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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzymatic Approach to Biodiesel Production

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The need for alternative energy sources that combine environmental friendliness with biodegradability, low toxicity, renewability, and less dependence on petroleum products has never been greater. One such energy source is referred to as biodiesel. This can be produced from vegetable oils, animal fats, microalgal oils, waste products of vegetable oil refinery or animal rendering, and used frying oils. Chemically, they are known as monoalkyl esters of fatty acids. The conventional method for producing biodiesel involves acid and base catalysts to form fatty acid alkyl esters. Downstream processing costs and environmental problems associated with biodiesel production and byproducts recovery have led to the search for alternative production methods and alternative substrates. Enzymatic reactions involving lipases can be an excellent alternative to produce biodiesel through a process commonly referred to alcoholysis, a form of transesterification reaction, or through an interesterification (ester interchange) reaction. Protein engineering can be useful in improving the catalytic efficiency of lipases as biocatalysts for biodiesel production. The use of recombinant DNA technology to produce large quantities of lipases, and the use of immobilized lipases and immobilized whole cells, may lower the overall cost, while presenting less downstream processing problems, to biodiesel production. In addition, the enzymatic approach is environmentally friendly, considered a "green reaction", and needs to be explored for industrial production of biodiesel.

KEYWORDS: Alcoholysis; biodiesel; bioenergy; fatty acid methyl esters; fatty acid alkyl esters; immobilized enzymes; interesterification; lipases; protein engineering; recombinant DNA; response surface methodology; transesterifcation

INTRODUCTION

There is renewed interest, and increased awareness, in alternative energy sources such as biodiesel, hydrogen, and bioethanol, for use in diesel engines, especially now that the current world dependency is heavily on petroleum and petrodiesel or fossil fuel. Energy security is another reason for alternative sources of fuels. As the price of petroleum fuel keeps rising everyday (currently around $65–67/barrel, approaching $70/barrel), the incentive for research and use of alternative fuels must also rise. Political, global warming, greenhouse gases, environmental, health, predicted shortage of fossil fuel, and economic issues and/or concerns dictate that we look elsewhere for fuel/energy to drive diesel engines. The use of biodiesel is not new, as Rudolph Diesel, in 1911, first used vegetable oil (groundnut/peanut oil) to power the diesel engine (*1*), that is, an engine that required compression and then ignition. The use of 100% pure vegetable or animal fats to power diesel has several drawbacks such as high fuel viscosity, low power output, thickening or gelling of the lubricating oil, oxidative stability, and low volatility resulting in carbon deposits due to incomplete combustion, to name a few. To circumvent some of these problems, the pyrolysis of fat (thermal decomposition at high temperatures to reduce the molecular size for use as biodiesel) to lower viscosity, microemulsions (vegetable oils, esters, and cosolvents such as short-chain alcohols, as dispersing agents) to lower viscosity, dilution of oil with solvents to lower viscosity, and transesterification with short-chain alcohols to form lower-chain fatty acid alkyl esters, FAAE (to increase volatility and decrease viscosity, although viscosity depends on fatty acid composition), have been employed. Transesterification appears to be the simplest and the best route to produce biodiesel, in large quantity, with physical characteristics that

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approximate fossil diesel with little or no deposit formation after combustion in diesel engines. Although several researchers have reviewed biodiesel production through chemical and enzymatic reactions (*1–11*), only very limited reviews were devoted to the enzymatic approach to biodiesel (*3, 6, 7, 12*). Our aim is to bring to light the many potentials of using enzymes, particularly lipases, for the future production of biodiesel. The potential for improving enzyme activity and efficiency through immobilization, enzyme regeneration, use of whole cells, protein engineering, and the production of recombinant lipases in large quantities, with the hope to reduce the overall cost of enzymatic biodiesel production, is discussed. In the future, lipase will be the catalyst of choice for biodiesel production. Presently, it is better than base- and acid-catalyzed reactions because it requires less energy to produce and little or no downstream processing. Both base- and acid-catalyzed transesterification will require neutralization.

CHEMICAL VERSUS ENZYMATIC ALCOHOLYSIS APPROACH

The commercial process commonly used to produce biodiesel is the chemical process that utilizes alkaline catalysts to convert vegetable oil or fat and methanol to fatty acid methyl esters, FAME. The basic catalysts employed are sodium or potassium hydroxide because they are relatively inexpensive (*13, 14*). However, they form soap, which consumes the catalyst, decreases yield, thereby making purification and isolation of the FAME difficult. Glycerol, a coproduct of the chemical alcoholysis reaction must be removed. Usually, a stoichiometric excess (normal stoichimetry requires a 3:1 molar ratio of methanol/vegetable oil) of substrates, that is, a molar ratio of 6:1 (methanol:vegetable oil), is preferred to increase methyl ester yield, and the reaction can be completed in a few hours at 40–65 °C. Other basic catalysts employed are sodium or potassium alkoxides, as well as their carbonates. Sodium methoxide, for example, is moisture-sensitive, and the substrates (oil and alcohol) must be essentially anhydrous (free of water) for the reaction to proceed to the right to a great extent without hydrolysis of the products. Sodium methoxide (0.5 mol %) is very reactive and can easily give >98% alkyl ester in 30 min. Acid catalysts can be, but are rarely, used because they are corrosive and result in slower reactions and a low yield of FAME. Vegetable oils with high free fatty acid contents are better esterified with acid catalysts. No soap is formed with acid catalysts, but higher temperature and higher substrate molar ratios may be needed. The preferred acid catalysts are sulfuric (*13*), hydrochloric, sulfonic, and organic-sulfuric acids because they give high yields of fatty acid alkyl esters. Acid catalysts require a substrate molar ratio of up to 30:1 at 55–80 °C with a 0.5–1 mol % catalyst concentration to yield approximately 99% biodiesel in 50 h (*6*). Other acid-based catalysts such as methanolic HCl or methanolic H_2SO_4 that are routinely used to prepare FAME for gas chromatographic analysis of fatty acids could be used for biodiesel production (*15*). In general, using chemical catalysts results in a FAME yield > 98%.

Transesterification or alcoholysis reaction can be carried out with enzymes, and numerous examples abound in the literature (*16–24*). There is a current interest in using lipases as the biocatalyst to commercially convert vegetable oils and fats to FAME as biodiesel fuel, since it is more efficient, highly selective, involves less energy consumption (reactions can be carried out in mild conditions), and produces less side products or waste (environmentally favorable). Most of the recent research involved determining the best enzyme source and optimizing the reaction conditions (substrate molar ratio, solvent (*20*) or no solvent (*21*), temperature, water content (*19, 20, 23*), free fatty acid level (*18*), percent conversion, acyl migration (*25*), and substrate flow rate in packed bed bioreactors) to improve the yield of biodiesel comparable to base-catalyzed reactions and for possible industrial scale-up and use. Enzymes have several advantages over chemical catalysts such as mild reaction conditions; specificity, reuse; and enzymes or whole cells can be immobilized, can be genetically engineered to improve their efficiency, accept new substrates, are more thermostable, and are considered natural, and the reactions they catalyze are considered "green" reactions. A major problem with lipase reaction with methanol is enzyme inactivation by methanol. This problem has been studied and presumably solved by Shimada et al. (*7*), who reported that the stepwise addition of methanol will alleviate methanol inactivation of *Candida antarctica* lipase and results in 90% yield of FAME from waste oil. This enzyme was stable for 100 d and could be reused up to 50 times without a significant loss of activity. Few studies have considered the nature of the alcohol used in the transesterification reaction. The addition of cosolvents such as *t*-butanol (*26*) appeared to prevent methanol inactivation of the lipase. Enzyme-catalyzed alcoholysis reactions can be performed in the presence or absence of a solvent, and it requires less energy and practically no purification to obtain FAME compared to base-catalyzed alcoholysis, where soap formation presents downstream processing disadvantages. Biocatalyst removal, if immobilized lipase is used, is by simple filtration or is not required if a packed bed bioreactor is used for the continuous production of biodiesel. In general, enzyme catalysts at 4–10 wt % result in FAME yields of 55–97% in 3–120 h at 30–50 °C (*1*).

The common methods for the analysis of FAME are by gas chromatography, thin layer chromatography, reverse-phase high-performance liquid chromatography, gas chromatography–mass spectrometry, and high-performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry. For more information on the methods of analyses of biodiesel and the products of the esterification reactions, see excellent reviews by Meher et al. (*11*) and Türkan and Kalay (*27*), since this is not the focus of the current review. Characteristics of biodiesel fuel are available elsewhere (*2–9*) but usually involve measuring the content of residual triacylglycerols (TAG), diacylglycerols, monoacylglycerols, free fatty acids, free glycerol, and alcohol; kinematic viscosity at 30–40 °C; density; flash point; cold filter plugging point; cetane number/index; acid value; caloric content; ash, water, and sulfur, contents; cloud point (CP); pour point (PP); carbon residue; and copper strip corrosion, by using various American Society for Testing and Materials (ASTM) methods. It should be noted that the fuel property specifications, requirements, limits, and analysis methods vary from country to country.

SOURCES OF RENEWABLE OILS AND FATS

A major source of fuel energy comes from nonrenewable, potentially exhaustible, resources such as fossil fuels (natural gas, petroleum, and coal). These sources have environmental and toxic problems, and they are not biodegradable.

1. Plant-Derived Oils. Plant-derived oils (TAG) as a substitute raw material for diesel were considered a good energy supply because they are carbon dioxide neutral. This stems from the fact that green plants grow through the photosynthesis process with CO_2 as a carbon source. Therefore, the combustion of plant-derived oils will release carbon dioxide which has

Table 1. Fatty Acid Composition of Possible Biodiesel Renewable Raw Materials (*1, 8, 34, 39, 75, 76*)

	fatty acids (%)													
oils/fats	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$	$C_{22:1\ n9}$	$C_{22:0}$	$C_{24:0}$	others	total SFA and UFA[a]
almond kernel	6.5	0.5	1.4	70.7	20.0								0.9	SFA, 7.9; UFA, 91.2
bay laurel leaf	25.9	0.3	3.1	10.8	11.3	17.6							31	SFA, 29; UFA, 40
borage	12.9	0.2	4.3	19.1	39.0	18.7	0.3	3.5		2.0				SFA, 17.5; UFA, 82.5
coconut	5.0		3.0	6.0									65	SFA, 8; UFA, 6
corn	11.7		1.9	25.2	60.6	0.5	0.2							SFA, 13.8; UFA, 86.3
cottonseed	28.3		0.9	13.3	57.5									SFA, 29.2; UFA, 70.8
crambe	2.1		0.7	18.9	9.0	6.9	2.1		0.8	58.5		1.1		SFA, 6.8; UFA, 93.3
groundnut	8.5		6.0	51.6	26.0									SFA, 14.5; UFA, 77.6
hazelnut kernel	4.9	0.2	2.6	81.4	10.5								0.3	SFA, 7.5; UFA, 92.1
jatropha	16.4	1.0	6.2	37.0	39.2		0.2							SFA, 22.8; UFA, 77.2
karanj	10.2		7.0	51.8	17.7	3.6	1.6	1.2			5.4	1.5		SFA, 25.7; UFA, 74.3
linseed	5.0		2.0	20.0	18.0	55.0								SFA, 7; UFA, 93
olive	11.8	1.5	2.7	74.2	8.5	0.7	0.4	0.3						SFA, 14.9; UFA, 85.2
palm oil	42.6	0.3	4.4	40.5	10.1	0.2							1.1	SFA, 47; UFA, 51.1
peanut	11.4		2.4	48.3	32.0	0.9	1.3		2.5			1.2		SFA, 18.8; UFA, 81.2
poppy seed	12.6	0.1	4.0	22.3	60.2								0.8	SFA, 16.6; UFA, 82.6
rapeseed	3.5		0.9	64.4	22.3	8.2								SFA, 4.4; UFA, 94.9
rice bran	11.7–16.5		1.7–2.5	39.2–43.7	26.4–35.1		0.4–0.6					0.4–0.9		SFA, 13.8–19.9; UFA, 66–79.4
safflower seed	7.3	0.1	1.9	13.5	77.0								0.2	SFA, 9.2; UFA, 90.6
sesame	13.0		4.0	53.0	30.0									SFA, 17; UFA, 83
soybean	11.4		4.4	20.8	53.8	9.3	0.3							SFA, 16.1; UFA, 83.9
sunflower	7.1		4.7	25.5	62.4		0.3							SFA, 12.1; UFA, 87.9
tallow	29.0		24.5	44.5										SFA, 53.5; UFA, 44.5
walnut kernel	7.2	0.2	1.9	18.5	56.0	16.2								SFA, 9.1; UFA, 90.9
wheat grain	20.6	1	1.1	16.6	56.0	2.9							1.8	SFA, 21.7; UFA, 76.5

[a] SFA = saturated fatty acids; UFA = unsaturated fatty acids.

previously been fixed through photosynthesis. Plant-derived fuels are renewable, inexhaustible, nontoxic, and biodegradable, with an energy content similar to that of fossil diesel fuel. Obviously, obtaining fuel from vegetable oils and fats is more expensive than doing so from petroleum-based fuels at the present time. This is due, in part, to the competition between vegetable oils and fats for biodiesel production with that for the food, feed, and oleochemical industries. Genetic engineering may help lower the cost in the near future as new crops will be developed with a high oil content specifically for nonfood use, especially from underutilized crops and plants. Although, if petroleum prices continue to increase, then biodiesel from used frying oils, deodorizer distillates, and plant oils will become competitive and commercially feasible. The source of oil crops will depend on their availability and varies by regions and countries, but these crops must be cheap and easy to grow, capable of large-scale production (that is, high yield/acre), and rich in oil percentage, and have a low cost of production (*1*). The fatty acid composition of the source oil or fat is important in biodiesel because, in the winter, oils containing more saturated fatty acids than unsaturated fatty acids may solidify and clog the fuel lines. The fatty acid composition of some of the biodiesel raw materials are shown in **Table 1**. Refined oils are more expensive but have a low production scale. Of all the vegetable oils available, high oleic acid containing oils are preferred because of the increased stability of their alkyl esters on storage and improved fuel properties (*1*). Soybean, palm kernel, cottonseed, sunflower, safflower, rapeseed, peanut/groundnut, and castor bean oils are the more commonly used oils in biodiesel production, although algal oils have potential for biodiesel production (*28*).

2. Waste Oils and Fats. Waste oils and fats (*19, 20*), used frying oils, lard, beef tallow, yellow grease (*29*), and other hard stock fats can also be used in preparing biodiesel. Used frying oils, while cheap, may have some disadvantages because of the contents of high polymerization products, high free fatty acid contents, susceptibility to oxidation, and high viscosity. Therefore, preliminary treatment such as the use of adsorbent materials (such as magnesium silicates) to reduce the free fatty acid content and polar contaminants may be necessary to improve the oil quality prior to transesterification to produce biodiesel catalyzed by a basic catalyst. Poor-quality oils may inactivate the basic catalysts or even enzyme catalysts.

Other potential alternative energy sources under consideration are nuclear, hydrogen, wind, solar, and hydropower. Blends of fuels such as ethanol and gasoline (gasohol), biodiesel and fossil diesel (for example, B20 = 20% B100 and 80% diesel), biodiesel and vegetable oils, or pure 100% biodiesel (B100) and 100% vegetable oils are examples of worldwide efforts to reduce dependency on 100% petroleum products. It has been suggested that biodiesel from vegetable oils or their blends has the following advantages over conventional diesel or petroleum: it is renewable, biodegradable, oxygenated, and less- or nontoxic; has a low sulfur content and higher cetane numbers; produces less smoke and particulates; produces lower carbon monoxide and hydrocarbon emissions; has a low aromatic content and higher heat content of about 88% of number 2 diesel fuel; and is readily available (*8, 12, 30*).

3. Microbial Oils. Microalgal oils represent another cheap source of renewable raw materials for biodiesel production that has received little or no attention. Algal oils are largely produced through substrate feeding and heterotrophic fermentation. Li et al. (*28*) reported the production of biodiesel on a large scale using the oils from microalga, *Chlorella* protothecoids, in bioreactors. The lipid content of the microalga was increased up to 44–48% of the cell dry weight. The oils were then used to produce biodiesel (98% conversion to FAAE) by a reaction catalyzed by immobilized *Candida* sp. lipase at a substrate molar ratio of 3:1 and a reaction time of 12 h. The product was said to be comparable to conventional biodiesel in physical properties. Luo et al. (*31*) used a novel psychrophilic lipase from *Pseudomonas fluorescens*, a 1,3-specific lipase, to produce biodiesel (92% yield) in 12 h but at 20 °C, which is lower than normal for most lipases (35–50 °C). A genetic engineering

technique was used to engineer this lipase to act at lower temperatures by cloning the lipase gene, lipB68, and overexpressing it in *Escherichia coli* BL21 (DE3) to obtain a recombinant protein which was subsequently purified and used in catalysis. Thus, the engineering and production of lipases capable of catalyzing biodiesel production at low temperatures will result in huge energy savings and lower the cost of enzymatic biodiesel production.

SOURCE OF ACYL ACCEPTORS IN TRANSESTERIFICATION

Transesterification is the process of exchanging acyl groups between an ester and an acid (acidolysis), between an ester and another ester (interesterification or ester–ester interchange), or between and ester and an alcohol (alcoholysis) (*32*). For biodiesel production through alcoholysis, methanol is most commonly used to produce FAME. Methanol is cheaper than ethanol and more reactive and more volatile as FAME compared to fatty acid ethyl esters (FAEE). Ethanol is preferred because it is considered more renewable than methanol and because it is obtained from agricultural products and hence is more environmentally friendly. Of course, the main purpose of alcoholysis is to reduce the viscosity of the fat and increase volatility and FAME combustion in a diesel engine without any engine modification. However, methanol may be potentially toxic. Other acyl group acceptors for the alcoholysis reaction are ethanol, propanol, isopropanol (*16, 33, 34*), butanol, branched-chain alcohols (*35–37*), *t*-butanol (*26, 38*), and octanol (*6*). Very few articles explored the use of other acyl acceptors other than alcohols (**Table 2**). Acyl acceptors such as methyl or ethyl acetate (ester–ester interchange) can be used to react with TAG to produce FAAE as biodiesel (*39*). The major and common routes to biodiesel synthesis are depicted in the equations below. All reactions can be catalyzed by a base, acid, or a lipase as catalyst.

Direct Esterification:

$R_1CO–OH + R–O–H \leftrightarrows R_1CO–OR + H_2O$

fatty acid + alcohol $\leftrightarrows$ fatty acid alkyl ester + water

Note, water must be removed to increase the yield. This is possible but not common for biodiesel preparation

Alcoholysis:

$RCO–OR_1 + R_2–O–H \leftrightarrows RCO–OR_2 + R_1–O–H$

$TAG + 3CH_3OH \leftrightarrows 3\ FAME + glycerol$

Note, glycerol must be removed for base-catalyzed reactions. Neutralization is required for basic and acidic catalysts. This method has easier product recovery with an enzyme as the catalyst.

Ester–Ester Interchange:

$R_1CO–OR_2 + R_3CO–OR_4 \leftrightarrows R_1CO–OR_4 + R_3CO–OR_2$

TAG + 3 methyl acetate $\leftrightarrows$ 3 methyl ester + triacetylglycerol

Note, no glycerol is produced in this process. It is very easy to recover methyl or alkyl ester. Triacetylglycerol (triacetin) is an antifungal agent and can also be used as a fixative in perfumery or as a solvent in basic dyes.

In the previous reactions, R_1, R_2, R_3, and R_4 represent the alkyl chain of the acyl group or alcohol; TAG is triacylglycerol; and FAME is fatty acid methyl ester, assuming complete conversion of the substrates to products. All the reactions above are completely reversible.

SOURCE OF LIPASES

Lipases are found in all living organisms and are broadly classified as intracellular and extracellular. They are also classified on the basis of the sources from which they are obtained, such as microorganism, animal, and plant. Lipases can be produced in high yields from microorganisms such as bacteria and fungi. In practice, microbial lipases are commonly used by the industry. The selection of a lipase for lipid modification is based on the nature of modification sought, for instance, position-specific modification of triacylglycerol, fatty-acids-specific modification, modification by hydrolysis, and modification by synthesis (direct synthesis and transesterification). The literature survey showed the use of lipases from some of the following sources. Microbial lipases are derived from *Aspergillus niger*, *Bacillus thermoleovorans*, *Candida cylindracea*, *Candida rugosa*, *Chromobacterium viscosum*, *Geotrichum candidum*, *Fusarium heterosporum*, *Fusarium oxysporum*, *Humicola lanuginose*, *Mucor miehei*, *Oospora lactis*, *Penicillium cyclopium*, *Penicillium roqueforti*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Rhizopus arrhizus*, *Rhizopus boreas*, *Rhizopus thermosus*, *Rhizopus usamii*, *Rhizopus stolonifer*, *Rhizopus fusiformis*, *Rhizopus circinans*, *Rhizopus delemar*, *Rhizopus chinensis, Rhizopus japonicus* NR400, *Rhizopus microsporus*, *Rhizomucor miehei*, *Rhizopus nigricans*, *Rhizopus niveus*, *Rhizopus oryzae*, *Rhizopus rhizopodiformis*, *Rhizopus stolonifer* NRRL 1478, *Rhodotorula rubra*, and *Staphylococcus hyicus*, to name a few (*40*). Animal sources are from pancreatic lipases, and plant lipases are from papaya latex, oat seed lipase, and castor seed lipase (*40*).

MECHANISM OF LIPASE REACTIONS

Enzymes, including lipases, have a specific, active three-dimensional structure in an aqueous environment with polar groups exposed and nonpolar groups buried inside. Unlike other enzymes, the nature of a lipolytic reaction catalyzed by lipases is very complex, in which the lipid substrates are water-insoluble (*40*). The need for some water to maintain and activate lipase and the immiscibility of lipids in water make the reaction media heterogeneous by forming a liquid–liquid interface. The interface is the point where the lipase can access the substrate and catalyze the reaction. Lipase activity can be easily influenced by the nature of the interface, interfacial properties, and interfacial area. The interface activates the enzyme by adsorption, which aids the opening of the lid on the catalytic site (*40, 69*). All types of interfaces, such as solid–liquid, liquid–liquid, or liquid–gas, can influence the activity due to the interfacial hydrophobicity. An increase in interfacial area increases the amount of enzyme adsorbed onto the interface, and that is why an increase in interfacial area increases the activity of the enzyme in a lipid/water heterogeneous system. Adsorption of the enzyme onto the interface initiates a sequence of events before complete catalysis can be achieved. Adsorption leads to activation and substrate binding followed by catalysis. The accumulation of reaction products on the interface reduces the interfacial pressure, which in turn corresponds to a high surface energy. These effects are undesirable because they exert a denaturing effect on the enzyme molecule, although it is well tolerated by lipase.

LIPASE REACTIONS ARE REVERSIBLE

Both intra- and extracellular lipases are designed to catalyze hydrolytic reactions since the living cells are made up of and surrounded by a water-rich environment. Water plays an important role as a medium to disperse the enzyme molecule and participates as a cosubstrate in hydrolysis (*40*). A reduction in water content may not affect the direction of hydrolysis as

Table 2. Enzymatic of Biodiesel Production by Transesterification of Plant Oils and Fats

oil or fat source	catalyst	acyl acceptor	solvent system	yield (%)	references
Jatropha curcas (jatropha) oil	immobilized *Candida antarctica* lipase B (Novozym-435)	ethyl acetate	solvent-free	91.3%	Modi et al. (*39*)
Pongamia pinnata (karanj) oil				90%	
Helianthus annus (sunflower) oil				92.7%	
soybean oil	recombinant LipB68 (*Pseudomonas fluorescens*)	methanol	*n*-heptane	92%	Luo et al. (*31*)
crude jatropha (*Jatropha curcas*) oil	immobilized *Candida antarctica* lipase B (Novozym-435)	propan-2-ol	hexane	92.8%	Modi et al. (*36*)
karanj (*Pongamia pinnata*) oil				91.7%	
sunflower (*Helianthus annus*) oil				93.4%	
cottonseed oil	*Candida antarctica* lipase	methanol	*t*-butanol	97%	Royon et al. (*38*)
waste-activated bleaching earth	*Candida cylindracea* lipase	methanol	diesel oil	~100%	Kojima et al. (*47*)
soybean oil	*Rhizopus oryzae* lipase	methanol	water-containing system without an organic solvent	80–90 wt %	Kaieda et al. (*43*)
soybean oil	immobilized *Pseudomonas cepacia* lipase (lipase PS)	methanol	solvent-free	67 mol %	Noureddini et al. (*51*)
		ethanol		65 mol %	
renewable oil	immobilized *Candida antarctica* lipase B (Novozym-435)	methyl acetate	solvent system	92%	Xu et al. (*49*)
soybean oil	*Rhizopus miehei* lipase (lipozyme IM-77)	methanol	*n*-hexane	92.2%	Shieh et al. (*74*)
palm kernel oil	*Pseudomonas cepacia* lipase (lipase PS-30)	ethanol	solvent-free	72%	Abigor et al. (*52*)
		t-butanol		62%	
		l-butanol		42%	
		n-propanol		42%	
		iso-propanol		24%	
		methanol		15%	
coconut oil		l-butanol		40%	
		iso-butanol		40%	
		l-propanol		16%	
		ethanol		35%	
		methanol		traces	
cottonseed oil	immobilized *Candida antarctica* lipase B (Novozym-435)	methanol	*t*-butanol	97%	Royon et al. (*38*)
soybean oil	preincubated immobilized *Candida antarctica* lipase B (Novozym-435) by methyl oleate for 0.5 h	methanol	solvent-free	97%	Samukawa et al. (*59*)
rice bran oil	*Cryptococcus* spp. S-2	methanol	aqueous medium	80%	Kamini and Iefuji (*45*)
soybean oil	*Candida antarctica* lipase	methanol	solvent-free	93.8%	Watanabe et al. (*42*)
cottonseed oil	immobilized *Candida antarctica* lipase B (Novozym-435)	methanol	solvent-free	91.5%	Köse et al. (*37*)
palm oil	*Rhizopus oryzae* lipase	methanol	waste-activated bleaching earth	55%	Pizarro and Park (*19*)
rapeseed oil	*Candida rugosa* lipase	2-ethyl-1-hexanol	solvent-free	97%	Linko et al. (*54*)
mowrah, mango, kernel, sal	immobilized *Mucor miehei* lipase (lipozyme IM-20)	C_4–$C_{18:1}$ alcohols	solvent-free	86.8–99.2%	De et al. (*77*)
sunflower oil	immobilized *Mucor miehei* lipase (lypozyme)	ethanol	solvent-free	83%	Selmi and Thomas (*78*)
fish oil	*Candida antarctica* lipase	ethanol	solvent-free	100%	Breivik et al. (*79*)
recycled restaurant grease	*Pseudomonas cepacia* lipase (lipase PS-30) + *Candida antarctica* lipase (lipase SP-435)	ethanol	solvent-free	85.4%	Wu et al. (*29*)
tallow, soybean, rapeseed oil	*Rhizopus miehei* lipase (lipozyme IM-60)	primary alcohols	hexane	94.8–98.5%	Nelson et al. (*35*)
	Candida antarctica lipase (lipase SP-435)	secondary alcohols	hexane	61.2–83.8%	
	Rhizopus miehei lipase (lipozyme IM-60)	methanol	solvent-free	19.4%	
	Rhizopus miehei lipase (lipozyme IM-60)	ethanol	solvent-free	65.5%	
sunflower	*Psedomonase fluorescens* lipase	methanol	solvent-free	3%	Mittelbach (*80*)
		methanol	petroleum ether	79%	
		ethanol	solvent-free	82%	

long as the water activity, a_w, is maintained at 1.0. A reduction in water activity below 1.0 affects the equilibrium constant of the system, and the direction of hydrolysis is changed to synthesis, in which water molecules will be produced to shift the system towards higher water activity. The application of water-immiscible solvents such as *n*-hexane in lipid modification serves two purposes, namely, (1) the ability to control the water content and therefore the water activity and (2) the possibility to modify high-temperature-melting lipids at low temperatures by solubilizing them. Other advantages of using water-immiscible solvents in lipid modification include (a) easy process control in large-scale production by reducing the viscosity of the oil, (b) keeping the enzyme in an insoluble form, (c) ease of enzyme recovery and reuse, (d) easy recovery of the products, and (e) increased enzyme stability due to a low water content and hence increased productivity and low cost of the final product (*40*). Biodiesel can be enzymatically produced in organic solvent or in no solvent.

CONDITIONS THAT AFFECT LIPASE-CATALYZED PRODUCTION OF BIODIESEL

The yield of biodiesel products through lipase catalysis is modulated by the substrate ratio (alcohol/oil), alcohol type, temperature of the reaction, water content, purity of the triacylglycerol, and enzymes' and whole cells' immobilizations. High concentrations or the addition of the required ratio of methanol/oil all at once have an inhibitory effect on certain lipases. A three-step or stepwise addition of methanol was proposed (*41*) and reviewed by Shimada et al. (*7*) in an attempt to increase the yield of biodiesel beyond the theoretical 66%. With this method, waste oil was converted to biodiesel at greater than 90% yield, and the lipase remained active for several days (more than three months) without an adverse loss of activity. Used frying oils and crude and waste oils from the refinery contain free fatty acids, phospholipids, and other impurities that may inactivate lipase during the transesterification reaction. Thus, the quality of the source oil or fat is important for efficient conversion to biodiesel. Watanabe et al. (*42*) found that nondegummed soybean oil containing phospholipids did not support catalysis by *Candida antarctica* lipase, but the degummed soybean oil was converted to biodiesel by lipase catalysis in 93.8% yield. A three-step methanolysis procedure was used, and the lipase retained activity after 25 times of reuse. **Table 2** shows the yield and the acyl acceptors reported for lipase-catalyzed transesterification to produce biodiesel fuel. Kaieda et al. (*17*) reported the effect of water and methanol on biodiesel production. In their report, *Candida rugosa*, *Pseudomonas cepacia*, and *Pseudomonas fluorescens* lipases were able to catalyze the conversion of soybean oil to biodiesel in the presence of high water content (up to 20%), indicating that a suitable amount of water may alleviate lipase inactivation by methanol. The reactions were carried out at 35 °C with 5% lipase. In particular, it was noted that *P. cepacia* exhibited methanol tolerance even at 2–3 molar equivalents of methanol/soybean oil. This type of lipase will find use in future biodiesel production and needs to be explored. A solvent-free system in which *Rhizopus oryzae* lipase catalyzed the methanolysis of soybean oil in the presence of 4–30 wt % water was reported in 80–90 wt % yield of FAME (*43*). A step-wise addition of methanol increased the FAME yield due to acyl migration, even though the enzyme is 1,3-specific. A kinetic model for the enzymatic methanolysis of vegetable oils for biodiesel has been proposed (*44*). This model is applicable to both continuous and batch enzymatic processes. Pizarro and Park (*19*) extracted vegetable oils from waste-bleaching-earth-containing oils and used that for *Rhizopus oryzae* lipase catalyzed production of biodiesel in the presence of a high water content. Methanol was added in one step, and the lipase was not inactivated by the methanol, indicating protection of the lipase by the high water content. Kamini and Iefuji (*45*) also reported that a crude lipase (S-2) from the yeast *Cryptococcus* spp. was able to tolerate up to 80% by weight water in the methanolysis of various vegetable oils to yield 80.2% FAME in 120 h. The molar ratio of methanol/oil was 4:1, and the reaction was performed at 30 °C. The researchers also demonstrated that it is possible to use low-value oils such as waste oils from a crude oil refinery as suitable renewable raw material for biodiesel production. Waste plant oil and *Candida rugosa* lipase were added to methanol, organic solvent, and activated bleaching earth (ABE) and used to synthesize biodiesel from rapeseed oil present in waste ABE. The addition of a 0.7 ratio of ABE/ABE + rapeseed oil increased the FAME yield 9-fold without methanol inactivation of the lipase, because methanol was adsorbed onto the ABE (*20*). These researchers (*21*) also performed similar experiments in a batch solvent-free system and were able to reuse the enzyme nine times without a significant loss of lipase activity. Chang et al. (*46*) used a recombinant *Staphylococcus epidermidis* lipase to catalyze fatty acid ester synthesis in an aqueous environment, and hence it can be used for biodiesel production in aqueous solutions. Kojima et al. (*47*) also reported a successful conversion of waste ABE oil extracts to FAME while using diesel oil as the organic solvent. *Candida rugosa* lipase (10%) was stable in the diesel solvent and resulted in a 100% yield of FAME without the need to remove the solvent from the desired biodiesel product. *Candida rugosa* has five isoforms of lipase which have been recently cloned and overexpressed in *Pichia pastoris* (*48*) and patented by our group. We have recently discovered that the recombinant *C. rugosa* lipase2 (lip2), among the isoforms tested, is very effective for biodiesel production (FAME) from soybean oil at a 93% yield (unpublished results).

To overcome the methanol inhibitory problem, different acyl acceptors such as ethyl and methyl acetates or even their anhydrides may be valuable. In one such study by Du et al. (*22*), methyl acetate, a novel acyl acceptor, was used to produce biodiesel from soybean oil, catalyzed by Novozym 435, in a 92% yield. The molar ratio of methyl acetate/soybean oil was 12:1, and the temperature of the reaction was 40 °C. Xu et al. (*49*) also used methyl acetate as an acyl acceptor and *Candida antarctica* lipase (30% by weight) to produce 92% methyl ester in 10 h at the same 12:1 molar ratio of substrates and the same temperature. The major advantages of this interesterification or ester interchange reaction over the alcoholysis reaction were that no glycerol (downstream separation problem for chemical catalysis) was formed; the method is suitable for both crude and refined, bleached, and deodorized (RBD) oils; and the operational stability of the lipase was great and could be reused up to 100 batches without a loss of activity. The byproduct of the interesterification was triacetylglycerol (triacetin), which has a higher value than glycerol and could help reduce the cost of biodiesel production (*22*). Modi et al. (*39*) used ethyl acetate for biodiesel production from crude oils of *Jatropha curcas* (jatropha), *Pongamia pinnata* (karanj), and *Helianthus annus* (sunflower) by immobilized Novozym 435-catalyzed interesterification to yield 91.3, 90, and 92.7% FAEE, respectively (**Table 2**). A molar ratio of 11:1 ethyl acetate/oil, a temperature of 50 °C, and 12 h of reaction time were employed. With ethyl acetate as an acyl acceptor, the reaction was repeated 12 times without a loss of significant enzyme activity, whereas with

ethanol as the acceptor, the enzyme activity was completely lost after six cycles. FAEEs were separated from the reaction products with column chromatography, using a column packed with 30 g of 60–120 mesh silica gel by eluting with 600 mL of hexane/diethyl ether, 99.5:0.5, v/v (*39*). The use of a two carbon acyl acceptor such as ethanol or ethyl acetate will lead to a biodiesel with a higher cetane number, lower pour and cloud points, higher flash and combustion points which will improve cold starts, and lower smoke opacity and exhaust temperatures (*39*). A study of the kinetics of the interesterification first suggested a ping-pong Bi–Bi with a substrate inhibition mechanism, but further examination revealed three consecutive and reversible reactions, with the first-step reaction being the rate-limiting step (*50*). Intermediates such as diacyl monoacetylglycerol and monoacyl diacetylglycerol were detected in the reaction.

A novel organic solvent, *tert*-butanol (*t*-butanol), was used in the presence of methanol to catalyze the formation of biodiesel from rapeseed oil (*26, 38*). Also, a combination of two lipases (3% lipozyme TL IM, 1,3-specific, and 1% Novozym 435, nonspecific) was employed in the reaction described by Li et al. (*26*). A volume ratio of *t*-butanol/rapeseed oil of 1:1 and a molar ratio of methanol/rapeseed oil of 4:1 were used to perform the reaction at 35 °C for 12 h to yield 95% biodiesel. No lipase activity loss was observed even after 100 d, representing 200 cycles of use, and the process was suitable for both waste (70% FFA content) and RBD (1.8% FFA) oils. The presence of molecular sieves in the dehydrated waste oil resulted in an over 90% yield of biodiesel. Royon et al. (*38*) reported a batch methanolysis yield of 97% FAME after 24 h at 50 °C with a reaction mixture that contained 32.5% *t*-butanol, 13.5% methanol, 54% cottonseed oil, and 0.017 g/oil and *Candida antarctica* as the biocatalyst. With a continuous fixed-bed bioreactor system at a substrate flow rate of 9.6 mL/h/g of enzyme, a 95% yield of FAME was obtained. The bioreactor was used over 500 h without a significant decrease in ester yield. It was hypothesized that the *t*-butanol acted as a solvent to solubilize both methanol and the glycerol and, therefore, was not a substrate for the lipases, which hardly utilizes tertiary alcohols. Modi et al. (*36*) used propan-2-ol, a branched-chain alcohol, as an acyl acceptor for biodiesel production from crude oils of *Jatropha curcas* (jatropha), *Pongamia pinnata* (karanj), and *Helianthus annus* (sunflower) by an immobilized Novozym 435-catalyzed alcoholysis reaction. The authors used 10% by oil weight of the enzyme, an alcohol/oil ratio of 4:1, and reacted the mixture at 50 °C for 8 h to obtain 92.8, 91.7, and 93.4% fatty acid propyl ester yields, respectively, from the above crude oils. Enzyme requirements for alcohols, and their amounts, as acyl acceptor in the alcoholysis reaction may vary. Novozym 535 and Lipozyme TL IM were found to prefer ethanol and methanol, respectively (*34*). Novozym 435 was preferred because it gave a higher conversion and retained 85% of its activity after being reused nine times in a solvent-free batch mode. Methanol and ethanol were used for the alcoholysis of soybean oil by *Pseudomonas cepacia* lipase immobilized in a sol–gel support (*51*). Methyl and ethyl esters were produced in 67 and 65 mol % yields, respectively, using 7.5:1 methanol/oil and 15.2:1 ethanol/oil ratios at 35 °C in the presence of 0.3–0.5 g of water. The immobilized lipase performed consistently better than the free lipase. Abigor et al. (*52*) used PS-30 lipase from Amano Enzyme (Lombard, IL) to prepare biodiesel from Nigerian lauric oils such as coconut and palm kernel oils by alcoholysis with various alcohols. Ethanol gave the highest yield of 72% ethyl ester followed by *t*-butanol at 40 °C in 8 h. Kose et al. (*37*) converted cottonseed oil to biodiesel with both short-chain primary and secondary alcohols, with isoamyl alcohol giving the best yield (94%). The condition for the *Candida antarctica* lipase catalyzed alcoholysis was 30% immobilized lipase, a 4:1 alcohol/oil ratio, and incubation at 50 °C for 7 h. Triolein was reacted with butanol under solvent-free conditions to produce oleyl alkyl esters (butyl oleate) as biodiesel (*53*). The yield of the alkyl ester reached 100% in 6 h when *Pseudomonas cepacia* lipase was used at 40 °C at a butanol/triolein ratio of 3:1. Part of the reason for complete alcoholysis reaction may be because of the reactivity of the *P. cepacia* lipase or the high purity of the triolein (99.8%) substrate used compared to those of vegetable oils and used oils, which contain other minor components and impurities. In a related work on biodiesel performance, Linko et al. (*54*) synthesized 1-butyl oleate, using various lipases, for use as an additive in biodiesel to improve engine performance at cold winter temperatures by decreasing the viscosity of the biodiesel. It should be noted that, in cold weather, the properties of biodiesel change, and this is based on the level of saturation of the fatty acids. Such biodiesel shows a high cloud point (i.e., the temperature at which fuel becomes cloudy due to solidification), and therefore clogs the fuel lines, and a high pour point (temperature at which fuel stops flowing). Both CP and PP depend on the saturated and unsaturated fatty acid esters that make up the biodiesel mixture (*53*). **Table 1** shows the fatty acid composition of biodiesel raw materials, especially the total saturated and unsaturated fatty acids. Tallow (53.5%) and palm oil (47%) contain the highest amounts of saturated fatty acids and, thus, may not be suitable for winter/cold weather biodiesel production without blending or an additive. Oils with the highest oleic acid contents, such as hazelnut kernel (61.4%), olive oil (74.2%), and almond kernel (70.7%), may be the most suitable for winter biodiesel production. In terms of total unsaturation, rapeseed (94.9%), crambe (93.3%), and linseed oil (93.0%) contain the highest. But linseed oil contains 55% 18:3 and crambe 58.5% 22:1, and these may have stability (linseed) and substrate utilization (crambe) problems with lipase catalysts. Therefore, different lipases must be used, because of their fatty acid chain-length specificity, for different biodiesel production. Recombinant DNA technology may play a role in re-engineering the lipases to take new substrates and efficiently convert them to biodiesel for cold and warm weather use. To prevent crystallization problems during winter, fuel additives such as butyl oleate (*54*) are added or branched-chain alcohols (such as isoamyl alcohol) are used as the acyl acceptor to decrease the solidification point, CP, and PP of the biodiesel.

Lipase-catalyzed reactions in supercritical carbon dioxide as the solvent have been reported (*55, 56*) and may prove useful for biodiesel production in terms of savings in downstream processing costs. This is because product purification is not necessary. Noncatalytic and catalytic supercritical methanolysis of vegetable oils to produce biodiesel have been reviewed by Demirbas (*8*).

FUTURE PROSPECTS FOR LIPASE-CATALYZED PRODUCTION OF BIODIESEL

The potential for using lipases as biocatalysts for biodiesel are enormous. Advantages of using lipase include ease of product recovery; low energy and temperature requirements; ease of enzyme recovery; mild reaction conditions of pH, temperature, and pressure; regeneration and reuse of the enzyme several times; use of reactors for continuous production; thermal stability at a relatively low temperature of operation; operational

stability of the enzyme; flexibility of accepting various substrates and alcohols; and reaction in solvent and solvent-free systems; lipases allow reactions in systems that contain acceptable levels of water and can esterify free fatty acids present in the crude, waste oils, and used frying oils as well as deodorizer distillates; immobilization allows reuse, confers stability, and allows higher enzyme loading for faster reaction; enzymes are natural, and recombinant lipases with enhanced or altered activities can be mass-produced after overexpression in relevant microorganisms, therefore making the overall process economically viable. Both 1,3-specific and nonspecific lipases can catalyze biodiesel production in good yields (**Table 2**). The 1,3-specific lipases are purported to increase reaction yield via acyl migration of the fatty acids to the other positions of the glycerol. This acyl migration was not caused by the lipase itself, but supposedly by the silica gel used in the immobilization process (*25*). Some disadvantages include high cost (at the moment; it could change, as described below), inactivation by acyl acceptors such as methanol, inactivation by minor components in the crude oil and waste oils, desorption from immobilization support, and fouling in packed bed bioreactors.

Ways to reduce the cost of the enzyme process may involve immobilization of whole cells and pretreatment of extracellular lipases to be more tolerant to short-chain alcohols such as methanol. Chen and Wu (*57*) pretreated immobilized *Candida antarctica* lipase by immersion in 2-butanol or *t*-butanol, and the activity of the nearly deactivated immobilized enzyme was regenerated. Indeed, the activity of pretreated Novozym 435 increased 10-fold compared to the nonpretreated enzyme. The enzyme, after being completely deactivated by methanol, was washed with 2-butanol and *t*-butanol to restore its activity to 56 and 75% of the original, respectively (*57*). Therefore, washing or pretreatment of lipases with 2-butanol or *t*-butanol is one way to improve the activity of used lipase for biodiesel production at a reduced cost. Preincubation of immobilized *Candida antarctica* lipase in methyl oleate for 0.5 h and in soybean oil for 12 h resulted in a FAME yield of 97% from soybean oil in 3.5 h with the stepwise addition of a 0.33 molar equivalent of methanol at 0.25–0.4 h intervals. Other reports on enzyme pretreatment to improve yield or activity of the lipase are available (*58, 59*).

LIPASE ENGINEERING

Lipases are fatty-acid chain-length-specific, substrate-specific, and regio- and enantioselective, and therefore biodiesel production will require different types of lipases and substrates from the various available renewable resources. Those lipases which are found suitable for biodiesel production can be produced in large quantities, while those that are not so selective and active can be engineered to improve their biocatalytic activity for biodiesel production through the means described below.

The use of recombinant DNA technology (genetic engineering) to produce large quantities of recombinant lipases will help lower the enzyme cost, and hence the economic hurdle that previously made the enzymatic approach to biodiesel production unattractive. Genetic engineering can be used to improve thermostability, fatty acid chain length specificity, substrate specificity, alcohol chain length specificity, methanol and ethanol tolerance, pH stability, and productivity for use in biodiesel production. We have engineered and produced an isoform/isoenzyme of *Candida rugosa* lipase (CRL) named lip2 (*60*), which may be useful in biodiesel production. Lip2 showed high activity toward long-chain alcohols in the esterification of myristic acid and possesses unique and remarkable catalytic properties different from the crude CRL and lip4. The native lip2 preferred the hydrolysis of long-chain triolein, while the recombinant lip2 showed preference for the hydrolysis of short-chain TAG. Thus, the chain-length preference was different for the native and recombinant lipases, and this may be attributed to the glycosylation, the additional N-terminal peptide, or amino acid substitutions between the recombinant and native lip2 (*60*). Recombinant lip2 was overexpressed in *Pichia pastoris* (*48*). Site-directed mutagenesis is very useful for the production of pure lipase isoforms of *C. rugosa* and will continue to provide insight into lipase catalysis and specificity (*60*). By applying gene shuffling (directed evolution) or rational design protein engineering (see reviews in refs *60 and 61*), the catalytic efficiency of lipases can be improved. The crystal structures of most lipases have been solved, allowing enzyme biotechnologists and biochemists to apply rational engineering to design new lipases with new functions or to improve the properties of existing lipases. Manipulating culture conditions and the use of recombinant DNA technology are possible means of obtaining novel lipases with unique functions and different isoforms of lipases such as CRL isoforms (*60*). Conventional overexpression systems such as *E. coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae* are frequently used to overexpress genes of interest (*61*). Lipase activities can be optimized for biotechnological processes by directed evolution (*62, 63*). *Rhizopus oryzae* lipase was overexpressed in *Saccharomyces cerevisiae*, and after drying, the whole cell was used to catalyze a solvent-free methanolysis reaction to produce biodiesel (*64*).

The use of whole cells immobilized in biomass support (*65*) requiring no expensive purification as with free or immobilized enzymes and the designing of lipases that will tolerate methanol through genetic engineering will undoubtedly lower the cost of biodiesel production on an industrial scale (*2*).

Some researches have shown that microwave treatment (*66*) and nanotechnology may indeed improve enzyme activity. Parker et al. (*66*) microwave-irradiated the hydrated cutinase enzyme at 50 °C and used it in the presence of organic solvent to catalyze and enhance (2–3-fold over conventional heating) cutinase activity, which resulted in an increased yield of butyl butyrate from the substrates (butanol and ethyl butyrate). The effect of microwave irradiation on the enzyme was considered nonthermal. The operational and thermal stability (*67*) of the lipase and the ability to reuse the enzyme several times are very important in the future commercialization of enzymatic biodiesel production. Of course, enzyme immobilization is known to improve its stability and makes reuse possible (*68–71*). Shimada et al. (*7*) demonstrated that immobilized *Candida antarctica* lipase could be used for up to 100 d for biodiesel synthesis through alcoholysis reaction without a significant loss of activity.

RESPONSE SURFACE METHODOLOGY (RSM)

RSM is a powerful statistical experimental design used to optimize reaction processes with a minimal number of experiments while avoiding a one-variable-at-a-time approach. This process may be found useful in optimizing the reaction conditions that affect biodiesel production to increase yield and for possible industrial adaptation. It is applicable to both chemical and enzymatic catalysis. RSM has been used in both biodiesel production and other applications (*29, 72, 73, 81, 82*). Shieh et al. (*74*) used *Rhizomucor lipase* (Lipozyme IM-77) to catalyze the alcoholysis of soybean oil with methanol to obtain a 92 wt % conversion to biodiesel in 6.3 h at 37 °C using a methanol/soybean oil molar ratio of 3.4:1 in the presence of

5.8% added water. RSM was also used to optimize the use of immobilized whole cells of *Rhizopus oryzae*, IFO4697, to catalyze the methanolysis of soybean oil to biodiesel in 72% yield (*73*). The authors added *t*-butanol to stabilize the lipase and to prevent methanol inactivation of the intracellular lipase which was used in 10 batches without a significant loss of activity. The cells were immobilized in biomass support particles. The methanol/soybean molar ratio was 5.2, and the system contained 3.1% water and 12% dry biomass of whole cells.

CONCLUSIONS

Biodiesel production can be done through the use of acid-, base-, supercritical carbon dioxide (*8*), or lipase-catalyzed reactions. Alcoholysis (exchange of alkyl alcohol with glycerol from triacylglycerol) is the preferred mechanism for converting renewable fats and oils to biodiesel as fatty acid alkyl esters. Fatty acid methyl esters are commercially produced using methanol, vegetable oil such as soybean oil, and sodium hydroxide as the chemical catalyst. There is a current interest in using immobilized lipases or immobilized whole cells as "green" alternatives to chemical catalysts to produce biodiesel industrially. However, the cost of the final enzymatic product remains a hurdle compared to the cheaper alternative of using chemical catalysis. Using the tools of recombinant DNA technology, it is possible to increase the supply of suitable lipases for biodiesel production. Protein engineering and site-directed mutagenesis may be used to alter the enzyme–substrate specificity, stereospecificity, and thermostability, or to increase their catalytic efficiency, which will benefit biodiesel production, and lower the cost of the overall process. Indeed, a thermostable and short-chain alcohol-tolerant lipase suitable for biodiesel production was recently cloned (*83*), and more research efforts should be focused in modifying and cloning more lipases. Lipases can be immobilized to increase their operational stability, making reuse several times possible, and the alcoholysis process economically feasible and competitive with conventional processes.

Genetic engineering can also be used to engineer new crops and improve the oil levels in existing crops, to provide sufficient renewable raw materials for biodiesel production. Specific crops and arable land may be dedicated to biodiesel production while others will be for feed and food to avoid artificial scarcity of each.

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