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REVIEW

Biodiesel Fuel Production by Transesterification of Oils

HIDEKI FUKUDA,[1]* AKIHIKO KONDO,[2] AND HIDEO NODA[3]

Division of Molecular Science, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan,[1] Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan,[2] and Kansai Chemical Engineering Co. Ltd., 9-7-2 Minaminanamatsu, Amagasaki 660-0053, Japan[3]

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Biodiesel (fatty acid methyl esters), which is derived from triglycerides by transesterification with methanol, has attracted considerable attention during the past decade as a renewable, biodegradable, and nontoxic fuel. Several processes for biodiesel fuel production have been developed, among which transesterification using alkali-catalysis gives high levels of conversion of triglycerides to their corresponding methyl esters in short reaction times. This process has therefore been widely utilized for biodiesel fuel production in a number of countries. Recently, enzymatic transesterification using lipase has become more attractive for biodiesel fuel production, since the glycerol produced as a by-product can easily be recovered and the purification of fatty methyl esters is simple to accomplish. The main hurdle to the commercialization of this system is the cost of lipase production. As a means of reducing the cost, the use of whole cell biocatalysts immobilized within biomass support particles is significantly advantageous since immobilization can be achieved spontaneously during batch cultivation, and in addition, no purification is necessary. The lipase production cost can be further lowered using genetic engineering technology, such as by developing lipases with high levels of expression and/or stability towards methanol. Hence, whole cell biocatalysts appear to have great potential for industrial application.

[**Key words:** biodiesel fuel, transesterification, lipase, whole cell biocatalyst, supercritical fluid]

Alternative fuels for diesel engines are becoming increasingly important due to diminishing petroleum reserves and the environmental consequences of exhaust gases from petroleum-fuelled engines. A number of studies have shown that triglycerides hold promise as alternative diesel engine fuels (1–8). However, the direct use of vegetable oils and/or oil blends is generally considered to be unsatisfactory and impractical for both direct-injection and indirect-type diesel engines. The high viscosity, acid composition, and free fatty acid content of such oils, as well as gum formation due to oxidation and polymerization during storage and combustion, carbon deposits, and lubricating oil thickening are some of the more obvious problems (9, 10). Consequently, considerable effort has gone into developing vegetable oil derivatives that approximate the properties and performance of hydrocarbon-based diesel fuels. Problems encountered in substituting triglycerides for diesel fuels are mostly associated with their high viscosity, low volatity, and polyunsaturated character (10). Three main processes have been investigated in attempts to overcome these drawbacks and allow vegetable oils and oil waste to be utilized as a viable alternative fuel: pyrolysis, micro-emulsification, and transesterification.

* Corresponding author. e-mail: fukuda@cx.kobe-u.ac.jp
phone: +81-(0)78-803-6192 fax: +81-(0)78-803-6206

Pyrolysis refers to chemical change caused by the application of thermal energy in the presence of an air or nitrogen sparge. Many investigators have studied the pyrolysis of triglycerides with the aim of obtaining products suitable for diesel engines (11–14). Thermal decomposition of triglycerides produces compounds of several classes, including alkanes, alkenes, alkadienes, aromatics, and carboxylic acids. Different types of vegetable oils reveal large differences in composition when they are thermally decomposed. Pyrolyzed soybean oil, for instance, contains 79% carbon and 12% hydrogen (12). It also has low viscosity and a high cetane number compared to pure vegetable oils. However, while pyrolyzed vegetable oils possess acceptable amounts of sulphur, water, and sediment, as well as giving acceptable copper corrosion values, they are unacceptable in terms of ash, carbon residues, and pour point. In addition, though the products are chemically similar to petroleum-derived gasoline and diesel fuel, the removal of oxygen during thermal processing also eliminates any environmental benefits of using an oxygenated fuel (9).

The use of microemulsions with solvents such as methanol, ethanol, and 1-butanol has also been studied as a means of solving the problem of high viscosity of vegetable oils (15–17). Microemulsions are isotropic, clear or translucent thermodynamically stable dispersions of oil, water, a surfactant, and often a small amphiphilic molecule, called a cosur-

factant (15). Ziejewski *et al*. (17) prepared an emulsion of 53.3% (v/v) alkali-refined and winterized sunflower oil, 13.3% (v/v) 190-proof ethanol and 33.4% (v/v) 1-butanol. This nonionic emulsion had a viscosity of 6.31×10^{-6} m^2/s at 40°C, a cetane number of 25, a sulfur content of 0.01%, free fatty acids of 0.01%, and an ash content of less than 0.01%. Lower viscosities and better spray patterns were obtained by increasing the amount of 1-butanol. Schwab *et al*. (15) reported that 2-octanol was an effective amphiphile in the micellar solubilization of methanol in triolein and soybean oil. However, in a laboratory screening endurance test, irregular injector needle sticking, heavy carbon deposits, incomplete combustion and an increase of lubricating oil viscosity were reported (17).

Transesterification, also called alcoholysis, is the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis, except that an alcohol is employed instead of water. Suitable alcohols include methanol, ethanol, propanol, butanol, and amyl alcohol. Methanol and ethanol are utilized most frequently, especially methanol because of its low cost and its physical and chemical advantages. This process has been widely used to reduce the viscosity of triglycerides, thereby enhancing the physical properties of renewable fuels to improve engine performance (18). Thus, fatty acid methyl esters (known as biodiesel fuel) obtained by transesterification can be used as an alternative fuel for diesel engines.

The properties of biodiesel and diesel fuels are compared in Table 1 (10, 19, 20). Biodiesel fuels produced from various vegetable oils have viscosities close to those of diesel. Their volumetric heating values are a little lower, but they have high cetane numbers and flash points. Since the characteristics of biodiesel are generally similar to those of diesel, the former is a strong candidate to replace diesel if the need arises.

Among the attractive features of biodiesel fuel are (i) it is plant-, not petroleum-derived, and as such its combustion does not increase current net atmospheric levels of CO_2, a "greenhouse" gas; (ii) it can be domestically produced, offering the possibility of reducing petroleum imports; (iii) it is biodegradable; and (iv) relative to conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, and, under some conditions, nitrogen oxides. It is well established that biodiesel affords a substantial reduction in SOx emissions and considerable reductions in CO, hydrocarbons, soot, and particulate matter (PM). There is a slight increase in NOx emissions, which can be positively influenced by delaying the injection timing in engines (19–25). Yamane *et al*. (19) recently reported that a biodiesel fuel with good ignitability, such as one with a high methyl oleate content, gives lower levels of NO, hydrocarbons, HCHO, CH_3CHO, and HCOOH, and also that soot formation is suppressed, since biodiesel is an oxygenated fuel having an O_2 mass fraction of 10%. In addition, Sheehan *et al*. (22) carried out life cycle analyses and found that the benefit of using biodiesel is proportionate to the level of blending with petroleum diesel. The overall life cycle emissions of CO_2 from 100% biodiesel fuel are 78.45% lower than those of petroleum diesel, and a blend with 20% biodiesel fuel reduces net CO_2 emissions by 15.66%. Substituting 100% biodiesel for petroleum diesel in buses reduces the life cycle consumption of petroleum by 95%, while a 20% blend of biodiesel fuel causes the life cycle consumption of petroleum to drop 19%.

As a consequence of its advantages, there is considerable interest in exploring and developing the use of biodiesel fuel. This paper reviews technologies relating to biodiesel fuel production by transesterification using acid- and alkali-catalysis, lipase enzyme, and supercritical fluid, focusing particularly on enzymatic production from the viewpoint of industrial application.

TRANSESTERIFICATION KINETICS FOR CHEMICAL AND ENZYMATIC CATALYSTS

Chemical catalyst The transesterification reaction with alcohol represented by the general equation shown in Fig. 1a consists of a number of consecutive, reversible reactions as shown in Fig.1b. The first step is the conversion of triglycerides to diglycerides, which is followed by the conversion of diglycerides to monoglycerides and of monoglycerides to glycerol, yielding one methyl ester molecule from

TABLE 1. Physical and chemical properties of biodiesel

Vegetable oil methyl ester	Kinematic viscosity (mm^2/s)	Cetane number	Lower heating value (MJ/*l*)	Cloud point (°C)	Flash point (°C)	Density (g/*l*)	Sulfur, (wt%)
Peanut[a]	4.9 (37.8°C)	54	33.6	5	176	0.883	—
Soybean[a]	4.5 (37.8°C)	45	33.5	1	178	0.885	—
Soybean[b]	4.0 (40°C)	45.7–56	32.7	—	—	0.880 (15°C)	—
Babassu[a]	3.6 (37.8°C)	63	31.8	4	127	0.879	—
Palm[a]	5.7 (37.8°C)	62	33.5	13	164	0.880	—
Palm[b]	4.3–4.5 (40°C)	64.3–70	32.4	—	—	0.872–0.877 (15°C)	
Sunflower[a]	4.6 (37.8°C)	49	33.5	1	183	0.860	—
Tallow[a]	—	—	—	12	96	—	—
Rapeseed[b]	4.2 (40°C)	51–59.7	32.8	—	—	0.882 (15°C)	—
Used rapeseed[c]	9.48 (30°C)	53	36.7	—	192	0.895	0.002
Used corn oil[c]	6.23 (30°C)	63.9	42.3	—	166	0.884	0.0013
Diesel fuel[b]	12–3.5 (40°C)	51	35.5	—	—	0.830–0.840 (15°C)	—
JIS-2D[c] (Gas oil)	2.8 (30°C)	58	42.7	—	59	0.833	0.05

[a] Ref. 10.
[b] Ref. 20.
[c] Ref. 19.

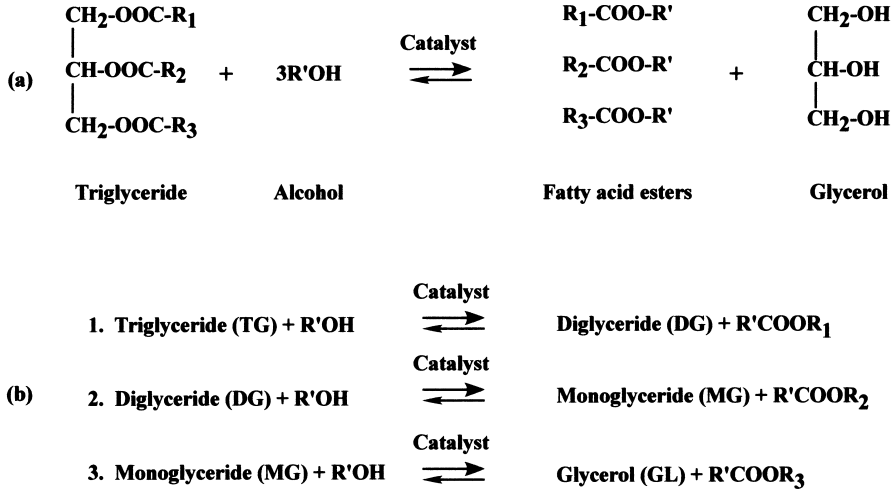

FIG. 1. Transesterification of triglyceride with alcohol. (a) General equation; (b) three consecutive and reversible reactions. R_1, R_2, R_3 and R′ represent alkyl groups.

each glyceride at each step (26, 27).

Several researchers have reported the kinetics for both acid- (26–29) and alkali-catalyzed (26, 27) transesterification reactions. Dufek *et al*. (28) studied the acid-catalyzed esterification and transesterification of 9(10)-carboxystearic acid and its mono- and di-methyl esters. Freedam *et al*. (26) reported the transesterification reaction of soybean oil and other vegetable oils with alcohols, examining the effects of the type of alcohol, molar ratio, type and amount of catalyst, and reaction temperature on rate constants and reaction order. With acid or alkali catalysis, the forward reaction followed pseudo-first-order kinetics for butanol : soybean oil = 30 : 1. However, with alkali catalysis the forward reaction followed consecutive, second-order kinetics for butanol : soybean oil = 6 : 1. The reaction of methanol with soybean oil at a 6:1 molar ratio with 0.5% sodium methoxide was a combination of second-order consecutive and fourth-order shunt reactions.

Enzymatic catalyst The kinetics of triglyceride transesterification with methanol, *i.e* methanolysis, catalyzed by *Ryzopus oryzae* lipase appears to be in accordance with a successive reaction mechanism (30). That is, triglycerides and partial glycerides are first hydrolyzed by lipase to partial glycerides and free fatty acids, respectively, after which methyl esters are synthesized from free fatty acids and methanol (see Fig. 1b). This suggests that, unlike in the case of alkali-catalyzed methanolysis, free fatty acids contained in used oils can be easily converted to methyl esters.

ACID-CATALYZED OR *IN SITU* TRANSESTERIFICATION

Acids used for transesterification include sulfuric, phosphoric, hydrochloric, and organic sulfonic acids. Although transesterification by acid catalysis is much slower than that by alkali catalysis (9, 10, 31), acid-catalyzed transesterification is more suitable for glycerides that have relatively high free fatty acid contents and more water (31, 32). Aksoy *et al*. (32) reported that it was necessary to perform transesterification under an acidic condition when the oil component was a low grade material such as sulphur olive oil. In general, the ethyl esters of monounsaturated or short-chain fatty acids with 2% sulfuric acid should make good alternative fuels (33).

In situ transesterification differs from the conventional reaction in that the oil-bearing material contacts acidified alcohol directly instead of reacting with purified oil and alcohol. That is, extraction and transesterification proceed within the same process, the alcohol acting both as an extraction solvent and an esterification reagent. *In situ* transesterification of sunflower oil with acidified methanol produces fatty acid methyl esters in yields significantly greater than those obtained from the conventional reaction with pre-extracted seed oil (34, 35). Kildiran *et al*. (36) have proposed *in situ* transesterification of soybean oil, while Özgül and Türkay (37) reported the *in situ* esterification of rice bran oil with different monohydric alcohols, exploiting the advantage of simultaneous easy extraction of neutral lipids from seeds and bran when *in situ* transesterification is carried out.

ALKALI-CATALYZED TRANSESTERIFICATION

Alkalis used for transesterification include NaOH, KOH, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. Alkali-catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst (38), and is thus most often used commercially.

Effects of moisture and free fatty acids For alkali-catalyzed transesterification, the glycerides and alcohol must be substantially anhydrous because water causes a partial reaction change to saponification, which produces soap (39). The soap consumes the catalyst and reduces the catalytic efficiency, as well as causing an increase in viscosity, the formation of gels, and difficulty in achieving separation of glycerol. Ma *et al*. (40) suggested that the free fatty acid content of the refined oil should be as low as possible, below 0.5%, and Feuge and Grose (41) also stressed

the importance of oils being dry and free of free fatty acids. Freedman *et al*. (31) reported that ester yields were significantly reduced if the reactants did not meet these requirements; sodium hydroxide or sodium methoxide reacted with moisture and carbon dioxide in the air, diminishing their effectiveness.

Effect of molar ratio Another important variable affecting the ester yield is the molar ratio of alcohol to vegetable oil. The stoichiometry of the transesterification reaction requires 3 mol of alcohol per mole of triglyceride to yield 3 mol of fatty esters and 1 mol of glycerol (see Fig. 1a). Higher molar ratios result in greater ester conversion in a shorter time. In the transesterification of peanut oil with ethanol, a 6:1 molar ratio liberated significantly more glycerol than a ratio of 3:1 (41). Freedman *et al*. (31) studied the effect of molar ratios (from 1:1 to 6:1) on ester conversion with vegetable oils. Soybean, sunflower, peanut and cotton seed oils behaved similarly, with the highest conversion being achieved at a 6:1 molar ratio. Krisnangkura and Simamaharnnop (42) transesterified palm oil at 70°C in an organic solvent with sodium methoxide as a catalyst and found that the conversion increased with increasing molar ratios of methanol to palm oil. Thus, a molar ration of 6:1 is normally used in industrial processes to obtain methyl ester yields higher than 98% on a weight basis (41, 43).

Effect of catalyst type Sodium methoxide has been found to be more effective than sodium hydroxide, presumably because a small amount of water is produced upon mixing NaOH and MeOH (31, 44). Alcantara *et al*. (45) transformed three fatty materials — bean oil, used frying oil, and tallow — with sodium methoxide into two different types of products by transesterification and amidation reactions with methanol and diethylamine, respectively. Amides enhance the ignition properties of petrochemical diesel fuel. However, sodium hydroxide and potassium hydroxide (46) are also able to catalyze transesterification, and because of their cheapness, are widely used in industrial biodiesel production.

ENZYMATIC TRANSESTERIFICATION BY LIPASE

Although chemical transesterification using an alkali-catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction.

Both extracellular and intracellular lipases are also able to effectively catalyze the transesterification of triglycerides in either aqueous or nonaqueous systems, and as shown in Table 2, enzymatic tranesterification methods can overcome the problems mentioned above. In particular, it should be noted that the by-product, glycerol, can be easily recovered without any complex process, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters. On the other hand, in general the production cost of a lipase catalyst is significantly greater than that of an alkaline one.

Use of extracellular lipases Various types of alcohols — primary, secondary, and straight- and branched-chain — can be employed in transesterification using lipases as catalysts (Table 3). Linko *et al*. (47) have demonstrated the production of a variety of biodegradable esters and polyesters with lipase as the biocatalyst. In the tranesterification of rapeseed oil with 2-ethyl-1-hexanol, 97% conversions of esters was obtained using *Candida rugosa* lipase powder. De *et al*. (48) investigated the conversion of fatty alcohol esters (C_4–$C_{18:1}$) using immobilized *Mucor miehei* lipase (Lipozyme IM-20) in a solvent-free system. The percentage of molar conversions of all corresponding alcohol esters ranged from 86.8 to 99.2%, while the slip melting points of the esters were found to increase steadily with increasing alcohol chain length (from C_4 to C_{18}) and to decline with the incorporation of unsaturation for the same chain length (as from C_{18} to $C_{18:1}$).

Transesterification of the triglycerides sunflower oil, fish oil, and grease with ethanol, *i.e* ethanolysis, has also been studied. In each case, high yields beyond 80% could be achieved using the lipases from *M. miehei* (49), *Candida antarctica* (50), *Pseudomonas cepacia* (51), respectively.

Nelson *et al*. (52) investigated the abilities of lipases in transesterification with short-chain alcohols to give alkyl esters. The lipase from *M. miehei* was the most efficient for converting triglycerides to their alkyl esters with primary alcohols, whereas that from *C. antarctica* was the most efficient for transesterifying triglycerides with secondary alcohols to give branched alkyl esters. Maximum conversions of 94.8–98.5% for the primary alcohols methanol, ethanol, propanel, butanol, and isobutanol, and of 61.2–83.8% for the secondary alcohols isopropanol and 2-butanol were obtained in the presence of hexane as a solvent. In solvent-free reactions, however, yields with methanol and ethanol were lower than those obtained with hexane; in particular, the yield with methanol decreased to 19.4%.

Mittelbach (53) reported transesterification using the primary alcohols methanol, ethanol, and 1-butanol, with and without petroleum ether as a solvent. Although the ester

TABLE 2. Comparison between alkali-catalysis and lipase-catalysis methods for biodiesel fuel production

	Alkali-catalysis process	Lipase-catalysis process
Reaction temperature	60–70°C	30–40°C
Free fatty acids in raw materials	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

TABLE 3. Enzymatic transesterification reactions using various types of alcohols and lipases

Oil	Alcohol	Lipase	Conversion (%)	Solvent	Ref.
Rapeseed	2-Ethyl-1-hexanol	*C. rugosa*	97	None	47
Mowrah, Mango, Kernel, Sal	C_4–$C_{18:1}$ alcohols	*M. miehei* (Lipozyme IM-20)	86.8–99.2	None	48
Sunflower	Ethanol	*M. meihei* (Lypozyme)	83	None	49
Fish	Ethanol	*C. antarctica*	100	None	50
Recycled restaurant grease	Ethanol	*P. cepacia* (Lipase PS-30) + *C. antarctica* (Lipase SP435)	85.4	None	51
Tallow, Soybean, Rapeseed	Primary alcohols[a]	*M. miehei* (Lipozyme IM60)	94.8–98.5	Hexane	52
	Secondary alcohols[b]	*C. antarctica* (SP435)	61.2–83.8	Hexane	
	Methanol	*M. miehei* (Lipozyme IM60)	19.4	None	
	Ethanol	*M. miehei* (Lipozyme IM60)	65.5	None	
Sunflower	Methanol	*P. fluorescens*	3	None	53
	Methanol		79	Petroleum ether	
	Ethanol		82	None	
Palm kernel	Methanol	*P. cepacia* (Lipase PS-30)	15	None	54
	Ethanol		72	None	

[a] Methanol, ethanol, propanol, butanol, and isobutanol.
[b] Isopropanol and 2-butanol.

yields with ethanol and 1-butanol were relatively high, even in reactions without a solvent, with methanol only traces of methyl esters were obtained. Abigor *et al*. (54) also found that in the conversion of palm kernel oil to alkyl esters using *P. cepacia* lipase, ethanol gave the highest conversion of 72%, while only 15% methyl esters was obtained with methanol. Lipases are known to have a propensity to act on long-chain fatty alcohols better than on short-chain ones (55, 56). Thus, in general, the efficiency of the transesterification of triglycerides with methanol (methanolysis) is likely to be very low compared to that with ethanol in systems with or without a solvent.

Effective methanolysis using extracellular lipase Recently, however, effective methanolysis reactions using extracellular lipase have been developed by several researchers, which are summarized in Table 4. Shimada *et al*. (57) found that immobilized *C. antarctica* lipase (Novozym 435) was the most effective for methanolysis among lipases they tested. Since the enzyme was inactivated by shaking in a mixture containing more than 1.5 molar equivalents of methanol against the oil, they developed a method of adding methanol stepwise to avoid lipase inactivation. As a result, more than 95% of the ester conversion was maintained even after 50 cycles of the reaction. In mixtures containing more than 1.5 molar equivalents of methanol, the excess amount of methanol remained as droplets dispersed in the oil. Because lipase may be inactivated when it contacts these methanol droplets, the authors stress that a reaction system in which methanol dissolves completely is necessary for the lipase-catalyzed methanolysis of oils. They also point out that the stepwise methanolysis procedure is suitable for adoption as an industrial process. Watanabe *et al*. (58) demonstrated effective methanolysis using two-step batch and three-step flow reaction systems with Novozym 435. The methyl ester content in the final-step elute reached 90–93%, and the lipase could be used for at least 100 d in both reaction systems without any significant decrease in the conversion. The effect of pretreatment of Novozym 435 on metha-

TABLE 4. Effective methanolysis processes with both extracellular and intracellular lipases

Lipase	Regiospecificity	Process and operation	Methyl ester content (%)	Reaction time (h)	Ref.
C. antarctica[a] (Novozym435)	None	Repeated fed-batch operation[d]	96–98	48	57
		Continuous operation[e]	92–94	7[h]	58
		Fed-batch operation[d,f]	87	3.5	59
C. rugosa[b], *P. cepacia*[b], *P. fluorescence*[b]	None	Fed-batch operation[d]	80–100	80–90	60
R. oryzae (F-AP15)[b]	1(3)-Regiospecific	Fed-batch operation[d]	80–90	70	30
R. oryzae IFO4697[c]	1(3)-Regiospecific	Fed-batch operation[d]	80–90	72	61
		Repeated fed-batch operation[d,g]	70–80	72	62

[a] Extracellular lipase immobilized by an ion-exchange resin.
[b] Extracellular lipase powder.
[c] Intracellular lipase in cells immobilized within BSPs (whole cell biocatalyst).
[d] Three-step addition of methanol; a reaction mixture of oil/methanol (1:1, mol/mol) was fed in each step.
[e] Continuous three-step flow reaction; a reaction mixture of oil/methanol (1:3, mol/mol) was fed into each column.
[f] Pretreatment of immobilized lipase with methyl oleate and soybean oil.
[g] Treatment of immobilized cells within BSPs using glutaraldehyde.
[h] Overall residence time.

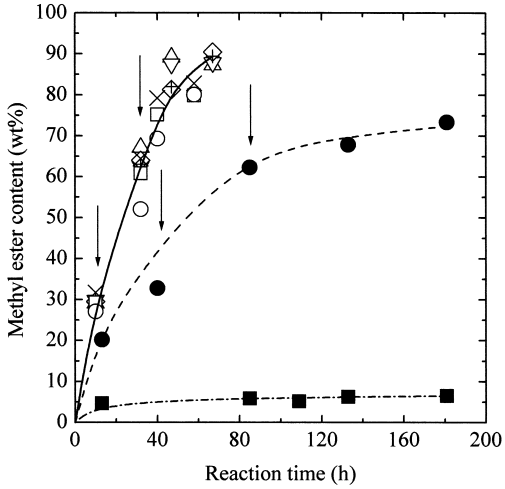

FIG. 2. Time courses of methyl ester content with 210 IU *Rhizopus oryzae* lipase added as (closed square) 0.6, (closed circle) 0.9, (open triangle) 1.2, (reverse open triangle) 1.5, (open rhombus) 1.8, (+) 2.4, (×) 3.0, (open square) 6.0, and (open circle) 9.0 ml enzyme solutions. The reaction mixture containing soybean oil 28.95 g, distilled water 0.6–9.0 ml (=2–30 wt% by weight of the initial substrate), and methanol 1.05 g, was incubated at 35°C with shaking at 150 oscillations/min in a bioshaker. Methanol (1.05 g) was added twice at the times indicated by arrows.

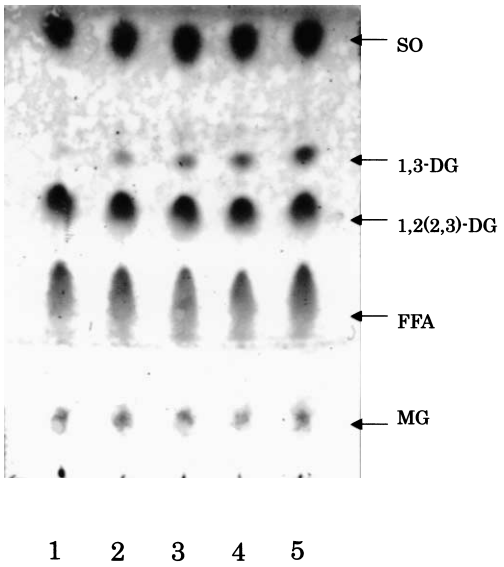

FIG. 3. TLC analysis of spontaneous acyl migration from the *sn*-2 position to the *sn*-1 or *sn*-3 position of partial glycerides. Soybean oil (2.5 g) hydrolyzed for 2 h and then washed 5 times with distilled water to completely remove lipase was incubated with 0.4 ml distilled water at 35°C with shaking at 150 oscillations/min in a bioshaker. Lane 1, first two hours of oil hydrolysis; lanes 2–5, hydrolyzed oil after 13, 20, 38, and 62 h incubation, respectively. Abbreviations: SO, soybean oil; MG, monoglyceride; DG, diglyceride; FFA, free fatty acid.

nolysis for biodiesel fuel production was investigated by Samukawa *et al*. (59). Methanolysis progressed much faster when Novozym 435 was preincubated in methyl oleate for 0.5 h and subsequently in soybean oil for 12 h. As a result, the methyl ester content in the reaction mixture reached over 97% within 3.5 h by stepwise addition of 0.33 molar equivalents of methanol at 0.25–0.4 h intervals.

Kaieda *et al*. investigated the methanolysis of soybean oil with both non-regiospecific (60) and 1(3)-regiospecific (30) lipases in a water-containing system without an organic solvent. Among the non-regiospecific lipases, those from *C. rugosa*, *P. cepacia* and *P. fluorescens* displayed significantly high catalytic ability. In particular, the *P. cepacia* lipase yielded high methyl ester contents in a reaction mixture with up to 2 or 3 molar equivalents of methanol to oil, which is attributed to the *P. cepacia* lipase having substantial methanol tolerance (60).

The *R. oryzae* lipase, which exhibits 1(3)-regiospecificity (63–66), is also effective for the methanolysis of soybean oil. As shown in Fig. 2, the lipase efficiently catalyzed methanolysis in the presence of 4–30% water in the starting material, but the enzyme was nearly inactive in the absence of water. Despite of the use of 1(3)-regiospecificity lipase, the methyl ester content in the reaction mixture reached 80–90% by stepwise additions of methanol to the reaction mixture. To investigate the possibility of spontaneous acyl migration in a water-containing system, the change in the 1,3-diglyceride content with incubation time after the hydrolysis of soybean oil was analyzed (30). Figure 3 shows TLC results for samples obtained after incubation for 13, 20, 38, and 62 h, including the sample obtained during the initial hydrolysis (lane 1). Although no 1,3-diglyceride was observed at the beginning of the incubation, spots indicating the presence of 1,3-diglyceride were detected from 13 h onwards, and the 1,3-diglyceride concentration increased with increasing incubation time. This indicates that acyl migration from the *sn*-2 position to the *sn*-1 or *sn*-3 position in partial glycerides can occur spontaneously in a water-containing system without lipase. Thus, a high methyl ester content above 80% was achieved.

Use of intracellular lipase as a whole cell biocatalyst

Bioconversion can be carried out using extracellular or intracellular enzymes, but extracellular enzymes require purification by procedures that may be too complex for practical use. Furthermore, enzymes recovered through such operations are generally unstable and expensive. Consequently, there has been considerable research into the direct use of whole cells as biocatalysts (67–69).

To utilize whole cell biocatalysts in a convenient form, cells should be immobilized in such a way that they resemble ordinary solid-phase catalysts used conventionally in synthetic chemical reactions. Among many available immobilization methods, a technique using porous biomass support particles (BSPs), developed by Atkinson *et al*. (70), has several advantages over other methods in terms of industrial application: (i) no chemical additives are required, (ii) there is no need for preproduction of cells, (iii) aseptic handling of particles is unnecessary, (iv) there is a large mass transfer rate of substrate and production within BSPs, (v) the particles are reusable, (vi) the particles are durable against mechanical shear, (vii) bioreactor scale-up is easy, (viii) costs are low compared to other methods. Because of its advantageous features, the BSP technique has been applied successfully in a wide variety of microbial (71–85), animal (86–94), insect (95), and plant cell (96–101) systems.

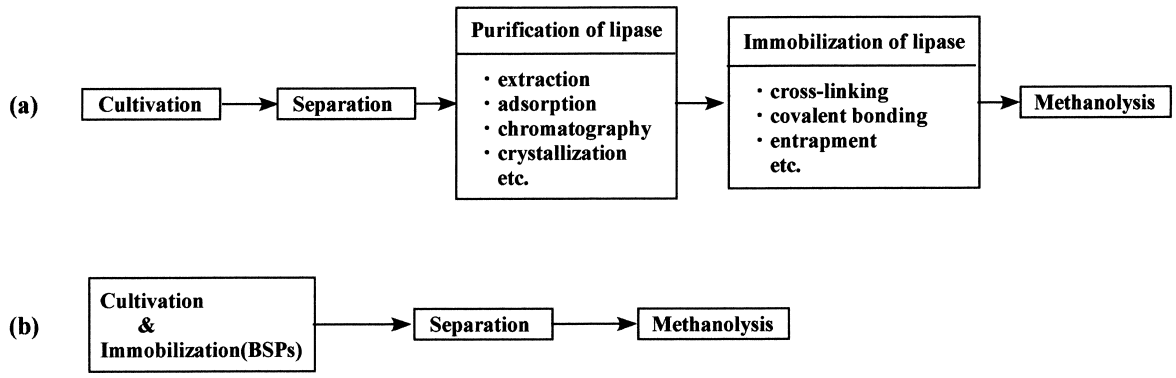

FIG. 4. Comparison of lipase production processes for methanolysis with extracellular (a) and intracellular (b) lipases.

In Fig. 4, the extracellular and intracellular (whole cell biocatalyst) lipase production processes are compared. Unlike in the case of extracellular lipase, no purification or immobilization processes are needed in preparing whole cell biocatalysts with BSPs, since immobilization can be achieved spontaneously during batch cultivation.

Utilizing *R. oryzae* cells immobilized within BSPs as a whole cell biocatalyst, Ban *et al*. (61) investigated the culture conditions for lipase production, and the effects of cell pre-treatment and water content in the reaction mixture on methanolysis. As shown in Fig. 5, *R. oryzae* cells easily became immobilized within the polyurethane foam BSPs during batch cultivation. Addition of olive oil or oleic acid to the culture medium as a substrate-related compound significantly benefited the intracellular lipase activity, while no glucose was necessary. As a result, when methanolysis was carried out with stepwise additions of methanol using BSP-immobilized cells in the presence of 10–20% water, the methyl ester content in the reaction mixture reached 80–90% without any organic solvent pretreatment (Fig. 6). This level of methyl ester production is almost the same as that achieved using extracellular lipase (30). To stabilize *R. oryzae* cells, cross-linking treatment with 0.1% glutaraldehyde solution was examined (62). As shown in Fig. 7, the lipase activity of the cells thus obtained was maintained during six batch cycles without any significant decrease, with the methyl ester content in each cycle reaching 70–83% within 72 h. Without the glutaraldehyde treatment, the activity decreased gradually with each cycle, to give a methyl ester content of only 50% at the 6th batch. These findings indicate that the use of whole cell biocatalysts immobilized within the BSPs (61, 62) offers a promising means of biodiesel fuel production for industrial application because of the simplicity of the lipase production process as well as the stability of lipase activity over a long period.

TRANSESTERIFICATION USING SUPERCRITICAL FLUIDS

With the aim of developing a novel methanolysis process for oil without using any catalyst, Saka and Kusdiana (102) made a fundamental study of biodiesel production in supercritical methanol. They demonstrated that preheating to a temperature of 350°C and treatment for 240 s in supercriti-

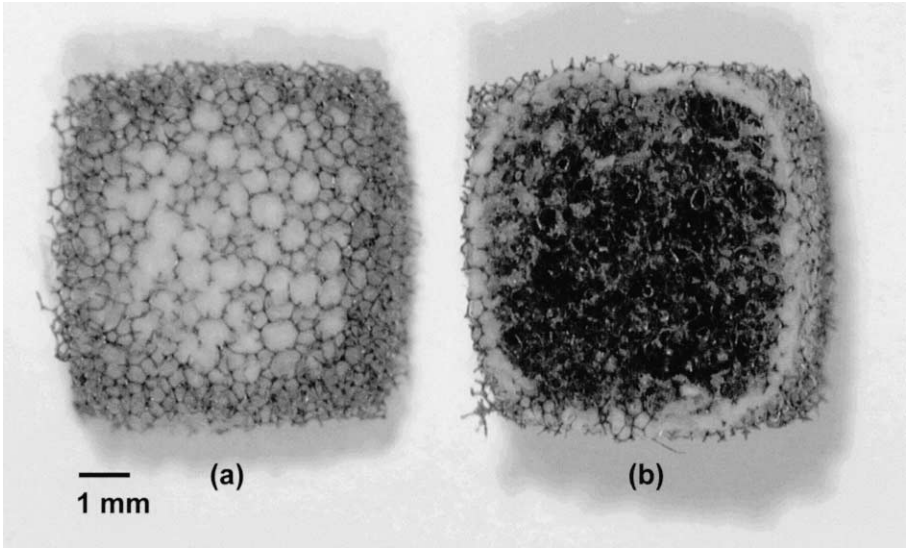

FIG. 5. Micrographs of surface (a) and cross-section (b) of a 6-mm cubic biomass support particle (voidage >97%; pore size, 50 pores per linear inch). Medium: polypepton 70 g/*l*; $NaNO_3$ 1.0 g/*l*; KH_2PO_4 1.0 g/*l*; $MgSO_4 \cdot 7H_2O$ 0.5 g/*l*; olive oil 30 g/*l*. Culture conditions: medium volume, 100 ml; temperature, 35°C; cultivation time, 80–90 h; shaking, 150 oscillations/min.

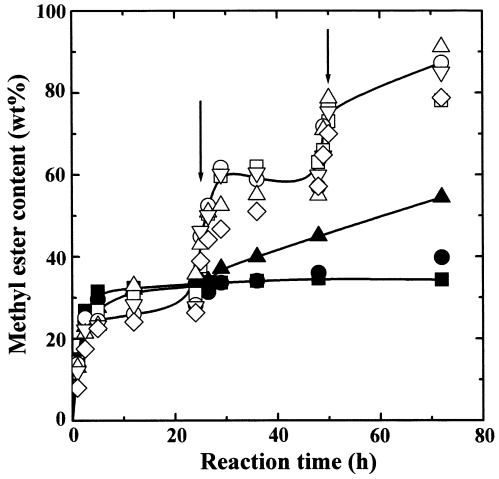

FIG. 6. Time courses of methyl ester content in methanolysis with BPS-immobilized cells in the presence of (closed square) 0, (closed circle) 0.2, (closed triangle) 0.3, (open square) 0.4, (open circle) 1.0, (open triangle) 1.5, (reverse open triangle) 2.0, and (open rhombus) 3.0 ml water in the initial reaction mixture. The reaction mixture containing soybean oil 9.65 g, 0.1 M acetate buffer (pH 5.6) 0–3.0 ml (=0–30 wt% by weight of the initial substrate), and methanol 0.35 g, was incubated with 50 pieces of BSPs at 35°C in a 50-ml screw-cap bottle on a reciprocal shaker (150 oscillations/min; amplitude 70 mm). Methanol (0.35 g) was added twice at the times indicated by the arrows.

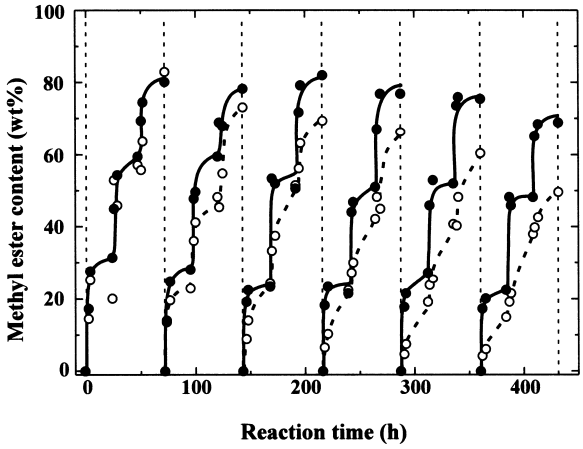

FIG. 7. Time courses of methyl ester contents in a repeated methanolysis reaction using BSP-immobilized cells with and without glutaraldehyde treatment (0.1% solution×1 h at 25°C). The procedure for immobilization within the BSPs was the same as described in Fig. 5. Methanolysis was carried out for 6 batch cycles in the presence of 15% water in the reaction mixture. Otherwise, the conditions were the same as described in Fig. 6. Symbols: (closed circle) treated cells; (open circle) untreated cells.

cal methanol were sufficient to convert rapeseed oil to methyl esters. Moreover, while the methyl esters produced were basically the same as those obtained in the conventional method with a basic catalyst, the methyl ester yield of the supercritical methanol method was higher. Kinetic analyses of the reactions in subcritical and supercritical methanol revealed that the rate of rapeseed oil conversion to methyl esters increased dramatically in the supercritical state. A reaction temperature of 350°C and a molar ratio of methanol to rapeseed oil of 42 to 1 were considered to be the best conditions (103). Since supercritical methanol has a hydrophobic nature with a lower dielectric constant, non-polar triglycerides can be well solvated with supercritical methanol to form a single phase oil/methanol mixture. However, liquid methanol is a polar solvent and has hydrogen bondings between OH oxygen and OH hydrogen to form methanol clusters. Thus, the oil to methyl ester conversion rate was found to increase dramatically in the supercritical state.

Free fatty acids contained in crude oils and fats could also be converted efficiently to methyl esters in supercritical methanol, leading to increase of the total yield of methyl esters from used oils (104). In addition, because the process is non-catalytic, the purification of products after the transesterification reaction is much simpler and more environmentally friendly compared with the alkali-catalyzed method in which all the catalyst and saponified products have to be removed to obtain biodiesel fuel. However, the supercritical methanol method requires a high temperature of 350°C and a pressure of 45 MPa, and in addition, large amount of methanol is necessary. Therefore, to employ this method in industrial application further investigations of production process such as continuous operation and scale-up are needed.

CONCLUSIONS AND FUTURE PROSPECTS

In recent years, biodiesel has become more attractive as an alternative fuel for diesel engines because of its environmental benefits and the fact that it is made from renewable resources. Used oils can also be utilized for making biodiesel fuel, thus helping to reduce the cost of wastewater treatment in sewerage systems and generally assisting in the recycling of resources (105). Biodiesel fuel production now exceeds 100,000 tonnes per year in several countries, including Belgium, France, Germany, Italy, and the United States (106). In Japan, small quantities of biodiesel fuel are now being produced from used oils in many localities.

For the production of biodiesel fuel, an alkali-catalysis process has been established that gives high conversion levels of oils to methyl esters, and at present this is the method that is generally employed in actual biodiesel production. However, it has several drawbacks, including the difficulty of recycling glycerol and the need for either removal of the catalyst or wastewater treatment. In particular, several steps such as the evaporation of methanol, removal of saponified products, neutralization, concentration, etc., are needed to recover glycerol as a by-product.

To overcome these drawbacks, which may limit the availability of biodiesel fuel, enzymatic processes using both extracellular and intracellular lipases have recently been developed. In Fig. 8, comparative flow diagrams for biodiesel fuel production by the alkali- and a lipase-catalysis processes are presented. The latter process is much simpler since recovery of unreacted methanol and wastewater treatment are unnecessary. In addition, only a simple concentration is required to recover glycerol.

Since the cost of lipase production is the main hurdle to commercialization of the lipase-catalyzed process, several attempts have been made to develop cost-effective systems. To avoid serious degradation of lipase activity in the pres-

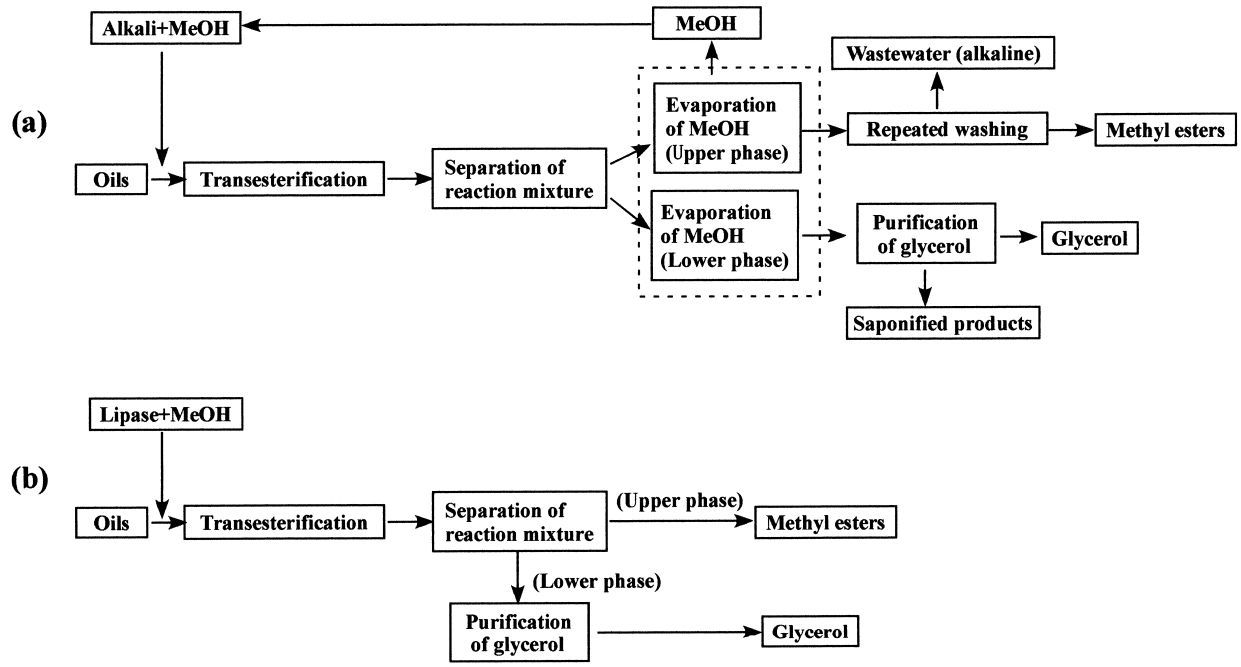

FIG. 8. Flow diagrams comparing biodiesel production using the alkali- (a) and lipase-catalysis (b) processes.

ence of a high concentration of methanol, a novel operation with stepwise addition of methanol has been developed (30, 57–60). The use of intracellular lipase as a whole cell biocatalyst (61, 62) is also an effective way to lower the lipase production cost, since complex purification is not necessary.

Another useful approach to reducing the production cost is to use solvent-tolerant lipases. Several microorganisms that produce solvent-tolerant lipases, either 1,3-specific from *Fusarium* sp. (107) or non-specific from *Pseudomonas* and *Bacillus* sp. (108–112), have been reported. These lipases are stable in most water-immisible solvents, but their stability generally decrease somewhat in water-misible solvents such as methanol and ethanol. However, activity of the lipase from *Fusarium heterosporum* was found to slightly increase in the presence of a low concentration of methanol (107).

Further enhancement of lipase production may be achieved by genetic engineering. High levels of expression of lipases from several microorganisms (113–115) have been successfully achieved using *Saccharomyces cerevisiae* as the host. Among these the lipase cDNA from *F. heterosporum* increased lipase productivity 3-fold over that of the original strain (114).

In the light of these findings, combining a whole cell biocatalyst with the use of a recombinant microorganism can be expected to considerably decrease the cost of lipase production. Such a novel system offers a promising process prospect for industrial biodiesel fuel production.

REFERENCES

1. **Bartholomew, D.:** Vegetable oil fuel. J. Am. Oil Chem. Soc., **58**, 286A–288A (1981).
2. **Pryde, E. H.:** Vegetable oil as diesel fuel: overview. J. Am. Oil Chem. Soc., **60**, 1557–1558 (1983).
3. **Adams, C., Peters, J. F., Rand, M. C., Schroer, B. J., and Ziemke, M. C.:** Investigation of soybean oil as a diesel fuel extender: endurance tests. J. Am. Oil Chem. Soc., **60**, 1574–1579 (1983).
4. **Peterson, C. L., Auld, D. L., and Korus, R. A.:** Winter rape oil fuel for diesel engines: recovery and utilization. J. Am. Oil Chem. Soc., **60**, 1579–1587 (1983).
5. **Strayer, R. C., Blake, J. A., and Craig, W. K.:** Canola and high erucic rapeseed oil as substitutes for diesel fuel: preliminary tests. J. Am. Oil Chem. Soc., **60**, 1587–1592 (1983).
6. **Engler, C. R., Johnson, L. A., Lepori, W. A., and Yarbrough, C. M.:** Effects of processing and chemical characteristics of plant oils on performance of an indirect-injection diesel engine. J. Am. Oil Chem. Soc., **60**, 1592–1596 (1983).
7. **Shay, E. G.:** Diesel fuel from vegetable oils: status and opportunities. Biomass Bioenerg., **4**, 227–242 (1993).
8. **Ziejewski, M. and Kaufman, K. R.:** Laboratory endurance test of a sunflower oil blend in a diesel engine. J. Am. Oil Chem. Soc., **60**, 1567–1573 (1983).
9. **Ma, F. and Hanna, M. A.:** Biodiesel production: a review. Bioresour. Technol., **70**, 1–15 (1999).
10. **Srivastava, A. and Prasad, R.:** Triglycerides-based diesel fuels. Renew. Sust. Energ. Rev., **4**, 111–133 (2000).
11. **Grossley, T. D., Heyes, T. D., and Hudson, B. J. F.:** The effect of heat on pure triglycerides. J. Am. Oil Chem. Soc., **39**, 9–14 (1962).
12. **Schwab, A. W., Dykstra, G. J., Selke, E., Sorenson, S. C., and Pryde, E. H.:** Diesel fuel from thermal decomposition of soybean oil. J. Am. Oil Chem. Soc., **65**, 1781–1786 (1988).
13. **Alencar, J. W., Alves, P. B., and Craveiro, A. A.:** Pyrolysis of tropical vegetable oils. J. Agric. Food Chem., **31**, 1268–1270 (1983).
14. **Billaud, F., Dominguez, V., Broutin, P., and Busson, C.:** Production of hydrocarbons by pyrolysis of methyl esters from rapeseed oil. J. Am. Oil Chem. Soc., **72**, 1149–1154 (1995).
15. **Schwab, A. W., Bagby, M. O., and Freedman, B.:** Preparation and properties of diesel fuels from vegetable oils. Fuel,

66, 1372–1378 (1987).
16. **Pryde, E. H.:** Vegetable oils as fuel alternatives — symposium overview. J. Am. Oil Chem. Soc., **61**, 1609–1610 (1984).
17. **Ziejewski, M., Kaufman, K. R., Schwab, A. W., and Pryde, E. H.:** Diesel engine evaluation of a nonionic sunflower oil — aqueous ethanol microemulsion. J. Am. Oil Chem. Soc., **61**, 1620–1626 (1984).
18. **Clark, S. J., Wangner, L., Schrock, M. D., and Piennaar, P. G.:** Methyl and ethyl soybean esters as renewable fuels for diesel engines. J. Am. Oil Chem. Soc., **61**, 1632–1638 (1984).
19. **Yamane, K., Ueta, A., and Shimamoto, Y.:** Influence of physical and chemical properties of biodiesel fuel on injection, combustion and exhaust emission characteristics in a DI-CI engine. Proc. 5th Int. Symp. on Diagnostics and Modeling of Combustion in Internal Combustion Engines (COMODIA 2001). Nagoya, p. 402–409 (2001).
20. **Varese, R. and Varese, M.:** Methyl ester biodiesel: opportunity or necessity? Inform, **7**, 816–824 (1996).
21. **Körbitz, W.:** The biodiesel market today and its future potential, p. 3–14. *In* Martini, N. and Schell, J. S. (ed.), Plant oils as fuels. Springer-Verlag, Heidelberg (1998).
22. **Sheehan, J., Camobreco, V., Duffield, J., Graboski, M., and Shapouri, H.:** An overview of biodiesel and petroleum diesel life cycles. Report of National Renewable Energy Laboratory (NREL) and US-Department of Energy (DOE). Task No. BF886002, May (1998).
23. **Schäfer, A.:** Vegetable oil fatty acid methyl esters as alternative diesel fuels for commercial vehicle engines, p. 29–46. *In* Martini, N. and Schell, J. S. (ed.), Plant oils as fuels. Springer-Verlag, Heidelberg (1998).
24. **Syassen, O.:** Diesel engine technologies for raw and transesterified plant oils as fuels: desired future qualities of the fuels, p. 47–63. *In* Martini, N. and Schell, J. S. (ed.), Plant oils as fuels. Springer-Verlag, Heidelberg (1998).
25. **Sams, T.:** Exhaust components of biofuels under real world engine conditions, p. 64–77. *In* Martini, N. and Schell, J. S. (ed.), Plant oils as fuels. Springer-Verlag, Heidelberg (1998).
26. **Freedman, B., Butterfield, R. O., and Pryde, E. H.:** Transesterification kinetics of soybean oil. J. Am. Oil Chem. Soc., **63**, 1375–1380 (1986).
27. **Noureddini, H. and Zhu, D.:** Kinetics of transesterification of soybean oil. J. Am. Oil Chem. Soc., **74**, 1457–1463 (1997).
28. **Dufek, E. J., Butterfield, R. O., and Frankel, E. N.:** Esterification and transesterification of 9(10)-carboxystearic acid and its methyl esters. Kinetic studies. J. Am. Oil Chem. Soc., **49**, 302–309 (1972).
29. **Eckey, E. W.:** Esterification and interesterification. J. Am. Oil Chem. Soc., **33**, 575–579 (1956).
30. **Kaieda, M., Samukawa, T., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Nomoto, F., Ohtsuka, K., Izumoto, E., and Fukuda, H.:** Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent. J. Biosci. Bioeng., **88**, 627–631 (1999).
31. **Freedman, B., Pryde, E. H., and Mounts, T. L.:** Variables affecting the yields of fatty esters from transesterified vegetable oils. J. Am. Oil Chem. Soc., **61**, 1638–1643 (1984).
32. **Aksoy, H. A., Kahraman, I., Karaosmanoglu, F., and Civelekoglu, H.:** Evaluation of Turkish sulphur olive oil as an alternative diesel fuel. J. Am. Oil Chem. Soc., **65**, 936–938 (1988).
33. **Klopfenstein, W. E. and Walker, H. S.:** Efficiencies of various esters of fatty acids as diesel fuels. J. Am. Oil Chem. Soc., **60**, 1596–1598 (1983).
34. **Harrington, K. J. and D'Arcy-Evans, C.:** A comparison of conventional and *in situ* methods of transesterification of seed oil from a series of sunflower cultivars. J. Am. Oil Chem. Soc., **62**, 1009–1013 (1985).
35. **Harrington, K. J. and D'Arcy-Evans, C.:** Transesterification *in situ* of sunflower seed oil. Ind. Eng. Chem. Prod. Res. Dev., **24**, 314–318 (1985).
36. **Kildiran, G., Özgül, S., and Türkay, S.:** *In-situ* alcoholysis of soybean oil. J. Am. Oil Chem. Soc., **73**, 225–228 (1996).
37. **Özgül, S. and Türkay, S.:** *In situ* esterification of rice bran oil with methanol and ethanol. J. Am. Oil Chem. Soc., **70**, 145–147 (1993).
38. **Formo, M. W.:** Ester reactions of fatty materials. J. Am. Oil Chem. Soc., **31**, 548–559 (1954).
39. **Wright, H. J., Segur, J. B., Clark, H. V., Coburn, S. K., Langdon, E. E., and DuPuis, R. N.:** A report on ester interchange. Oil Soap, **21**, 145–148 (1944).
40. **Ma, F., Clements, L. D., and Hanna, M. A.:** Biodiesel fuel from animal fat. Ancillary studies on transesterification of beef tallow. Ind. Eng. Chem. Res., **37**, 3768–3771 (1998).
41. **Feuge, R. O. and Grose, T.:** Modification of vegetable oils. VII. Alkali catalyzed interesterification of peanut oil with ethanol. J. Am. Oil Chem. Soc., **26**, 97–102 (1949).
42. **Krisnangkura, K. and Simamaharnnop, R.:** Continuous transmethylation of palm oil in an organic solvent. J. Am. Oil Chem. Soc., **69**, 166–169 (1992).
43. **Fillieres, R., Benjelloun-Mlayah, B., and Delmas, M.:** Ethanolysis of rapeseed oil: quantitation of ethyl esters, mono-, di-, and triglycerides and glycerol by high performance size-exclusion chromatography. J. Am. Oil Chem. Soc., **72**, 427–432 (1995).
44. **Hartman, L.:** Methanolysis of triglycerides. J. Am. Oil Chem. Soc., **33**, 129–132 (1956).
45. **Alcantara, R., Amores, J., Canoira, L., Fidalgo, E., Franco, M. J., and Navarro, A.:** Catalytic production of biodiesel from soy-bean oil, used frying oil and tallow. Biomass Bioenerg., **18**, 515–527 (2000).
46. **Nye, M. J., Williamson, T. W., Deshpande, S., Schrader, J. H., Snively, W. H., Yurkewich, T. P., and French, C. R.:** Conversion of used frying oil to diesel fuel by transesterification: preliminary tests. J. Am. Oil Chem. Soc., **60**, 1598–1601 (1983).
47. **Linko, Y.-Y., Lämsä, M., Wu, X., Uosukainen, W., Sappälä, J., and Linko, P.:** Biodegradable products by lipase biocatalysis. J. Biotechnol., **66**, 41–50 (1998).
48. **De, B. K., Bhattacharyya, D. K., and Bandhu, C.:** Enzymatic synthesis of fatty alcohol esters by alcoholysis. J. Am. Oil Chem. Soc., **76**, 451–453 (1999).
49. **Selmi, B. and Thomas, D.:** Immobilized lipase-catalyzed ethanolysis of sunflower oil in solvent-free medium. J. Am. Oil Chem. Soc., **75**, 691–695 (1998).
50. **Breivik, H., Haraldsson, G. G., and Kristinsson, B.:** Preparation of highly purified concentrates of eicosapentaenoic acid and docosahexaenoic acid. J. Am. Oil Chem. Soc., **74**, 1425–1429 (1997).
51. **Wu, W. H., Foglia, T. A., Marmer, W. N., and Phillips, J. G.:** Optimizing production of ethyl esters of grease using 95% ethanol by response surface methodology. J. Am. Oil Chem. Soc., **76**, 517–521 (1999).
52. **Nelson, L. A., Foglia, A., and Marmer, W. N.:** Lipase-catalyzed production of biodiesel. J. Am. Oil Chem. Soc., **73**, 1191–1195 (1996).
53. **Mittelbach, M.:** Lipase catalyzed alcoholysis of sunflower oil. J. Am. Oil Chem. Soc., **67**, 168–170 (1990).
54. **Abigor, R., Uadia, P., Foglia, T., Haas, M., Jones, K., Okpefa, E., Obibuzor, J., and Bafor, M.:** Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils. Biochem. Soc. Trans., **28**, 979–981 (2000).
55. **Shimada, Y., Sugihara, A., Nakano, H., Kuramoto, T., Nagao, T., Gemba, M., and Tominaga, Y.:** Purification of docosahexaenoic acid by selective esterification of fatty

acids from tuna oil with *Rhizopus delemar* lipase. J. Am. Oil Chem. Soc., **74**, 97–101 (1997).
56. **Shimada, Y., Sugihara, A., Minamigawa, Y., Higashiyama, K., Akimoto, K., Fujikawa, S., Komemushi, S., and Tominaga, Y.:** Enzymatic enrichment of arachidonic acid from *Mortierella* single-cell oil. J. Am. Oil Chem. Soc., **75**, 1213–1217 (1998).
57. **Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H., and Tominaga, Y.:** Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc., **76**, 789–793 (1999).
58. **Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H., and Tominaga, Y.:** Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc., **77**, 355–360 (2000).
59. **Samukawa, T., Kaieda, M., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., and Fukuda, H.:** Pretreatment of immobilized *Candida antarctica* lipase for biodiesel fuel production from plant oil. J. Biosci. Bioeng., **90**, 180–183 (2000).
60. **Kaieda, M., Samukawa, T., Kondo, A., and Fukuda, H.:** Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. J. Biosci. Bioeng., **91**, 12–15 (2001).
61. **Ban, K., Kaieda, M., Matsumoto, T., Kondo, A., and Fukuda, H.:** Whole cell biocatalyst for biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles. Biochem. Eng. J., **8**, 39–43 (2001).
62. **Ban, K., Hama, S., Nishizuka, K., Kaieda, M., Matsumoto, T., Kondo, A., Noda, H., and Fukuda, H.:** Repeated use of whole cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. J. Mol. Catal. B: Enz. (2002). (in press)
63. **Okumura, S., Iwai, M., and Tsujikawa, T.:** Positional specificities of four kinds of microbial lipases. Agr. Biol. Chem., **40**, 655–660 (1976).
64. **Macrae, A. R.:** Lipase-catalyzed interesterification of oils and fats. J. Am. Oil Chem. Soc., **60**, 291–294 (1983).
65. **Matori, M., Asahara, T., and Ota, Y.:** Positional specificity of microbial lipases. J. Ferment. Bioeng., **72**, 397–398 (1991).
66. **Scheib, H., Pleiss, J., Stadler, P., Kovac, A., Potthoff, A. P., Haalck, L., Spener, F., Paltauf, F., and Schmid, R. D.:** Rational design of *Rhizopus oryzae* lipase with modified steleoselectivity toward triadylglycerols. Protein Eng., **11**, 675–682 (1998).
67. **Kondo, A., Liu, Y., Furuta, M., Fujita, Y., Matsumoto, T., and Fukuda, H.:** Preparation of high activity whole cell biocatalyst by permeabilization of recombinant flocculent yeast with alcohol. Enzyme Microb. Technol., **27**, 806–811 (2000).
68. **Liu, Y., Hama, H., Fujita, Y., Kondo, A., Inoue, Y., Kimura, A., and Fukuda, H.:** Production of *S*-lactoylgluthathione by high activity whole cell biocatalysts prepared by permeabilization of recombinant *Saccharomyces cerevisiae* with alcohols. Biotechnol. Bioeng., **64**, 54–60 (1999).
69. **Liu, Y., Fujita, Y., Kondo, A., and Fukuda, H.:** Preparation of high-activity whole cell biocatalysts by permeabilization of recombinant yeasts with alcohol. J. Biosci. Bioeng., **89**, 554–558 (2000).
70. **Atkinson, B., Black, G. M., Lewis, P. J. S., and Pinches, A.:** Biological particles of given size, shape, and density for use in biological reactors. Biotechnol. Bioeng., **21**, 193–200 (1979).
71. **Xing, X.-H., Honda, H., Shiragami, N., and Unno, H.:** A model analysis of microbial retainment process in porous support particles in a fluidized-bed wastewater treatment reactor. J. Chem Eng. Jpn., **25**, 89–95 (1992).
72. **Huysman, P., Meenen, P. V., Assche, P. V., and Verstraete, W.:** Factors affecting the colonization of non porous and porous packing materials in model upflow methane reactors. Biotechnol. Lett., **5**, 643–648 (1983).
73. **Fynn, G. H. and Rankin, S. W.:** Methanogenesis by methanogen species immobilised on reticulated foam biomass support particles. Biotechnol. Lett., **9**, 67–70 (1987).
74. **Fukuzaki, S., Nishio, N., and Nagai, S.:** The use of polyurethane foam for microbial retention in methanogenic fermentation of propionate. Appl. Microbiol. Biotechnol., **34**, 408–413 (1990).
75. **Fukuda, H., Turugida, Y., Nakajima, T., Nomura, E., and Kondo, A.:** Phospholipase production using immobilized cells of *Streptoverticillium cinnamoneum*. Biotechnol. Lett., **18**, 951–956 (1996).
76. **Kobayashi, T., Tachi, K., Nagamune, T., and Endo, I.:** Production of penicillin in a fluidized-bed bioreactor using urethane foam as carriers. J. Chem. Eng. Jpn., **23**, 408–413 (1990).
77. **Furuta, H., Arai, T., Hama, H., Shiomi, N., Kondo, A., and Fukuda, H.:** Production of glucoamylase by passively immobilized cells of a flocculent yeast, *Saccharomyces diastaticus.* J. Ferment. Bioeng., **84**, 169–171 (1997).
78. **Liu, Y., Kondo, A., Ohkawa, H., Shiota, N., and Fukuda, H.:** Bioconversion using immobilized recombinant flocculent yeast cells carrying a fused enzyme gene in an 'intelligent' bioreactor. Biochem. Eng. J., **2**, 229–235 (1998).
79. **Webb, C., Fukuda, H., and Atkinson, B.:** The production of cellulase in a spouted bed fermentor using cells immobilized in biomass support particles. Biotechnol. Bioeng., **28**, 41–50 (1986).
80. **Fukuda, H. and Morikawa, H.:** Enhancement of γ-linolenic acid production by *Mucor ambiguus* with nonionic surfactants. Appl. Microbiol. Biotechnol., **27**, 15–20 (1987).
81. **Anselmo, A. M. and Novais, J. M.:** Biological treatment of phenolic wastes: comparison between free and immobilized cell systems. Biotechnol Lett., **14**, 239–244 (1992).
82. **Vassileve, N., Kautola, H., and Linko, Y.-Y.:** Immobilized *Aspergillus terreus* in itaconic acid production from glucose. Biotechnol. Lett., **14**, 201–206 (1992).
83. **Kautola, H., Rymowicz, W., Linko, Y.-Y., and Linko, P.:** Itaconic acid production by immobilized *Aspergillus terreus* with varied metal additions. Appl. Microbiol. Biotechnol., **35**, 154–158 (1991).
84. **Ziomek, E., Kirkpatric, N., and Reid, I. D.:** Effect of polydimethyl siloxane oxygen carriers on the biological bleaching of hardwood kraft pulp by *Trametes versicolor.* Appl. Microbiol. Biotechnol., **35**, 669–673 (1991).
85. **Kautola, H. and Linko, Y.-Y.:** Fumaric acid production from xylose by immobilized *Rhizopus arrhizus* cells. Appl. Microbiol. Biotechnol., **31**, 448–452 (1989).
86. **Matsushita, T., Ijima, H., Koide, N., and Funatsu, K.:** High albumin production by multicellular spheroids of adult rat hepatocytes formed in the pores of polyurethane foam. Appl. Microbiol. Biotechnol., **36**, 324–326 (1992).
87. **Matsushita, T., Hidaka, H., Kamihata, K., Kawakubo, Y., and Funatsu, K.:** High density culture of anchorage-dependent animal cells by polyurethane foam packed-bed culture systems. Appl. Microbiol. Biotechnol., **35**, 159–164 (1991).
88. **Matsushita, T., Ketayama, M., Kamihata, K., and Funatsu, K.:** Anchorage-dependent mammalian cell culture using polyurethane foam as a new substratum for cell attachment. Appl. Microbiol. Biotechnol., **33**, 287–290 (1990).
89. **Miyoshi, H., Yanagi, K., Fukuda, H., and Ohshima, N.:** Long-term continuous culture of hepatocytes in a packed-bed reactor utilizing porous resin. Biotechnol. Bioeng., **43**,

635–644 (1994).
90. **Yamaji, H. and Fukuda, H.:** Growth kinetics of animal cells immobilized within porous support particles in a perfusion culture. Appl. Microbiol. Biotechnol., **42**, 531–535 (1994).
91. **Yamaji, H. and Fukuda, H.:** Continuous IgG production by myeloma cells immobilized within porous support particles. J. Ferment. Bioeng., **83**, 489–491 (1997).
92. **Yamaji, H., Fukuda, H., Nojima, Y., and Webb, C.:** Immobilization of anchorage-independent animal cells using reticulated polyvinyl formal resin biomass support particles. Appl. Microbiol. Biotechnol., **30**, 609–613 (1989).
93. **Yamaji, H. and Fukuda, H.:** Long-term cultivation of anchorage-independent animal cells immobilized within reticulated biomass support particles in a circulating bed fermentor. Appl. Microbiol. Biotechnol., **34**, 730–734 (1991).
94. **Ogawa, T., Kamihira, M., Terashima, S., Yasuda, K., Iijima, S., and Kobayashi, T.:** High cell density cultivation of anchorage-dependent cells using a novel macroporous cellulosic support. J. Ferment. Bioeng., **74**, 27–31 (1992).
95. **Yamaji, H., Tagai, S., Sakai, K., Izumoto, E., and Fukuda, H.:** Production of recombinant protein by baculovirus-infected cells in immobilized culture using porous biomass support particles. J. Biosci. Bioeng., **89**, 12–17 (2000).
96. **Corchete, P. and Yeoman, M. M.:** Biotransformation of (-)-codeinone to (-)-codeine by *Papaver somniferum* cells immobilized in reticulate polyurethane foam. Plant Cell Rep., **8**, 128–131 (1989).
97. **Ishida, B. K.:** Improved diosgenin production in *Dioscorea deltoidea* cell cultures by immobilization in polyurethane foam. Plant Cell Rep., **7**, 270–273 (1988).
98. **Mavituna, F. and Park, J. M.:** Growth of immobilized plant cells in reticulate polyurethane foam matrices. Biotechnol. Lett., **7**, 637–640 (1985).
99. **Koge, K., Orihara, Y., and Furuya, T.:** Effect of pore size and shape on the immobilization of coffee (*Coffea arabica* L) cells in porous matrices. Appl. Microbiol. Biotechnol., **36**, 452–455 (1992).
100. **Pak, Y. H., Seo, W. T., and Liu, J. R.:** Enhanced production of shikonin by *Lithospermum erythrorhizon* cells immobilized in polyurethane foam matrices. J. Ferment. Bioeng., **70**, 317–321 (1990).
101. **Rhodes, M. J. C., Smith, J. I., and Robins, R. J.:** Factors affecting the immobilization of plant cells on reticulated polyurethane foam particles. Appl. Microbiol. Biotechnol., **26**, 28–35 (1987).
102. **Saka, S. and Kusdiana, D.:** Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. Fuel, **80**, 225–231 (2001).
103. **Kusdiana, D. and Saka, S.:** Kinetics of transesterification in rapeseed oil to biodiesel fuel as treated in supercritical methanol. Fuel, **80**, 693–698 (2001).
104. **Kusdiana, D. and Saka, S.:** Methyl esterification of free fatty acids of rapeseed oil as treated in supercritical methanol. J. Chem. Eng. Jpn., **34**, 383–387 (2001).
105. **Murayama, T.:** Evaluating vegetable oils as a diesel fuel. Inform, **5**, 1138–1145 (1994).
106. **Krawczyk, T.:** Biodiesel-alternative fuel makes inroads but hurdle remain. Inform, **7**, 800–815 (1996).
107. **Shimada, Y., Koga, C., Sugihara, A., Nagao, T., Takada, N., Tsunasawa, S., and Tominaga, Y.:** Purification and characterization of a novel solvent-tolerant lipase from *Fusarium heterosporum*. J. Ferment. Bioeng., **75**, 349–352 (1993).
108. **Sugihara, A., Ueshima, M., Shimada, Y., Tsunasawa, S., and Tominaga, Y.:** Purification and characterization of a novel thermostable lipase from *Pseudomonas cepacia*. J. Biochem., **112**, 598–603 (1992).
109. **Iizumi, T., Nakamura, K., and Fukase, T.:** Purification and characterization of a thermostable lipase from newly isolated *Pseudomonas* sp. KWI-56. Agric. Biol. Chem., **54**, 1253–1258 (1990).
110. **Sugihara, A., Tani, T., and Tominaga, Y.:** Purification and characterization of a novel thermostable lipase from *Bacillus* sp. J. Biochem., **109**, 211–216 (1991).
111. **Ogino, H., Miyamoto, K., Yasuda, M., Ishimi, K., and Ishikawa, H.:** Growth of organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 in the presence of various organic solvents and production of lipolytic enzyme in the presence of cyclohexane. Biochem. Eng. J., **4**, 1–6 (1999).
112. **Ogino, H., Watanabe, F., Yamada, M., Nakagawa, S., Hirose, T., Noguchi, A., Yasuda, M., and Ishikawa, H.:** Purification and characterization of organic solvent-stable protease from organic solvent-tolerant *Pseudomanas aeruginosa* PST-01. J. Biosci. Bioeng., **87**, 61–68 (1999).
113. **Bertolini, M. C., Schrag, J. D., Cygler, M., Ziomek, E., Thomas, D. Y., and Vernet, T.:** Expression and characterization of *Geotrichum candidum* lipase I gene. Eur. J. Biochem., **228**, 863–869 (1995).
114. **Nagao, T., Shimada, Y., Sugihara, A., and Tominaga, Y.:** Expression of lipase cDNA from *Fusarium heterosporum* by *Saccharomyces cerevisiae*: high-level production and purification. J. Ferment. Bioeng., **81**, 488–492 (1996).
115. **Takahashi, S., Ueda, M., Atomi, H., Beer, H. D., Bornscheuer, U. T., Schmid, R. D., and Tanaka, A.:** Extracellular production of active *Rhizopus oryzae* lipase by *Saccharomyces cerevisiae*. J. Ferment. Bioeng., **86**, 164–168 (1998).