Conversion of Vegetable Oil to Biodiesel Using Immobilized *Candida antarctica* Lipase

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ABSTRACT: Biodiesel derived from vegetable oils has drawn considerable attention with increasing environmental consciousness. We attempted continuous methanolysis of vegetable oil by an enzymatic process. Immobilized *Candida antarctica* lipase was found to be the most effective for the methanolysis among lipases tested. The enzyme was inactivated by shaking in a mixture containing more than 1.5 molar equivalents of methanol against the oil. To fully convert the oil to its corresponding methyl esters, at least 3 molar equivalents of methanol are needed. Thus, the reaction was conducted by adding methanol stepwise to avoid lipase inactivation. The first step of the reaction was conducted at 30°C for 10 h in a mixture of oil/methanol (1:1, mol/mol) and 4% immobilized lipase with shaking at 130 oscillations/min. After more than 95% methanol was consumed in ester formation, a second molar equivalent of methanol was added and the reaction continued for 14 h. The third molar equivalent of methanol was finally added and the reaction continued for 24 h (total reaction time, 48 h). This three-step process converted 98.4% of the oil to its corresponding methyl esters. To investigate the stability of the lipase, the three-step methanolysis process was repeated by transferring the immobilized lipase to a fresh substrate mixture. As a result, more than 95% of the ester conversion was maintained even after 50 cycles of the reaction (100 d).

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KEY WORDS: Biodiesel, *Candida antarctica* lipase, immobilized enzyme, methanolysis, vegetable oil.

Biodiesel (fatty acid methyl esters) is produced by alcoholysis of vegetable oils and animal fats. The main advantages of biodiesel are its biodegradability, renewability, and improved exhaust emissions. Because of these environmental advantages, biodiesel has drawn considerable attention. Currently, biodiesel is produced from vegetable oils in Europe and North America (1,2), and from waste edible oil in Japan. At present, chemical methods are used for biodiesel production, but these methods have drawbacks, such as, difficulties in the recovery of glycerol, the need for removal of catalyst, and the energy-intensive nature of the processes. Furthermore, oils containing free fatty acids and/or water are incompletely transesterified using chemical methods. A biochemical approach can overcome these problems, but has not been adopted industrially because of the high cost of the enzyme catalyst. The establishment of a continuous production process using an immobilized enzyme is strongly needed to decrease the production cost of biodiesel using this approach.

Several studies report alcoholyses of vegetable oils and animal fats with primary and secondary alcohols and straight- and branched-chain alcohols using lipases as catalysts (3–6). However, these reports do not contain the continuous methanolysis of oils and fats in an organic solvent-free environment. A continuous reaction system without organic solvent is necessary for the industrial production of biodiesel. Our preliminary studies showed that the incomplete methanolysis of oils in a mixture without organic solvent was attributed to inactivation of the enzyme, and that stepwise addition of methanol protected the enzyme from inactivation. This paper describes the factors affecting methanolysis of vegetable oils by *Candida antarctica* lipase for the continuous production of biodiesel.

MATERIALS AND METHODS

Materials. Vegetable oil (a mixture of soybean and rapeseed oils) was donated by Showa Sangyo Co. Ltd. (Tokyo, Japan). The molar amount of the oil was calculated from its saponification value. Methanol and tricaprylin were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan) and Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), respectively. *Rhizopus delemar* and *Aspergillus niger* lipases were gifts from Tanabe Seiyaku Co. Ltd. (Osaka, Japan) and Amano Pharmaceutical Co. Ltd. (Aichi, Japan), respectively. *Fusarium heterosporum* lipase was prepared as described previously (7): Ammonium sulfate was added to the culture filtrate to 80% saturation, and the resulting precipitate was dialyzed against water. The lipases were immobilized on a ceramic carrier SM-10, donated from NGK Insulators Ltd. (Aichi, Japan), according to our previous paper (8). Immobilized *C. antarctica* lipase (Novozym 435) and *Rhizomucor miehei* lipase (Lipozyme

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IM60) were obtained from Novo Nordisk (Bagsvaerd, Denmark).

Methanolysis. A standard reaction mixture consisted of the oil, methanol, and immobilized lipase. The reaction was conducted in a 20- or 50-mL screw-capped vessel at 30°C with shaking at 130 oscillations/min.

Analysis. The content of methyl esters (ME) in the reaction mixture was quantified on a Hewlett-Packard 5890 gas chromatograph equipped with a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Forsom, CA) using tricaprylin as an internal standard. The column temperature was raised from 150 to 300°C at 10°C/min and then maintained for 3 min. The injector and detector temperatures were 250 and 320°C, respectively. The composition of ME, monoglycerides (MG), diglycerides (DG), and triglycerides (TG) in the reaction mixture was analyzed on a DB-1ht capillary column (0.25 mm × 15 m; J&W Scientific). The column temperature was raised from 200 to 370°C at 15°C/min and then maintained for 13 min. The injector and detector temperatures were 250 and 380°C, respectively. The content of glycerol in the ME layer was measured as follows. An equal amount of water was added to the ME fraction (>95%), and the water layer recovered after shaking the mixture vigorously. The glycerol dissolved in the water was measured by glycerokinase pyruvate kinase/lactate dehydrogenase assay system (F-Kit Glycerol; Boehringer Mannheim, Mannheim, Germany) according to the supplier's protocol. Water content in the reaction mixtures was determined by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan).

RESULTS

Screening for a lipase suitable for methanolysis of vegetable oil. Vegetable oil was subjected to methanolysis using several immobilized lipases as catalysts (Table 1). Immobilized *Rhizopus* and *Fusarium* lipases required preactivation with a small amount of water before use (8). Accordingly, the first reaction was conducted using an oil/methanol (1:1, mol/mol) mixture containing 2% water, and subsequent reactions were done by transferring the enzyme to a fresh substrate mixture without adding water. In the first reaction, *Candida* and *Rhizomucor* lipases catalyzed the alcoholysis somewhat, but the ester conversions by the other lipases were less than 10%. When the reactions were repeated with fresh substrate, ester conversion with *Candida* lipase increased gradually and reached 33.1% in the fourth reaction, whereas ester conversion with *Rhizomucor* lipase was constant. One molar equivalent of methanol against the oil was present in the reaction mixture, showing that 99.3% methanol (33.1/33.3) was consumed for the conversion of the oil to its corresponding ME. Because *Candida* lipase gave the highest ester conversion among the lipases tested, it was selected for further study.

Effect of water content on methanolysis of vegetable oil. Nelson *et al*. (6) showed that *C. antarctica* lipase did not require pretreatment with water for alcoholysis to occur. To confirm that the pretreatment is also unnecessary in our reaction system, the immobilized lipase was shaken in mixtures of oil/methanol containing various amounts of water (Fig. 1). When the reaction was done without adding water (water content, 320 ppm), ester formation was 30.7% after 6 h, and 92.1% of the methanol in the mixture was consumed. Ester formation after 6-h reaction decreased with increasing water content in the mixture, and ester formation after 24-h reaction was 27.5%, even though 0.7% water was present. These results showed that preactivation with water was not necessary for immobilized *C. antarctica* lipase. Water in the mixture decreased the reaction rate but did not affect the equilibrium of the reaction.

After the first reaction in the mixture with added water, subsequent reactions were repeated by transferring the lipase to a fresh substrate mixture without adding water. Table 2 shows the extent of ester formation and hydrolysis after 6 h in the first

TABLE 1
Methanolysis of Vegetable Oil by Immobilized Lipases[a]

	Conversion (%)[b]			
Lipase	First	Second	Third	Fourth
Rhizomucor miehei	19.1	21.8	18.6	19.4
Candida antarctica	16.0	26.6	30.6	33.1
Rhizopus delemar	2.0	0.5	0.3	0.5
Fusarium heterosporum	8.7	1.8	0.5	0.6
Aspergillus niger	0.4	0.5	0.5	0.6

[a]The first reaction was conducted in a mixture of 10 g oil/methanol (1:1, mol/mol), 0.4 g immobilized lipase, and 0.2 mL water. Subsequent reactions were repeated by transferring the lipase to a fresh oil/methanol mixture without adding water.
[b]The ratio of methyl esters converted to the oil used as a substrate.

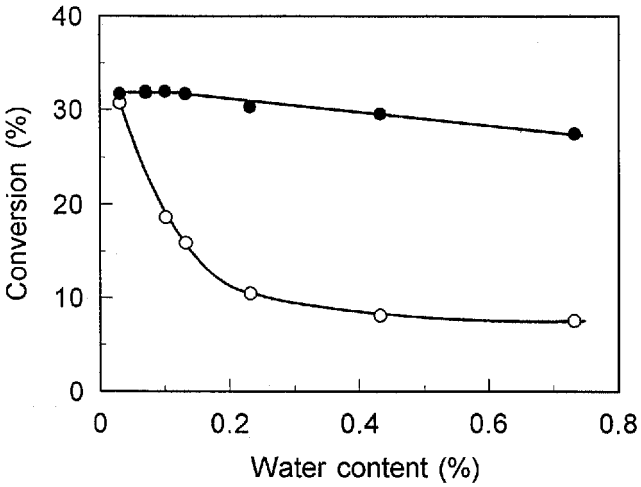

FIG. 1. Effect of water content on methanolysis of vegetable oil by *Candida antarctica* lipase. A reaction mixture of 10 g oil/methanol (1:1, mol/mol), 0.4 g immobilized *Candida* lipase and various amounts of water was shaken at 30°C. The water content in the substrate mixture before the reaction was measured by Karl Fischer titration. Ester conversion is expressed as the ratio of methyl esters converted to the oil used as a substrate. ○, Ester conversion after 6-h reaction; ●, ester conversion after 24-h reaction.

TABLE 2
Inhibition of Methanolysis of Vegetable Oil by Water[a]

	First reaction		Fifth reaction		
Water[b] (%)	Conversion[c] (%)	Hydrolysis (%)	Conversion[c] (%)	Hydrolysis (%)	Water[d] (ppm)
0	31.9	1.1	33.2	0.7	374
0.2	10.5	2.7	32.8	0.8	421
0.7	7.5	4.3	31.5	0.7	353
2.0	6.7	5.7	31.9	0.7	319

[a]The first reaction was performed at 30°C for 24 h in a mixture of 10 g oil/methanol (1:1, mol/mol), 0.4 g immobilized *Candida antarctica* lipase and indicated amount of water. Subsequent reactions were repeated with a fresh oil/methanol mixture without adding water. The results after 6-h reaction are presented.
[b]Amount of water added in the first reaction.
[c]The ratio of methyl esters converted to the oil used as a substrate.
[d]Water content in the mixture of the fifth reaction.

and fifth reactions. The percentage hydrolysis increased by adding water, whereas ME formation decreased. The addition of 2% water resulted in 5.7% hydrolysis and 6.7% ester formation after 6-h reaction. The excess water was removed by repeating the reaction four times with oil/methanol mixture without adding water (five times in total). As a result, hydrolysis decreased to the level of the reaction without adding water, and the ester formation increased. From these results, we conclude that lipase inhibition by water is reversible.

Effect of methanol content on methanolysis of vegetable oil. The oil was alcoholyzed at 30°C for 24 h with various amounts of methanol (Fig. 2). When more than 1.5 molar equivalents of methanol were present initially in the oil mixture, methanolysis decreased. The lipase was transferred to the mixture of oil/methanol (1:1, mol/mol) after the 24-h reaction in the 1:3 (mol/mol) mixture, and the alcoholysis repeated for 24 h. But restoration of lipase activity was not observed, indicating that the loss in activity was irreversible inactivation by methanol.

Other factors affecting methanolysis of vegetable oil. The oil was allowed to react at 30°C with 1 molar equivalent of methanol using 1 to 10% by weight of the lipase. The ester conversion reached 29.9% after 6 h in the reaction with 4% of the lipase. When the reaction time was extended to 24 h, more than 30% of the ester conversion was achieved with 2% of the lipase. Methanolysis of the oil was conducted over the temperature range of 20 to 60°C (Fig. 3). The ester conversion at 6 h increased with increasing temperature, with the optimal reaction temperature of 50 to 60°C. However, when the reaction was extended to 24 h, the ester conversion reached 30.5% even at 20°C. On the basis of the above results, the conditions other than reaction time were determined as follows: a mixture of oil/methanol (1:1, mol/mol) and 4% immobilized *Candida* lipase was incubated at 30°C at 130 oscillations/min.

Time course of methanolysis of vegetable oil. At least 3 molar equivalents of methanol are required for the complete conversion of the oil to its corresponding ME. The lipase, however, was inactivated by adding more than 1 molar equivalent of methanol, and methanolysis ceased. Hence, the reaction was performed by adding 1 molar equivalent of methanol every 24 h. A typical time course is shown in Figure 4. In the first step reaction in oil/methanol (1:1, mol/mol), ester conversion reached 32.5% at 7 h. With the addition of a second molar equivalent of methanol after 24 h, ester conversion reached 65.4% after 7 h (total, 31 h). A third molar equivalent of methanol was added again after a total of 48 h, and the reaction continued. After 24 h (72 h total), 98.4% of the oil was converted to its corresponding ME, showing that the stepwise addition of methanol was effective for complete conversion of the oil.

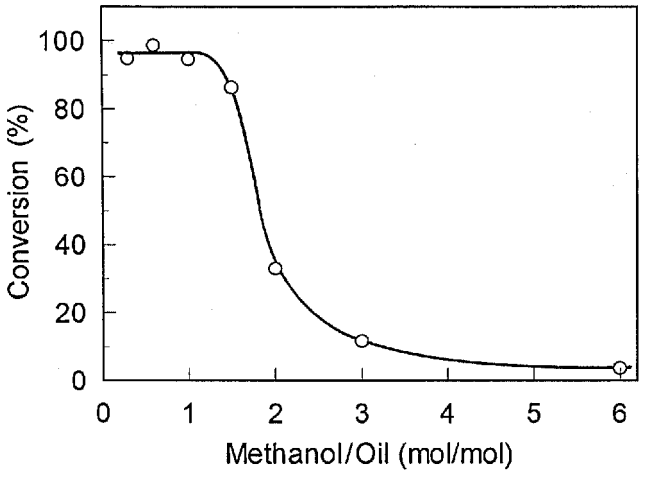

FIG. 2. Effect of methanol content on methanolysis of vegetable oil. A mixture of 10 g oil/methanol and 0.4 g immobilized lipase was shaken at 30°C for 24 h. The conversion was expressed as the amount of methanol consumed for the ester conversion of the oil (when the molar ratio of methanol/oil was less than 3), and as the ratio of methyl ester to the oil (more than 3).

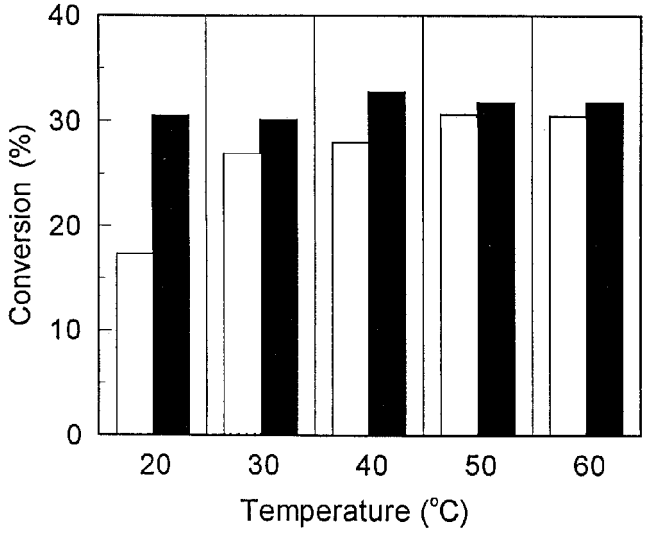

FIG. 3. Effect of temperature on methanolysis of vegetable oil. A mixture of 10 g oil/methanol (1:1, mol/mol) and 0.4 g immobilized *Candida antarctica* lipase was shaken at the temperature indicated. □, Ester conversion after 6 h; ■, ester conversion after 24 h.

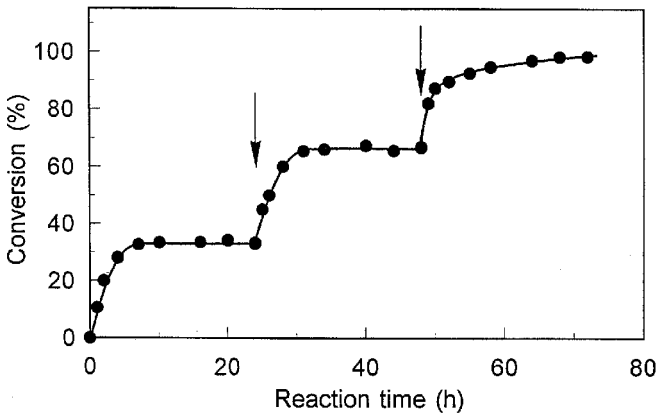

FIG. 4. Time course of methanolysis of vegetable oil. A reaction mixture of 28.95 g oil, 1.05 g methanol (the molar ratio of methanol to the oil, 1:1), and 1.2 g immobilized *Candida* lipase was shaken at 30°C. After 24- and 48-h reactions, 1.05 g methanol was added as indicated with arrows.

Continual conversion of vegetable oil to corresponding ME. To shorten the reaction time, the three-step reaction was conducted as follows: The first step was performed in a mixture of oil/methanol (1:1, mol/mol) for 10 h; the second molar equivalent of methanol was added and the reaction was continued for 14 h; after the third addition of methanol, the reaction was continued for 24 h. This procedure shortened the total reaction time from 72 to 48 h. The 48-h three-step reaction was repeated by transferring the enzyme to a fresh oil/methanol mixture. As shown in Table 3, ester conversion decreased slightly with increasing cycle number, but >95% conversion was maintained even after 50 reaction cycles. The content of glycerides remaining in the reaction mixture was low, with partial glycerides being main components and triglycerides minor components. In addition, concomitant hydrolysis was negligible when the reaction was done using substrates containing less than 400 ppm water. These results confirmed that lipase inactivation was avoided by the stepwise addition of methanol.

The reaction mixture containing >95% ME easily separated into two layers with standing. The glycerol content in the ME layer and the ME content in the glycerol layer were 0.05 and 0.23%, respectively. These results show that glycerol and ME can be separated merely by letting the reaction mixture stand.

TABLE 3
Continual Conversion of Vegetable Oil to Corresponding Methyl Esters by Immobilized *Candida antarctica* Lipase

Cycle[a]	Composition (%)				Hydrolysis (%)
	ME[b]	MG[c]	DG[d]	TG[e]	
1	97.4	1.6	1.0	n.d.[f]	1.0
5	97.0	1.6	1.4	n.d.	0.5
10	97.5	1.4	1.1	n.d.	0.4
20	96.7	1.6	1.4	0.3	0.2
30	96.3	1.7	1.6	0.4	0.2
50	96.2	1.7	2.1	n.d.	0.2

[a]One cycle of reaction: the first step, for 10 h in a mixture of 10 g oil/methanol (1:1, mol/mol); the second step, for 12 h after adding the second molar equivalent of methanol; the third step, for 24 h after adding the third molar equivalent of methanol.
[b]Methyl esters.
[c]Monoglycerides.
[d]Diglycerides.
[e]Triglycerides.
[f]Not detectable.

DISCUSSION

We have described the continuous production of biodiesel from vegetable oil by repeating the three-step reaction with stepwise addition of methanol. The process presented here has the following features: (i) glycerol and ME layers readily separate by standing the reaction mixture; (ii) the enzymatic process is easier to conduct than the chemical process; (iii) the problem of cost may be solved by continuous reaction in a fixed-bed reactor; and (iv) the risk of explosion can be eliminated since no solvent is required. When these advantages are considered, an enzymatic process may be more effective than a chemical process.

In general, it is known that lipases act on long-chain fatty alcohols better than on short-chain fatty alcohols (9,10). This property may be correlated with inactivation of the enzyme in addition to alcohol selectivity. Indeed, *Candida* lipase was inactivated in the mixture containing >1.5 molar equivalents of methanol in the oil (Fig. 2). Additionally, one molar equivalent of methanol or less readily dissolves in the oil. In mixtures containing >1.5 molar equivalents of methanol, the excess amount of methanol remained as droplets dispersed in the oil. When the lipase contacts these methanol droplets, it may be inactivated. It was reported that methanolysis of oils occurred in a mixture with *n*-hexane even though 3 molar equivalents of methanol was present (6). This result can be explained if there was the complete dissolution of the methanol in the medium. A reaction system in which methanol dissolves completely is necessary for the lipase-catalyzed methanolysis of oils.

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