS OF SEAWEED LIPID COMPOUNDS

26.3.1. General Lipids

The occurrence of PUFAs in general n-3 fatty acids is a unique feature of marine algae lipids and the PUFAs have considerable health and economic significance. The important physiological effects of PUFAs of marine origin, especially EPA and DHA, have also been reviewed by Bhaskar *et al.*, (2006b). Harada and Kamei (1997) studied the selective cytotoxicity of 8 species of marine algae extracts to several human leukemic cell lines. The extract of red alga, *Amphiroa zonata* exhibited strong cytotoxicity to all human leukemic cell lines tested, and murine leukemic cells L1210 at the concentrations of 15 to 375 μg/ml. The extract from a brown alga *Dilophus okamurae* with weak selective cytotoxic activity to L1210 cells exhibited not only strong cytotoxicity to L1210, but also to human leukemic cells, HL60 and MOLT-4 at 50 μg/ml. The extract from a green alga *Cladophoropsis vaucheriaeformis* with most selective cytotoxicity, did not show cytotoxicity to any human leukemic cell lines tested at 50 μg/ml. Thus the red alga *Amphiroa zonata* might be a suitable natural

Figure 26.4. Fucosterol and other structurally related sterols isolated from seaweeds. (A) Fucosterol; (B) 24ξ-hydroperoxy-24-vinyl cholesterol; (C) 29-hydroperoxystigmasta-5,24(28)-dien-3β-ol; (D) 24-ethylcholesta-4,24(28)-dien-3-one; (E) 24ξ-hydroperoxy-24-ethylcholesta-4,28(29)-dien-3-one; (F) 24-ethylcholesta-4,24(28)-dien-3,6-one; (G) 24ξ-hydroperoxy-24-ethylcholesta-4,28(29)-dien-3,6-dione; (H) 24ξ-hydroperoxy-6β-hydroxy-24-ethylcholesta-4,28(29)-dien-3-one; and (I) 6β-hydroxy-24-ethylcholesta-4,24(28)-dien-3-one. For details readers may refer to Sheu *et al.* (1999).

source for the development of anti-cancer agents without side-effects. Yamaguchi *et al.* (2001) studied the effect of various algae on bone calcification in the femoral-metaphyseal tissues of rats. Bone calcium content significantly increased by the administration of *U. pinnatifida, S. horneri, E. bicyclis*, or *C. scmitziana*. Bone alkaline phosphatase activity, which is an enzyme for calcification, was significantly enhanced by administration of *S. horneri* or *G. amansi, S. horneri* extract had an anabolic effect on bone calcification *in vivo* and *in vitro*.

Bhaskar *et al.* (2004c) investigated the fatty acid composition of total lipids and different lipid classes of brown algae *Sargassum marginatum*. Phospholipids were found to be higher in PUFA among all the lipid classes analysed. Phopholipids exhibited cytotoxic activity as low as 20 μg/ml on human promyelocytic leukemia (HL-60) cells (Fig. 26.5). Marine algae are well known as a rich source of polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids. Despite their high content of highly unsaturated fatty acids (HUFA), which are very susceptible to oxidation, their quality was not changed during storage (Sakata, 1997). It is believed that marine algae are protected against oxidative deterioration by certain antioxidant systems and a number of studies have been conducted to verify and evaluate the antioxidant activity of marine algae. Some unsaturated fatty acids have been reported to play an effective role in antioxidant activity.

26.3.2. Conjugated Fatty Acids

Interest in seaweeds has been on the rise owing to the recognition of important bioactive molecules like conjugated fatty acids, pigments (especially fucoxanthin) and polyunsaturated fatty acids (PUFAs) that have profound physiologi-

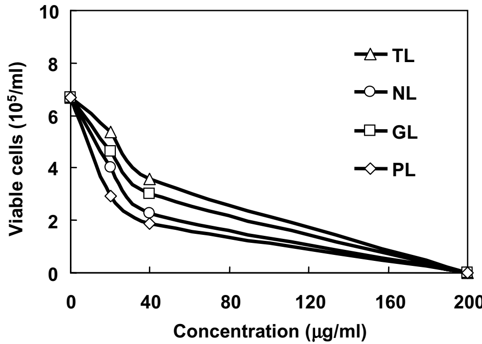

Figure 26.5. Effect of concentration (μg per ml) of total lipids and each lipid class of *S. marginatum* on the viability of HL-60 cells after 72 hour incubation at 37 °C. TL—Total lipids; NL—Neutral lipids; GL—Glycolipids; PL—Phospholipids. Source: Bhaskar *et al.*, 2004c.

cal effects in the treatment of tumors and other related problems (Bhaskar *et al.*, 2004c; Suzuki *et al.*, 2001; Kotake-Nara *et al.*, 2001; Hosokawa *et al.*, 2004; Yasui *et al.*, 2005). The occurrence and important physiological effects of conjugated fatty acids of both aquatic and terrestrial plants has been recently reviewed (Bhaskar *et al.*, 2006a).

There are few reports on the inhibitory effect of conjugated polyenes on the growth of cancer cell lines. Begin *et al.* (1988) reported the toxic effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on several kinds of tumor cells; other polyunsaturated fatty acids, *i.e.*, arachidonic acid (22:4n-6), α-linolenic acid (18:3n-3), and γ-linolenic acid (18:3n-6) have cytotoxic action on several tumor cell lines at concentrations above 50 μM. Further, Tsuzuki *et al.* (2004) demonstrated that the anticarcinogenic effect of CLN are directly associated with lipid peroxidation. They transplanted human colon cancer cells (DLD-1) into nude mice, and CLA (9c, 11t and 10t, 12c- 18:2) and CLN (9c, 11t, 13t-18:3) were administered to animals. Tumor growth was suppressed by the supplementation of CLA and CLN, and the extent of suppression was CLN >9c, 11t-CLA.>10t, 12c-CLA, in that order. Furthermore, DNA fragmentation was enhanced and lipid peroxidation increased in tumor cells of the CLN-fed mouse. Thus this study indicates the possibility of seaweeds as potential sources of anticancer substances.

26.3.3. Fucoxanthin

Carotenoids have beneficial effects in cancer chemoprevention (Astrog, 1997). Fucoxanthin, one of the the major carotenoids in brown algae, is known to exhibit antitumor activity against human neuroblastoma GOTO cells (Okuzumi *et al.*, 1990). Inhibitory effects on mouse duodenum and on skin carcinogenesis have also been reported (Okuzumi *et al.*, 1993; Satomi *et al.*, 1996).

The carotenoids act as either antioxidants or as prooxidants, depending on the environment. Fucoxanthin has a unique structure, including an unusual allenic bond and 5,6-monoepoxide in its molecule. Epoxy beta carotene, neoxanthin, halocynthiaxanthin, and fucoxanthin containing epoxide in their molecules induced a remarkable reduction in the growth of leukemia and prostrate cancer cells (Nishino *et al.*, 1992; Hosokawa *et al.*, 1999; Duitsman *et al.*, 1999; Kotake-Nara *et al.*, 2001). Maoka *et al.* (2007) characterized fucoxanthin and fucoxanthin esters in the Chinese Surf clam, *Matra chinensis* on the basis of ^{1}H NMR and FAB-MS spectra. ^{1}H NMR revealed that the hydroxyl group at C-3 in fucoxanthin and fucoxanthinol was acylated. 3′-o-Acylated compounds such as fucoxanthinol 3-ester or fucoxanthinol 3,3′-diester were not found in the clam. The fatty acids esterified with fucoxanthin and fucoxanthinol were identified as C24:6, C22:6, C20:0, C20:1, C18:0, C18:1, C16:0, C16:1 and C14:0 by FAB-MS data. Mori *et al.* (2003) isolated metabolites of fucoxanthin from brown algae *Scytosiphon lomentaria*, and the structure of the new compound was determined by NMR. Some of the compounds isolated by them are presented in Figure 26.3.

Fucoxanthin has been reported to be very effective in inducing apoptosis in human colon cancer cells. In a study screening the anti-proliferative activity of seafood extracts on tumor cells, Fucoxanthin from brown algae, *Undaria pinnatifida*, was found to be the active principle (Kotake-Nara *et al.*, 2001). In another study dealing with the antiproliferative activity of fucoxanthin on HL-60 cells (Hosokawa *et al.*, 1999), fucoxanthin exhibited an activity higher than that of β-carotene, inhibiting the proliferation of cancer cells at a concentration as low as 22.6 μM. Further, in HL-60 cells treated with fucoxanthin for 24h, viable cell numbers decreased in a dose-dependent manner (Fig. 26.6). The strong inhibitory effect of fucoxanthin has been also confirmed using human prostrate cancer cells (Kotake-Nara *et al.*, 2001). In their study, Kotake-Nara and colleagues charted the effect of fifteen kinds of carotenoids (phytoene, phytofluene, lycopene, β-carotene, β-cryptoxanthin, α-carotene, canthaxanthin, astaxanthin, capsanthin, luetin, zeaxanthin, violoxanthin, neoxanthin, and fucoxanthin) present in food stuffs had on the growth of the human prostrate cell cancer lines (PC-3, DU 145 and LNCap). Among the carotenoids evaluated, they reported that neoxanthin and fucoxanthin caused a remarkable reduction in the growth of prostrate cancer cells. DNA fragmentation revealed that these two carotenoids apparently reduced the cell viability by inducing apoptosis.

A study on the effect of fucoxanthin on the viability of human cancer cells Caco-2, revealed that after 72 hr of incubation with 7.6 mM of fucoxanthin, the number of viable cells decreased by 39% compared to the control (Fig. 26.7).

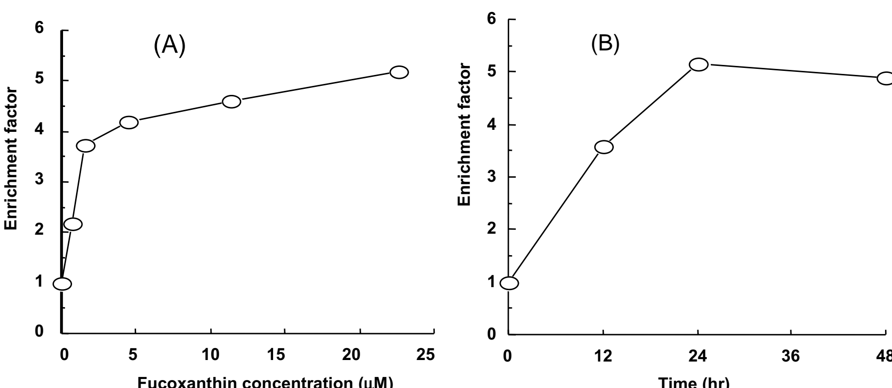

Figure 26.6. Effect of fucoxanthin on DNA fragmentation in HL-60 cells. HL-60 cells (1 – 105 cells/ml) were incubated with different concentration of fucoxanthin for 24 h (A) and different incubation time with 11.3 mM fucoxanthin (B). DNA fragmentation was analyzed by sandwich ELISA with biotin labeled anti-histone antibody and peroxidase conjugated anti-DNA antibody. The level of DNA fragmentation was expressed as an enrichment factor defined as absorbance (405 nm) of cells treated with fucoxanthin relative to that of untreated cells. Source: Hosokawa *et al.*, 1999.

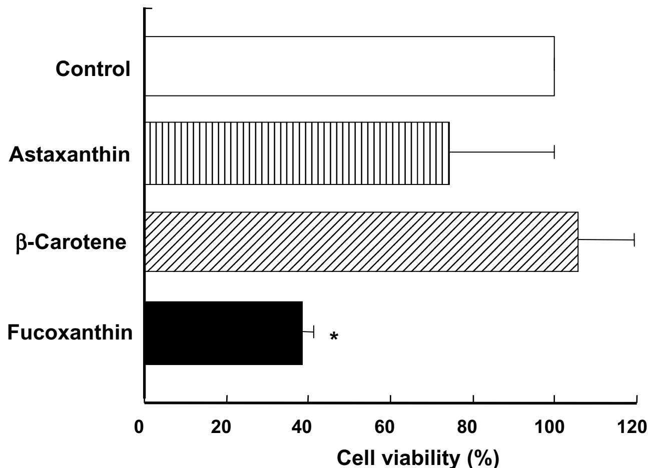

Figure 26.7. Comparision of viability of Caco-2 cells incubated with fucoxanthin, astaxanthin or (-carotene. Caco-2 cells were incubated with 7.6 μM each carotenoids for 72 h. Cell viability was measured by WST-1 assay. Values are means ± SD (n=3/4). The asterisk indicates a value significantly different from the control value ($P < 0.01$). Source: Hosokawa *et al.*, 2004.

Fucoxanthin has also been reported to reduce the viability of human colon cancer cell lines (DLD-1 and HT-29 cells) in a dose- and time-dependent manner. Caco-2 cells were more sensitive to fucoxanthin followed by DLD-1 and HT-29 cells (Hosokawa *et al.*, 2004). Fucoxanthin suppresses the level of Bcl-2 protein, which is responsible for the suppression of programmed cell death as a survival factor (Hockenbery *et al.*, 1990; Levy *et al.*, 2003). DNA fragmentation induced by fucoxanthin has been reported to be partially inhibited by a caspase inhibitor Z-VAD-fmk. However, since Z-VAD-fmk diminished DNA fragmentation by only 40%, the apoptosis signaling in Caco-2 cell by fucoxanthin seems to be mediated by caspase-dependent and independent pathways. Further, fucoxanthin may also regulate the redox signals, and then facilitate the progression as apoptosis through Bcl-2 protein suppression and caspase dependent and independent pathways (Hosokawa *et al.*, 2004). The combination effect of fucoxanthin and troglitazone on the reduction of Caco-2 cell viability was demonstrated by Hosokawa *et al.* (2004).

Obesity, the excessive accumulation of body fat, is a major risk factor for diseases like hypertension, diabetes arthritis, and cardiovascular diseases. Uncoupling protein (UCP1) is a key anti-obesity molecule. UCP1 expression in brown adipose tissue (BAT) is a significant component of whole body energy expenditure: dysfunction threre contributes to the development of obesity (Lowell *et al.*, 1993). Recently, the anti-obesity effect of edible seaweed carotenoids, fucoxanthin, through the protein gene expression of UCP1 in

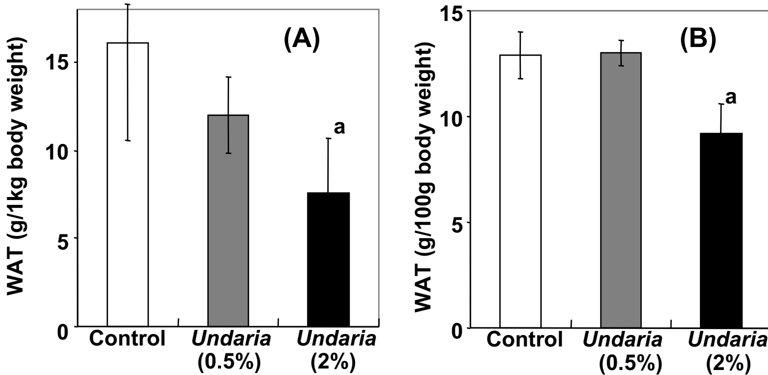

Figure 26.8. Weight of WAT of Mice Fed Fucoxanthin, *Undaria* Glycolipids and Control Diet.
[a]Significant different from control ($P < 0.01$). The dietary fats were 13% soybean oil (control), 12.6% soybean oil + 0.4% fucoxanthin, and 11.2% soybean oil + 1.8% *Undaria* glycolipid. Source: Maeda *et al.*, 2005.

white adipose tissue (WAT) was reported by Maeda *et al.* (2005). In that study, a mixture of 7% soybean oil (control), 6.5% soybean oil + 0.5% Undaria lipids, and 5% soybean oil + 2% undaria mlipids was fed to rats, and the weight of WAT was significantly lower in 2% Undaria lipids-fed rats than in control group (Fig. 26.8). In 0.5% Undaria lipids-fed rats, the weight of the WAT was not significantly lower than in the control group. Further, body weight of mice fed the 2% Undaria lipid was significantly lower ($P < 0.05$) than that of the control group, although there were no significant differences in the dietary mean daily intake between the groups. Fucoxanthin-rich fraction and *Undaria* glycolipids fraction were administered to obese KK-Ay mice. The WAT weight of fucoxanthin rich fraction- fed mice was significantly lower than that of control mice. However, there was no difference in WAT weight of mice fed *Undaria* glycolipids and the control diet. This result indicates that the antiobesity effect is largely due to fucoxanthin as the active component in the *Undaria* lipids.

UCP1 is a dimeric protein present in the inner mitochondrial membrane of BAT, and it dissipates the pH-gradient generated by oxidative phospholyration, releasing chemical energy as heat. UCP1 is exclusively expressed in BAT, where the gene expression is increased by cold, adrenergic stimulation, β_3-agonists, retinoids, and thyroid hormones. UCP1 seems to be a key molecule for anti-obesity. It is usually expressed only in BAT; adult humans have very little BAT, and most of their fat is stored in WAT. Considered as breakthrough discoveries for an ideal therapy of obesity, the regulation of UCP1 expression in tissues other than BAT by food constituents would be important. From this viewpoint, the anti-obesity effect of edible seaweed carotenoid, fucoxanthin,

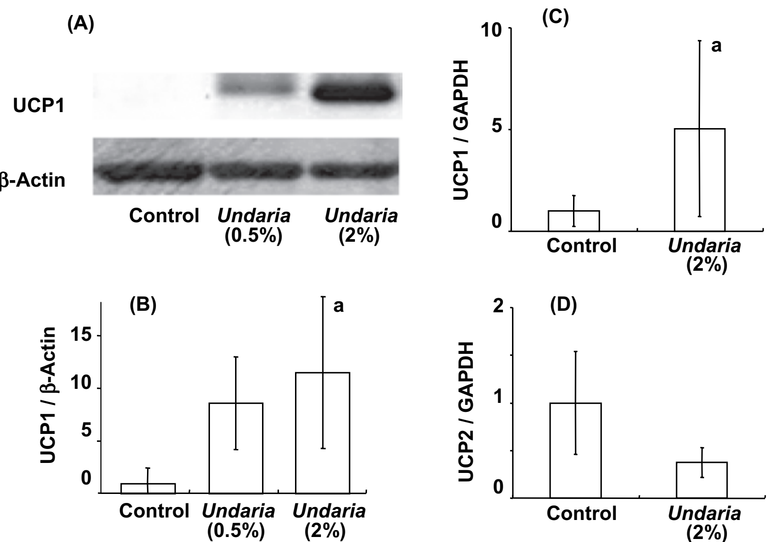

Figure 26.9. UCP1 and UCP2 Expressions in WAT of Mice Fed *Undaria* Lipids and Control Diet. (A), Western blot analysis of UCP1. (B), UCP1 protein expression. (C), UCP1 mRNA expression. (D), UCP2 mRNA expression. [a]Significant different from control ($P < 0.05$). Source: Maeda *et al.*, 2005.

is very interesting, as its activity depends on the protein and gene expressions of UCP1 in WAT. As demonstrated by Figure 29.9, UCP1 expression was found in WAT of *Undaria* lipids-fed mice, although there was little expression in that in thecontrol mice. Expression of UCP1 mRNA was also found in WAT of *Undaria* lipids-fed mice, but little in the control (Figure 26.9). UCP1 is a specific protein which induces fat oxidation and conversion of the energy to heat. Therefore, the decrease in WAT weight of *Undaria* lipids-fed mice would be due to the adaptive thermogenesis through UCP1 expression in WAT. UCP1 expression in WAT was also found in fucoxanthin-fed mice, but little expression of UCP1 was found in WAT of mice fed *Undaria* glycolipids and control diets. This result confirmed the anti-obesity activity of seaweed carotenoid, fucoxanthin, through up-regulation of UCP1 expression in WAT. That fucoxanthin induces both protein and mRNA expressions of UCP1 in WAT is a clue for new dietary anti-obesity therapy. While an enormous amount of data has been collected on thermogenesis in BAT through UCP1 expression, therehad been no information on UCP1 expression in WAT induced by a diet component until the above report. An excessive accumulation of fat in WAT induces some diseases such as Type II diabetes. Direct heat production by fat oxidation in WAT, therefore, will reduce risk of these diseases in humans. For more information on physiological effects of fucoxanthin, see Miyashita (2007).

26.3.4. Fucosterol

Clinical studies have demonstrated that dietary intake of plant sterols (as part of normal diet, or as a supplement) may help to reduce blood cholesterol levels (Kamal-Eldin *et al.*, 1998; Dunford and King, 2000). It has been suggested that sterols have anti inflammatory, antibacterial, antifungal, antiulcerative and antitumor activity (Beveridge *et al.*, 2002). The oxygenated fucosterols also exhibited cytotoxicity against various cancer cell lines (Rocha *et al.*, 2007). The oxygenated sterols isolated from a brown seaweed, *Turbinaria conoides*, exhibited cytotoxicity towards several cancer cell lines. The compounds showed significant bioactivity as their ED50 values were $\leq 4.0\,\mu g/mL$ indicating the potential to recover such bioactive substances naturally (Sheu *et al.*, 1999).

26.3.5. Antioxidant properties

Antioxidant activity of marine algae may arise from carotenoids, tocopherols and polyphenols. These compounds directly or indirectly contribute to inhibition or suppression of free radical generation. The lipophylic extracts from 16 species of seaweeds showed potential antioxidant activities proportional to the content of unsaturated fatty acids (Huang and Wang, 2004). Indole compounds isolated from marine algae have proven to exert inhibitory effect on lipid oxidation (Takahashi *et al.*, 1998). Investigations on dimethylsulphoniopropionate have recently revealed that this compound from marine algae could serve as an effective antioxidant (Athukorala *et al.*, 2005). And some unknown compounds present in marine algae may also act as active constituents in inhibiting lipid oxidation.

Various extraction methods have been used to release these identified and unidentified antioxidant substances from marine algae. Solvent extraction methods employ different solvent systems depending on the solubility of the desired bioactive materials in certain solvents. More recently, enzyme assisted extraction has been proposed to prepare potential natural water soluble antioxidants from marine algae. Enzymes such as carbohydrases and proteases are used to macerate the tissues of the algae, break down the cell walls to release interior compounds (Heo *et al.*, 2005a, 2005b). Mori *et al.* (2003) found that the methanol extract of marine brown algae *Sargassum micracanthum* inhibited oxiditation in rat liver homogenates. A red alga *Grateloupia filicina* was reported to contain compounds with high antioxidant efficacy equal to or better than that of commercial antioxidants such as BHA, BHT and α- tocopherol, thus its use as an natural antioxidant in food formulations was suggested (Athukorala *et al.*, 2003a, 2003b, 2005). Mediterranean marine algae of genus Cystoseria were found to possess antioxidant activity comparable to that of α-tocopherol (Foti *et al.*, 1994). Furthermore, water, methanol, and ethanol extracts of an edible seaweed *Hizikia fusiformis* showed significant ROS scavenging activity, indicating that it might prove to be a valuable source of both water soluble and fat soluble antioxidants (Siriwardhana *et al.*, 2003). Enzy-

matic extracts from various brown algae were reported to exert a positive effect in reducing oxidative damage to DNA (Heo *et al.*, 2005a,b; Park *et al.*, 2005).

26.4. PREPARATION OF SEAWEED LIPIDS FOR INDUSTRIAL USE

26.4.1. Resources

Total production of aquatic plants, including seaweeds, in 2004 was 15.36 MMT, to which aquacultured seaweeds contributed almost 91%, while capture (harvested from the wild) contributed 9% (FAO, 2006). Seaweeds as a group contributed >80% of the total global production of aquatic plants through culture. There are about 10,500 species of seaweeds divided into three main class *viz.*, green (*Chlorophytes*), brown (*Phaeophytes*), and red (*Rhodophytes*). Brown seaweed is the major class, contributing about >63% of the total culture production in 2004, followed by red (36%), and green (<1%). There are about 2200 species of brown algae, 6500 species of red, and 8000 species of green algae, and most are found in cold water. The brown color results from the dominance of the xanthophyll pigment, fucoxanthin, which masks other pigments including chlorophyll-a and chlorophyll-c, β-carotene, and other xanthophylls. There are about 2200 species of brown algae: most are marine and found in cold water.

The color of red algae results from the dominance of the pigments phycoerythrin and phycocyanin. With walls of cellulose, agar, and carrageenan, the long chain polysaccharides enjoy widespread commercial use. A very important group of red algae is the coralline, which secrete calcium carbonate on the surface of their cells. Corallines have been used in bone replacement therapies. There are about 6500 species of red algae, most of which are marine. The red algae *Kappaphycus* and *Betaphycus* are now the most important sources of carrageenan, commonly used ingredients in foods.

The coloration in green algae is due to chlorophyll a and b. Most of the species are aquatic, and are found commonly in fresh water and marine habitats; some are terrestrial, growing in soil, trees, or rocks. Some are symbiotic with fungi, others are symbiotic with animals, *e.g.*, the fresh water coelenterate Hydra has a symbiotic species of chlorella. Chlorella is sold as a health supplement (Wong and Cheung, 2000).

The principle problem of algae as sources of lipids is their high water (70–90%) and low lipid content. Despite their lipid content (~7%), the fatty acids of marine algae have aroused considerable interest among researchers for their nutritional value to other marine organisms, occurrence of bioactive conjugated fatty acids, and potential medical applications. The annual production of carotenoids from seaweeds is estimated to be approximately 100 million tons, of which fucoxanthin, the main carotenoids from brown seaweed, contributes more than 10% (Matsuno, 2001).

26.4.2. Other Resources (Microalgae)

As an alternative to usual marine oils, PUFA can be obtained from micro algae (Figure 26.1) and microbes. PUFAs in microorganisms are usually synthesized from the starting monounsaturated fatty acid, oleic acid, by the same enzyme mechanisms found in higher organisms. There are mainly two reactions involved in the pathways: chain elongation, and desaturation (Figure 26.10). However, in the context of this section, we will examine only microalgae as an important alternative for PUFA production.

Depending on species and environmental conditions, the lipid content of microalgae may vary between a few percent to over 80% of the biomass on a dry weight basis (Yongmanitchai and Owen, 1989). These microorganisms accumulate over 20–25% lipid on a dry biomass. Oleaginous microorganisms store lipids mainly in the form of triacylglycerols. Various eukaryotes can accumulate large amounts of triacylglycerols (Ratledge, 2001). Fatty acid composition of microorganisms, both qualitatively and quantitatively, is influenced by environmental conditions. Parameters such as media composition, aeration, light intensity, and light-dark cycle, temperature, and culture age all play a major role in biosynthesis and accumulation of PUFAs in most microorganisms. Microalgae oil or single cell oil (SCO) production via fermentation is a new concept (Ratwan, 1991). In the SCO process, microorganisms that are able to produce the desired oil are cultivated in a bioreactor (Sijtsma and de Swaaf,

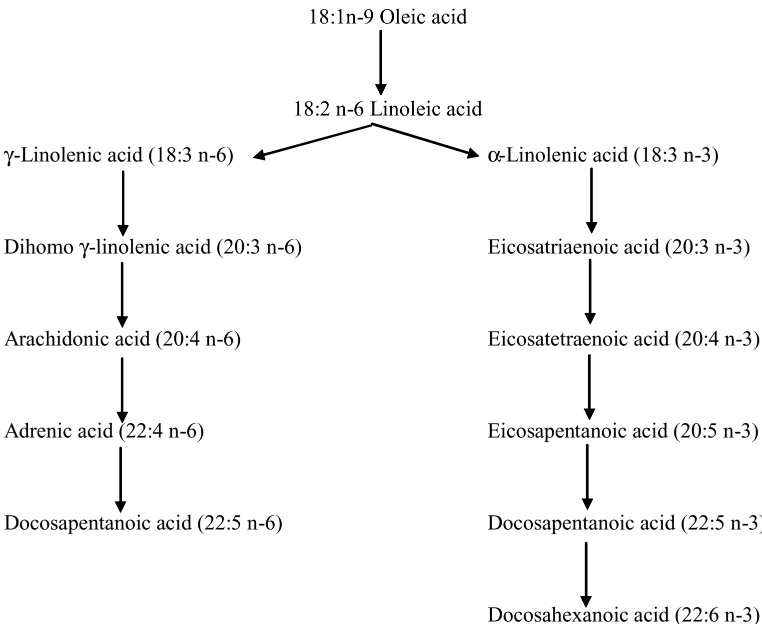

Figure 26.10. Polyunsaturated fatty acid (PUFA) synthesis pathways in microbes.

2004). In industrial scale fermentation, cells are harvested at maximum volumetric productivity, followed by drying and further processing of the oil (Kyle, 1997). Since heterotrophic cultivation is independent of light, this production system possesses many advantages such as axenical operation, optimal controlled condition, increased reproducibility, higher biomass concentrations, and straightforward scale-up of the fermentation process (Chen, 1996). High levels of DHA are found in heterotrophic marine algae including *Traustochitrium, Schizochytrium* and *Crypthecodinium cohnii* species. Fatty acid composition of microorganisms, both qualitatively and quantitatively, is influenced by environmental conditions. Parameters such as media composition, aeration, light intensity, and light-dark cycle, temperature, and culture age all play a major role in biosynthesis and accumulation of PUFAs in most of the microorganisms. Algal oils have many benefits in functional foods due to their n-3 fatty acids and the lack of environmental toxins. Algae usually contain one specific PUFA rather than a mixture of various PUFA.

Some lower fungi and algae of class *Dinophyceae are* good sources of DHA. Macroscopic algae such as the *Rhodophyceae,* which contain substantial amount of EPA, are potential sources of omega-3 fatty acids for production of marine culture (Yongmanitchai and Ward, 1989). Recently, a Japanese firm patented a method for the production of EPA concentrate from marine Chlorella extract. Marine unicellular algae, *Chlorella minutissima*, contains very high EPA content (45% of total fatty acids) at its optimal culture conditions. This particular species is a promising source of EPA for commercial purpose. EPA concentrate obtained from this alga was claimed to be superior to those from fish oil because it has no unfavorable fish flavor (Yongmanitchai and Ward, 1989).

Algal oils have many benefits in functional foods due to their high n-3 fatty acids and lack of environmental toxins. Alga usually contains one specific PUFA rather than mixture of various ones. This gives the algal oil added advantage compared to fish oils, which contain mixtures of PUFA. In addition, PUFA can be purified more easily (and more economically) (Ratledge, 2001). Belarbi *et al.* (2000) have developed a low cost process for recovering esterified eicosapentaenoic acid (EPA) from microalgae, recovering over 70% of the EPA content in the esterified crude extract at purities exceeding 90%. The recovery scheme utilized either wet or freeze dried algal biomass. The process consists of three main steps: *i)* simultaneous extraction and transesterification of algal biomass, *ii)* argenated silica gel chromatography of crude extract, and *iii)* removal of pigments by a second column chromatographic step. Argenated silica gel chromatography recovered about 70% of the EPA ester present in the crude fatty ester mixture. Compared to the green algae *M. subterraneus*, the diatom *P. tricornutum* has important advantages as a potential commercial producer of EPA. The quality of microalgal EPA compares favorably with that of fish oil. Compared to free fatty acid, EPA ester is more stable in storage. Shelf life is extended by storing in hexane. The silver contamination in the final purified EPA is negligibly small (<210 ppb) (Belarbi *et al.*, 2000).

26.5. CONCLUSION

Seaweed is one of the important marine living resources, and bountiful in several biofunctional molecules of marine origin. With a unique structure and biochemical composition, seaweed is a potential candidate for exploiting several biomolecules that exhibit multifunctional properties in the form of food, energy, medicine, and cosmetics, and in biotechnological applications. Although innumerable data exists on the physiological benefits afforded by seaweed, not much has been done industrially to exploit and utilize the seaweed biomolecules. Effort is needed to establish feasible recovery methods to enhance the utility of these important functional molecules.

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CHAPTER 27

Enzymatic Production of Marine-derived Protein Hydrolysates and Their Bioactive Peptides for Use in Foods and Nutraceuticals

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27.1. INTRODUCTION

Enzymatic hydrolysis has long been used to produce functional protein hydrolysates from vegetable and milk proteins; however it also shows great potential for use in marine products. During the 1960s and '70s, initial research began into the utilization of fishery by-products through the production of marine-

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derived protein hydrolysates. Use of these bioactive compounds in the food and feed industries shows promise as a way to increase the value of these traditionally underutilized by-products (Dong *et al.*, 2005). One of the first published studies on marine-derived protein hydrolysates was conducted with papain, and was focused on the production of peptones for fermentation media (Sripathy *et al.*, 1962). Since then, this area of research has grown significantly, attracting the interest of both scientists and food processors, largely due to an increased demand for utilization of limited marine resources along with an increasing world population.

As food ingredients, protein hydrolysates possess numerous functional properties, including water-holding capacity, gelation, foam stability, and emulsion capacity (Khan *et al.*, 2003; Sathivel *et al.*, 2005). Moreover, many studies have demonstrated that protein hydrolysates show an excellent amino acid balance, good digestibility, rapid uptake, and the presence of certain bioactive peptide components. They have potential use in a variety of applications including food additives such as emulsifiers and foaming agents, plant nutrients, fertilizers, animal feeds, and protein supplements (Kristinsson and Rasco, 2000b; Sathivel *et al.*, 2005). Currently, marine-derived protein hydrolysates are widely used as feed for a variety of farmed animals and cultured fish. Although this technology has not shown significant economic benefits thus far, recent products developed for human consumption have met with some success in the worldwide market (Kristinsson, 2007; Kristinsson and Rasco, 2000b).

Food materials, including fish, contain the precursors to these bioactive peptides, which can be formed *in vitro* or *in vivo* by enzymatic hydrolysis (Korhonen and Pihlanto 2003). During the past decade, a number of studies have reported on the many physiological properties of these bioactive peptides. The aim of this review is to describe the production of novel peptides derived from marine protein hydrolysates, elucidating the underlying mechanisms of physiological and biofunctional activity that are particular to individual bioactive peptides. The general sources and production of protein hydrolysates will be discussed, followed by a discussion of the nutraceutical properties of protein hydrolysates and their associated bioactive peptides.

27.2. ENZYMATIC PRODUCTION OF MARINE-DERIVED PROTEIN HYDROLYSATES

27.2.1. Potential Sources of Protein Hydrolysate

Currently, only about 50 to 60% of the total marine catch is used for direct human consumption, and annual discards from world fisheries have been estimated to be approximately 25–30 million metric tons (Sovik and Rustad, 2005). Novel means of processing are required to convert fishery by-products into more marketable and acceptable forms (Benjakul and Morrissey, 1997).

Isolation of functional compounds, such as protein hydrolysates and their related biopeptides, shows high potential as a way to increase the utilization of these valuable resources. To date, the by-products/wastes from several fish species have been exploited for commercial production of protein hydrolysate, with the most common source being fillet by-products. These discards—bones, tails, and heads—account for about 22% of the raw material, and can contain considerable amounts of high-quality protein (Gbogouri *et al.*, 2004). Frames of a variety of fish species have been utilized for this purpose, with some examples being cod (Jeon *et al.*, 1999; Liaset *et al.*, 2000; Slizyte *et al.*, 2005b) Atlantic salmon [(*Salmo salar*) (Gbogouri *et al.*, 2004; Liaset *et al.*, 2000)], red salmon [(*Oncorhynchus nerka*) (Sathivel *et al.*, 2005)], yellowfin sole [(*Limanda aspera*) (Jun *et al.*, 2004; Jung *et al.*, 2006b)], and hoki [(*Johnius belengerii*) (Kim *et al.*, 2007)].

Significant amounts of by-product can be also obtained through the surimi-production line, inclluding viscera, head, skin, bone and some muscle tissue., Generated during processing, these can be as high as 70% of the original raw material depending on the method of meat extraction from the carcass (Benjakul and Morrissey, 1997). By-products from other marine industries that have also been used for protein hydrolysate production, include the hepato-pancreas from squid processing (Ono *et al.*, 2002; Ono *et al.*, 2004) and solid wastes (mantle, gills, gonads, digestive gland, and substandard muscle) from scallop processing (Mukhin and Novikov, 2001). Discards of marine crustaceans including deep-water prawn (*Pandalus borealis*) and king crab [(*Paralithodes camtschaticus*), (Mukhin and Novikov, 2001)] have also been utilized as a source of protein hydrolysate. Another opportunity for improvement in the utilization of fish by-products is with spawned salmon. Large quantities of upstream chum salmon are harvested in Hokkaido, Japan, with total landings every year amounting to more than 2 million fish; however, spent females and males are generally discarded, causing considerable harm to the surrounding ecosystem. These salmon muscles are high in protein and low in lipid, which is advantageous for production of protein hydrolysate.

Next to by-products, underutilized fish species are the second major source for marine-derived protein hydrolysate. In addition to increasing utilization of limited marine resources, these species could also help to expand market opportunities. Some currently underutilized species–sharks such as Spiny dog fish (*Squalus acanthias*) and pelagic thresher [(*Alopias pelagicus*), (Nomura *et al.*, 2002)] for example—have great potential as sources of high-quality protein (Diniz and Martin, 1997). Another underutilized species, the giant squid (*Dosidicus gigas*), has a low economic value because of its large soft mantel but possesses an unique amino acid composition (Rajapakse *et al.*, 2005b).

27.2.2. Hydrolysis with Proteolytic Enzymes

Proteolytic enzymes hydrolyze the peptide bonds in proteins and polypeptides. These proteases are separated into two main groups: those that can

cleave N- or C- terminal peptide bonds (exopeptidases), and those that can cleave internal peptide bonds [(endopeptidases), (Shahidi and Kamil, 2001; Sumantha *et al.*, 2006)]. A combination of enzymes with endo- and exopeptidase activity is often used in commercial hydrolysis applications to achieve a more thorough degradation (Adler-Nissen, 1986; Kristinsson, 2007). Most commercially available enzymes for protein hydrolysis are derived from plants and microorganisms; however, these enzymes are also present in fish viscera and muscle. Some of the most common include digestive proteolytic enzymes (Je *et al.*, 2005a), alcalase (Kim *et al.*, 2007; Klompong *et al.*, 2007; Li *et al.*, 2006; Sathivel *et al.*, 2005; Slizyte *et al.*, 2005c), flavourzyme (Kim *et al.*, 2007; Klompong *et al.*, 2007; Nilsang *et al.*, 2005), neutrase (Kim *et al.*, 2007; Slizyte *et al.*, 2005b), protamex (Liaset *et al.*, 2002), and thermolysin (Ono *et al.*, 2006).

Digestive proteolytic enzymes have been used in a wide range of applications. Pepsin, trypsin, and α-chymotrypsin are the most commonly used digestive proteolytic enzymes in the field of protein hydrolysate production. Pepsin is an acidic and aspartic protease with an optimum pH between 1.0 and 4.0 (Adler-Nissen, 1986). It preferentially cleaves proteins at the carboxylic groups of aromatic amino acids, such as phenylalanine and tyrosine, and does not cleave bonds containing leucine, aspartic acid, and glutamic acid-COOH (Adler-Nissen, 1986). Trypsin is a pancreatic protease with substrate specificity based on positively-charged lysine and arginine side chains. Categorized as a serine proteinase, trypsin has a maximum activity at alkaline pH. The conformation of trypsin is well ordered between pH 7.0 and 8.0, but is significantly less ordered at more acidic or alkaline pH values (Simon *et al.*, 2001). Like trypsin, α-chymotrypsin can be also categorized as a pancreatic serine proteinase, and the optimum pH of this protease is 8.0 (Whitaker, 1994). α-chymotrypsin catalyzes the hydrolysis of peptide and ester bonds at the carboxyl group of a variety of amino acids, mostly those with aromatic or long aliphatic side chains, such as phenylalanine, tyrosine, and tryptophan.

Several alkaline proteases are produced by utilizing alkaliphilic *Bacillus*. One of the most, alcalase, is prepared by submerged fermentation of a selected strain of *B. Licheniformis* produced by Novozymes (Bagsvaerd, Denmark). The optimal temperature for this enzyme is between 55 and 70 °C (depending on the type of substrate), and optimum pH values are between 6.5 and 8.5 (Slizyte *et al.*, 2005c). Flavourzyme is a fungal protease/peptidase complex also produced by Novozymes, using the submerged fermentation of a selected strain of *Aspergillus oryzae*. It can be used for the hydrolysis of proteins under neutral or slightly acidic conditions. The optimal working conditions for this enzyme are pH 5.0–7.0, with the optimal temperature around 45–50 °C. This protease can be categorized as an exopeptidase that particularly cleaves amino acids from the C-termini of peptides and proteins by utilizing a catalytic triad of serine, histidine, and aspartic acid in their active sites (Blinkovsky *et al.*, 1999). Neutrase is a metalloproteinase manufactured by Novozymes produced by a selected strain of *Bacillus subtilis*. The optimum pH of this protease is

7.0, with increased stability in the presence of Ca^{2+}. It preferentially catalyzes the hydrolysis of peptide bonds with leucine and phenylalanine (Adler-Nissen, 1986). Neutrase is generally useful in cases where proteins must be broken down into peptides either moderately or more extensively (Slizyte *et al.*, 2005b). It has an optimum temperature of 45–55 °C and an optimum pH of 5.5–5.7 (Zapelena *et al.*, 1997). These enzymes fulfill the purity demands for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives and Food Chemicals Codex.

Thermolysin, originally derived from *Bacillus thermoproteolyticus*, can be categorized as a neutral metalloprotease. The optimum conditions for this enzyme are pH 7.0 and 60–80 °C. Thermolysin also requires catalytically active zinc,and calcium for thermostability (Whitaker, 1994). When these conditions are met, heat-stable thermolysin specifically hydrolyzes peptide bonds involving isoleucine, leucine, valine, and phenylalanine (Adler-Nissen, 1986). It has been extensively utilized in the production of antihypertensive peptides which will be discussed later in this review.

Protein hydrolysis can also occur via autolytic activity, with trypsin, chymotrypsin, and other digestive enzymes principally responsible for this reaction (Lopetcharat *et al.*, 2001). Wako and others (1996) reported production of bioactive peptides from squid liver and mantle muscle by applying autolysis at 37 °C. Wu and co-workers (2003) also reported production of protein hydrolysates from mackerel (*Scomber austriasicus*) prepared by a combination of autolytic and commercially available enzymes, Protease N. However, the study concluded that degree of hydrolysis was much higher in antioxidative peptides prepared by Protease N compared to those from autolysis. The major setback to the use of endogenous enzymes is that very little control is possible, as visceral material can vary substantially in activity and enzyme levels (Kristinsson 2007; Shahidi *et al.*, 1995). Consequently, most of the products of autolysis have thus far been utilized only in animal feed or fertilizer. However, one fish protein hydrolysate product, a fish sauce, is prepared using autolysisand is commonly used as a flavor enhancer or salt replacement in a variety of food applications (Tungkawachara *et al.*, 2003).

27.2.3. Production of Protein Hydrolysates

A variety of processes for recovering marine-derived hydrolysates with enzymes have been reported. The main principle of the process is similar in the majority of published studies, with slight modifications depending on the target compounds and functionalities. The general outline of the process is shown in Figure 27.1. High concentrations of lipids and pro-oxidants in the raw materials of fatty fish such as pelagics (*e.g.,* mackerel and sardines), are prone to lipid oxidation during the hydrolysis reaction. This may result in discolorations and off-flavors in the final products. To minimize this problem, antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) can be added prior to the hydrolysis reaction at levels of 0.01%

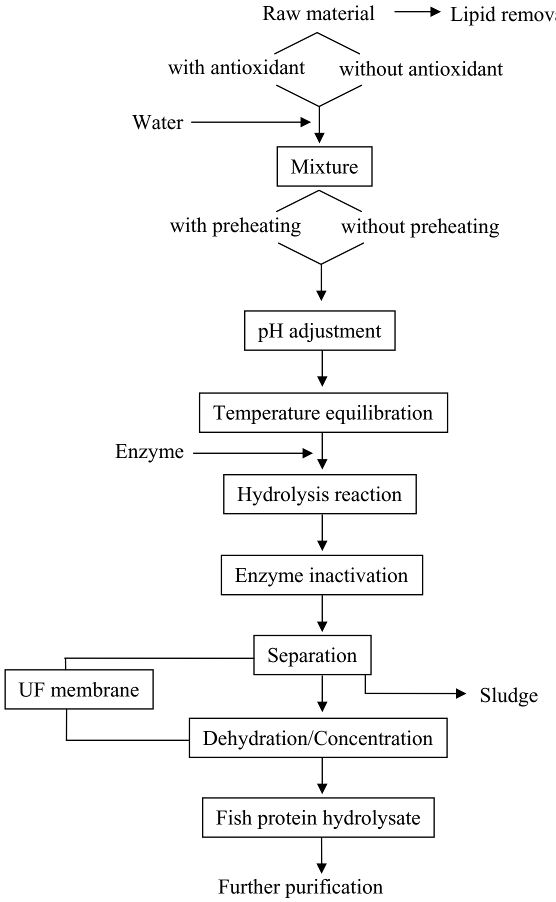

Figure 27.1. Protein hydrolysis for recovery of marine-derived peptides.

(w/w) of lipid content in the mince (Liceaga-Gesualdo and Li-Chan, 1999). Depending on species and lipid content, some studies actually de-fat the raw materials as the initial step. Klompong and colleagues (2007) reported the production of protein hydrolysate with a high antioxidant activity from yellow stripe trevally. They described lipid removal from the raw material using isopropanol at a ratio of 1:4 (mince:solvent, w/v); the residue was defatted at 75 °C for 90 m, the supernatant was removed, and the precipitate was dried at room temperature. Candido and Sgarbieri (2003) also reported removal of lipid from Nile tilapia by utilizing cold ethanol, and extracting the lipid with hot ethanol (50 °C). The lipid content should be lower than 0.5% to prevent noticeable alteration of the lipids during storage (Spinelli *et al.*, 1972).

Following de-fatting, the next step in hydrolysate production is to homogenize the mixture with water. This is sometimes followed by a preheating step, depending on the state of the raw material. Typically, fish by-products contain high levels of spoilage bacteria and digestive enzymes, which can possibly result in reduction of the final product quality. Thus, preheating (around 95 °C) can inactivate such organisms and endogenous enzymes, especially when the sample was not freshly prepared (Kristinsson, 2007; Slizyte *et al.*, 2005a). Next, the pH and temperature of the mixture should be adjusted according to the optimum conditions for enzymatic activity. Commercially available food grade enzyme preparations are commonly supplied in liquid or powder form, which makes them easy and safe to handle. After the addition of proteolytic enzymes, catalysis takes place as three consecutive reactions: *i)* formation of the Michaelis complex between the substrate and the enzyme, *ii)* cleavage of the peptide bond to liberate peptides, and *iii)* a nucleophilic attack on the remains of the complex, to promote separation from the other peptide and to reconstitute the enzyme (Adler-Nissen, 1986). This series of reactions is carried out rapidly with the proteins in the mixture, and results in a significant reduction in the viscosity of the slurry (Kristinsson, 2007). As shown in Figure 27.2, the amount of hydrolysate produced depends on the properties of the enzymes utilized, along with reaction times. After hydrolysis, the mixture should be heated at a temperature range between 80 °C to 100 °C for a prolonged period in order to inactivate proteolytic enzymes by irreversible denaturation.

Sludge, including unhydrolyzed proteins and lipid, can be separated by centrifugation or filtration. Commonly, 3-4 fractions can be obtained: *i)* the sludge (water-insoluble portion) on the bottom, *ii)* protein hydrolysate (water-soluble compounds), *iii)* the oil layer on top, and *iv)* in some cases, an emulsion layer (Slizyte *et al.*, 2005b). Further fractionation can be applied with ultrafiltration (UF) membranes that have different molecular weight cut-offs depending on the target peptides. After fractionation, the protein hydrolysate undergoes dehydration, which is generally accomplished with freeze-drying. Candido and Sgarbieri (2003) used a three step process to isolate protein hydrolysates from Nile tilapia. First, protein concentrate was prepared to remove most of the lipids, then the hydrolysis reaction was carried out with Flavourzyme, and finally, the resulting protein hydrolysates were fractionated by centrifugation (600 g, 15 m). Fractionation resulted in a final product that was lower in total lipids and higher in minerals.

When the ultimate objective is to produce bioactive peptides for particular purposes, such as antioxidative or antihypertensive activities, the purification of target peptides from protein hydrolysate can be carried out using UF membranes with or without chromatographic techniques. Jun *et al.* (2004) reported successful preparation of protein hydrolysates from yellowfin sole frame by first using extracted mackerel intestine crude enzyme at pH 10.0 and 50 °C, followed by treatment with pepsin at pH 2.0 and 37 °C. The resultant hydrolysate was further fractionated through five different UF membranes with

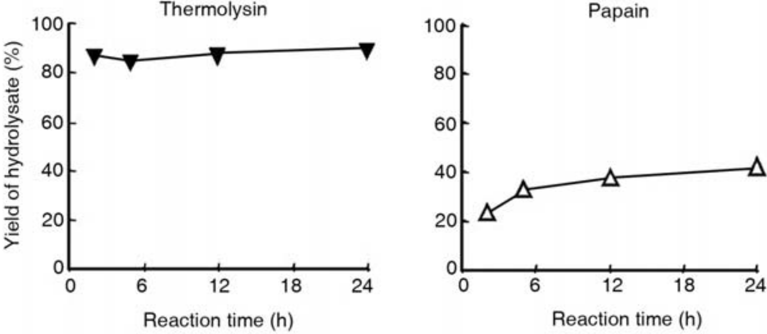

Figure 27.2. Time course of hydrolysate yield.
(Hydrolysis was carried out on defatted salmon flesh with 5% protease at 37 °C.)

molecular weight cut-offs of 30, 10, 5, 3, and 1 kDa. This enabled purification of the antioxidative peptides, which were determined to be 13 kDa, using high-performance liquid chromatography (HPLC) and a gel permeation chromatography column. A step-wise elution with distilled water/ethanol can be also applied in order to isolate peptides with antihypertensive activity, as described by Ono and colleagues (2003, 2006). Isolated hydrolysate from upstream chum salmon muscle was loaded on an octa-decyl silica gel column (ODS, Cosmosil 140 C18-OPN, Nacalai Tesque, Inc., Kyoto, Japan) and eluted with a series of 600 mL aliquots of 10, 25, 50 and 99.5% ethanol. This allowed for the separation of individual fractions, which were then assayed for inhibitory activity against angiotensin-converting enzyme (ACE). Further purification was carried out by applying the active fraction to a Sephadex G-25 column (Amersham Co., Uppsalsa, Sweden) and then eluting with distilled water. The active peaks were then separated by reversed phase HPLC. Likewise, muscle hydrolysate from pelagic thresher was gel-filtered on a Sephadex LH-20 column (Amersham Pharmacia Biotech, Tokyo, Japan) with 30% methanol. This resulted in a successful separation of the active fractions on a diethylaminoethyl-Toyopearl 650M column [(Tosoh, Tokyo, Japan); (Nomura *et al.*, 2002)].

27.3. PHYSIOLOGICAL PROPERTIES OF PROTEIN HYDROLYSATES AND ASSOCIATED BIOACTIVE PEPTIDES

Many studies have been published focusing on the functionality of marine-derived protein hydrolysate for food applications, such as solubility, emulsifying capacity, foaming capacity, water holding capacity, and oil holding capacity (Candido and Sgarbieri, 2003; Gbogouri *et al.*, 2004; Kristinsson and Rasco, 2000a; Liceaga-Gesualdo and Li-Chan, 1999; Slizyte *et al.*, 2005a). For an excellent overview of such functionalities the reader is referred to a review paper

by Kristinsson and Rasco (2000b). However, this section will focus solely on the use of protein hydrolysates and associated bioactive peptides in nutraceutical applications, with a special emphasis on marine-derived peptides. When present as a part of the native protein source, bioactive peptides are inactive; however, once released, these peptides function as regulatory compounds with hormone-like activity (Vermeirssen *et al.*, 2004). These peptides can be liberated from the parent protein during processes such as gastrointestinal digestion, food processing, and enzymatic hydrolysis (Clare and Swaisgood, 2000). Following further purification, the peptides that demonstrate health-promoting activities can be used in nutraceutical applications. This section will discuss the role of these bioactive peptides in nutraceuticals, with particular focus on their antihypertensive, antioxidant, and physiological properties. Amino acid abbreviations will be expressed according to the IUPAC-IUB Joint Commission on Biochemical Nomenclature.

27.3.1. Antihypertensive Properties

27.3.1.1. ACE Inhibitory Activity Hypertension was first identified as one of the most typical cardiovascular risk factors in the late 1950s, and since then, has been established as an international problem, affecting with 15–20% of adults worldwide (Je *et al.*, 2006; Mann and Oddou, 2001). The renin-angiotensin system is a key regulator of cardiac and vascular functions, including blood pressure, with renin producing decapeptide angiotensin I from angiotensinogen (Nakano *et al.*, 2006). The inactive form of decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) can be converted by ACE into a potent vasoconstrictor, octapeptide antiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Angiotensin II can be further converted to angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe), which controls the production of aldosterone. ACE also inactivates the depressor bradykinin (Je *et al.*, 2005b; Sturrock *et al.*, 2004). The results of ACE activity are increases in blood pressure via vasoconstriction, increases in systemic resistance, and stimulated secretion of aldosterone to promote sodium and water resorption in the kidneys (Kitts and Weiler, 2003). ACE is primarily found in tissues, including the blood vessels, heart, kidney, brain, and adrenal gland. A small percentage (<10%) of ACE is also located in the plasma (Swamy *et al.*, 2003). Antihypertensive peptides, known as ACE inhibitors, play a pivotal role in regulating the renin-angiotensin, kallikrein-kinin, and immune systems (Minervini *et al.*, 2003). ACE inhibitory peptides exert an antihypertensive function by first becoming resistant to further digestion by digestive-tract endopeptidases (Kitts and Weiler, 2003). They partially or totally resist hydrolysis, and, because of their lowmolecular size, may enter peripheral blood intact to exert systemic effects (Yoshikawa *et al.*, 2000). As illustrated in Figure 27.3, inhibition of ACE represses the conversion from angiotensin I to angiotensin II. This approach is thus far one of the most efficient methods for suppressing high blood pressure (Saiga *et al.*, 2006). Interestingly, recent findings have indicated that this suppression system

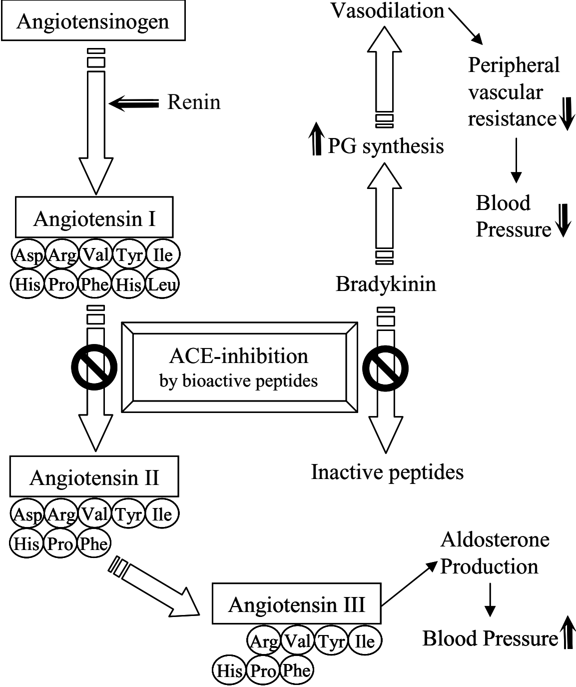

Figure 27.3. Dual effects of ACE inhibition.

is far more complex than originally described, and the presence and roles of additional compounds (*e.g.*, angiotensin 1–7 and angiotensin IV) has been revealed over the last few years (Smith and Turner 2004).

The major nutraceutical application of marine-derived bioactive peptides has been ACE inhibition, and a partial list of identified bioactive peptides is given in Table 27.1. Fish protein has been reported to be an advantageous starting material for preparation of ACE inhibitory peptide hydrolysates (Curtis *et al.*, 2002). Marine-derived protein hydrolysate tends to contain high concentrations of cysteine, methionine, and arginine, all of which help reduce hypertension (Kristinsson, 2007). Such marine-derived peptides could be used as potent functional food additives and represent a healthier and natural alternative to ACE inhibitor drugs (Li *et al.*, 2004). Currently, the bioactive oligopeptides from dried bonito and sardine muscle have been approved as Foods for Specified Health Use by the Ministry of Health and Welfare in Japan.

27.3.1.2. Antihypertensive Effects In vitro *In vitro*, ACE inhibitory activity is generally analyzed using synthetic substrates that have amino-substituted tri and dipeptides of hippuryl-His-Leu (Fahmi *et al.*, 2004; Jung *et al.*, 2006b).

TABLE 27.1. Partial list of ACE inhibitory peptides from marine sources.

Protein source	Treatment	Peptide	IC50 (μM)*	Reference
Pelagic thresher muscle	Thermolysin	Val-Trp	1.68	Nomura *et al.*, 2002
		Met-Trp	3.76	
		Ile-Lys-Trp	0.54	
		Leu-Trp-Ala	12.7	
		Val-Ser-Trp	23.2	
		Val-Thr-Arg	135.9	
		Phe-Arg-Val-Phe-Thr-Pro-Asn	9.59	
Salmon muscle	Thermolysin	Phe-Leu	13.6	Ono *et al.*, 2003, 2006
		Leu-Phe	383.2	
		Ala-Trp	6.4	
		Val-Trp	2.5	
		Met-Trp	9.8	
		Ile-Trp	4.7	
		Leu-Trp	17.4	
		Trp-Ala	277.3	
		Trp-Val	500.5	
		Trp-Met	98.6	
		Trp-Leu	34.1	
Dried bonito	Thermolysin	Ile-Val-Gly-Arg-Pro-Arg-His-Glu-Glu	6.2	Yokoyama *et al.*, 1993
		Ala-Leu-Pro-His-Ala	10	
		Phe-Gln-Pro	12	
		Leu-Lys-Pro-Asn-Met	17	
		Asp-Tyr-Gly-Leu-Tyr-Pro	62	
		Ile-Lys-Pro-Leu-Asn-Tyr	43	

TABLE 27.1. ***Continued***

Protein source	Treatment	Peptide	IC50 (μM)*	Reference
Sardine muscle	Alkaline protease	Met-Phe	44.7	Matsufuji *et al.*, 1994
		Arg-Tyr	51	
		Leu-Tyr	38.5	
		Tyr-Leu	82	
		Ile-Tyr	10.5	
		Gly-Arg-Pro	20.0	
		Gly-Trp-Ala-Pro	3.86	
Sea bream scale	Alkaline protease	Gly-Tyr	265	Fahmi *et al.*, 2004
		Val-Tyr	16	
		Gly-Phe	708	
		Val-Ile-Tyr	7.5	
Alaska pollack skin	Alcalase + protease + collagenase	Gly-Phe-Lys	2.65	Byun and Kim, 2001
		Gly-Phe-Met	17.13	
Red algae	Acid + pepsin	Ile-Tyr	2.65	Saetsuna, 1998
		Met-Lys-Tyr	7.26	
		Ala-Lys-Tyr-Ser-Tyr	1.52	
		Leu-Arg-Tyr	5.06	
Squid liver and mantle muscle	Autolysis	Tyr-Ala-Leu-Pro-His-Ala	9.8	Wako *et al.*, 1996
		Gly-Tyr-Ala-Leu-Pro-His-Ala	27.3	

*Concentration of ACE inhibitor required to inhibit 50% of ACE activity.

Spectrophotometry is used to determine the IC_{50} value, the concentration of hydrolysates that causes a 50% reduction of ACE activity.

A variety of proteolytic enzymes have been tested for their ability to liberate bioactive peptides with high ACE inhibitory activity. Thus far, the majority of published studies have pointed to thermolysin as the most effective enzyme for this purpose (Nomura *et al.*, 2002; Ono *et al.*, 2006; Yokoyama *et al.*, 1992). Yokoyama *et al.* (1992) concluded that the peptides obtained by using thermolysin demonstrated the highest potent inhibitory activity, with an IC_{50} of 29 μg/ml, in comparison with the peptides prepared with trypsin (161 μg/ml), chmotrypsin (117 μg/ml), or pepsin (47 μg/ml). Similarly, a study conducted by Ono *et al.* (2002) demonstrated that thermolysin-prepared peptides showed the highest ACE inhibitory activity compared with peptides prepared using pepsin, trypsin, chymotripsin, protease Type I, protease Type IV, protease Type XXII, acidic protease, alkaline protease, or neutral protease.

Bioactive peptides obtained by enzymatic hydrolysis are complex mixtures that contain one or more active constituents at low concentrations. Peptides with the most ACE inhibitory activity are typically 2-5 amino acids in length and often contain one or more of the following: an aromatic amino acid (tyrosine), a branched-chain amino acid (isoleucine), or proline (Curtis *et al.*, 2002). Cheung *et al.* (1980) reported that peptides with competitive ACE inhibition activity have aromatic amino acids at the C-terminal and branched-chain aliphatic amino acids at the N-terminal. However, a more recent study suggested that inhibitory peptides possess an aliphatic amino acid reside in the C-terminal (Byun and Kim, 2001). Some studies have shown that ACE inhibitory ability is largely dependent on peptide size, with small molecular weight peptides being more effective (Kristinsson, 2007). It has also been suggested that the optimum molecular weights of most of the ACE inhibitory peptides in the hydrolysate should be less than 3 kDa (He *et al.*, 2006). Jung *et al.* (2006b) found that fractionation of an α-chymotrypsin digest of yellowfin sole resulted in different ACE inhibitory activities depending on molecular weight. Among three ranges of molecular weight (30-10 kDa, 10-5 kDa, below 5kDa) separated by a UF membrane bioreactor system, the highest ACE inhibitory activity was found in the lowest molecular weight fractions (<5 kDa) with an IC_{50} value of 0.883 mg/ml. The fraction was further purified by consecutive chromatographic techniques, and the ACE inhibitory peptide was determined to have a molecular weight of 1.3 kDa, consist of eleven amino acids (Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu), and have an IC_{50} value of 28.7 μg/ml. Eleven ACE inhibitory peptides with chain lengths of 2–4 amino acids were isolated from sardine muscle hydrolysate prepared with alkaline protease. Among them, Lys-Trp was the most potent inhibitor, with an IC_{50} value of 1.63 μg/ml (Matsufuji *et al.*, 1994). Matsui *et al.* (1993) reported the isolation of ACE inhibitory peptides from sardine muscle by hydrolysis with Alcalase which possessed an IC_{50} value of 250 μg/mL. Further purification resulted in isolation of a more potent inhibitor fraction with an IC_{50} value of 83 μg/mL.

Fahmi *et al.* (2004) reported production of protein collagen hydrolysate prepared from sea bream scales with alkaline protease. The most active peptide purified was Val-Ile-Tyr (IC_{50} = 7.5 μM) followed by Val-Tyr (IC_{50} = 16 μM), and it was demonstrated that the ACE inhibitory activities of the purified peptides were 5-20 times higher than that of the unpurified hydrolysate. Likewise, a study conducted by Yokoyama *et al.* (1992) demonstrated that protein hydrolysates prepared with thermolysin, with subsequent purification by chromatographphy, showed higher ACE inhibition than their parental peptides. Among a total of eight ACE inhibitory peptides, a peptide consisting of Leu-Lys-Pro-Asn-Met (IC_{50} = 2.4 μM) was the most effective. Moreover, this peptide was further hydrolyzed into Leu-Lys-Pro (IC_{50} = 0.32 μM) by ACE itself, resulting in a peptide with eight-fold higher activity.

The amino acid sequence of a bioactive peptide is an essential factor in determining its ACE inhibition pattern. As shown in Table 27.1, both peptide size and sequence are important factors affecting the IC_{50} value. This has been reported in several studies, including investigations conducted by Ono and co-workers (2003, 2006) where a total of seven ACE inhibitory peptides were isolated from defatted chum salmon muscle. All of them were di-peptides with IC_{50} values ranging from 2.5 to 500.5 μg/ml, depending on amino acid sequences. The inhibition mode of ACE inhibitory peptides can be evaluated by Lineweaver-Burk plots analysis. Most of the reported peptides act as competitive inhibitors for ACE binding; however alternative inhibitory modes for dipeptides have been reported (Ono *et al.*, 2006; Sato *et al.*, 2002). The inhibition site of these peptides and its precise mechanism has not yet been clarified, and further investigation into the correlation between the inhibition mode and the structure of these peptides is necessary (Li *et al.*, 2004).

27.3.1.3. Antihypertensive Effects In vivo

27.3.1.3.a. Animal Trials There is a general consensus that marine-derived protein hydrolysates and associated bioactive peptides may play a significant role in regulating blood pressure *in vivo*. Antihypertensive effects can be measured in spontaneously hypertensive rats (SHR), which are genetically predisposed to have high blood pressure (Vermeirssen *et al.*, 2004). To determine the effects of oral administration of ACE inhibitory peptides, changes in systolic blood pressure (SBP) of SHR are generally measured by the tail-cuff method before and after treatment.

Oral administration of hydrolysates (doses of 500 and 2000 mg/kg of body weight) from upstream chum salmon muscle prepared with thermolysin resulted in significant reductions in the blood pressure of SHRs in comparison with control rats. Blood pressure in SHRs remained significantly lower than control rats for up to 8 h, with the maximum reduction occurring 4 h after administration, and levels returning to normal after 24 h (Ono *et al.*, 2003). This trend implies that the key constituents of the hypotensive effect were likely short-chain peptides from protein hydrolysates. In contrast, if the main

constituents were the long-chain peptides, maximum reductions in blood pressure would be expected approximately 6 to 8h after administration,because these peptides should be converted to true ACE inhibitors by digestion *in vivo*.

In a study conducted by Je *et al.* (2005c), the antihypertensive effects of purified peptide from fermented blue mussel (*Mytilus edulis*) sauce was evaluated by oral administration at a dose of 10mg/kg body weight. The SBP was measured at 1, 2, 3, 6, and 9h after administration. SBP levels were reported to drop significantly after 3h, after which point the levels remained constant up to 6h. These results were comparable to those obtained with Captopril, a commercially available drug for antihypertension treatment, which significantly lowered SBP 1 to 6h after administration. Studies to elucidate the ACE inhibitory activity of dried bonito oligopeptides reported production and isolation of eight ACE inhibitory peptides (Fujita and Yoshikawa, 1999; Yokoyama *et al.*, 1992). A peptide consisting of Leu-Lys-Pro-Asn-Met possessed long-lasting and dose-dependent antihypertensive activity after oral administration in SHRs. In a follow-up study, Fujita *et al.* (2001) reported that ultra-filtration of the concentrated oligopeptides from dried bonito resulted, after oral administration, in a two-fold increase in both ACE inhibitory activity and antihypertensive activity in the SHRs, compared with the original non-purified oligopeptides. In another study, thermolysin was used to isolate bioactive peptides from dried bonito (Yokoyama *et al.*, 1992). Peptides containing the amino acid sequence Ile-Lys-Pro were reported to successfully inhibit elevations in blood pressure when given intravenously at a dose of 10mg/kg of body weight. Similarly, a peptide consisting of Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly showed an equivalent effect on normotensive rats. A study conducted by Fujita *et al.* (2001) demonstrated an antihypertension effect with a concentrated thermolysin digest of dried bonito hydrolysate. The minimum effective dose of this peptide was 125mg/kg, and SBP could be reduced by 10mmHg after an oral dose of 290mg/kg.

Itou and Akahane (2004) reported an antihypertensive effect of *heshiko*, a fermented mackerel product prepared by autolysis, on SHRs. A comparison on the effects of oral administration of *heshiko* and mackerel extract to SHRs (5, 10 or 50mg/kg) revealed that *hesiko* had higher antihypertensive activity (SBP reduction of 36mmHg) compared to raw mackerel extract (SBP reduction of 12mmHg). Minimal doses of *heshiko* extract were found to be effective at reducing SBP, which decreased significantly 2 to 4h post-administration and returned to original levels after 8h. Long-term administration resulted in significant reductions in SBP during the first 28 days of administration, after which point the SBP levels remained low for another 28 days until the treatment was discontinued. Oral administration of 300mg/kg body weight of bioactive peptides derived from sea bream scale also resulted in significant decreases in the blood pressure of SHRs. These peptides possessed higher ACE inhibitory activity *in vitro* than the commercial hypertension drug enalapril maleate (Fahmi *et al.*, 2004). A study undertaken by Suetsuna (1998)

demonstrated isolation of peptides with a tyrosine residue at the C-terminus (Ile-Tyr, Met-Lys-Tyr, Ala-Lys-Tyr-Ser-Tyr, and Leu-Arg-Tyr). After oral administration, these dipeptides caused prolonged reduction of SBP whereas dipeptides with phenylalanine at the carboxyl-terminus caused a more rapid but shorter reduction of SBP. Jung *et al.* (2006b) tested antihypertensive activity of the purified peptides from yellowfin sole by measuring the change of SBP at 1, 2, 3, 6 and 9 h after oral administration of 10 mg/kg body weight. A reduction of SBP (22 mmHg) was observed 3 h after administration, after which point the SBP level remained constant up to 9 h. The SBP reduction caused by the purified peptides was equivalent to that observed with the commercially available drug, Captopril, indicating a high inhibitory effect on hypertension. The isolated peptide was composed of hydrophobic amino acids at the C-terminal and had an IC_{50} of 22.3 μM. The study suggested that ACE was strongly influenced by the C-terminal tripeptide sequence of the substrate, indicating that peptides having hydrophobic amino acids at these positions might be potent inhibitors.

Thus far, it has been difficult to establish a direct relationship between ACE inhibitory activity in *vitro* and antihypertensive activity in *vivo* (Vermeirssen *et al.*, 2004), partially because some peptides with weak ACE inhibitory activities *in vitro* show strong antihypertensive activities after oral or intravenous administration. Leu-Lys-Pro-Asn-Met peptide (IC_{50} = 2.4 μM) isolated from dried bonito was found to be hydrolyzed by ACE, producing a peptide of Leu-Lys-Pro (IC_{50} = 0.32 μM) with eight-fold higher ACE inhibitory activity relative to the parent peptide or the Leu-Lys-Pro-Asn-Met peptide. SBP was significantly reduced after oral administration in SHRs, with a minimum effective dose of 8 mg/kg. The maximum reduction in blood pressure occurred after 4 h and these levels remained steady up to 6 h after oral administration. As for Leu-Lys-Pro peptide, SBP reduction was found with a minimum dose of 2.25 mg/kg. The maximum reduction in blood pressure was observed 2 h after oral administration, with a return to the original SBP levels within 6 h of administration. Fujita and Yoshikawa, studying the problem, suggested that the Leu-Lys-Pro-Asn-Met peptide exerts long-lasting antihypertensive activity and called it a prodrug-type ACE-inhibitory peptide to indicate that it exhibited smaller IC_{50} values than those of the parent peptides (Fujita and Yoshikawa, 1999).

27.3.1.3.b. Clinical Trials In contrast to the determination of antihypertensive effects in SHRs, few clinical trials have been conducted. A randomized double-blind placebo-controlled study with 29 human volunteers reported a significant antihypertensive effect on mildly hypertensive subjects by means of ACE inhibition (Kawasaki *et al.*, 2000). High-normal and mild essential hypertensive subjects were orally administrated 100 ml of liquid containing 3 mg Val-Tyr, a prominent ACE inhibitor isolated from sardine muscle hydrolysate. Reductions in SBP and diastolic blood pressure (DBP) of 9.7 mmHg

and 5.3 mmHg, respectively, were observed after 1 week, and reductions of 9.3 mmHg and 5.2 mmHg were observed after 4 weeks. As a follow-up study, Matsui *et al.* (2002) reported an antihypertensive effect of the same dipeptide (Val-Tyr) obtained from sardine muscle via hydrolysis with alkaline protease (*Bacillus iicheniformis*), followed by further fractionation with chromatographic techniques. Isolated dipeptide (0, 3, 6 or 12 mg) was dissolved in 100 ml water, and clinically healthy and normotensive male subjects were administered one of the four drinks. The study revealed that the antihypertensive effect of this peptide was dose-dependent in human plasma and maximum absorption was observed 2 h after a single oral administration (Vermeirssen *et al.*, 2004).

In another study, the antihypertensive activity of thermolysin digests from dried bonito in hypertensive and borderline hypertensive subjects was tested (Fujita and Yoshikawa, 1999). Thirty subjects were assigned to two groups: one group received the digest by oral ingestion (3 g/day) and the other group received a placebo. This treatment was carried out over the course of 8 weeks, and then the groups were switched for another 8 weeks. Reductions in blood pressure of 12.7 mmHg and 12.4 mmHg, respectively, were observed for the 2 groups following completion of the treatment period. In a follow-up crossover study that was placebo-controlled, double-blind, and randomized, Fujita *et al.* (2001) tested the effect of long-term ingestion of concentrated peptides from dried bonito containing the ACE inhibitory peptide Leu-Lys-Pro-Asn-Met. This study involved sixty-one borderline and mildly hypertensive subjects that were administered the bioactive peptide at a dose of 1.5 g/day. After the first 5 week period, reductions of 11.7 mmHg and 6.9 mmHg were observed in SBP and DBP levels, respectively. The peptide isolated from dried bonito exhibited long-lasting antihypertensive activity without any side effects in terms of body mass index and heart rate.

27.3.2. Antioxidant Properties

27.3.2.1. Antioxidants in Food Systems Lipid oxidation, the reaction of oxygen with unsaturated fatty acids in biological systems and foods, has attracted great interest in the past few decades (Rajapakse *et al.*, 2005b). Free radicals-mediated lipid oxidation is considered to be one of the major setbacks to providing processed foods with high quality and acceptability. Rancid flavor, off-odor, toxic compounds, and discoloration are unavoidable in most food processing lines, and such factors contribute to quality deterioration of stored foods and shorten the shelf life (Ranathunga *et al.*, 2006). Consumption of oxidized foods has detrimental effects to human health, causing severe diseases such as hepatomegaly, or necrosis of epithelial tissues (Saiga *et al.*, 2003). To prevent the deterioration of food due to lipid oxidation, natural antioxidants (such as α-tocopherol) or synthetic antioxidants (such as BHT and BHA) can be used in food and biological systems. Although synthetic

antioxidants are effective and inexpensive in comparison with natural antioxidants, their use has become somewhat restricted due to the potential risks to human health (Ranathunga *et al.*, 2006).

Because synthetic antioxidants have become less acceptable in recent years (Das *et al.*, 2006), there has been increasing interest in the use of naturally-occurring, safe antioxidative agents (Bekhit *et al.*, 2003; Ranathunga *et al.*, 2006). As a result, numerous studies have been conducted to reveal antioxidant properties in peptides derived from hydrolyzed food proteins, including marine-derived peptides. The ability of peptides to inhibit deleterious changes caused by lipid oxidation appears to be largely associated with certain amino acid residues within peptides. These include tyrosine, methionine, histidine, lysine, and tryptophan, all of which are capable of chelating pro-oxidative metal ions (Pena-Ramos and Xiong, 2001). Also peptides containing basic amino acids are known to be electron acceptors, giving them the ability to take on electrons from the free radicals formed during the oxidation of unsaturated fatty acids (Chen and Decker, 1994). Some amino acids (*e.g.*, arginine, citrulline, glycine, taurine, and histidine), small peptides (*e.g.*, carnosine and anserine), and nitrogenous metabolites (*e.g.*, creatine and uric acid) directly scavenge oxygen free radicals (Fang *et al.*, 2002). In fact, all amino acids have been shown to have antioxidative activity in some systems, likely a result of the antioxidant nature of the $R\text{-}NH_3$ group, and dependent on the molecular weight of the peptides (Je *et al.*, 2005a). Uchida and Kawakishi (1992) reported significant antioxidative activity in a histidine-containing peptide, and demonstrated that this peptide had a higher activity due to the imidazole ring of histidine. It was suggested that the presence of the imidazole ring increases the metal chelating and lipid radical-trapping abilities of the molecule, and, because of the high hydrophobic affinity of histidine, increased the interaction between the peptides and fatty acids. For instance, the naturally-occurring dipeptides carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-L-1-methylhistidine) have peroxyl radical-trapping abilities and can act as reducing agents by chelating metal ions, quenching singlet oxygen, and binding hydroperoxides (Kohen *et al.*, 1988).

27.3.2.2. Marine-Derived Peptides as Antioxidants A variety of marine-derived bioactive peptides possess strong antioxidative activities against the peroxidation of lipids or fatty acids. Low molecular weight peptides are expected to hold high antioxidant activity, as they can easily react with lipid radicals and thereby reduce radical-mediated lipid peroxidation (Ranathunga *et al.*, 2006). Rajapakse *et al.* (2005b) isolated unique low molecular weight peptides high in hydrophobic amino acids from giant squid. The study utilized three main gastrointestinal proteases (pepsin, trypsin, and α-chymotrypsin). Trypsin and α-chymotrypsin digests showed the highest lipid peroxidation inhibitory activity, which was further supported by an *in vitro* lipid peroxidation inhibition assay using linoleic acid. The pepsin digest, on the other hand, showed relatively low antioxidant activity. Next, the two most potent peptides

in the trypsin digest, Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da), and Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da), were isolated by fractionation. Protein hydrolysates from mackerel were prepared with a crude protease derived from *Aspergillus oryzae*, followed by subsequent lactic acid bacterial fermentation (Yin *et al.*, 2005). Compared to crude hydrolysates from minced mackerel, this method considerably increased the antioxidant ability of mackerel hydrolysates in the presence of linoleic acid (from 10% to 42%), DPPH scavenging capacity (from 38% to 85%), reducing power (from 0.58% to 0.83%), Fe^{2+} chelating capability (from 42% to 72%), and trolox equivalent antioxidant capacity (from 2.5 to 3.5 mM).

A peptide with a molecular weight of 672 Da and high antioxidative activity was isolated from Alaska pollock frame (Je *et al.*, 2005c). The marine peptide had an amino acid sequence of Leu-Pro-His-Ser-Gly-Tyr and, according to analysis with electron spin resonance spectroscopy, was able to scavenge 35% of hydroxyl radicals at 53.6 μM. Je *et al.* (2005a) also reported preparation of antioxidative peptides from hoki frame. Pepsin digests from hoki were further fractionated using a UF membrane with molecular weight cutoffs of 10, 5, 3 and 1 kDa. In this case, the 1–3 kDa fraction showed the highest antioxidative activity in a linoleic acid emulsion system. Also, the 1–3 kDa peptides possessed the highest scavenging effects for DPPH and superoxide anion radials. Mendis *et al.* (2005) reported isolation of an antioxidative peptide with a molecular weight of 797 Da from gelatin hydrolysates prepared with trypsin from hoki. In comparison with pepsin and α-chymotrypsin digests, the peptide obtained with trypsin showed the highest scavenging activities on superoxide and DPPH radicals assessed by ESR spectroscopy. The purified peptide (His-Gly-Pro-Leu-Gly-Pro-Leu) possessed a strong radical scavenging ability and inhibited peroxidation of linoleic acid at levels similar to the highly active synthetic antioxidant, BHT.

Antioxidative peptides were purified from the tryptic hydrolysate of conger eel (*Conger myriaster*) muscle utilizing a UF membrane reactor with four different molecular weight cutoffs [(>5 kDa, 3–5 kDa, 1–3 kDa, <1 kDa); (Ranathunga *et al.*, 2006)]. The fraction with molecular weights under 1 kDa showed the highest antioxidative property, and further purification of these peptides was carried out with chromatography. Sequence determination revealed that the isolated peptide consisted of Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn, and had a molecular weight of 928 Da. This peptide scavenged hydroxyl radicals and carbon-centered radicals at IC_{50} values of 74.1 μM and 78.5 μM, respectively. Kim *et al.* (2007) reported the isolation of a peptide with a molecular weight of 1801 Da from hoki (*Johnius belengerii*) by gastrointestinal digestion. Among the proteolytic enzymes tested (pepsin, trypsin, papain, α-chymotrypsin, Alcalase, and Neutrase), pepsin showed the highest antioxidative activity in assays of lipid peroxidation inhibition and free-radical scavenging, utilizing the electron spin resonance spin-trapping technique. Further purification using the UF membrane followed by chromatographic methods resulted in isolation of a peptide with the amino acid sequence

Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn. This peptide showed higher inhibitory activity than that of α-tocopherol and effectively quenched a variety of free radicals (DPPH, hydroxyl, peroxyl, and superoxide radicals). Reduction of *t*-butylhydroperoxide-induced cytotoxicity on human embryonic lung fibroblasts, and the protection of free-radical-induced DNA damage were also reported. Complexes of free amino acids and peptides from mackerel (*Scomber austriasicus*) prepared by autolysis with protease N (Amano Phermaceutical Co., Nagoya, Japan) showed significant antioxidant properties (Wu *et al.*, 2003) as measured by the ability of the compounds to inhibit oxidation in a linoleic acid system, scavenging effect on DPPH free radical, and reducing power. A strong correlation was reported between the level of peptides present and the antioxidant activity observed. The peptide fraction with a molecular weight of 1400 Da possessed stronger antioxidant activity compared to smaller molecular weight fractions (900 and 200 Da).

Production of gelatin hydrolysates from Alaska pollock skin was carried out by consecutive digestions with Alcalase, Pronase E, and collagenase, utilizing a three-step recycling membrane reactor (Kim *et al.*, 2001). Peptides with high antioxidative activity that were obtained during the second step were hydrolyzed by Pronase E, and showed a 58% inhibition of linoleic acid peroxidation. Their molecular weights ranged from 1.5 to 4.5 kDa. From this mixture, a purified peptide consisting of 16 amino acids (Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly) was isolated. This peptide was notable in that it contained a Gly residue at the C-terminus, and the repeating motif Gly-Pro-Hyp. Researchers theorized that these properties help give the compound a high antioxidative activity, as reflected by a strong inhibition of peroxidation of linoleic acid, along with enhancement of the cell viability of cultured liver cells. A noteworthy synergistic antioxidant effect of gelatin hydrolysates combined with α-tocopherol was also reported.

Following protein hydrolysis, an additional reaction can be carried out to obtain plastein, a water-soluble, heat stable antioxidant. Plastein is a mixture of higher molecular weight protein-like substances formed by the condensation of lower-molecular weight peptides, such as protein hydrolysates, during the plastein reaction (Yamashita *et al.*, 1971). A study conducted by Ono *et al.* (2004) assessed the production of plastein from squid hepatopancreas and indicated its high potential as a commercial antioxidant due to its thermostability, tastelessness, and high solubility in water.

27.3.3. Other Bioactive Properties

Besides ACE-inhibitory and antioxidative activities, other biological properties have been reported for protein hydrolysates and their associated bioactive peptides. For example, earlier studies demonstrated a hypolipidemic effect of fish protein (Bergeron and Jacques, 1989; Zhang and Beynen, 1993). A recent study by Tanaka *et al.* (2006) demonstrated production of protein hydrolysates from oyster prepared with aloase, an endoprotease from *Bacillus subtilis,* and

pancitase an exoprotease from *Aspergillus oryzae* (Yakult, Tokyo, Japan). The isolated peptides exhibited a number of bioactive properties, including hypolipidemic activity. Treatment of Sprague-Dawley rats, SHRs, and Otsuka Long-Evans Tokushima Fatty rats with the peptides resulted in significant reductions in hepatic triglyceride levels, suggesting that these peptides suppress fatty acid synthesis in the liver.

In research into the prevention and treatment of cardiac disease, a number of studies have demonstrated the hypocholesterolemic effects of soy protein hydrolysates in both animal and human models. Fish protein exhibited a similar effect in recent studies involving rat models. In the study, dietary intake of fish protein reduced blood lipid concentrations, and induced expression of hepatic genes involved in cholesterol homeostasis (Shukla *et al.*, 2006). Administration of peptides from salmon frames was reported to reduce total cholesterol Acyl-CoA cholesterol acyltransferase activity while increasing HDL cholesterol in the liver of zucker rats (Wergedhl *et al.*, 2004). The cholesterol-lowering effect of fish protein hydrolysate was suggested in part, due to a high ratio of methionine to glycine (Sugiyama *et al.*, 1986).

Besides hypolipidemic and hypocholesterolemic effects, the importance of anticoagulants and antiplatelets has been well recognized for the prevention of ischaemic events in patients with cardiovascular diseases. A novel peptide with anticoagulant and antiplatelet properties was enzymatically extracted from yellowfin sole [(*Limanda aspera*), (Rajapakse *et al.*, 2005a)]. Further purification of the peptide revealed a molecular weight of 12.01 kDa, and a single-chain monomeric conformation, suggesting that yellowfin sole peptides inhibit the factors that contribute to coagulation.

Some studies have shown that marine-derived peptides are capable of accelerating absorption of calcium. Jung *et al.* (2005) reported an increase calcium absorption when bone phosphopeptides fractionated from hoki (*Johnius belengerii*). These phosphopeptides contained 23.6% phosphorus and had a molecular weight of 3.5 kDa leading the authors to propose that, potentially, because of their calcium-binding activity, these oligophosphopeptides could be utilized in the nutraceuticals industry. In a later study, peptides recovered from Alaska pollock backbone also showed a high affinity for calcium. Hydrolysis of Alaska pollock backbone was carried out using nine different proteolytic enzymes, where the highest degradation efficiency was found in the pepsin digest. The peptide (Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Ala-Met-Tyr-Thr-Leu-Val) with a molecular weight of 1442 Da exhibited the highest affinity for calcium ions on the surface of hydroxyapaptite crystals (Jung *et al.*, 2006a).

Peptides with immunoreactivities can be defined as regulatory peptides with immune functions. A study conducted by Fouchereau-Peron *et al.* (1999) reported the presence of immunoreactivities in peptide hormones derived from fish protein hydrolysates. This study focused on bioactive peptides with potential applications in therapeutics, with particular attention given to the caliotropic hormones derived from the calcitonin gene. Such peptides were

prepared from three different sources: *i*) cod stomach and viscera, *ii)* cod and shrimp head, and *iii)* sardine by either autolysis or hydrolysis with proteolytic enzymes (trypsin and Alcalase). Among them, the cod viscera extracts and sardine hydrolysates contained immunologically and biologically active calcitonin-gene-related peptide molecules. The potential use of these hormones in a variety of physiological functions, such as controlling inflammation, type II diabetes, and hypertension, was proposed by the authors.

Interestingly, a marine-derived peptide product called Stabilium 200 has been reported to reduce anxiety in humans and to improve memory and learning performances in both rats and humans. Stabilium 200 is a derivative of the commercial product PC 60, which is a mixture of fish protein hydrolysates from mainly cod and mackerel. Bernet *et al.* (2000) reported that the marine-derived Gabolysat PC 60 showed anxiolytic properties by exhibiting diazepam-like effects on stress responsiveness of the rat pituitary-adrenal system and sympathoadrenal activity.

Antiproliferative activity is yet another possible bioactive function of marine-derived peptides. Mackerel hydrolysate prepared with an enzymatic reaction, followed by lactic acid bacterial fermentation, was reported to stimulate the proliferation of both human hybridoma HB4C5 and mouse macrophage J774.1 cells (Yin *et al.*, 2005). Likewise, fish protein hydrolysates obtained from blue whiting, cod, plaice, and salmon exerted a significant antiproliferative activity on human cancer cell lines *in vitro*. Additional anticancer peptides, with a molecular weight of 440.9 Da, were discovered in the hydrophobic fraction of anchovy peptides. These peptides induced apoptosis in human U937 lymphoma cells through the increase of caspase-3 and caspase-8 activity (Lee *et al.,* 2003, 2004).

27.4. CONCLUSIONS

Marine-derived protein hydrolysates are generally obtained from fish processing by-products, as well as from underutilized fish species. The hydrolysates have different properties and characteristics depending on a variety of factors, such as enzyme type, pH, temperature and enzyme/substrate ratio. Further fractionation by UF membranes can be applied to obtain different fractions of peptides with preferred molecular weights. For nutraceutical purposes, chromatographic techniques are often applied to purify bioactive peptides isolated from different fractions. Fish protein hydrolysates and their associated bioactive peptides possess numerous functional and physiological properties. Most studies on the bioactivity of marine-derived peptides have focused on ACE inhibitory, followed by antioxidative, properties. Along with molecular weight, amino acid sequence is a crucial factor in determining the ACE inhibition pattern and antioxidative function. Numerous studies have suggested the utilization of marine-derived peptides in the food and nutraceutical industries. These commercial applications of marine-derived bioactive pep-

tides show promise both for promoting a healthy population, and for improving utilization of our limited marine resources.

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CHAPTER 28

Bioengineering and Application of Glucose Polymers

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28.1. INTRODUCTION

Glucose polymers exist in nature in various forms. Amylose is a mostly linear polymer of α-1,4 linked glucose with rare α-1,6 linked branched points and is the minor component of starch. Amylopectin, the major component of starch, has short amylose chains connected together with α-1,6 linkages to form a characteristic cluster structure. Higher plants accumulate starch as an energy reserve, while the equivalent molecule in animals and some microorganisms is glycogen. Glycogen is also a branched glucan, and the reserve carbohydrate in animals and microorganisms. Glycogen differs from amylopectin in the number and organization of α-1,6 branch linkages, and in the absence of

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cluster structure. Glucose polymers also exist in a cyclic form, *e.g.*, cyclic α-1,4 glucans with degree of polymerization from 6 to 8 are well known as cyclodextrins. These polymers are different not only in their structure but also in their physical and chemical properties and functions, indicating that their exploitations depend on their structure.

There is increasing interest in engineering glucose polymers with distinct structure and properties to find novel applications, which cannot be achieved by the existing glucose polymers. Amylose, for example, is a functional and ecological biomaterial and is expected to be used in various industries. However, pure amylose is currently not available for industrial purposes, since the separation of natural amylose from amylopectin is difficult. Recent progress in the areas of molecular biology, glycobiology, and genome analysis has facilitated the design and manufacture of tailor-made carbohydrate molecules for various industrial needs. We have been studying glucose polymers with controlled molecular size and structure (Fujii *et al.*, 2003). This paper reviews our progress toward producing tailor-made glucose polymers.

28.2. ENZYMES FOR ENGINEERING GLUCOSE POLYMERS

Enzymes which produce or cleave glucosidic linkages include, hydrolytic, phosphorolytic, and transglycosidic enzymes. Hydrolytic enzymes, the most extensively studied, have already been used to manufacture wide range of mono- and oligosaccharides. Hydrolytic enzymes are very useful low-molecular weight carbohydrates, but not appropriate for producing high molecular weight polysaccharides: phosphorolytic and transglycosidic enzymes are very promising and useful in those applications. Although quite a few phosphorolytic enzymes and transglycosidic enzymes have been identified and subjected to experimental work, only a limited number are utilized in manufacturing. The phosphorylases and transglycosidases used in our study are summarized in the following section.

28.2.1. Phosphorylases

Phospholylases, as summarized in Table 28.1, catalyze the reversible phosphorolysis of polysaccharides or oligosaccharides, and produce phosphorylated mono-saccharides. Among such enzymes α-glucan phosphorylase (GP, EC 2.4.1.1), sucrose phosphorylase (SP, EC 2.4.1.7) and cellobiose phosphorylase (CBP, EC 2.4.1.20) are of great interest, since they can produce α-glucose 1-phosphate (α-G1P) from three major biomasses: starch, sucrose, and cellulose. Only these three are described in this paper, a comprehensive review of other phosphorylases can be found in Kitaoka and Hayashi (2002).

28.2.1.1 α-Glucan Phosphorylase GP catalyzes the reversible phosphorolysis of α-1,4 glucan into α-G1P and α-1,4 glucan, short one unit as shown in equation 28.1:

TABLE 28.1. List of carbohydrate-processing phosphorolytic enzymes.

EC No.	Name	Product
2.4.1.1	(α-glucan) phosphorylase	α-Glc1-P
2.4.1.7	Sucrose phosphorylase	α-Glc1-P
2.4.1.8	Maltose phosphorylase	β-Glc1-P
2.4.1.20	Cellobiose phosphorylase	α-Glc1-P
2.4.1.30	1,3-β-Oligoglucan phosphorylase	α-Glc1-P
2.4.1.31	Laminaribiose phosphorylase	α-Glc1-P
2.4.1.49	Cellodextrin phosphorylase	α-Glc1-P
2.4.1.64	Trehalose phosphorylase	β-Glc1-P
2.4.1.97	β-1,3-Glucan phosphorylase	α-Glc1-P
2.4.1.211	Lacto-N-biose phosphorylase	α-Gal1-P
2.4.1.216	Trehalose 6-phosphate phosphorylase	β-Glc1-P
2.4.1.230	Kojibiose phosphorylase	β-Glc1-P
2.4.1.231	Trehalose phosphorylase	α-Glc1-P
n.d.	Chitobiose phosphorylase	α-GlcNAcl-P

Source: Kitaoka, Tian, and Nishimoto, 2005 (with slight modification).

$$(1,4\text{-}\alpha\text{-D-glucan})n + Pi \leftrightarrow (1,4\text{-}\alpha\text{-D-glucan})n\text{-}1 + \alpha\text{-G1P} \quad \text{[Eq. 28.1]}$$

GP is widely distributed in microorganisms, plants, and animals, and seems to share a similar catalytic mechanism (Palm *et al.*, 1985; Fukui *et al.*, 1982; Hudson *et al.*, 1993; Schinzel and Nidetzky, 1999). Although all GP belong to a large highly homologous group that includes glycogen phosphorylase from bacteria, yeast, and animals, starch phosphorylase from plants, and maltodextrin phosphorylases from bacteria, these enzymes differ in their modes of regulation and their substrate preferences (Palm *et al.*, 1985; Xavier *et al.*, 1999). It has been reported that the smallest primer molecule for the glucan synthetic reaction of GP is maltotetraose, with maltopentaose being the smallest effective substrate for glucan degradation. These are generally believed to be the common features of all GPs. Recently however, GPs from *T. aquaticus* and some other thermophilic bacteria were found to have distinct substrate specificity, where maltotriose is the smallest primer for glucan synthesis and maltotetraose is the smallest substrate for glucan degradation (Takaha *et al.*, 2001). Therefore we divide GPs into two subgroups that can be distinguished by their primary structure and substrate specificity (Takaha *et al.*, 2001). GPs from potato and *T. aquaticus* represent each subgroup, respectively.

Potato-type L GP is one of the most extensively studied enzyme for glucan synthesis, but is not suitable for practical application because of its low thermal stability and sensitivity to proteolytic degradation. As has been described above, we have been studying GP from several thermophilic microorganisms, including *Bacillus stearothermophilus* (Takata *et al.*, 1998) and *T. aquaticus* (Takaha *et al.*, 2001), and has been subjected to produce glucose polymers (Fujii *et al.*, 2003). We have also challenged to enhance the properties of potato-type L GP by random and site-directed mutagenesis, and successfully

obtained a variant enzyme with improved thermostability and resistance to proteolytic degradation (Yanase *et al.*, 2005).

28.2.1.2. Sucrose Phosphorylase SP catalyzes the reversible phosphorolysis of sucrose into α-G1P and fructose as shown in equation 28.2:

$$\text{sucrose} + \text{Pi} \leftrightarrow \text{fructose} + \alpha\text{-GIP} \qquad \text{[Eq. 28.2]}$$

SP is not ubiquitous; it is only found in some mesophilic bacteria, including *Streptococcus mutans* (Koga *et al.*, 1991) and *Leuconostoc mesentroides* (Kitao *et al.*, 1993). While the physiological function of this enzyme is not clearly understood, it is thought to be involved in the metabolism of extracellular sucrose. Since there is no SP gene identified in the published genome sequence of thermophilic organisms, we decided to engineer practical SP with increased thermal stability. Introducing random mutagenesis into the structural gene of SP from *S. mutans*, eight amino acid substitutions were identified as effectively -r enhancing thermal stability. Site-directed mutagenesis was then carried out to obtain SP with all eight amino acid substitutions which significantly retained the enhanced thermal stability (Fujii *et al.*, 2006).

28.2.1.3. Cellobiose Phosphorylase CBP catalyzes the reversible phosphorolysis of cellobiose into α-G1P and glucose as shown in equation 28.3:

$$\text{cellobiose} + \text{Pi} \leftrightarrow \text{glucose} + \alpha\text{-G1P} \qquad \text{[Eq. 28.3]}$$

This enzyme, found in some bacteria which can metabolize cellulose, is theorized to be involved in the utilization of extracellular cellobiose produced by the action of cellulase (Kitaoka and Hayashi, 2002). This enzyme phosphorolyzes cellobiose but not cellotriose or anything larger. Cellooligosaccharides larger than cellotriose were phosphorolyzed by another phosphorylase called cellodextrin phosphorylase (EC 2.4.1.49).

28.2.2. Glucan Transferases

Glucan transferases which work on α-1,4 glucan, or produce α-1,4 glucan, are summarized in Table 28.2. We are especially interested in the enzyme which will cleave one glucosidic linkage of α-1,4 glucan and transfer the digested glucan fragment to other glucan, to form a new glucosidic linkage. The two most important enzymes within the scope of our objectives are 4-α-glucanotransferase (4αGT, EC 2.4.1.25) and branching enzyme (BE, EC 2.4.1.18).

28.2.2.1. 4-α-Glucanotransferase 4αGT (EC 2.4.1.25) catalyze the transfer of glucan chain from one α-1,4 glucan molecule to another as shown in equation 28.4:

TABLE 28.2. List of α-1,4-glucan-processing glucan transferases.

EC No.	Accepted name	Systematic name
EC 2.4.1.2	Dextrin dextranase	1,4-α-D-glucan:1,6-α-D-glucan 6-α-D-glucosyltransferase
EC 2.4.1.4	Amylosucrase	Sucrose:1,4-α-D-glucan 4-α-D-glucosyltransferase
EC 2.4.1.18	1,4-α-glucan branching enzyme	1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase
EC 2.4.1.19	Cyclomaltodextrin glucanotransferase	1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing)
EC 2.4.1.24	1,4-α-glucan 6-α-glucosyltransferase	1,4-α-D-glucan:1,4-α-D-glucan(D-glucose) 6-α-D-glucosyltransferase
EC 2.4.1.25	4-α-glucanotransferase	1,4-α-D-glucan:1,4-α-D-glucan 4-α-D-glycosyltransferase
EC 2.4.1.139	Maltose synthase	α-D-glucose-1-phosphate:α-D-glucose-1-phosphate 4-α-D-glucosyltransferase (dephosphorylating)
EC 2.4.1.161	Oligosaccharide 4-α-D-glucosyltransferase	1,4-α-D-glucan:1,4-α-D-glucan 4-α-D-glucosyltransferase
EC 5.4.99.15	(1-4)-α-D-glucan 1-α-D-glucosylmutase	(1-4)-α-D-glucan 1-α-D-glucosylmutase
EC 5.4.99.16	Maltose α-D-glucosyltransferase	Maltose α-D-glucosylmutase

$$(\alpha\text{-1,4 glucan})m + (\alpha\text{-1,4 glucan})n \leftrightarrow (\alpha\text{-1,4 glucan})m\text{-}x + (\alpha\text{-1,4 glucan})n + x \quad \text{[Eq. 28.4]}$$

The enzyme activity is widely distributed in bacteria, yeasts, plants, and animals, and catalyzed by at least five structurally distinct enzyme groups (Takaha and Smith, 1999) as shown in Table 28.3. In addition to the inter-molecular transglycosylation reaction expressed in equation (4), 4αGT also catalyze the intramolecular transglycosylation reaction (cyclization reaction) of α-1,4 glucan to produce cyclic α-1,4 glucan as shown in equation 28.5:

$$(\alpha\text{-1,4 glucan})n \leftrightarrow \text{cyclic } (\alpha\text{-1,4 glucan})x + (\alpha\text{-1,4 glucan})n\text{-}x \quad \text{[Eq. 28.5]}$$

We have tested various 4αGTs for their ability to produce cyclic α-1,4 glucan from amylose, and now have the view believe that a cyclization reaction is the common feature of this enzyme (Fujii *et al.*, 2003; Yanase *et al.*, 2002). However, the smallest cyclic glucan produced is greatly dependent on the type of enzyme employed (Table 28.2). In addition to two transferase activities described above, some 4αGTs also exhibit week hydrolitic activity (Fujii *et al.*, 2005a).

28.2.2.2. Branching Enzyme BE catalyzes the transfer of the glucan chain from one α-1,4-glucan molecule to a glucan acceptor, to form a new α-1,6 linkage. The enzyme activity is widely distributed in bacteria, yeasts, plants, and mammals, and is responsible for the formation of branch structure in amylopectin and glycogen. We have discovered that branching enzymes from *B. stearothermophilus* (Takata *et al.*, 1994) and *Aquifex aeolicus* (Takata *et al.*, 2003) catalyze a cyclization reaction of amylose or amylopectin and produce glucans with cyclic structure (Takata *et al.*, 1996a, 1996b, 1997).

28.3. SYNTHESIS OF LINEAR α-1,4 GLUCAN

GP catalyzes a transfer of the glucose moiety from α-G1P to maltooligosaccharide primer, and can produce linear α-1,4 glucan by repeating this activity. It has been reported that linear amylose can be synthesized using potato GP, and the Mw of amylose can be controlled by the α-G1P / primer molar ratio (Kitamura, 1996). Themostable GP from *T. aquaticus* (Takaha *et al.*, 2001) and genetically engineered potato GP with three amino acid substitutions can also be employed to produce amylose from α-G1P. In any case tested, essential linear amylose was produced with a yield of around 90%, and the molecular size was strictly controlled by the α-G1P / primer molar ratio (Yanase *et al.*, 2007; Ohdan *et al.*, 2005). All these results demonstrate that amylose production from α-G1P by GP is a good system for manufacturing amylase; the only problem is the expense of the substrate, α-G1P.

TABLE 28.3 Classification and characteristics of 4-α-glucanotransferases.

Enzyme Group	CAZy family [a]	Smallest donor [b]	Smallest acceptor [b]	Smallest transferred unit [b]	Smallest cyclic glucan (DP)	
(1) CGTase (EC 2.4.1.19)	GH13					
-*Bacillus macerans*		G3 [c]	G [c]	G	6	(French., 1957)
(2) D-enzyme / Amylomaltase	GH77					
-*Solanum tuberosum*		G3	G	G2 [c]	17	(Takaha *et al.*, 1996)
-*Escherichia coli*		G2	G	G	17	(Takaha and Smith, 1999)
-*Thermus aquaticus*		G2	G	G	22	(Terada *et al.*, 1999)
(3) Glycogen debranching enzyme	GH13					
-*Saccharomyces cerevisiae*		G4 [c]	G2	G2	11	(Yanase *et al.*, 2002)
(4) 4-α-glucanotransferase	GH13					
-*Thermotoga maritima*		G4	G2	G2	N.T. [d]	
(5) 4-α-glucanotransferase	GH57					
-*Thermococcus litoralis*		G2	G	G	16	(Imamura *et al.*, 2001)
-*Pyrococcus kodakaraensis*		G2	G	G	16	(Tachibana *et al.*, 1997)

[a] CAZy family name was obtained from Carbohydrate-Active Enzymes. Server at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html.
[b] Tested in the disroportionation reaction.
[c] G, G2, G3, G4 = glucose, maltose, maltotriose, maltotetraose, respectively.
[d] N.T. = not tested

One possible way to solve this problem is to combine another enzyme, like SP, which produces α-G1P for GP. Waldmann and colleagues reported the combined use of SP and GP for the production of amylose from sucrose (1986). In this system, SP catalyzes the phosphorolysis of sucrose to produce α-G1P and fructose, and the α-G1P is next used as a substrate of GP to produce amylose. An interesting feature of this SP-GP system is that Pi produced in the second GP reaction is used as a substrate for the first SP reaction. The cooperative action by the two phosphorylases proceeds continuously with a constant Pi concentration, without any inhibition caused by an accumulation of Pi. Based on this SP-GP system, we have now established the process to manufacture essentially linear amylose with controlled molecular size, by using thermostable variants of SP and GP (Yanase *et al.*, 2007; Ohdan *et al.*, 2007).

We next tried to provide α-G1P, not from sucrose, but from cellobiose by using CBP. When CBP from *Clostridium thermocellum* (Kim *et al.*, 2002) and GP were incubated with cellobiose in the presence of Pi and maltooligosaccharide primer, linear α-1,4 glucan was synthesized wth a yield (38.6 %) that was much lower than in the SP-GP method (Ohdan *et al.*, 2007). To improve the yield of amylose, mutarotase and glucose oxidase were added to the initial reaction mixture with the expectation that they would remove the glucose derived from the CBP reaction and consequently shift the equilibrium state to phosphorolysis. The yield of amylose was increased to 64.8% (Ohdan *et al.*, 2007).

At present cellobiose is not a practical substrate for amylose production, but the enzymatic degradation of cellulose is extensively studiedand the conversion of cellobiose into amylose by the CBP-GP system should be the important way to convert cellulosic biomass into value-added materials and products.

As has been described, the combined use of two phosphorylases is a powerful tool to convert one carbohydrate into another with a different structure. The idea of phosphorylase coupling was first examined by Waldmann *et al.* (1986), but had been employed for the synthesis of cellobiose from sucrose (Kitaoka *et al.*, 1992), laminaribiose from sucrose (Kitaoka *et al.*, 1993), trehalose from maltose (Yoshida *et al.*, 1995) and kojioligosaccharides from trehalose (Chaen *et al.*, 1999). Discovery of new phosphorylases and their application through phosphorylase coupling should be a promising area in polysaccharide and carbohydrate engineering.

28.4. SYNTHESIS OF CYCLIC α-1,4 GLUCAN

As described, cyclic α-1,4 glucan can be produced by the cyclization reaction of 4αGTs on amylose. The smallest cyclic glucan produced is dependent on the type of enzyme employed (Table 28.3). Yield of cyclic glucan appeared to

be determined by the extent of hydrolytic activity against the cyclization activity. When linear amylose with molecular mass of 320 kDa incubated potato D-enzyme (4αGT type II), cyclic α-1,4 glucan, with a yield of 97 %, was produced (Takaha *et al.*, 1996). However, the yield drastically decreases when the amylomaltase (4αGT typeII) from *T. aquaticus* (one of the most extensively studied enzymes) is employed (Fujii *et al.*, 2005a). It is suitable for manufacturing cyclic glucan because of its extremely high thermal stability, but its relatively high hydrolytic activity presents a problem. . We therefore introduced random saturation mutagenesis onto its structural gene, and obtained a variant enzyme (Y54G) whose hydrolytic activity is substantially removed (Fujii *et al.*, 2005b). Further investigation of this variant enzyme revealed that the mutated amino acid residue, Y54, constitutes a second glucan binding site on the surface of the enzyme, and determines its reaction specificity (Fujii *et al.*, 2007).

Glycogen debranching enzyme (4αGT typeIII) has 4αGT and amylo-1,6-glucosidase activity, and can produce cyclic α-1,4 glucan with the yield more than 50% from amylopectin (Yanase *et al.*, 2002). Cyclic glucan production from amylopectin by the action of the glycogen debranching enzyme is cost effective, since amylopectin is much less expensive than synthetic amylose produced from α-G1P. However, since we are now able to produce linear amylose from sucrose with SP-GP system, the glycogen debranching enzyme may no longer be a good production system for α-1,4 glucan. Direct conversion of sucrose into cyclic α-1,4 glucan by using SP-GP and 4αGT is now under investigation.

28.5. SYNTHESIS OF BRANCHED GLUCAN

Potentially, branched glucan can be produced by adding BE to the reaction mixture for amylose production. It can be produced by the combined action of GP and BE on G-1-P in the presence of adequate primer molecule, and the extent of branching can be controlled by the relative BE/GP activity ratio (Fujii *et al.*, 2003). Incubating three enzymes (SP, GP, and BE) with sucrose in the presence of a primer molecule can also produce branched gluten (Ryoyama *et al.*, 2004), and glucans with various molecular weights and branching patterns can be achieved by changing the sucrose/primer molar ratio and relative BE/GP activity ratio.

When BE from *B. stearothermophilus* or *A. aeolicus* were incubated with amylopectin, these enzymes did not introduce further branch linkages into amylopectin, but digested amylopectin into each cluster unit through cyclization (Takata *et al.*, 1996b, 1997).

Partially hydrolyzed starch is called dextrin, and it is used in various industrial applications. α-Amylase had been the enzyme used to produce dextrins; its molecular weight was controlled by the amount of α-amylase and its

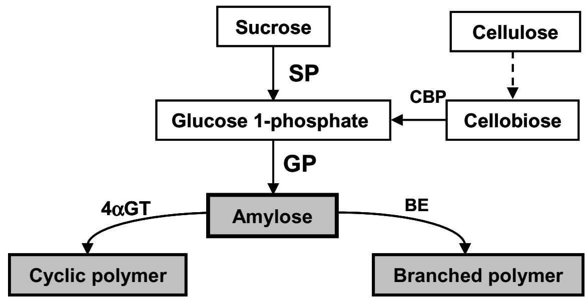

Figure 28.1. Engineering of glucose polymers.

reaction conditions. We found that cyclization of BE was an alternative way to degrade starch and to produce novel dextrin. The action of BE on amylopectin was restricted to the region connecting cluster units; the dextrin produced by BE had very narrow molecular weight distribution (weight average DP of 2500) as compared to dextrins produced by α-amylase (Fujii *et al.*, 2003). The branched cyclic dextrin produced by BE was highly soluble in water and its solution did not form precipitate even after repeated freeze/thaw cycles. These dextrins had relatively longer external chains than the conventional dextrins, which can interact or incorporate with various molecules. Branched cyclic dextrin has been used in the food industry as a carrier to produce oil and flavor powders, to protect polyunsaturated fatty acids from oxidation, and to mask bitter or sour tastes.

28.6. CONCLUSIONS

As described in this paper, we have developed systems to produce glucose polymers with linear, branched, cyclic structures either from sucrose or cellobiose (Fig. 28.1). The molecular weight and structure of glucose polymers can be controlled to a significant extent by the enzyme type, reaction conditions, and substrate. This cost effective system was achieved by the use of phosphorylase coupling and genetically engineered enzymes with enhanced properties, and we expect that the application of these glucose polymers will not be restricted to the food industry.

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CHAPTER 29

Peroxidase-Catalyzed Polymerization of Phenolic Compounds Containing Carbohydrate Residues

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29.1. INTRODUCTION

Peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is a prominent biocatalyst that polymerizes a wide range of aromatic compounds, generally through the following consecutive steps (Yamazaki and Yokota, 1973; Sakurada *et al.*, 1990):

$E + H_2O_2 \rightarrow$ Compound I $+ 2H_2O$ [Step 1]
Compound I $+ AH_2 \rightarrow$ Compound II $+ AH\cdot$ [Step 2]
Compound II $+ AH_2 \rightarrow E + AH\cdot$ [Step 3]
$AH + AH\cdot \rightarrow A_2H_2$ [Step 4]

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where E, Compound I, and Compound II are the resting (native) and two oxidized forms of the enzyme, respectively, and AH_2, AH· and A_2H_2 are the substrate electron donors, its free radical intermediates and the oxidized products, respectively. H_2O_2 serves as an electron acceptor in the first step; the free radicals generated from the substrates by the action of Compounds I and II in the second and third stepscouple each other non-enzymatically in the final step to produce the polymerized products. Since Saunders *et al.* found that horseradish peroxidase (HRP) produced insoluble polymers from various phenolic derivatives (Saunders *et al.*, 1964), a number of investigations have been conducted, not only to remove undesirable phenols in wastewater treatment, but also to prepare phenolic polymers, which are often difficult to synthesize by conventional chemical methods (Dordick *et al.*, 1987, 31; Akkara *et al.*, 1991; Oguch *et al.,* 2000; Mita *et al.*, 2001). In addition, such biocatalytic polymerizations are considered environmentally benign: their reactions proceed efficiently, selectively, under mild conditions and in aqueous solutions without toxic organic solvents.

Poly(hydroquinone) is a redox active polymer useful as a sensing material. However, the chemical and enzyme oxidation of hydroquinone (HQ) produces benzoquinone rather than polymers (Fig. 29.1). Electrochemical polymerization can afford poly(HQ), though the product has irregular phenylene linkages. In 1995, HRP-catalyzed polymerization of arbutin (4′-hydroxyphenyl β-D-glucoside, β-Arb) was reported by Wang *et al.* as a strategy to obtain poly(HQ) of regular units derived from a polymerized glycoside, poly(β-Arb), by the acid hydrolysis of the glucose (Glc) residues (Fig. 29.1) (Wang *et al.*,

Figure 29.1. Oxidative polymerization of HQ and β-Arb.
A, Chemical oxidation or enzyme reaction with HRP/H_2O_2; B, electrochemical reaction; C, enzyme reaction with HRP/H_2O_2.

1995). In previous studies, however, poly(β-Arb) or its carbohydrate residues had not been the focus of research interest (Wang *et al.*, 1995; Wang *et al.*, 1998). We applied the coupling action of HRP to obtain polymers, and related compounds with various glycosyl residues (Nakano *et al.*, 2005; Kiso *et al.*, 2007). Since such compounds, including poly(β-Arb), are regarded as "glyco-materials," the products may have potential applications, for example, in cell biology, or in the pharmaceutical and cosmetic industries. This article reviews our studies on the synthesis of various polymerized glycosides as well as some properties of the products.

29.2. POLYMERIZATION PROCESSES OF ARBS

Two hydroquinone glucosides, 4′-hydroxyphenyl α-D-glucoside (α-Arb) (Nishimura *et al.*, 1994; Sugimoto *et al.*, 2003) and β-Arb, were polymerized with HRP by the stepwise addition of H_2O_2. As shown in Figure 29.2, the reaction progressed responding to the supply of H_2O_2. Along with the polymerization, reaction solutions turned reddish-brown, a color typical for π-conjugated compounds. When the amount of H_2O_2 was almost equivalent to the initial Arbs, polymer yields peaked. After the endpoint, however, the polymer decreased slightly, attributable to the partial hydrolysis of the Glc side residues caused by a drop in the pH of the reaction solutions. Yields could be improved in the presence of a concentrated buffer of neutral pH. Excessive

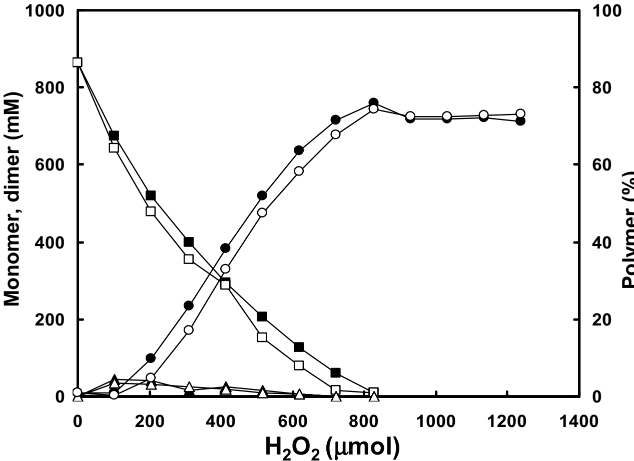

Figure 29.2. Progress of polymerization of α-Arb and β-Arb responding to the addition of H_2O_2.
Open circle, poly(α-Arb); closed circle, poly(α-Arb); open square, α-Arb; closed square, β-Arb; open triangle, di(α-Arb); closed triangle, di(β-Arb). The polymers were determined after precipitation with ethanol. The monomers and dimers were measured by HPLC.

H_2O_2 converts HRP into an over-oxidized inactive form (Shindler *et al.*, 1976), and, consequently, the immediate increase in the oxidant concentration resulted in considerable inactivation. For this reason, H_2O_2 had to be added to Arbs/HRP mixtures in divided portions. For the polymerization of Arbs, HRP showed higher activity than the enzymes from soybean and *Arthromyces*, while the three enzymes, the peroxidases from horseradish, soybean, and *Arthromyces*, polymerized β-Arb faster than α-Arb. Oxidation potentials (α-Arb, 821; β-Arb, 835 mV), determined by cyclic voltammetry, suggested almost the same electrochemical reactivity of Arbs. Accordingly, the different polymerization rates of Arbs were attributable not to their electrochemical reactivity, but rather to the substrate selectivity of the enzyme: the peroxidases recognized β-Arb as a better substrate.

Theoretical modeling was performed, based on the theiry that radical coupling caused polymerization among the monomers, as well as between the substrates of different DPs. The equal reactivity of these substrates was assumed, while the calculated model successfully reproduced most of the experimental results: an increase of the polymer with a decrease of the monomer and a temporal accumulation of the dimer responded to the addition of H_2O_2. The observed yield of the dimer, however, was much lower (<5%) than the 15% calculated. Our model may be improved if the reactivity of each oligomer is taken into consideration.

29.3. POLYMERIZED PRODUCTS OF ARBS

29.3.1. Oligomers

In an early stage of the polymerization, the oligomers were detected by HPLC or TLC, although the yields remained low, at 5–8% of the initial monomers, during the reaction (Fig. 29.3). The dimers, di(α-Arb) and di(β-Arb), had the same bi-phenyl structures, linked at the 3′-positions. Figure 29.4 shows the simulated structure of di-, penta-, and deca(α-Arb)s. Under the local minimum energy, the glucosyl group surrounded the poly(HQ) axis, which had stabilized structure by hydrogen bonds with each other. To explain the low accumulation of oligomers, electrochemical reactivity was compared in terms of the highest occupied molecular orbital (HOMO), calculated by the density functional theory. Increasing HOMO with DP predicted that the substrates would become more reactive as they polymerized more, because a higher HOMO generally signifies higher oxidative reactivity. In other words, the polymerized product (dimers) polymerizes more readily than the substrate (monomers). Accordingly, the low accumulation of oligomers is attributable to increasing oxidative reactivity.

Arbs inhibit tyrosinase, a key enzyme for melanogenesis, and Arbs are used in topical skin-lightning cosmetics. Unfortunately, however, the inhibition activities of the oligomers and polymers decreased compared to that of the

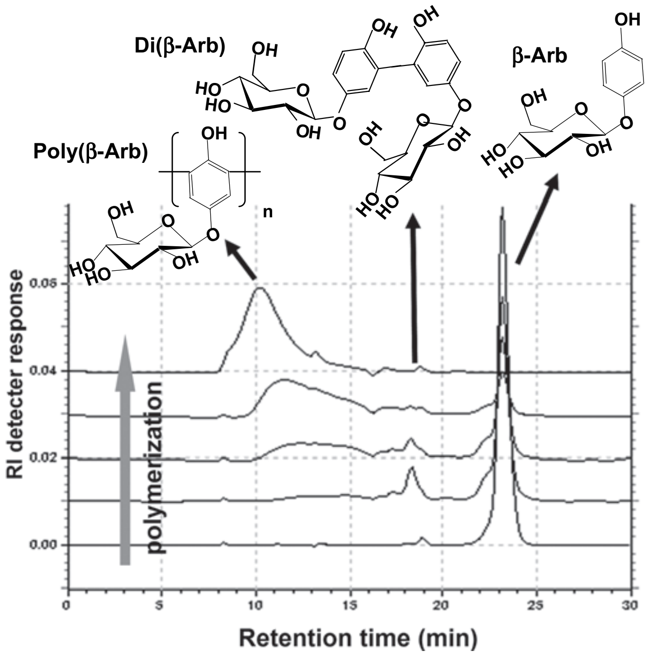

Figure 29.3. GPC-HPLC analysis of the polymerization products from β-Arb and their structures.
The reaction was progressed by the stepwise addition of H_2O_2.

monomers: The IC_{50} values of β-Arb, β-Arb oligomers (DP 2-4), and poly(β-Arb) for melanin production with the mouse melanoma B16 cells were approximately 120, 450, and 770 ppm, respectively. The dimers emit bluish fluorescence under UV radiation, while monomers and polymers are non-fluorescent. Excitation and emission occurred at 338 and 435 nm, respectively. On a TLC plate containing a fluorescent indicator, spots of the dimers, as well as of a few longer oligomers, were visualized under UV light. The oligomers showed a broad absorption at around 300 and 335 nm, which covered UVA (315–400 nm) and UVB (280–315 nm) regions, while the monomers showed simple absorption at around 280 nm. A potential application as a fluorescent labeling agent for sugar-binding molecules may be considered, if oligomer yields are improved.

29.3.2. Polymers

On GPC-HPLC, poly(Arb)s were distributed in a range corresponding to 0.5–25 kDa. The peak top positions corresponded to approximately 3.3–3.5 kDa, which was almost equivalent to a DP of 12. The DP of poly(Arb)s was also reasonably estimated as 10–20 from the viscosity, compared to those of polyethylene glycol standards, based on the assumption of equivalent

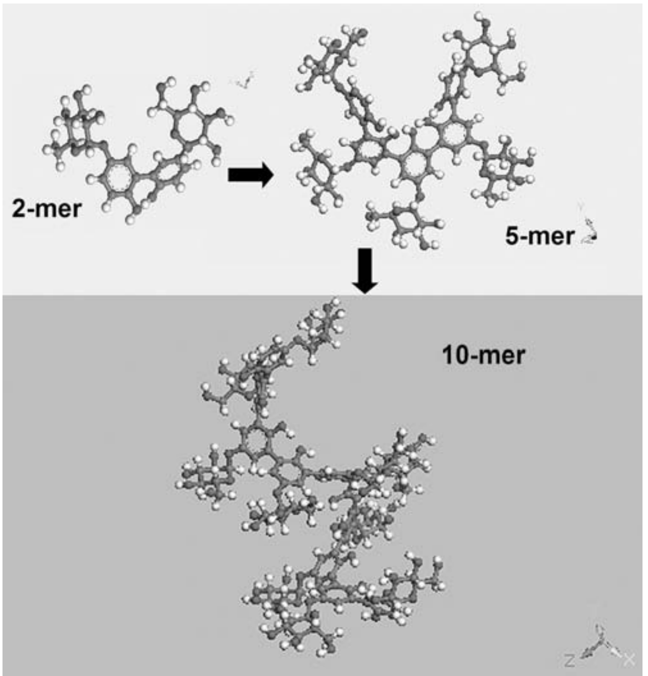

Figure 29.4. Simulated structure of oligomeric α-Arbs.
The structures were fully optimized by semi-empirical quantum calculation with VAMP program of Material Studio package (Accelrys Co.). PM3 Hamiltonian was used in the calculations.

molecular density. Meanwhile, MS analysis of poly(HQ)s derived from poly(Arb)s revealed the components distributed mainly at DP 7–17. Poly(α-Arb) contained more smaller components (DP < 10) than poly(β-Arb). There were no practical differences in the phenolic backbone structures of poly(α-Arb) and poly(β-Arb): NMR and IR analyses of poly(Arb)s, poly(HQ)s, and acetylated poly(HQ)s suggested that the neighboring HQ moieties were linked regularly at the 3′ and 5′-positions, as shown elucidated Fig. 29.1 (Wang *et al.*, 1995). No evidence was found for the involvement of the oxyphenylene (C-O-C) linkages. In addition, the structures of the dimers also supported this conclusion. Arbs are easily crystallized in needle-like figures, but the poly(Arb)s did not form a crystalline shape after the water-ethanol solution was air-dried. X-ray diffraction patterns of the powder showed no sharp peak but a broad signal, characteristic of amorphous materials.

The polymers exhibited lowered inhibition activity against melanogenesis, although toxicity on the melanoma cells decreased compared to the monomers. Poly(β-Arb) formed conjugates with several metal ions, especially lead acetate. Interaction of the polymer with sugars such as maltotetraose was also suggested by enhanced co-precipitation in the presence of ethanol (Kiso and

Nakano, 2004). These properties, as well as electrochemical ones, should facilitate new polymer applications.

29.4. GLYCOSYLATED DERIVATIVES OF POLY(ARB)

29.4.1. Enzyme Reactions for the Synthesis

We introduced heterogeneous side residues into poly(β-Arb) to expand its structural varieties as well as biological activities (Nakano *et al.*, 2005). The synthesis involved two successive enzyme reactions: Glycosylation of β-Arb, and subsequent polymerization by HRP (Fig. 29.5). In the first step, cyclodextrin glucanotransferase, β-galactosidase, and α-mannosidase were used to modify the Glc residue of β-Arb. The glycosylation yields were approximately 56, 30 and 25%, respectively. The main products were a series of α-1,4-linked glucosyl/maltooligosyl β-Arbs, β-1,4-linked galactosyl/galactobiosyl β-Arbs, and α-1,6-linked mannosyl β-Arb, respectively. In the second step, HRP efficiently polymerized glycosyl Arbs, together with the remaining β-Arb. GPC-HPLC (Fig. 29.6) showed that the reactions were efficient: the substrate (β-Arb) and the glycosylated products disappeared completely after the polymerization, and new peaks of polymers appeared at a shorter retention time. The polymerization occurred specifically to aromatic compounds, and

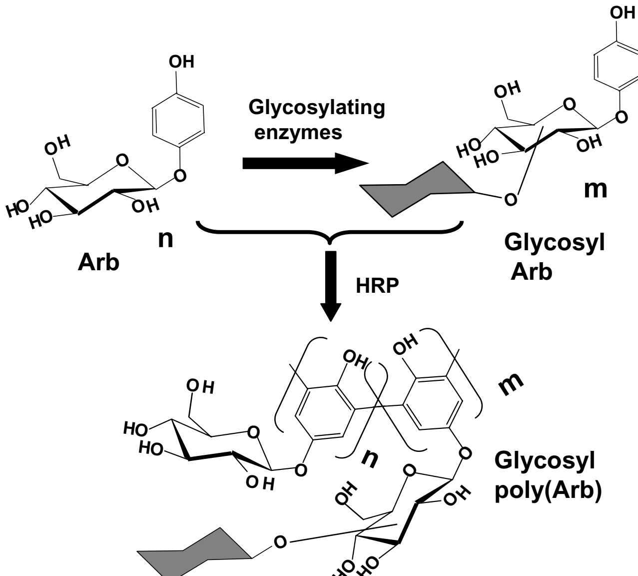

Figure 29.5. Scheme for the synthesis of glycosyl poly(Arb)s by enzymatic glycosylation followed by polymerization with HRP.
n = 4–7, m = 3–6. Shaded figures represent the glycosyl residues introduced.

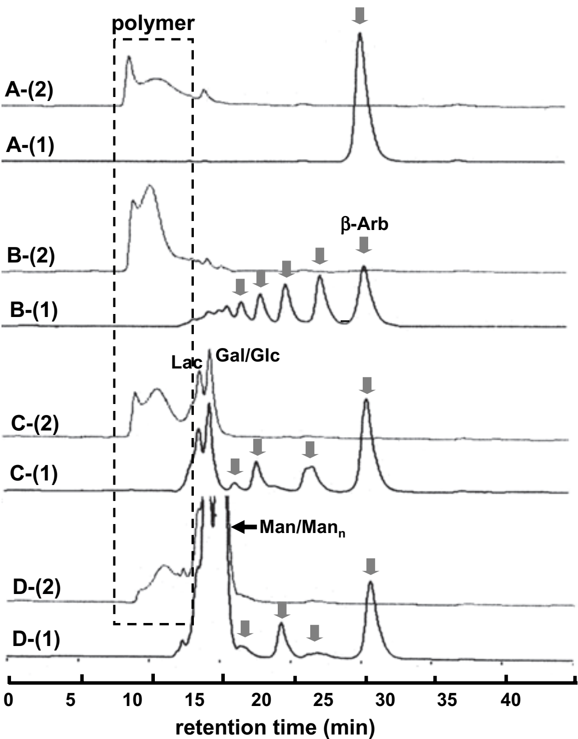

Figure 29.6. GPC-HPLC of the polymerization products from glycosylated Arbs. HRP and then H_2O_2 solution was added to the glycosylation mixtures drop by drop to become 1.5 mole equivalents to the glycosides. The peaks of Arb and glycosyl Arb(s) were indicated by shaded arrows. A-(1), Arb; A-(2), polymerization of A-(1); B-(1), α-glucosylated/maltooligosylated Arb(s); B-(2), polymerization of B-(1); C-(1), β-galactosylated Arb(s); C-(2), polymerization of C-(1); D-(1), α-mannosylated Arb(s); D-(2), polymerization of D-(1). Polymer peaks were surrounded by dotted squares.

the ordinal saccharides (Glc, lactose, and mannose) remained unchanged, despite existing at high concentrations.

29.4.2. Structures and Properties

The main chains of the glycosylated polymers were the same as that of poly(β-Arb), while the glucosyl/maltooligosyl, galactosyl, and mannosyl polymers possessed approximately 5–6, 3 and 2–3 of the introduced glycosyl residues in every 10 β-Arb units, respectively. These side residues influenced biological affinity: mannosylated polymer was mostly adsorbed to immobilized Concanavalin A (Con A) lectin gel and desorbed methyl α-mannoside, whereas 50–70% of the other polymers were not adsorbed. β-Galactosyl polymer was specifically adsorbed to immobilized Caster bean lectin (RCA_{120}) gel; other

polymers showed no interactions. These behaviors agreed with the specificity of the lectins. Furthermore, β-1,4-galactosylated β-Arb was isolated from the glycosylation reaction mixture, mixed with β-Arb at different ratios, and co-polymerized by HRP. The adsorbability of the resulting polymers to the Con A gel decreased, depending on galactose content. These results suggested that controlling the binding specificity is possible by choosing the structures and contents of glycosyl residues. Glycosyl poly(β-Arb)s are regarded as artificial glycopolymers. Polymers may enjoy potential usefulness in glycobiology or in pharmaceutical fields if the side residues can be appropriately designed.

29.5. COUPLING OF ARB AND GENTISATE

HRP catalyzed the coupling of β-Arb and 2,5-dihydroxybenzoate (sodium gentisate, GA) (Kiso *et al.*, 2007). Poly(β-Arb) was not synthesized in the presence of sufficient GA (approximately 1.5 times more than β-Arb). When the reaction mixture was allowed to stand for a few days, the coupling product precipitated gradually. In addition to β-Arb, similar coupling products were detected in the reactions with GA and dihydroxyphenyl glucosides. In the presence of β-Arb, other derivatives of hydroxybenzene and hydroxybenzoate revealed no such product. An approximate β-Arb/GA ratio of 1:2 was effective, and optimized yields were 50–60% more than the initial β-Arb. Insoluble Arb-GA was recovered in a good yield and in a pure state, simply by centrifugation followed by washing with water. The compound had a novel aglycone structure, in which the C3′-position of the HQ moiety of β-Arb linked with the C6-position of GA, and then intramolecular esterification occurred between the phenolic OH group of β-Arb moiety and the carboxyl group of GA moiety (Fig. 29.7). The intermediating compound has not been identified, and the esterification seems to be a slow step.

Figure 29.7. Scheme for the coupling reaction of β-Arb and GA.

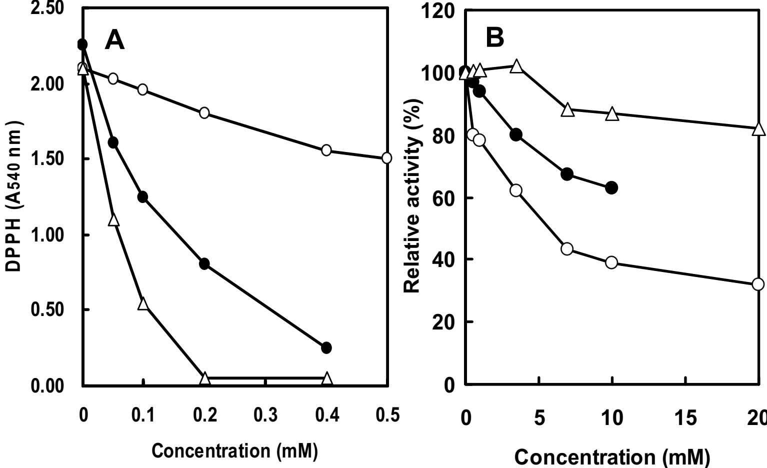

Figure 29.8. Activities of coupling product of β-Arb and GA.
A, anti-oxidation activity evaluated by the neutralizing of DPPH as a model radical compound; B, inhibition activity against mouse melanoma tyrosinase measured using L-DOPA as a substrate. Open circle, β-Arb; closed circle, the coupling product (Arb-GA); open triangle, GA.

The coupling product exerted anti-oxidation, as well as inhibition activity against tyrosinase, of which the former was much higher than that of β-Arb, and slightly lower than that of GA. For mushroom tyrosinase, meanwhile, Arb-GA showed greater inhibition than each starting substrate, and the inhibition against the mushroom enzyme was of the competitive type. However, the inhibition against mouse melanoma tyrosinase was at an average level compared to the strong inhibition ability of β-Arb (see Fig. 29.8). A lower inhibition against the mammalian tyrosinase is not necessarily favorable to its application to cosmetics.

We believe the coupling may be a potential method of enabling the specific and efficient modification of the aglycone structures of phenolic glycosides, without protection and de-protection steps.

29.6. CONCLUSIONS

A number of phenolic polymers were synthesized by oxidative coupling with HRP. Enzyme catalysis, which occurs in aqueous solutions, is advantageous to the reactions of water-soluble glycosyl substrates. Hence, enzymatic polymerization is useful for obtaining "glycopolymers" containing various carbohydrate residues. Enzymatic coupling is also available for the specific modification of the aglycone structures of phenolic glycosides. Such versatility can be attributed to the tolerance of the redox enzyme to changes in the glycosyl structures

of substrates; residues, anomeric linkages, and DPs. Advantages also exist in regio-selectivity to afford novel phenolic backbones, together with high efficiency and simple reaction systems. On the other hand, we encountered difficulties in controlling DP to obtain a certain oligomer in good yields, or elongated polymers of desired DPs. Despite such unresolved problems, however, we believe that our studies contribute to the utilization of HRP and relating redox enzymes for synthesizing useful glycomaterials.

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CHAPTER 30

Production of Lipase and Oxygenated Fatty Acids from Vegetable Oils

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30.1. INTRODUCTION

Much research has been focused on the efficient production of various metabolites using inexpensive carbon sources such as sugar, starch, whey, and vegetable oils (Kim, 2000). Vegetable oils are desirable inexpensive feedstocks for various bioproducts (Hou and Hosokawa, 2005). The yields from vegetable oils are high compared to those of other carbon sources, since they have more carbon atoms per weight (Akiyama *et al.*, 2003; Kahar *et al.*, 2004). For example,

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the theoretical yield coefficients of polyhydroxyalkanoate (PHA) production from vegetable oils are as high as 1.0 g-PHA per g-vegetable oils used, while the yield from glucose is only 0.32–0.48 g-PHA per g-glucose used (Yamane, 1992).

The United States produces more than 18 billion pounds of soybean oil annually, with a yearly carryover of more than 300 million pounds (Hou and Hosokawa, 2005). How to utilize this surplus oil effectively is a large economic issue in the agricultural community. Soybean oil is a relatively cheap raw material (22 to 25 cents per pound) and an attractive candidate for bioindustries (Hou and Hosokawa, 2005). The content of unsaturated fatty acids such as oleic and linoleic acids are 22% and 55% for soybean oil, 26% and 60% for corn oil, and 61% and 21% for canola oil, respectively. Various value-added oxygenated fatty acids have been produced from unsaturated fatty acids such as oleic and linoleic acid by biotransformation (Hou and Hosokawa, 2005; Hou, 2003). In this paper, we introduce an example of the high yield production of PHA from soybean oil or oleic acid through literature survey. We also summarize the production of two industrially important products from oleic acid: lipase by non-pathogenic yeast *Candida cylindracea*, and 10-ketostearic acid by *Flavobacterium* sp. DS5, by high cell density fed-batch culture techniques.

30.2. EXAMPLE OF HIGH YIELD PRODUCTION OF POLYHYDROXYALKANOATES FROM VEGETABLE OILS

PHAs are reserve polyesters accumulated as intracellular granules in various microorganisms (Lee, 1996). Some bacteria can accumulate PHA at levels exceeding 80% (w/w) of dry cells, usually under the condition of limiting nutritional elements such as N, P, S, O or Mg in the presence of excess carbon source. Recently, PHAs have been attracting attention because of their potential use as biodegradable thermoplastics. A large variety of different PHAs have been reported with different numbers of main chain carbon atoms and different types of pendent groups (Steinbuchel and Valentin, 1995). PHAs are classified into two groups according to the carbon numbers in their monomer structure: short-chain-length PHA (SCL-PHA), and medium-chain-length PHA (MCL-PHA).

The use of PHAs as substitutes for conventional non-degradable plastics in a wide range of applications has been hampered by the high production cost of PHAs compared with those of petrochemical-based polymers. PHA cost-reduction methods have included strain development, improving fermentation and separation processes, and use of less expensive substrates. Using sugars as carbon sources, SCL-PHA production with high productivity has been achieved in an optimized fermentation process (Kim *et al.*, 1994a, 1994b; Ryu *et al.*, 1997; Wang and Lee, 1997). In spite of such efforts, PHA production from sugars has an unsolvable disadvantage on a low PHA yield from carbon substrate. Poly(3-hydroxybutyrate) [P(3HB)] production from glucose usually yields a

range from 0.30–0.40 g-PHB per g-glucose used. The carbon source must be altered from sugar to other inexpensive sources that will allow bacteria to produce PHA with a high yield (Kahar *et al.*, 2004).

Kahar and collagues (2004) produced a P(3HB) homopolymer and a copolymer of 3HB with 5 mol% 3-hydroxyhexanoate, P(3HB-co-5 mol% 3HHx), by wild type strain H16 of *Ralstonia eutropha* and its recombinant strain, respectively, from soybean oil as a sole carbon source, using 10 L lab-scale fermentor. The PHA production by both strains was achieved with a high dry cell weight (118–138 g/L) and a high PHA content per dry cell weight (71–76%) to attain a high yield of PHA (ranging from 0.72 to 0.76 g-PHA per g-soybean oil used). Another advantage of using soybean oil for PHA production is that the net CO_2 emissions are lower than those of typical petrochemical polymers. Akiyama *et al.* (2003) assessed PHA production processes using soybean oil, based on the simulation data of total energy, net CO_2 emissions, and PHA production cost. The net CO_2 emissions of PHA production from soybean oil were less than 1.0 kg-CO_2/kg-plastic, which was very low compared to the production of petrochemical polymers (1.7–3.1 kg-CO_2/kg-plastic) if high yield production of PHA is achieved (Akiyama *et al.*, 2003).

MCL-PHA consists of 6 to 14 carbon atoms in their monomer structure, with various functional groups in the side chain. Rubbery and flexible, with low crystallinity, they can be used in a wide variety of applications which cannot be fulfilled by P(3HB) or other short chain length SCL-PHAs (Lee *et al.*, 1999b). High cell density fed-batch cultures of *Pseudomonas putida* were grown for the production of MCL-PHA, using oleic acid as a carbon source (Lee *et al.*, 2000). By applying phosphorus limitation, high cell concentrations of 141 g/L and PHA concentrations of 72.6 g/L were achieved in 38 h. The PHA content obtained was 51.4 wt%, which was lower than that reported for SCL-PHAs and MCL-PHAs obtained with *P. oleovorans* (75–80 wt%) (Kim *et al.*, 1994a, 1994b; Kim, 2002).

30.3. PRODUCTION OF LIPASE BY *CANDIDA CYLINDRACEA*

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) from various organisms have been used successfully as biocatalysts in bioorganic synthesis, hydrolysis, modifications of fats and oils, and transesterifications of triacylglycerols (Hou, 2002). Lipase produced by *C. cylindracea* has been one of the most widely used enzymes in synthetic organic chemistry, catalyzing the hydrolysis of carboxylic acid esters in aqueous medium, or the reverse reaction in organic solvents. It has a wide range of current and future applications, including in the food and flavor industries, in the production of ice cream and single cell protein, in the biocatalytic resolution of pharmaceuticals, in carbohydrate esters and amino acid derivatives, in biocide, biosensor, bioremediation, and biosurfactants, and in cosmetics (Benjamin and Pandey, 1998).

One of the most popular methods to achieve high cell density is fed-batch culture by controlling the nutrient feeding, which is often necessary for the

high yield and productivity of the desired product (Yamane and Shimizu, 1984; Lee *et al.*, 1999a). The rationale and various feeding strategies for, and examples of, high cell density fed-batch culture are all recently reviewed by Kim (2006). Since many of the commercially available lipase preparations are rather expensive, their industrial use is justified mainly for the manufacture of products of good commercial value (Mukherjee, 2005). While there have been numerous studies on the effects of environmental factors (carbon source, nitrogen source, surfactant, agitation speed, *etc.*) and cultivation modes (batch, fed-batch, and continuous culture) on lipase production by *C. cylindracea* (Ferrer *et al.*, 2001; Gordillo *et al.*, 1998a, 1998b; Sokolovska *et al.*, 1998), high cell density cultivations of *C. cylindracea* have been rare. Most cell concentrations obtained with *C. cylindracea* were less than 10 g/L. Kim and Hou (2006) carried out high cell density fed-batch cultures of *C. cylindracea* to increase cell concentration and extracellular lipase activity. Two feeding strategies, intermittent and stepwise feeding, were compared for cell growth and lipase production.

Figure 30.1 summarizes the results of fed-batch culture by *C. cylindracea* NRRL Y-17506, using oleic acid as a carbon source. From the intermittent feeding fed-batch culture, 52 g/L of a cell concentration was obtained at 138.5 h, which was about 10 times higher than that of flask culture. However, the final extracellular lipase activity was 6.3 U/ml, only 2 times the maximum lipase activity in the flask culture. The relatively low lipase activity was due to the high oleic acid concentration in culture broth (0–50 g/L). Stepwise feeding fed-batch cultures were carried out to simulate an exponential feeding and to investigate the effects of specific growth rate (0.02, 0.04 and 0.08 h^{-1}) on cell growth and lipase production. The highest final cell concentration obtained was 90 g/L when the set point of specific growth rate (μ_{set}) was 0.02 h^{-1}. High specific growth rate (0.04 and 0.08 h^{-1}) decreased extracellular lipase production in the later part of fed-batch cultures due to build-up of the oleic acid oversupplied. The highest extracellular lipase activity was 23.7 U/ml when μ_{set}

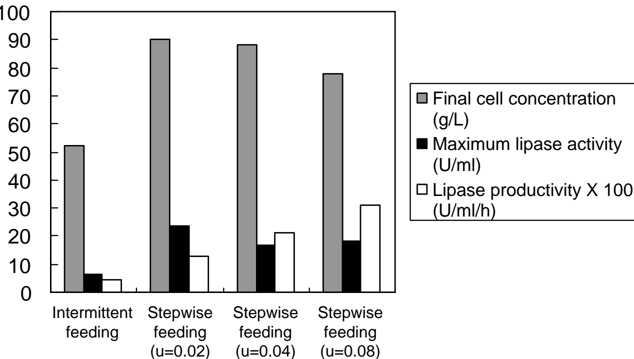

Figure 30.1. Summary of lipase production by fed-batch culture of *Candida cylindracea*. u: set point of specific growth rate (h^{-1}).

was $0.02\,h^{-1}$, while the highest lipase productivity was 0.31 U/ml/h at μ_{set} of $0.08\,h^{-1}$ due to the shorter culture time when it was calculated at a time of maximum lipase activity.

Major objectives of high cell density fed-batch culture are to maximize the product concentration, or the volumetric productivity. Considering the high purification cost, high product concentration is desired for low-volume-high-value products such as recombinant protein, while high productivity and product yield from raw materials are more important for economical production of high-volume-low-value products such as ethanol, organic acids, and PHA. Since lipase is a relatively expensive product, a culture method for the highest lipase activity is desirable and a value for μ_{set} of $0.02\,h^{-1}$ gave the highest lipase activity, even though lipase productivity was highest at a μ_{set} of $0.08\,h^{-1}$ (Kim and Hou, 2006).

30.4. PRODUCTION OF OXYGENATED FATTY ACIDS BY *FLAVOBACTERIUM* SP. DS5

Soybean oil and its component fatty acids can be converted to value-added products such as oxygenated fatty acids through bioprocesses. Alpha-, beta- and omega-hydroxy fatty acids, as well as dicarboxylic acids, have been produced by bioprocesses. Many novel oxygenated fatty acids have been discovered by Hou's group through microbial biotransformation (Hou and Hosokawa, 2005; Hou, 2003). Unsaturated fatty acids such as oleic, linoleic, and/or linolenic acids are converted by microbial enzymes to mono-, di-, and tri-hydroxy and other oxygenated fatty acids such as keto fatty acids. Keto and hydroxy fatty acids are useful industrial chemicals, used in plasticizer, surfactant, lubricant, and detergent formulations because of their special chemical properties, such as higher viscosity, and reactivity compared with other fatty acids. Oxygenated fatty acids can be used not only as specialty chemicals, but also as bioactive agents such as antifungal agents (Hou and Forman, 2000; Kato *et al.*, 1984; Masui *et al.*, 1989). One of the new products discovered by Hou's group, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) which was produced from *Pseudomonas aeruginosa* PR3, has anti-microbial activity. DOD was also tested as a raw material for the synthesis of industrial products such as rigid polyurethane foams (Hou and Hosokawa, 2005).

It has been reported that a microbial isolate, *Flavobacterium* sp. strain DS5, produced 10-ketostearic acid (10-KSA) from oleic acid in 85% yield (Hou, 1994a). The purified product was white, plate-like crystals melting at 79.2 °C. A small amount of 10-hydroxystearic acid (10-HSA) was also produced during the bioconversion, suggesting that oleic acid is converted to 10-KSA via 10-HSA, and the enzyme catalyzing the hydration is C-10 positional specific (Hou, 1994b, 1995). The DS5 bioconversion products from oleic, linoleic, α-linolenic, and γ-linolenic acid are all 10-hydroxy fatty acids. The optimum time, pH, and temperature for the production of 10-KSA have been reported in flask

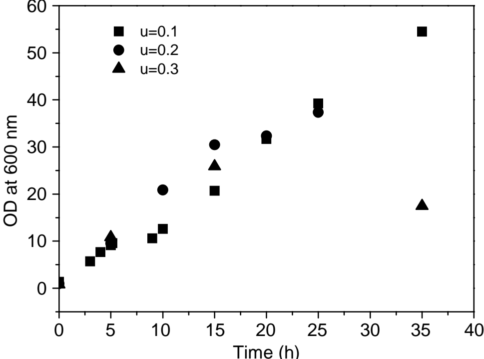

Figure 30.2. Effects of specific growth rate in stepwise feeding fed-batch culture on the growth of *Flavobacterium* sp. DS5.

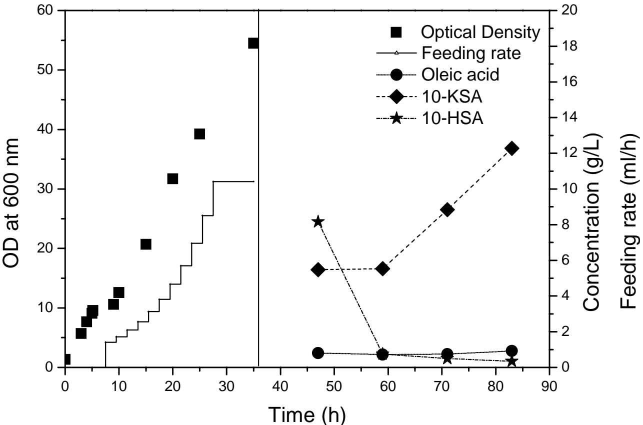

Figure 30.3. Time courses of fed-batch culture of *Flavobacterium* sp. DS5 with specific growth rate of 0.1 h^{-1} during the growth phase.

cultures (Hou, 1994a). To develop an industrial process for the production of these new oxygenated fatty acids, their productivity needs to be increased. We further investigated the effects of culture conditions on cell growth and 10-KSA production in flask cultures. The optimum concentrations of glucose and yeast extract, addition time, and volume of oleic acid for 10-KSA production were less than 20 g/L, more than 3 g/L, 18 h and 0.3 ml/50 ml, respectively. Fed-batch cultures were grown to improve 10-KSA production. The same stepwise feeding strategy used for lipase production by *C. cylindracea* was employed to grow cells to high density, and to produce 10-KSA. The set points of specific growth rate (μ_{set}) tested were 0.1, 0.2 and 0.3 h^{-1}, respectively. Figure 30.2 shows the effect of μ_{set} on cell growth in three fed-batch cultures. The highest cell concentration was obtained with μ_{set} of 0.1 h^{-1}. The optical density obtained

was 54.4 at 35 h, which was 5 to 10 times of final cell concentration in a typical flask culture. Higher specific growth rate (0.2 and 0.3 h^{-1}) decreased cell concentration, possibly due to the overfeeding of nutrients. Figure 30.3 shows the time courses of fed-batch culture with μ_{set} of 0.1 h^{-1}. Stepwise feeding was started at 7 h. When the optical density increased to 54.4 in 35 h, nutrient feeding was stopped and oleic acid was added. 10-HSA concentration reached 8 g/L at 47 h and then decreased. 10-KSA concentration started to increase with decreasing 10-HSA concentration, showing that 10-HSA converted to 10-KSA. The final 10-KSA concentration was 12 g/L in 83 h, which was 4 to 5 times that in flask culture (2.5 to 3.5 g/L).

30.5. CONCLUSION

Plant oils or their derived fatty acids are inexpensive renewable carbon sources. In addition, the theoretical yield coefficient of bioproducts (PHA) from plant oil and fatty acid is considerably higher than that from sugars. High cell density fed-batch cultures produced value-added products from soybean oil or oleic acid as the carbon source. PHAs with high yield were produced by fed-batch culture of *R. eutropha* or its recombinant strain from soybean oil. High cell concentrations obtained by fed-batch cultures from oleic acid improved lipase activity by *C. cylindracea* and 10-KSA by *Flavobacterium* sp. DS5, compared with those of flask cultures. There are still many industrially important value-added products that can be produced from inexpensive substrates such as soybean oil.

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CHAPTER 31

Production of Biologically Active Hydroxy Fatty Acids by *Pseudomonas aeruginosa* PR3

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31.1. INTRODUCTION

Hydroxy fatty acids (HFA), derived forms of fatty acids that carry hydroxyl groups, are considered important industrial products because the hydroxyl groups on fatty acids add special properties such as higher viscosity and reactivity, compared with other non-hydroxylated fatty acids. Those properties give hydroxy fatty acids new potential to be used in a wide range of industrial fields as resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. At present, castor oil and its derivatives are the only commercial sources of these hydroxy fatty acids. However, fluctuating supplies of and prices for castor oil have prompted a search for alternative raw materials by way of microbial conversion.

Since Wallen *et al.* (1962) reported the first bioconversion of oleic acid to 10-hydroxystearic acid by a Pseudomonad, microbial conversions of unsaturated fatty acids from different substrates by various microbial strains have been widely exploited to produce new, value-added products. Among the unsaturated fatty acids used for microbial production of hydroxy fatty acids, three (oleic, linoleic, and linolenic acids) were well studied as substrates to produce mono-, di-, and trihydroxy fatty acids. Recently, a bacterial strain *Pseudomonas aeruginosa* NRRL B-18602 (PR3) has been studied to produce hydroxy fatty acids from several fatty acid substrates. In this review, we introduce the production of hydroxy fatty acids from their corresponding fatty acid substrates by *P. aeruginosa* PR3 and their industrially valuable biological activities.

31.2. PRODUCTION OF HYDROXY FATTY ACIDS FROM UNSATURATED FATTY ACIDS BY PR3

31.2.1. Monohydroxy Fatty Acids

Production of a monohydroxy fatty acid from an unsaturated fatty acid by Pseudomonad was first reported by Wallen *et al.* (1962). They reported that Pseudomonad was able to hydrate oleic acid at the *cis*-9-double bond, resulting in the production of optically active l0-hydroxystearic acid (10-HAS) with a l4% yield. Another monohydroxy fatty acid was produced as an intermediate in the conversion of oleic acid to 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) by PR3 (Kim *et al.*, 2000a). The intermediate structure, after first introduction of a hydroxyl group, was identified as 10-hydroxy-8-octadecenoic acid (HOD), in which the C8 double bond was possibly in the *cis* form (Hou and Bagby, 1992). However, the rearranged double bond of HOD turned out to be *trans* rather than *cis* by NMR and IR analysis, and the carbon number 10 carrying a hydroxyl group represented the *S*-configuration with a minor isomer of *R*-form (Kim *et al.*, 2000b). The overall bioconversion pathway of oleic acid leading to DOD by strain PR3 was postulated as shown in Figure 31.1.

Figure 31.1. Schematic diagram of the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from oleic acid by *P. aeruginosa* PR3. HOD represents 10(S)-hydroxy-8(*E*)-octadecenoic acid.

31.2.2. Dihydroxy Fatty Acids

Hou *et al.* (1991) reported the discovery of a new compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), which was produced from oleic acid at a greater than 60% yield by PR3. Maximum DOD production was achieved after 48 hour incubation under pH 7.0, 30°C, and 150 rpm shaking speed. The yield of DOD production by strain PR3 was improved to over 80% through modifying the culture medium and reaction parameters (Kuo *et al.*, 1998). The production of a similar compound by *Pseudomonas* sp. 42A2 was reported and studied (De Andres *et al.*, 1994; Mercade *et al.*, 1988).

The production of DOD from oleic acid by PR3 is unique in that it involves an introduction of two hydroxyl groups at carbon numbers 7 and 10, and a rearrangement of the double bond from carbons 9 to 8. As shown in Figure 31.1, the substrate (oleic acid) is first converted to HOD during the introduction of a hydroxyl group at C10(*S*), and a double bond is shifted from C9 *cis* to C8 *trans*. The resulting plausible intermediate, HOD, is then subject to another hydroxylation at C7(*S*) resulting in DOD formation. It was reported that a C10 position-specific and *cis*-specific hydratase was involved in the hydration of unsaturated fatty acid by *Flavobacterium* sp. DS5, in which the C10 hydroxyl group was introduced with the removal of the C9 *cis* double bond, typical of hydration reactions of fatty acids (Hou, 1995a, 1995b). However, it was unlikely that a hydratase was involved in DOD formation by PR3 because the double bond at C9 of the substrate was retained as a shifted *trans* configuration at carbon 8 during the hydroxylation.

Another dihydroxy fatty acid, 10,12-dihydroxy-8(*E*)-octadecenoic acid (DHOD), was produced as an intermediate in the conversion of ricinoleic acid to 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) by PR3 (Fig. 31.2) (Kim

Ricinoleic acid
Hydroxylation with double bond migration
DHOD
Hydroxylation
TOD

Figure 31.2. Schematic diagram of the production of 7,10,12-triihydroxy-8(*E*)-octadecenoic acid (TOD) from ricinoleic acid by *P. aeruginosa* PR3. DHOD represents 10,12-dihydroxy-8(*E*)-octadecnoic acid.

et al., 2000c; Kuo *et al.*, 2001). Although two hydroxyl groups were present on the intermediate compound of TOD formation, the overall metabolic pathway was assumed to be the same as that involved in DOD formation from oleic acid, because both the substrates (oleic acid and ricinoleic acid) contained C18 fatty acid backbones with a *cis*-double bond at carbon 9 except for a hydroxyl group pre-introduced at C12 of ricinoleic acid. Maximum DHOD production was achieved after 30 h incubation under at 28 °C and pH 6.5.

Based on the postulated common metabolic pathway involved in DOD and TOD formation by PR3, it was assumed that palmitoleic acid containing a singular C9 *cis* double bond (a common structural property shared by oleic and ricinoleic acids), could be utilized by PR3 to produce hydroxy fatty acid. Bae *et al.* (2007) reported that palmitoleic acid could be utilized as a substrate for the production of hydroxy fatty acid by PR3. Structural analysis of the major product produced from palmitoleic acid by PR3 confirmed that strain PR3 could introduce two hydroxyl groups on carbon 7 and 9 with shifted migration of 9-*cis* double bond into 8-*trans* configuration, resulting in the formation of 7,10-dihydroxy-8(*E*)-hexadecenoic acid (DHD) (Fig. 31.3). The time course study of DHD production showed that DHD formation was time-dependently increased, and peaked at 72 h after the addition of palmitoleic acid as substrate. However, production yield of DHD (23%) from palmitoleic acid was relatively low when compared to that of DOD (70%) from oleic acid (Hou and Bagby, 1991).

The postulated bioconversion pathway of palmitoleic acid leading to DHD was very similar to those of DOD production from oleic acid (Fig. 31.1) (Kim *et al.* 2000a) and TOD from ricinoleic acid (Fig. 31.2) (Kim *et al.* 2000c). All pathways included the introduction of two hydroxyl groups next to the 8-*trans* double bond which was migrated from 9-*cis* configuration. These results

Palmitoleic acid

7,10-dihydroxy-8(E)-hexadecenoic acid (DHD)

Figure 31.3. Schematic diagram of the production of 7,10-dihydroxy-8(*E*)-hexadecenoic acid (DHD) from palmitoleic acid by *P. aeruginosa* PR3.

strongly suggested that the enzyme systems involved in DHD production from palmitoleic acid could be same as those for DOD and TOD production from their corresponding substrates. Based on these results, it was assumed that substrate specificity of this enzyme system could be attributed to the location of the *cis* double bond in the fatty acid.

There were limited trials to use palmitoleic acid as substrate for microbial conversion. *Flavobacterium* sp. DS5 produced 10-keto and 10-hydroxy products from palmitoleic acid (Hou, 1995b) and a filamentous fungus *Trichomonas* sp. AM076 converted palmitoleic acid to a small amount of 9,12-hexadecadienoic acid (Shirasata *et al.*, 1998). However there was no identified dihydroxy fatty acid product from those microorganisms.

31.2.3. Dihydroxy Fatty Acids from Triolein

Most HFA production by PR3 was done with free fatty acids as substrates. However, considering the structural properties, it was highly plausible that homo-triacylglyceride(specifically triolein) containing three oleic groups could be utilized as a substrate for the production of DOD by a microbial enzyme system which was involved in DOD production from oleic acid. In an effort to investigate the feasibility of triolein for the production of HFA by PR3, Chang *et al.* (2007) used triolein as a substrate, and reported that triolein could be efficiently utilized to produce DOD by PR3. According to their report, GC/MS and FTIR analysis of the major product from the bioconverted triolein confirmed that the major compound of the crude extract was DOD.

The fact that PR3 could utilize triolein to produce DOD indicated the possibility that lipase induction could release free fatty acids from triolein before it was used as substrate for DOD production. When Chang and colleagues monitored the time-dependent activity of extracellular lipase, they reported that triolein, not oleic acid, induced lipase activity. The induction of lipase activity was observed 12 h after substrate addition, and DOD production

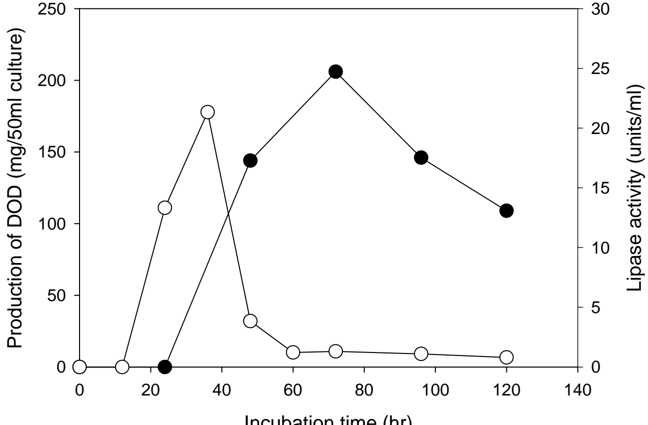

Figure 31.4. Time-coursed lipase induction (open circle) and DOD formation (closed circle) from triolein by *P. aeruginosa* PR3. Incubation time represented reaction time after triolein was added to the 24h-old culture.

started 12 h after lipase induction (Fig. 31.4). These results suggested that PR3 induced lipase activity to release free oleic acid from triolein prior to DOD formation.

The time course study of DOD production from triolein at 28 °C and pH 7.0 indicated that DOD production started 24 h after substrate addition and reached maximum at 72 h. This optimal incubation time was different from that of DOD production from oleic acid by the same strain PR3 (Hou and Bagby, 1991) in that DOD production peaked at 48 h after substrate addition. This discrepancy could be explained by the fact that PR3 required additional time to induce lipase activity for the release of oleic acid from triolein before the released oleic acid was used for DOD production. Based on the results of this study, the overall bioconversion pathway of triolein leading to DOD by strain PR3 was postulated as shown in Figure 31.5. Triolein was first hydrolyzed into oleic acid by the triolein-induced lipase, and then the released oleic acid was converted to DOD by PR3, during which two hydroxyl groups were introduced at C7 and C10, and a double bond was shifted from C9 *cis* to C8 *trans*.

Efficient carbon and nitrogen sources for the production of DOD from triolein were glucose, galactose, fructose, and yeast extract, respectively. Under optimized conditions, the maximum yield of DOD production represented 66% of substrate. These results demonstrated that natural vegetable oils, without being intentionally hydrolyzed, could be used as efficient substrates for the microbial production of value-added hydroxy fatty acids by PR3.

31.2.4. Trihydroxy Fatty Acids

There are reports about the production of certain 18 carbon-trihydroxy fatty acids from plants (Baur *et al.*, 1977; Dix and Marnett, 1985; Esterbauer and

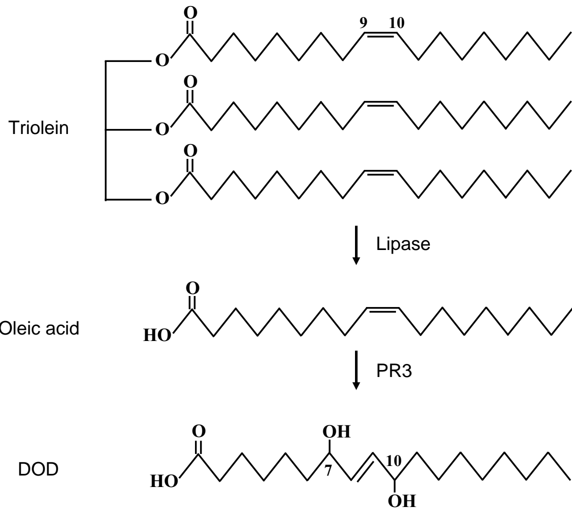

Figure 31.5. Schematic diagram of the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from triolein by *P. aeruginosa* PR3.

Schauenstein, 1977), animals (Funk and Powell, 1983), and by chemical methods (Gardner *et al.*, 1984). Of these trihydroxy fatty acids, 9,10,11-trihydroxy-12-octadecenoic acid (12-THOD), 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (11-THOD), and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (10-THOD) have gained special attention because of their biological activities. Kato *et al.* (1984) reported that the mixed hydroxy fatty acids isolated from the *Sasanishiki* rice plant suffering from rice blast disease exhibited a strong inhibition activity toward germination and elongation of the germ tube from conidia of rice blast fungus. Their structures were identified as 9*S*,12*S*,13*S*-trihydroxy-10-octadecenoic acid, and 9*S*,12*S*,13*S*-trihydroxy-10,15-octadecadienoic acid (Kato *et al.*, 1985, 1986). 10-THOD was also isolated from tubers of taro (*Colocasia antiquorum*) inoculated with *Ceratocystis fimbriata*, and showed activity against black rot fungus (Masui *et al.*, 1989).

Kim *et al.* (2006) reported that PR3 was able to convert linoleic acid into equimolar mixture of two compounds, 11-THOD and 10-THOD. The total amount of THODs produced in the culture increased with time and peaked at 72 h, with a maximum THOD production (220 mg/50 ml culture, 44% weight yield), after which the amount of THOD in the medium remained virtually unchanged up to 240 h, indicating that THODs were not further metabolized by strain PR3. This result was different from those reported for the production of DOD, TOD, and DHD from their corresponding substrates, in that all these products were further metabolized by PR3 after it peaked at 48 h or 72 h of incubation time. However, the production of 12,13,17-9(*Z*)-octadecenoic acid (THOA) from linoleic acid by *Clavibacter* sp. ALA2 showed similar results: THOA was not further metabolized (Hou, 1996). Various

conditional factors were investigated for the maximal production of THOD by PR3. THOD production increased with the substrate concentration and peaked at 500 mg, after which it decreased slightly and then plateaued at 1 g. The optimal reaction temperature and pH were 30–40 °C and pH 7.0, respectively. Maltose, starch, sucrose, and glucose were effective for THOD production, and sodium glutamate was most effective nitrogen source.

It was reported that lipid peroxide molecules(LOOH) decomposed into radicals in the presence of bivalent metal ions, in particular, iron or copper, since these ions generated intermediate radicals ($LO^{\bullet}$), leading to the formation of hydroxyl groups on fatty acids (Gardner, 1989). To address the effect of metal ions on the production of THODs by PR3, Kim *et al.* (2002) studied the dose-dependent effect of iron and copper ions on the production of THOD by PR3. According to their report, the requirement of the iron ion for THOD formation was essential. However, microbial growth was not significantly influenced by the concentration of iron ions tested. Even in the absence of a defined iron ion, cell density increased to 80% of the maximal value. For sufficient production of THOD from linoleic acid by PR3, there was a threshold concentration level of Fe^{+2} ions between 0.07 mM and 0.14 mM. This concentration range accounted for a substrate/Fe^{+2} ion molar ratio from 5100 to 2550. It was highly plausible to assume that existence of the threshold concentration level of Fe^{+2} ion for THOD production could be raised by the preference of two iron ions Fe^{+2}/Fe^{+3} in the equilibrium for discrete reactions by the enzyme system involved in this event.

They also investigated the effect of Fe^{+2} ions on the production of DOD and TOD from their corresponding substrates, for comparison with THOD production from linoleic acid by PR3. When Fe^{+2} ions were added at 0 mM, 0.07 mM, and 0.21 mM, THOD production increased greatly. However, TOD production from ricinoleic acid linearly decreased with the relatively low production level, and DOD production from oleic acid fluctuated. These results demonstrated that the enzyme of PR3 (possibly lipoxygenase) involved in the production of THOD from linoleic acid was different from that involved in the formation of DOD and TOD from their corresponding substrates. This supposition was supported when the key element of the substrate for the nonheme iron containing lipoxygenase was an 1,4-*cis,cis*-diene unit present in the structure of linoleic acid (Veldink and Vliegenthart, 1984), a structure is not shown in oleic and ricinoleic acids.

31.2.5. Other Hydroxy Fatty Acids

Some polyunsaturated fatty acids were tested for the production of hydroxy fatty acids by PR3 because polyunsaturated fatty acids contained a 1,4-*cis,cis*-diene unit which could serve as a key structural element for the hydroxylation by lipoxygenase. When eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) carrying 1,4-*cis,cis*-diene unit were used as substrates for bioconversion by PR3, new products were detected on TLC analysis as shown in

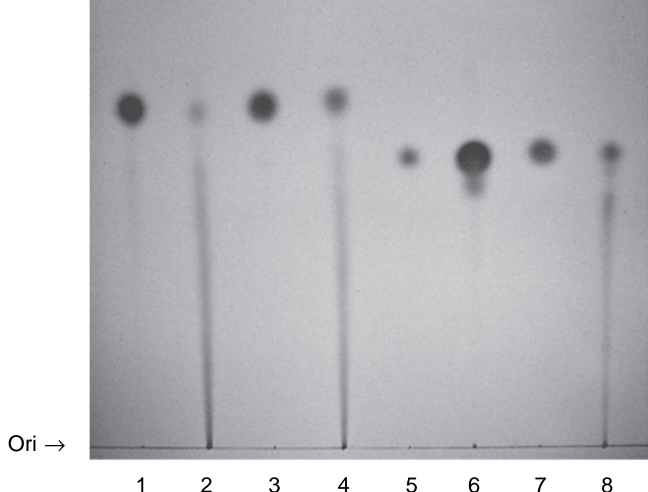

Figure 31.6. Thin layer chromatography analysis of products from the bioconversion of fatty acids by *P. aeruginosa* PR3. Lane numbers consecutively represented standard docosahexaenoic acid (DHA), bio-converted DHA, standard eicosapentaenoic acid (EPA), bio-converted EPA, standard conjugated linoleic acid (CLA), bio-converted CLA, standard linoleic acid (LA), and bio-converted LA.

Figure 31.6. Although new products were not clearly separated into spots, there were noticeable differences in band patterns between the bioconverted fatty acids and substrate fatty acids.

31.3. BIOLOGICAL ACTIVITIES OF HYDROXY FATTY ACIDS PRODUCED BY PR3

31.3.1. Bioconversion of Polyunsaturated Fatty Acids by PR3 and Their Oxidative Effects

According to the reports describing metabolic pathways involved in the conversion of linoleic acid to trihydroxy fatty acids, several intermediate reaction products, such as trihydroxy-, hydroperoxy-, dihydroxy-, and hydroxyepoxy-octadecenoate, were involved (Kato *et al.*, 1984, 1986). Those metabolites of linoleic acid showed distinct biological functions according to their intermediate structures, including mono-, di-, trihydroxy-octadecenoic acid, and hydroperoxy-, epoxy-forms (Kato *et al.*, 1984; Blair, 2001; Göbel *et al.*, 2002; Hou and Forman, 2000). In an effort to understand the overall mechanism involved in the varied biological functions of the complicated reaction metabolites of bio-converted polyunsaturated fatty acids, Kim *et al.* (2006) studied the oxidative activities on fish oil, of crude extracts produced by PR3 from

several polyunsaturated fatty acids. They tried to convert EPA, DHA, linoleic acid, and conjugated linoleic acid (CLA) using PR3. All fatty acid substrates tested, except CLA, showed complicated profiles of the bio-converted products in TLC analysis. Bio-converted linoleic acid represented a smeared band in the middle-upper area of the TLC plate, while EPA and DHA produced smeared bands in the lower part of the plate (Fig. 31.6), suggesting that bio-converted products from EPA and DHA were relatively more polar than those from linoleic acid. However, CLA carrying one *cis* and one *trans* double bond (mixture of *c*9, *t*11;43% and *t*10, *c*12;55%) in the fatty acid chain did not produce any particular new spots in the TLC analysis. GC analysis of all bio-converted fatty acids (except for CLA) presented complicated peak profiles containing more than 20 peaks.

They investigated the effect of bio-converted polyunsaturated fatty acids on the oxidation of fish oil containing a relatively high amount of polyunsaturated fatty acids, which were often found in the phospholipids of biological membranes. According to their results, oxidation of fish oil was greatly enhanced by the bioconverted linoleic acid, but not by the bioconverted CLA. And intact linoleic acid did not cause any additional oxidative effect on fish oil. Bio-converted EPA showed effects similar to those by bio-converted linoleic acid on fish oil oxidation. Bio-converted DHA, however, was more effective for the oxidation of fish oil than the other bio-converted fatty acids tested. These results, collectively, provided a hint about how the bio-converted fatty acids could exert biological activities. Changes in the physical state of membranes by variation in lipid types and oxidation on phospholipids can cause significant impacts, not only on membrane fluidity, but also on membrane proteins, which could result in significant damage to cellular functions.

31.3.2. Antibacterial Activities of the Bioconverted EPA and DHA

Since the bioconverted polyunsaturated fatty acids showed strong oxidative activities toward fish oil, their antibacterial activities were investigated against some foodborne pathogenic bacteria (Shin *et al.*, 2007). According to the results given in Tables 31.1 and 31.2, crude extracts of the bioconverted EPA and DHA showed great potential for antibacterial activities against Gram-positive bacteria such as *Bacillus subtilis*, *Listeria monocytogenes*, and *Staphylococcus aureus*, and Gram-negative bacteria such as *Enterobacter aerogenes*, *Escherichia coli*, *E. coli* O157:H7, *E. coli* O157:H7 (human), *P. aeruginosa*, *Salmonella enteritidis*, and *Salmonella typhimurium*. However, free EPA and DHA as negative controls had minor or negligible antibacterial effect. Gram-positive were more susceptible than Gram-negative bacteria to the bioconverted EPA and DHA, as they exhibited relatively higher antibacterial activities against *B. subtilis*, *L. monocytogenes*, *S. aureus* (ATCC 6538) and *S. aureus* (KCTC 1916) than other strains (Table 31.2). Growth inhibition by the crude extract of the bioconverted EPA and DHA was similar against Gram-positive bacteria, but the bioconverted extract of DHA was more effective than EPA

TABLE 31.1. The zone of growth inhibition of selected bacteria by crude extracts of the bioconverted EPA and DHA.

	Zone of inhibition (mm)	
Bacteria tested	EPA	DHA
Bacillus subtilis ATCC 6633	12	13
Listeria monocytogenes ATCC 19166	11	12
Staphylococcus aureus ATCC 6538	10	11
Staphylococcus aureus KCTC 1916	12	14
Pseudomonas aeruginosa KCTC 2004	10	12
Escherichia coli ATCC 8739	7	7
Escherichia coli O157:H7 ATCC 43888	8	8
Escherichia coli O157:H7 (human)	7	8
Enterobacter aerogenes KCTC 2190	7	7
Salmonella enteritidis KCCM 12021	7	7
Salmonella typhimurium KCTC 2515	7	8

TABLE 31.2. Minimal inhibitory concentration (MIC) of crude extracts (μg/ml) of the bioconverted EPA and DHA to inhibit the growth of selected bacteria.

	MIC (μg/ml)	
Bacteria tested	EPA	DHA
Bacillus subtilis ATCC 6633	350	350
Listeria monocytogenes ATCC 19166	350	350
Staphylococcus aureus ATCC 6538	500	500
Staphylococcus aureus KCTC 1916	500	500
Pseudomonas aeruginosa KCTC 2004	350[a]	250[b]
Escherichia coli ATCC 8739	2,350[a]	1,800[b]
Escherichia coli O157:H7 ATCC 43888	1,650[a]	1,350[b]
Escherichia coli O157:H7 (human)	1,800[a]	1,650[b]
Enterobacter aerogenes KCTC 2190	5,000[a]	4,800[b]
Salmonella enteritidis KCCM 12021	2,350[a]	1,650[b]
Salmonella typhimurium KCTC 2515	1,800[a]	1,650[b]

Note: Superscript a and b indicate statistically significant ($P < 0.05$) differences between bioconverted EPA and DHA against bacteria tested.

against Gram-negative bacteria as determined by minimum inhibitory concentration.

31.4. CONCLUSION

Various unsaturated fatty acids were efficiently used for the production of various hydroxy fatty acids by bacterial strain *P. aeruginosa* PR3. Among those unsaturated fatty acids, oleic acid, ricinoleic acid, linoleic acid, palmitoleic acid,

and triolein were well studied as substrates to produce mono-, di-, and trihydroxy fatty acids. Important environmental conditions for their production were also studied. The oxidative and antibacterial activities of bioconverted EPA and DHA produced by PR3 suggested that crude extracts of bioconverted EPA and DHA could be considered as promising antimicrobials in improving food safety by controlling foodborne pathogens.

31.5. ACKNOWLEDGMENT

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CHAPTER 32

Biotransformation of Oils to Value-added Compounds

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32.1. INTRODUCTION

Plant oils are excellent sources of some valuable compounds such as unsaturated fatty acids, phytosterols, squalene, pigments, antioxidants, vitamins, waxes, glycolipids, and lipoproteins. Plant oils could be employed for technological uses as biodiesel, lubricants, surfactants, emulsifiers, biopolymers, and so on. Vegetable oils also can serve as appropriate sources for the production of valuable compounds having applications in food, pharmaceutical. medical, and environmental fields. Attention has been focused on various types of value-added fatty acids (polyunsaturated fatty acids, conjugated fatty

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acids, hydroxy, keto, epoxy, branched and cyclic fatty acids), biosurfactants based on functional link between carbohydrates and selected lipid moieties (glycolipids, glycosylated and acylglycosylated sterols), flavour compounds, and so on.

Biotechnological transformation is powerful tool to effectively utilize a broad variety of plant oils, with the aim to modify their structure for the production of new lipid-based materials with demanded properties and functions. One method of plant oil transformation is based on the direct utilization by microorganisms. Employed oils can be converted to aimed compounds by submerged cultivation or oils, and/or oleaginous plant materials can be utilized during solid state fermentation to useful bioproducts enriched with demanded microbial products. Another biotransformation technique covers the enzymatic modification of oil components to structured lipids with biological properties.

There has been interest in lipid production by biotechnological routes for at least the last 70 years (Mukherjee, 2002). Nowadays microbial oil-related materials are highly specific, expensive to obtain from agricultural/animal sources and from possible chemical synthesis, or not commonly available by any other means. Although an amazing diversity of fatty acid structures occurs in microbial kingdom (Ratledge and Wilkinson, 1989), only some of these organisms can synthesize economically interesting fatty acids in sufficiently large quantities. The microorganisms or microbial enzymes that could be considered for plant oils transformation might provide the main stimulus for high value oil materials as potential commercial realities.

32.2. MICROORGANISMS AS A TOOL FOR OIL BIOTRANSFORMATION

Although the contribution of microorganisms to the oil industry has been nearly negligible, several benefits can be envisioned for these microbes (Certik and Shimizu, 1999a): *i)* their enormous growth rates on a wide variety of substrates allows the utilization and transformation of inexpensive oil materials, *ii)* oil biotransformation can be carried out throughout the year, since there is no seasonal or climatic dependence associated with it, *iii)* the active lipid-modifying apparatus can be simply regulated, *iv)* oil-based high value compounds can be easily tailored by unique biotransformation systems of selected microbial cells, *v)* microbes are the appropriate hosts into which foreign (plant or animal) genes could be cloned for the production of desired oil hitherto not found in microorganisms but which occur in other oil and fat sources , and *vi)* microorganisms are useful models for studying lipid biotransforming machinery.

32.3. BIOTRANSFORMATION OF OILS TO POLYUNSATURATED FATTY ACIDS (PUFAS)

Increasing demand for high-value lipids has focused commercial attention on the provision of suitable biosynthetic framework for their production. One of the main targets for microbial oil transformation is the construction of healthy and dietary important polyunsaturated fatty acids, such as γ-linolenic acid (18:3 ω-6; GLA), dihomo-γ-linolenic acid (20:3 ω-6; DGLA), arachidonic acid (20:4 ω-6; AA), eicosapentaenoic acid (20:5 ω-3; EPA), and docosahexaenoic acid (22:6 ω-3; DHA). Their applications in various fields (*e.g.*, PUFA-based nutraceuticals, therapeutics, etc.) coupled with their inadequacy from conventional agricultural and animal sources, has put attention on developing suitable biotechnologies to produce these compounds (Gill and Valivety, 1997a). Comparative successes with employing highly PUFA-synthesizing oleaginous microorganisms have enabled several processes to attain commercial levels (Certik and Shimizu, 1999b; Ratledge, 2004). The indication that a stable economic niche exists for microbial PUFAs has led several companies to subsidize development of these biotechnological methods. Rhône-Poulenc, Gist Brocades, Suntory, Idemitsu, Martek, and Lion have developed processes for fungal PUFAs. Algal approach to form PUFAs has been developed by Martek, Nisshin Oil Mills, Kelco International, Omega-Tech, Scotia Pharmaceutical, and Heliosynthese. On the other hand, Sagami and Nestle have examined bacterial PUFA-rich oils.

Particularly active in the synthesis of PUFAs are species of fungi belonging to Zygomycetes (Certik and Shimizu, 1999a; Ward and Singh, 2005). Oleaginous fungi producing PUFA could be economically valuable because the most of their PUFAs occur in the triacylglycerol fraction of their lipids. Microalgae are also potential sources of these fatty acids (Giridhar and Wu, 2007), however, microalgae require special growth conditions and harvesting/recovery techniques, so their use is not always economically feasible. Marine bacteria are another possible microbial source of ω-3 PUFAs, but PUFAs predominantly bound to phospholipid membranes cause additional difficulties for their recovery. The competition among fungi, microalgae, bacteria, and plants as sources of PUFAs will undoubtedly serve as a stimulus for all concerned, and it will be of considerable interest to see which routes eventually emerge as the economically preferred ones for the production of individual fatty acids.

The extensive research and development of PUFA production carried out over the past several years continues, basically aimed at improving the economic competitiveness of microbial lipids compared to plant- and animal-derived oils. Emphasis is on increasing the product value, using inexpensive oil substrates, screening for more efficient strains, and reducing the processing steps necessary for oil recovery from the cells. Screening of potential microorganisms for PUFA production is the first and an essential step limiting the number of strains for further study and practical use. These strains can be directly used in a laboratory-scaled optimization process. Two basic processes

have been developed for microbial production of PUFAs: submerged, and solid state fermentations (Certik and Shimizu, 1999a, 1999b). Simultaneously with fermentation trials, toxicological treatments of the strains and their metabolites must be performed to determine the safety of the product.

32.3.1. PUFA Production by Submerged Fermentation

Submerged cultivations for industrial use require a process that consists of several operation units from strain cultivation to oil refining. There are, however, three key operations which require special attention owing to the nature of oleaginous organisms: *i)* fermentation, *ii)* cell separation, and *iii)* oil extraction and refining (Certik and Shimizu, 1999a). Because the economic considerations of PUFA production are severely affected by the cost of raw material feedstock, selection of growth media (usually nitrogen-limited) is an important step. It should be noted that media adequate for the screening process are different from large-scale production media. The most cost efficient substrates are the waste materials from the food industry, preferable when the oil is intended for human consumption. After appropriate pretreatment, the substrate is either batch or continuously fermented under favorable conditions with sufficient amount of inoculum to yield biomass rich in PUFA-oil.

To obtain specific PUFA in high yield requires knowledge about the fermentation physiology of microorganisms for both oil biosynthesis and biotransformation (Certik, 2000). There are four distinct pathways coherent to each other involved in this process: *i) de novo* synthesis of fatty acids from cleaved polysaccharides, *ii)* incorporation of exogenous fatty acids into lipid structures, *iii)* desaturation and elongation of lipid sources, and *iv)* biohydrogenation, and partial or total degradation (β-oxidation) of fatty acids (Certik *et al.*, 1997). Thus, employing various oils to the mixture of substrates or direct utilization of oil-rich residues resulted in lipids consisting of high PUFA yields.

Certain fatty acids served as a useful substrate or precursor to achieve a high yield of commercially desirable PUFAs. Kamisaka *et al.* (1990), during experiments with *Mortierella ramannaina*, concluded that intracellular linoleic acid was incorporated mainly into phosphatidylcholine and phosphatidylserine where it was desaturated into GLA. Then produced GLA was gradually transferred, and accumulated in triacylglycerols. EPA-producing aquatic filamentous fungus *Saprolegnia* sp. synthesized odd chain PUFAs during growth on a medium supplemented with 13, 15 and 17 carbon fatty acids (Shirasaka *et al.*, 1995). The incorporation of linseed oil containing high levels of ALA promoted the production of EPA in *Mortierella* species. This is very promising from biotechnological point of view because there are various types of easily available natural oils containing ALA. In addition, the bioconversion of ALA to EPA is a cost-efficient temperature independent.

It should be noted that although the relative content of PUFAs in total lipids is often diminished in cells after the microbial assimilation of exogenous

oils, the total yield of PUFAs is considerably higher compared to cultivation without oil addition (Certik *et al.*, 1997). It seems to be that oil transformation by microbes is the most important and economically justified process for PUFA production.

32.3.2. PUFA Production by Solid State Fermentation (SSF)

The principal difficulty with submerged oil transformation for PUFA production has been in its marketing rather than in developing the large-scale fermentation and oil extraction process. The association of oleaginous fungi with solid state fermentations (SSF) has been tested to improve the commercial potential of microbial oils and thus create new perspectives for the economic competitiveness and market of microbial PUFAs. Solid state fermentation is a process in which microorganisms grow on a moist solid substrate in the absence of free water (Pandey, 2003). SSF simulates fermentation reactions occurring in nature and allows microbial utilization of raw agro-materials or byproducts of the agro-food industries. Because some oleaginous fungi simultaneously decrease anti-nutrient compounds in the substrates (*e.g.*, phytic acid) and partially hydrolyze substrate biopolymers, prefermented mass with a high content of PUFAs may be used as inexpensive food and feed supplement (Slugen *et al.*, 1994). Thus, SSF might provide the other opportunity to fill marketing claims in food, feed, pharmaceutical, veterinary, and environmental fields.

Biotechnological processes must be highly effective and competitive with other commonly used techniques to attain commercial feasibility. Because SSF can be carried out on a variety of agroindustrial materials and oil residues that have limited nutritional values, it is necessary to optimize the potential of microorganisms for the transformation of agroindustrial materials and oil residues into desired metabolites. Screening microorganisms has led to the selection of both *Mortierella isabellina* and *Thamnidium elegans* as producers of GLA (Slugen *et al.*, 1994; Certik *et al.*, 2006), and *Mortierella alpina* as a producer of DGLA, AA and EPA (Certik *et al.*, 2003a; Slavikova and Certik, 2005). Generally, the substrate surface is not only covered by the fungal mycelium during cultivation, but the fungal hyphae also penetrate into the substrates. Thus, fungal PUFAs are accumulated in the newly formed bioproduct, and their amount depends on the substrates, microorganisms, and cultivation conditions used.

Depending on the microorganism, various types of cereal substrates were employed during SSF experiments. Spent malt grains (SMG) were added to some substrates and served as an internal support. Substrates without SMG in most cases led to agglomeration of substrate particles and created a more compact mass, which in turn interfered with microbial respiration and negatively affected substrate utilization. The presence of SMG improved the bioconversion of linoleic acid from substrates to GLA (Certik *et al.*, 2006). Substrates with internal support not only provided better respiration and

aeration efficiency due to an increased inter-particle space, but also helped remove the heat generated during fermentation. It should be noticed that although PUFAs were synthesized more effectively by the addition of SMG to substrates, total PUFAs yield was also dependent on substrate/SMG ratio. An unbalanced ratio might provide limited surface for microbial attack and thus poorer availability of assimilable compounds (including oils) from substrates.

Emphasis is also put on the physiological regulation of microbial bioconversion. Fungi grew on a carbohydrate-containing substrate after the optimization of cultural conditions in constant lipid yield with the demanded fatty acid profile. The ability of the strains to utilize exogenous fatty acids opens new possibilities to prepare PUFAs with high yields. Because there is a stock of relatively inexpensive vegetable oils containing individual fatty acid precursors, SSF was applied for the microbial utilization of renewable agricultural oils to modify their properties for the production of value-added bioproducts with enhanced biological characteristics. Since certain fatty acids can serve as a substrate and precursor for individual fatty acid production, PUFA production by SSF could be useful for a newly developing market, as the risk to producers, and the investment cost may beunremarkable.

32.3.2.1. Bioproducts Enriched with γ-Linolenic Acid (GLA) Cultivating *Mortierella isabellina* on barley led to 18.6% of total oil in the bioproduct, corresponding to 18g GLA/kg bioproduct (Slugen, *et al.*, 1994). *Thamnidium elegans* also effectively utilized various types of cereals and other materials, enriching them with oil containing GLA. The improvement of GLA formation in the SSF process was achieved by following steps: *i)* the gradual elevation of carbon/nitrogen ratio with addition of glucose or whey, *ii)* the optimization of water activity, temperature, and oxygen availability, and *iii)* the transformation of exogenously added oils, with linoleic acid as a precursor of GLA (Certik *et al.*, 2002, 2003b). The final prefermented bioproduct contained 25% lipid, the total GLA amount yielded almost 10g/kg bioproduct. Because *T. elegans* possesses an active oil-biotransforming system, this strain was tested for its ability to convert directly oil-rich substrates (corn, sunflower seeds, linseeds, rapeseeds) to GLA. It was interesting that GLA biosynthesis was not improved by sunflower seeds containing linoleic acid as a precursor for GLA formation, but the best GLA yield (10g GLA/kg bioproduct) was achieved by the utilization and transformation of corn (Certik and Slavikova, 2005). This indicates that either some oil-rich substrates (sunflower seeds) might not be suitable material for *T. elegans*, or that corn contains, besides the fatty acids necessary for GLA synthesis, other compounds that might enhance GLA accumulation in fungal oil.

32.3.2.2. Bioproducts Enriched with Arachidonic Acid (AA) Solid-state fermentations were also employed to improve the market for fungal AA-rich bioproducts. Screening of many fungi has resulted in selection of *Mortierella*

alpina, that has also been used for the preparation of microbial oil with high AA content by submerged fermentations (Certik and Shimizu, 1999b; Ward and Singh, 2005). The growth of this fungus on agroindustrial materials during SSF was much slower compare with *T. elegans*, and prefermentation was completed after 10–14 days. Nevertheless, the search for optimal substrate revealed that *M. alpina* grown on a millet formed 57.4 g AA/kg bioproduct, or 49.1% AA in total fatty acids (Stredanska *et al.*, 1993). The same strain also effectively converted a mixture of wheat bran/spent malt grains (3 : 1, w/w) leading to bioproduct with 4.2% AA (Certik *et al.*, 2003a).

32.3.2.3. Bioproducts Enriched with Dihomo-γ-Linolenic Acid (DGLA) Basic cultivation of *M. alpina* usually leads to a standard fatty acid profile with a predominant concentration of AA and a low level of DGLA. Since bioconversion of DGLA to AA is catalyzed by Δ^5 desaturase, inhibition of this metabolic step is accompanied by a rapid increase of DGLA/AA ratio (Certik *et al.*, 1998). This strategy was applied during SSF for preparation of a bioproduct enriched with DGLA by *M. alpina*. The addition of sesame seeds (containing sesamin analogous, effective inhibitors of Δ^5 desaturase) to peeled barley rapidly enhanced DGLA levels in the bioproduct and the DGLA/AA ratio as well. However, when *M. alpina* utilized only crushed sesame seeds (characterized by sufficient amount of saccharides, proteins, and lipids), the DGLA/AA ratio in bioproduct after 13 days of cultivation reached a value of 2.9 that corresponded to almost 20 g DGLA/kg bioproduct. Subsequent cultivation for a further 5 days led to the final 21.3 g DGLA/kg bioproduct (Huong *et al.*, 1998; Certik *et al.*, 2003a).

32.3.2.4. Bioproducts Enriched with Eicosapentaenoic Acid (EPA) The SSF process has been developed to prepare EPA-rich bioproducts using fungi that can rapidly utilize, incorporate, and modify exogenously added oils. The ability of these strains to utilize exogenous fatty acids opens new possibilities to prepare biomaterials rich in PUFAs by SSF. Linseed oil, with about 57% α-linolenic acid, is a precursor of PUFAs belonging to ω-3 fatty acid family. Because *M. alpina* possesses an appropriate enzymatic apparatus for the possible transformation of α-linolenic acid to EPA (Certik *et al.*, 1998), linseed oil was added to the substrate to shift the formation of ω-6 fatty acids (AA) to ω-3 fatty acids (EPA). This led to the prefermentation of the mixture of peeled barley/linseed oil/spent malt grains (0.5 : 1 : 3, w/w) by *M. alpina* and simultaneously yielded 23.4 g EPA/kg bioproduct (17.8% EPA in oil) and 36.3 g AA/kg bioproduct (27.6% AA in oil) (Certik *et al.*, 2003a; Slavikova and Certik, 2005). Subsequent storage of the bioproduct at 10 °C for 7 days caused an increase of the ratio of the total w-6/w-3 fatty acids from 0.9 to 1.1, as well as an increase in the ratio of PUFA/saturated fatty acids from 6.0 to 7.8. Thus, such a strategy allows for the preparation of oils with a desirable ω-6/ω-3 PUFA ratio, leading to more beneficial human applications.

32.3.3. Regulation of Microbial Oil Transformations

Although the manipulation of microbial oil composition is a rapidly growing field of biotechnology, the use of microbial lipids is still insufficient to meet industrial demand. Modified microbial oils, yielding to the best commercial varieties, must have a broad production base to be competitive with other commodities. Therefore, the emphasis is on increasing the PUFA value by employing new biotechnological alternative strategies that could be combined with classical fermentations.

32.3.3.1. Mutation Techniques Although several wild oleaginous microorganisms are able to synthesize PUFA-rich oils, these strains have a limited ability to produce new PUFAs, or to increase existing PUFA formation. Mutation techniques resulting in the suppression or activation of specific desaturases and elongases are beneficial, not only for the production of tailor-made fatty acids, but for studying fatty acid biosynthesis in a microbial body (Certik *et al.*, 1998). Several mutants with modified fatty acid desaturases and fatty acid elongases have been characterized and employed for PUFA formation. The ability of these mutants to utilize exogenous fatty acids allows the high yield production of various PUFAs. For example, eicosatetraenoic acid (20:4 ω-3; ETA) was formed when Δ^5 or Δ^{5+12} desaturase-defective mutants grew in medium with the addition of a linseed oil contained a high ALA concentration. In addition, Δ^{12} desaturase-defective mutants efficiently converted exogenous ALA into EPA and increased the EPA/AA ratio in the fungal oil. Thus, the mutants are excellent tools for regulating exogenous fatty acid flow to targeted PUFAs. (For more details about fatty acid desaturase mutants, numerous papers have been published by Shimizu group.).

32.3.3.2. Molecular Engineering Progress in the genetic engineering of microorganisms has led to the speculation that certain oil components could become marketable commodities if the genomes of traditional oleaginous microbes were appropriately modified. The major challenges in modifying lipid composition are to change the degree of fatty acid unsaturation, and to insert new functional group/s into the fatty acid chain (*e.g.*, hydroxy, epoxy). Many of genes involved in fatty acid alternations (fatty acid desaturases, acyltransferases, and elongases) have been characterized and isolated (Warude *et al.*, 2006). Their expression into other microorganisms has resulted in the formation of new and not commonly found PUFAs. Such functional exchanges of fatty acid modifying genes from different origins are an important step for constructing novel microbial varieties synthesizing economically valuable fatty acids by their active biotransforming systems.

32.3.4. Enzymatic Biotransformations

Biotransformation of oils by enzymatic treatment is also an important and challenging area. The application of enzymes-catalysed reactions in many

industrial fields has led to incorporation of these technologies in the improvement and structuring of lipid composition from various sources. Employment of enzymatic routes to obtain desired PUFA oils is an attractive alternative to chemical processes because i) the PUFA profile of the product can be engineered by appropriate selection of biocatalysts and reaction conditions, and ii) he formation of undesirable coproducts is almost eliminated under the mild conditions characteristic for enzymatic conversions (Gandhi, 1997). In particular, enzymatic interesterifications are useful for modifying microbial TAGs, where their nutritional values are based, not only on the degree of unsaturation, but also on acyl chain length and the positional composition of individual PUFAs in the glycerol backbone. Additionally, sufficient lipase-assisted incorporation of ω-3 PUFAs to the oils containing ω-6 PUFAs allows the preparation of oils with desirable ω-6/ω-3 PUFA ratios, leading to more beneficial human applications (Gill and Valivety, 1997b; Ju *et al.*, 1998; Haraldsson, 2007). Therefore, these tailor-made TAGs, because of their superior assimilation and metabolism, make desirable compounds for nutritional supplements, infant formulas, and therapeutic agents.

The commercial practicability of enzyme-mediated bioconversions has been clearly demonstrated by producing highly purified PUFAs, unique TAGs, PUFA-based bioemulsifiers, and phospholipids for use in foods, nutraceuticals, biomedicals, pharmaceuticals, cosmetics, and drug-delivery devices (Gill and Valivety, 1997b). Potentially, lipase-based processing can provide access to bulk and refined PUFA feedstock suitable for further biological applications that have been hindered by the high cost of PUFAs refined by current chemical procedures. Microbial PUFAs could also be incorporated into seed and animal oils, providing inexpensive PUFA-fortified oils suitable for direct use. Finally, the future prospects of some oleaginous microorganisms are related to their special biotransforming systems, converting PUFAs into commercially highly valued oxygenated fatty acid derivatives (prostaglandins, leukotrienes, thromboxanes, hydroxy-PUFAs) and aroma or flavour compounds (Lamacka and Sajbidor, 1997; Gill and Valivety, 1997b).

32.4. BIOTRANSFORMATION OF OILS TO CONJUGATED LINOLEIC ACID (CLA)

Conjugated linoleic acid (CLA) is a generic name for a mixture of positional and geometric isomers of linoleic acid (9c, 12c-octadecadienoic acid, C18:2) with conjugated double bonds at 7 and 9, 8 and 10, 9 and 11, 10 and 12, 11 and 13, or 12 and 14 positions. This fatty acid, carried out by rumen bacteria in the ruminal process, arises along a stepped pathway and ends with the full saturation of linoleic acid into stearic acid. These naturally occurring groups of dienoic derivatives of linoleic acid (LA) are incorporated into the fat in beef and the milk of ruminants before the saturation process has been completed. Food products from ruminants, particularly

dairy products, are the major dietary source of CLA for humans (Khanal and Dhiman, 2004).

Conjugated metabolites of linoleic acid have been extensively studied during last few years. Of the various CLA isomers, the *cis*-9, *trans*-11, and the *trans*-10, *cis*-12 (c9, t11 and t10, c12) configurations are considered to be the most effective, attracting attention because of their anticarcinogenic, antiadipogenic, antiatherogenic, and antidiabetic health benefits (Banni *et al.*, 2003; Belury, 2003; Kritchevsky, 2003). CLA'srole in vitamin A metabolism (Carta *et al.*, 2002), bone modelling (Watkins *et al.*, 2003), and platelet aggregation and immune response (Cook *et al.*, 2003) has also been reported. Other novel applications of CLA are oriented on foods, skin care products, and cosmetics (Kapoor *et al.*, 2003). Some of the biological activity of the two major CLA isomers may result from their different involvement in fatty acid metabolism.

32.4.1. Techniques for CLA Synthesis

Increasing interest in these high-valued CLA metabolites, together with the necessity of a well-balanced diet has been observed for recent years. Nowadays, the most CLA is synthesized chemically by alkaline isomerization of oils rich in linoleic acid, and such CLA is sold as food supplement. However, a disadvantage of chemical preparation of CLA is a mixture of various unexpected CLA isomers. Demand for "natural" CLA metabolites has challenged biotechnological strategies employing special bacteria that are able to utilize and convert linoleic acid to aimed conjugated isomers (Kundrikova and Certik, 2005; Ogawa *et al.*, 2005, 2007). The fact that CLA is synthesized by ruminant bacteria has incrased interest in overproducing these biologicalcompounds by appropriate microorganisms. From this point of view, biotechnological techniques might bring about the more effective transformation of extracellular linoleic acid to CLA metabolites. Moreover, the introduction of biological reactions to CLA production may solve the problem of the by-production of unwanted CLA isomers. Thus, the "natural" formation of biologically active compounds is preferred to chemical synthesis.

32.4.2. Regulation of Microbial Conversion of Linoleic Acid to CLA

The first, important step in the bioconversion of linoleic acid into conjugated linoleic acid (CLA) is the preparation of an adequate amount of bacterial biomass with suitable physiological properties, such as the high activity of key enzymes reliable for the biotransformation. The second step is bioconversion of linoleic acid to CLA. LA conversion to CLA occurs in anaerobic conditions because the presence of oxygen promotes oxidative metabolism in some bacteria and results in lower CLA production (Ogawa *et al.*, 2001). Many bacterial strains of *Lactobacillus, Lactococcus, Streptococcus, Bifidobacteria, Propionibacterium, Butyrivibrio* and bacterial isolated from various sources have been

tested for CLA production. Three types of CLA isomers were formed: c9, t11/t9, c11-CLA (CLA 1); t9, t11-CLA (CLA 2): a t10, c12-CLA (CLA 3). Two types of hydroxy fatty acids—10-hydroxy-*cis*-octadecenoid acid (HY1) and 10-hydroxy-*trans*-octadecenoic acid (HY2)—have also been detected as intermediates of CLA biosynthesis. Screening experiments resulted in selection of three bacterial cultures, *L. plantarum* JCM 8341, *L. plantarum* DSMZ 2601, and *Enterococcus faecium* CCM 4231 (bacterial isolate from calf rumen). Bacterial cells taken from an earlier cultivation phase (24 h) transformed linoleic acid to CLA and HY (hydroxy octadecenoic acids) more effectively when compared with elder cells. Modification of transformation conditions modified the ratio of various CLA derivatives, and improved the yield of aimed CLA metabolite/s. An L-serine addition increased the ratio of CLA 1/ CLA 2, and stannum was discovered to be the best stimulator of CLA 1 biosynthesis. And while rapid HY accumulation occurred in 1,2- and 1,3-diacylglycerols, CLA isomers were mainly incorporated to monoacylglycerols. This findings might shed new light on the regulation of CLA overproduction by selected bacterial strains.

Other approaches to enhance the conversion of LA to CLA are found in immobilization techniques. Immobilized cells of *Lactobacillus reuteri* were 5.5 times more efficient in producing CLA than washed cells grown under optimized conditions (Lee *et al.*, 2003a, 2003b). Improved CLA production was also observed with the immobilized cells of *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* in polyacrylamide and chitosan (Lin *et al.*, 2005). The major CLA isomers produced by these immobilized cells were c9, t11-CLA and t9, t11-CLA.

Although many studies on fatty acid conversion to CLA have been performed, the mechanism of CLA formation has not been elucidated in detail. Ogawa *et al.* (2001) indicated that the transformation of linoleic acid to CLA using washed cells of *Lactobacillus acidophilus* AKU 1137 is not a single one-step isomerization of non-conjugated diene to a conjugated diene, but involves the production of hydroxy fatty acids, *i.e.*, 10-hydroxy-trans-octadecaenoic and 10-hydroxy-cis-octadecaenoic acids. Further attention to the optimization of fermentation conditions should improve the conversion of hydroxy fatty acids into CLA. Ricinoleic acid was investigated as a substrate for CLA production, and *L. plantarum* JCM 1551 transformed more than 70% of the acid to CLA that consisted of only two isomers: c9, t11-CLA (21%), and t9, t11-CLA (79%) (Ando *et al.*, 2003). Kishino *et al.* (2002) and Ogawa *et al.* (2007) tested the additions of other fatty acids (linoleic, α-linolenic, oleic and ricinoleic acids, and castor oil) for CLA production from ricinoleic acid by the washed cells of *Lactobacillus plantarum* AKU 1009a. It is interesting that while α-linolenic acid (0.6 mg/mL) increased CLA biosynthesis from ricinoleic acid, LA and the combination of LA with α-linolenic acid decreased the CLA formation from ricinoleic acid. Because free form of ricinoleic acid is a suitable substrate for CLA production, castor oil (with about 88% ricinoleic acid in the form of triacylglycerol) can be used as a substrate only with help of lipases (Ando *et al.*, 2004).

32.5. CONCLUSIONS

The biotechnological transformation of plant oils to value-added compounds undoubtedly depends on their commercial potential and a demand for unusual oil-derived metabolites can be produced by conventional means. Current achievements with microbial PUFA- or CLA-rich oils confirm the potential of biotechnologies to create a new economic competitiveness and market for these oils. Biotechnological strategies are focused on novel microbial strains and biotransforming techniques for biosynthesis of economically valuable lipid metabolites. Further perspectives are connected with the ability of microbial cells to perform a huge number of oil-biotransforming reactions, resulting in the formation of commercially interesting products (microbial hydroxy-PUFAs, prostaglandins, leukotrienes, and thromboxanes) which are extraordinarily expensive to produce by chemical synthetic routes. Additional possibilities lie in the ability of microbial cells to produce unusual lipid classes (such as hydroxyalkanoates, wax esters, cerebrosides, glycolipids, and sterol derivatives) or to perform a huge number of oil-biotransformating reactions resulting in the formation of compounds useful in the flavour, fragrance, pharmaceutical, and fine-chemical arenas. The extremely broad range of oil-biotransforming products in industry (surfactants and emulsifiers, soaps, paints and varnishes, texturizing agents, lubricants and plasticizers in cosmetics, textiles, plastics, oil drilling fluids, *etc.*), nutrition (margarines and butters, nutraceuticals) and medicine (various PUFA-containing drugs) is breathtaking and opens a large number of possibilities for academic research as well as demonstrates the enormous potential of their applications.

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