

Continuous Production of Biodiesel Fuel from Vegetable Oil Using Immobilized *Candida antarctica* Lipase

Yomi Watanabe^a, Yuji Shimada^{a,*}, Akio Sugihara^a, Hideo Noda^b,
Hideki Fukuda^c, and Yoshio Tominaga^a

^aOsaka Municipal Technical Research Institute, Osaka 536-8553, Japan, ^bKansai Chemical Engineering Co. Ltd., Hyogo 660-0053, Japan, and ^cDivision of Molecular Science, Graduate School of Science and Technology, Kobe University, Hyogo 657-8501, Japan

ABSTRACT: *Candida antarctica* lipase is inactivated in a mixture of vegetable oil and more than 1:2 molar equivalent of methanol against the total fatty acids. We have revealed that the inactivation was eliminated by three successive additions of 1:3 molar equivalent of methanol and have developed a three-step methanolysis by which over 95% of the oil triacylglycerols (TAG) were converted to their corresponding methyl esters (ME). In this study, the lipase was not inactivated even though 2:3 molar equivalent of methanol was present in a mixture of acylglycerols (AG) and 33% ME (AG/ME33). This finding led to a two-step methanolysis of the oil TAG: The first-step was conducted at 30°C for 12 h with shaking in a mixture of the oil, 1:3 molar equivalent of methanol, and 4% immobilized lipase; the second-step reaction was done for 24 h after adding 2:3 molar equivalent of methanol (36 h in total). The two-step methanolysis achieved more than 95% of conversion. When two-step reaction was repeated by transferring the immobilized lipase to a fresh substrate mixture, the enzyme could be used 70 cycles (105 d) without any decrease in the conversion. From the viewpoint of the industrial production of biodiesel fuel production, the two-step reaction was conducted using a reactor with impeller. However, the enzyme carrier was easily destroyed, and the lipase could be used only several times. Thus, we attempted flow reaction using a column packed with immobilized *Candida* lipase. Because the lipase packed in the column was drastically inactivated by feeding a mixture of AG/ME33 and 2:3 molar equivalent of methanol, three-step flow reaction was performed using three columns packed with 3.0 g immobilized lipase. A mixture of vegetable oil and 1:3 molar equivalent of methanol was fed into the first column at a constant flow rate of 6.0 mL/h. The eluate and 1:3 molar equivalent of methanol were mixed and then fed into the second column at the same flow rate. The final step reaction was done by feeding a mixture of eluate from the second column and 1:3 molar equivalent of methanol at the same flow rate. The ME content in the final-step eluate reached 93%, and the lipase could be used for 100 d without any decrease in the conversion.

Paper no. J9352 in *JAOCs* 77, 355–360 (April 2000).

KEY WORDS: Biodiesel fuel, *Candida antarctica*, continuous reaction, fixed-bed bioreactor, immobilized enzyme, lipase, methanolysis, vegetable oil.

*To whom correspondence should be addressed at Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536-8553, Japan. E-mail: shimaday@omtri.city.osaka.jp

Fatty acid methyl esters (biodiesel fuel) originating from vegetable oils and animal fats have drawn attention in the last decade as a renewable, biodegradable, and nontoxic fuel. Because of these environmental advantages, biodiesel fuel is expected as a substitute for conventional fossil fuel and has been industrially produced from vegetable oils in North America and Europe (1,2) and from waste edible oils in Japan by chemical processes. Chemical processes give high conversion of triacylglycerols (TAG) to their corresponding methyl esters (ME) in short reaction times but have drawbacks such as being energy intensive, difficulty in recovering glycerol, the need for removal of alkaline catalyst from the product and treatment of alkaline wastewater, and the interference of the reaction by free fatty acids and water. Enzymatic methods can overcome these problems but have not been industrialized because of the high cost of enzyme. We have been trying to develop the continuous methanolysis of vegetable oil TAG in a organic solvent-free system for the purpose of practical application of enzymatic method.

Several reports describe enzymatic alcoholysis of TAG. When ethanol, isopropanol, butanol, and long-chain alcohols were used as substrates, TAG were efficiently converted to their fatty acid esters even in solvent-free systems (3–11). However, the efficiency of methanolysis was very low, although it was high in a system containing *n*-hexane (9). We previously reported that the irreversible inactivation of *Candida antarctica* lipase by methanol caused the low conversion of vegetable oil TAG to ME in a solvent-free system, and that the lipase was not inactivated in the presence of 1:3 molar equivalent of methanol against total fatty acids in the oil (12). The reaction was thus conducted by three successive additions of 1:3 molar equivalent of methanol after consumption of methanol. The three-step reaction converted more than 95% fatty acids in the oil to their corresponding ME. To adopt the stepwise methanolysis as an industrial process, it is needed to further increase the reaction efficiency and/or to develop a continuous flow reaction system with fixed-bed bioreactor.

This paper shows that *Candida* lipase is not inactivated when 2:3 molar equivalent of methanol is present in a mixture of acylglycerols (AG) and 33% ME. On the basis of this

finding, we devise two-step batch reaction. In addition, continuous conversion of vegetable oil TAG to ME is accomplished by three-step flow reaction with three fixed-bed bioreactors, although two-step flow reaction is not successful because of the irreversible inactivation of the lipase.

MATERIALS AND METHODS

Materials. Vegetable oil (a mixture of soybean and rapeseed oils) was donated by Showa Sangyo Co. Ltd. (Tokyo, Japan). Molar amount of the oil TAG 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. Immobilized *C. antarctica* lipase (Novozym 435) was obtained from Novo Nordisk (Bagsvaerd, Denmark).

Methanolysis of vegetable oil. Batch reaction was performed at 30°C in a 20- or 50-mL screw-capped vessel with shaking at 130 oscillations/min using 4% immobilized *Candida* lipase as a catalyst. Two-step batch reaction was conducted as follows: The first step was done in a mixture of vegetable oil and 1:3 molar equivalent of methanol against total fatty acids in the oil, and 2:3 molar equivalent of methanol was then added after consumption of methanol. Continual batch reaction was repeated by transferring the lipase to a fresh substrate mixture.

Flow reaction was conducted at 30°C using two or three columns packed with 3.0 g immobilized *Candida* lipase (15 × 80 mm). Three-step flow reaction was performed as follows: The first step was done by feeding a substrate mixture of the oil and 1:3 molar equivalent of methanol with a peristaltic pump. The substrate of the second-step reaction was prepared by removing glycerol (lower layer) after settlement overnight (named glycerol-free eluate), and fed into the second column after mixing with 1:3 molar equivalent of methanol. The third-step methanolysis was done by feeding a mixture of the glycerol-free second-step eluate and 1:3 molar equivalent of methanol to the third column. On the other hand, two-step flow reaction was performed as follows: The first step was done under the same conditions as those of the three-step flow reaction, and the second step was done by feeding a mixture of the glycerol-free first-step eluate and 2:3 molar equivalent of methanol to the second column.

Preparation of a mixture of AG and ME. Methanolysis was performed using three 100-mL screw-capped vessels. A mixture of 72.37 g vegetable oil, 2.63 g methanol (1:3 molar equivalent against total fatty acids in the oil), and 3.0 g immobilized *Candida* lipase was admitted into each vessel and shaken for 12 h at 30°C and 130 oscillations/min. The methanolysis was repeated 10 times by transferring the lipase to a fresh substrate mixture. All reaction mixtures were combined, and the glycerol layer was removed after settlement overnight. The amount of the resulting mixture of AG and ME was 2,070 g, and the ME content was 33.2%. The mixture is named AG/ME33.

A mixture (75 g) of AG/ME33, 1:3 molar equivalent of

methanol, and 3.0 g immobilized *Candida* lipase was shaken under the same conditions as those of the preparation of AG/ME33. The methanolysis was repeated 10 times by transferring the lipase to a fresh substrate mixture. Glycerol in the reaction mixture was removed as described above. The amount of the resulting mixture was 680 g, and the ME content was 65.9%. The mixture is named AG/ME66.

Analysis. The ME content 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, Folsom, CA) using tricaprylin as an internal standard. The composition of ME, monoacylglycerols (MAG), diacylglycerols (DAG), and TAG in the reaction mixture was analyzed on a DB-1ht capillary column (0.25 mm × 15 m; J&W Scientific). The analyses on both columns were performed under the conditions described elsewhere (12). Water content was measured by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan).

RESULTS

Effect of methanol content on methanolysis of AG in a mixture containing 33% ME. Immobilized *Candida* lipase was irreversibly inactivated in the presence of more than 1:2 molar equivalent of methanol against total fatty acids in vegetable oil TAG (12). A part of methanol remained as droplets dispersed in the oil. We presumed that lipase was inactivated by the methanol droplets in the substrate, but not by dissolved methanol. Because methanol solubility in the mixture of oil and ME is higher than that in the oil alone, the lipase may not be inactivated even in the presence of more than 1:2 molar equivalent of methanol in a mixture of AG and 33% ME (AG/ME33). Thus AG/ME33 was used as a substrate and AG were alcoholized at 30°C for 24 h with various amounts of methanol using 4% immobilized *Candida* lipase (Fig. 1). Methanol was soluble up to 2:3 molar equivalent in AG/ME33, and equal molar equivalent of methanol did not reduce the conversion of AG to ME as expected. After AG/ME33 was alcoholized with more than 2:3 molar equivalent of methanol, the enzyme was transferred to a fresh substrate mixture of AG/ME33 and 2:3 molar equivalent of methanol, and the subsequent reactions were done under the same conditions. The initial velocities of the reactions with lipases, which were used in the presence of 1:1, 5:3, and 7:3 molar equivalent of methanol, decreased to 88, 66, and 3% of that of the reaction with lipase used in the presence of 2:3 molar equivalents of methanol, respectively. These findings showed that more than equal amount of methanol against total fatty acids in the reaction mixture irreversibly inactivated the lipase even though AG/ME33 was used as a substrate.

Two-step methanolysis of vegetable oil TAG. Methanol is one of the two substrates in the methanolysis of TAG. In general, the velocity of enzyme reaction depends on the concentration of substrate at the limited concentration. Hence, the higher concentration of methanol is effective for the increase

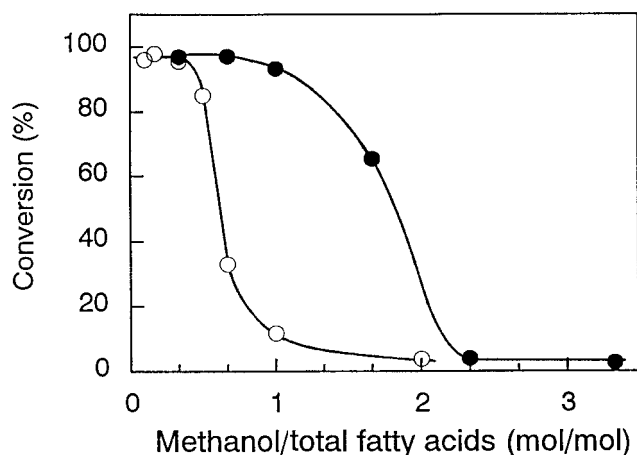


FIG. 1. Methanol inhibition of immobilized *Candida antarctica* lipase in the presence of methyl esters. One reaction mixture (○) was composed of vegetable oil, methanol, and immobilized lipase, and the other mixture (●) was composed of acylglycerols/33% methyl esters mixture (AG/ME33), methanol, and immobilized lipase. The reaction mixture (10 mL) was shaken at 30°C for 24 h together with 4% immobilized lipase. Methanol amount was expressed as the molar ratio to the amount of total fatty acids in the system. The conversion was expressed as the amount of methanol consumed for the ester conversion of acylglycerols (when the amount of methanol is less than that of fatty acids in acylglycerols), and as the molar ratio of methyl esters to total fatty acids in the system (when the amount of methanol is more than that of fatty acids in acylglycerols).

in the methanolysis velocity. However, because more than 1:2 molar equivalent of methanol inactivated the lipase in the reaction using vegetable oil and methanol as substrates, the first-step methanolysis was conducted at 30°C in a mixture of the oil, 1:3 molar equivalent of methanol, and 4% immobilized *Candida* lipase. Methanol was completely consumed after 7 h, and 33% of fatty acids in the oil was converted to ME (Fig. 2). As shown in Figure 1, the lipase was stable in a mixture of AG/ME33 and 2:3 molar equivalent of methanol against total fatty acids in the reaction mixture. Therefore, 2:3 molar equivalent of methanol was added in the 7-h reaction mixture, and the time course was compared with that of the reaction with 1:3 molar equivalent of methanol (Fig. 2). As expected, the velocity of reaction with 2:3 molar equivalent methanol was faster than that of the reaction with 1:3 molar equivalent of methanol, and 96.1% of fatty acids in the oil were converted to their ME after 20 h (27 h in total). Meanwhile, because the addition of second 1:3 molar equivalent of methanol resulted in the complete consumption of methanol after 10 h (17 h in total), the third 1:3 molar equivalent of methanol was added again in the reaction mixture. The velocity of the third-step methanolysis was slow compared with those of the first and second steps, and it took 20 h for the reaction to reach 96.5% conversion (37 h in total). These results show that two-step methanolysis saves 27% of the reaction time compared with three-step methanolysis.

Continual two-step batch reaction. Continual two-step batch methanolysis was performed as follows: The first-step

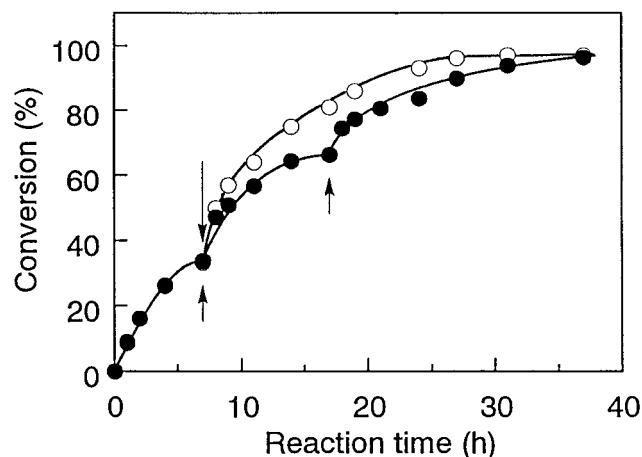


FIG. 2. Time courses of two- and three-step methanolyses of vegetable oil triacylglycerols. Two-step reaction (○): A mixture of 28.95 g vegetable oil, 1.05 g methanol (1:3 molar equivalents against the total fatty acids), and 4% immobilized *Candida* lipase was incubated at 30°C with shaking. After 7-h reaction, 2.10 g methanol (2:3 molar equivalents) was added in the reaction mixture. Three-step reaction (●): The first-step reaction was conducted under the same conditions as those of two-step reaction, and 1.05 g methanol (1:3 molar equivalents) was added after 7- and 17-h reactions. Downward and upward arrows indicate the addition of 2:3 and 1:3 molar equivalent of methanol, respectively.

reaction was conducted at 30°C for 12 h in a mixture of 9.65 g oil, 0.35 g methanol (1:3 molar equivalent against total fatty acids), and 4% of immobilized *Candida* lipase, and the second step was done for 24 h after adding 0.70 g methanol (2:3 molar equivalent). The 36-h two-step reaction was repeated by transferring the lipase to a fresh first-step oil/methanol mixture. More than 95% of conversion was maintained during 70 cycles (105 d), and the remaining AG were mainly partial AG and not TAG. The hydrolysis extent was less than 0.25%, although the substrate mixture contained 250–450 ppm of water. We have reported that more than 95% of conversion was maintained during 50 cycles (100 d) in three-step methanolysis: the first step, 10 h; the second step, 14 h; and the third step, 24 h (48 h in total) (12). The 36-h two-step methanolysis increased the productivity of 25% with the same lipase durability.

With the aim of industrial application of enzymatic method to the production of biodiesel fuels, the two-step batch reaction was performed using a 1-L reactor with impeller. But, the lipase carrier was easily destroyed by the shearing force, and the immobilized lipase could be used only a few times. Hence, we next attempted continuous flow reaction with a fixed-bed bioreactor to use the lipase for a long period.

Two-step flow reaction with fixed-bed bioreactor. Two kinds of substrate mixtures were fed into columns packed with 3.0 g of the lipase (15 × 80 mm) at 30°C and a flow rate indicated; one substrate mixture consisted of vegetable oil and 1:3 molar equivalent of methanol against total fatty acids, and the other mixture consisted of AG/ME33 and 2:3 molar equivalent of methanol (Table 1). In the reaction with the former substrates, the conversion increased as the flow rate de-

TABLE 1
Effect of Flow Rate on Two-Step Methanolysis of Vegetable Oil Triacylglycerols with Fixed-Bed Reactor^a

Substrate		Flow rate (mL/h)	ME content ^d (%)
ME content ^b (%)	Methanol content ^c (molar equivalent)		
0	1:3	5.9	33.2
		9.8	30.4
		18.4	21.5
		39.5	11.0
		80.2	9.4
33	2:3	4.1	91.6
		6.0	91.2
		9.5	65.9
		18.1	45.8
		40.4	33.2

^aThe preparation of fixed-bed reactor and the operation conditions were described in the text.

^bThe content of methyl esters (ME) in glycerides.

^cThe content of methanol is expressed as the molar equivalent against the total fatty acids.

^dThe content of ME in the reaction mixture eluted from the column.

creased. Methanol was almost consumed at a flow rate of 5.9 mL/h (5.3 g/h), and the conversion reached 33.2%. In the reaction with the latter substrates, the conversion rate increased with decreasing the flow rate, but the conversion of the oil TAG was 91.6% even though the flow rate was reduced to 4.1 mL/h (3.6 g/h).

A substrate mixture of AG/ME33 and 2:3 molar equivalent of methanol was fed into a column packed with 3.0 g immobilized lipase at a constant flow rate of 4.4 mL/h for 10 d, and the ME content in the eluate was assayed. The content was 91.2% at the first day, but decreased to 51.6 and 34.9% after 5 and 10 d, respectively. The immobilized lipase was then taken out of the column completely, and shaken at 30°C in 75 g mixture of the oil and 1:3 molar equivalent of methanol (Fig. 3). While the reaction with 3.0 g of the fresh immobilized lipase consumed 98.5% methanol (the ME content, 32.8%) after 10 h, the used enzyme showed only a slight activity. This result shows that the decrease in the conversion at the second step of two-step flow reaction is due to the irreversible inactivation of the lipase.

Continuous three-step flow reaction with fixed-bed bioreactor. Because immobilized *Candida* lipase was not inactivated at the lower concentration of methanol, we attempted methanolysis of vegetable oil TAG by three-step flow reaction. Table 2 shows the effect of flow rate on the second- and third-step reactions. The reaction was conducted at 30°C, and the flow rate indicated using a column packed with 3.0 g immobilized lipase. When a mixture of AG/ME33 and 1:3 molar equivalent of methanol was fed into the column to investigate the effect of flow rate on the second-step reaction, the ME content increased as the flow rate decreased and reached 65.0% at a flow rate of 5.7 mL/h (5.0 g/h). The effect of flow rate on the third-step reaction was also investigated using a mixture of AG/ME66 and 1:3 molar equivalent of methanol

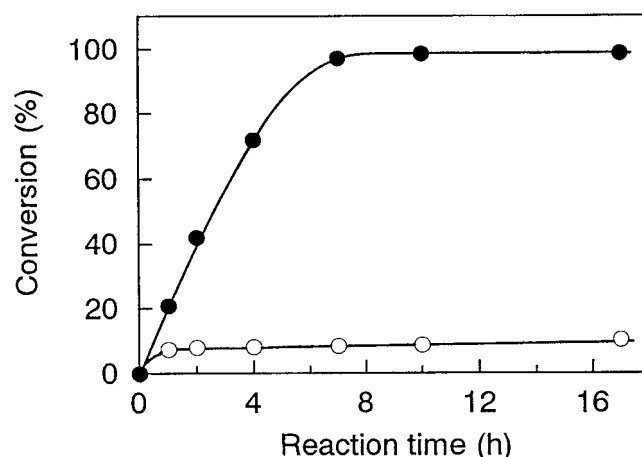


FIG. 3. Activity of immobilized *Candida* lipase used in two-step flow reaction. The flow reaction was done by feeding a substrate mixture of AG/ME33 and 2:3 molar equivalent of methanol against the total fatty acids, as described in the text. The lipase used in the flow reaction (○) and fresh lipase (●) were used as catalysts in methanolysis of vegetable oil. A mixture of 72.37 g vegetable oil, 2.63 g methanol (1:3 molar equivalent against the total fatty acids), and 3.0 g immobilized lipase was incubated at 30°C with shaking. The conversion was expressed as the amount of methanol consumed for the ester conversion of the oil triacylglycerols. For abbreviation see Figure 1.

as substrates. As a result, the ME contents were reached 93.2 and 94.3% at a flow rate of 6.0 (5.2) and 4.5 mL/h (3.9 g/h), respectively.

On the basis of above results, three-step flow reaction was conducted at 30°C using three columns packed with 3.0 g immobilized lipase. A mixture of vegetable oil and 1:3 molar equivalent of methanol was continuously fed into the first column at a constant flow rate of 6.0 mL/h. The eluate was set-

TABLE 2
Effect of Flow Rate on Three-Step Methanolysis of Vegetable Oil Triacylglycerols with Fixed-Bed Reactor^a

Substrate		Flow rate (mL/h)	ME content ^d (%)
ME content ^b (%)	Methanol content ^c (molar equivalent)		
33	1:3	5.7	65.0
		10.0	63.7
		19.6	59.5
		42.0	51.3
		80.0	38.1
66	1:3	4.5	94.3
		6.0	93.2
		11.8	88.0
		21.0	82.9
		41.0	75.6

^aThe preparation of fixed-bed reactor and the operation conditions were described in the text.

^bThe content of ME in glycerides.

^cThe content of methanol is expressed as the molar equivalent against the total fatty acids.

^dThe content of ME in the reaction mixture eluted from the column. See Table 1 for abbreviation.

TABLE 3
Continuous Conversion of Vegetable Oil Triacylglycerols to Corresponding ME by a Three-Step Flow Reaction Using Immobilized *Candida antarctica* Lipase^a

Day	Composition (wt%)						
	First step	Second step	Third step				FFA
	ME	ME	ME	MAG	DAG	TAG	
1	30.4	63.7	93.7	2.4	2.8	0.9	0.2
10	32.7	64.7	94.2	2.3	2.9	0.4	0.2
30	32.7	63.6	93.9	2.4	2.5	1.0	0.2
50	32.0	64.2	92.3	2.9	3.4	1.2	0.2
75	32.3	65.1	93.0	2.6	3.6	0.7	0.1
100	32.2	64.5	92.6	3.0	3.3	0.9	0.2

^aReaction conditions were described in the text. ME, methyl esters; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols; FFA, free fatty acids.

tled overnight to remove glycerol. The second 1:3 molar equivalent of methanol was added in the glycerol-free eluate, and the substrate mixture was then fed continuously into the second column at the same flow rate, 6.0 mL/h. The final step reaction was conducted by continuously feeding a mixture of the glycerol-free eluate and 1:3 molar equivalent of methanol into the third column at the same flow rate. The ME contents in the eluate from each column are shown in Table 3. While three-step flow methanolysis was continued for 100 d, the conversions of the oil TAG to ME did not decrease. The conversions in the first, second, and third steps were 32, 64, and 93%, respectively. The ME content in the final product of flow reaction was slightly lower than that in the product of batch reaction. The acid value of the final-step eluate was less than 0.6, although the substrate mixture contained 250–500 ppm of water. In addition, remaining glycerides in the final-step eluate were partial AG as observed in two-step batch reaction. These results show that immobilized *Candida* lipase also works efficiently in three-step methanolysis without any decrease in the conversion.

DISCUSSION

We have shown that vegetable oil TAG was efficiently methanolized by two-step batch reaction and three-step flow reaction with immobilized *Candida* lipase, and that the lipase could be used for at least 100 d in both reaction systems without any significant decrease in the conversion. High cost of lipase is one of the most serious problems for the application of enzymatic process to the industrial production of biodiesel fuel from vegetable oil. However, the two processes developed in this study, especially continuous three-step flow reaction, can reduce the cost of lipase and shed light on the industrial production of biodiesel fuel by enzymatic method.

The addition of 2:3 molar equivalent of methanol did not inactivate *Candida* lipase at the second step of two-step batch reaction (Fig. 1) but inactivated the lipase at the second step of two-step flow reaction (Fig. 3). This discrepancy may be explained by the effect of by-product of methanolysis, glyc-

erol. In the flow reaction with fixed-bed reactor, quite a lot of glycerol remained in the bottom of the column because of its high viscosity. The glycerol disturbs the diffusion of substrates to lipase molecule, and the decrease in reaction efficiency gradually increases the unreacted methanol. Excess amount of methanol migrates from AG/ME layer to glycerol layer, and the lipase is inactivated by higher concentration of methanol in glycerol layer. On the other hand, the amount of methanol in two-step batch reaction is fixed, and the immobilized lipase is not covered with glycerol by quick shaking. Therefore, the lipase in two-step batch reaction may not be inactivated by methanol.

One cycle of two-step batch reaction was conducted for 36 h with 4% immobilized lipase. Thus the daily amount of substrates converted to ME by 1 g lipase is 16.7 g/g/d. Meanwhile, three-step flow reaction was done at the flow rate of 6.0 mL/h (5.2 g/h) using three columns packed with 3 g lipase. The daily amount of substrates converted to ME by 1 g lipase is thus calculated as 13.9 g/g/d, and the two-step batch reaction was more effective than three-step flow reaction. However, the lipase carrier was easily destroyed in a reactor with impeller, and the enzyme could be used only a few times in spite of being stable for long period. This result shows that a special type of fluid-bed bioreactor is necessary for the two-step batch reaction. On the other hand, continuous flow reaction using a fixed-bed bioreactor does not destroy the carrier of the lipase and is generally useful for the reduction of production cost. In addition, the immobilized lipase used in the three-step batch reaction described in our previous paper (12) is still active, showing that the enzyme is stable for at least 2 yr. These facts suggest that the cost for producing biodiesel fuel by the three-step flow reaction becomes lower than that by chemical process.

ACKNOWLEDGMENT

This work was supported by the New Energy and Industrial Technology Development Organization.

REFERENCES

1. Krawczyk, T., Biodiesel: Alternative Fuel Makes Inroads but Hurdles Remain, *INFORM* 7:800–815 (1996).
2. Cvengros, J., and Z. Cvengrosova, Quality Control of Rapeseed Oil Methyl Esters by Determination of Acyl Conversion, *J. Am. Oil Chem. Soc.* 71:1349–1352 (1994).
3. Mittelbach, M., Lipase-Catalyzed Alcoholysis of Sunflower Oil, *Ibid.* 67:168–170 (1990).
4. Linko, Y.-Y., M. Lämsä, A. Huhtala, and P. Linko, Lipase-Catalyzed Transesterification of Rapeseed Oil and 2-Ethyl-1-hexanol, *Ibid.* 71:1411–1414 (1994).
5. Linko, Y.-Y., M. Lämsä, X. Wu, E. Uosukainen, J. Seppälä, and P. Linko, Biodegradable Products by Lipase Biocatalysis, *J. Biotechnol.* 66:41–50 (1998).
6. Show, J.-F., and D.-L. Wang, Lipase-Catalyzed Ethanolysis and Isopropanolysis of Triglycerides with Long-Chain Fatty Acids, *Enzyme Microb. Technol.* 13:544–546 (1991).
7. Breivik, H., G.G. Haraldsson, and B. Kristinsson, Preparation of Highly Purified Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid, *J. Am. Oil Chem. Soc.* 74:1425–1429 (1997).

8. Wu, W.H., T.A. Foglia, W.N. Marmer, and J.G. Phillips, Optimizing Production of Ethyl Esters of Grease Using 95% Ethanol by Response Surface Methodology, *Ibid.* 76:517–521 (1999).
9. Nelson, L.A., T.A. Foglia, and W.N. Marmer, Lipase-Catalyzed Production of Biodiesel, *Ibid.* 73:1191–1195 (1996).
10. Selmi, B., and D. Thomas, Immobilized Lipase-Catalyzed Ethanolsis of Sunflower Oil in a Solvent-Free Medium, *Ibid.* 75:691–695 (1998).
11. De, B.K., D.K. Bhattacharyya, and C. Bandhu, Enzymatic Synthesis of Fatty Alcohol Esters by Alcoholysis, *Ibid.* 76:451–453 (1999).
12. Shimada, Y., Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, and Y. Tominaga, Conversion of Vegetable Oil to Biodiesel Using Immobilized *Candida antarctica* Lipase, *Ibid.* 76:789–793 (1999).

[Received August 12, 1999; accepted January 13, 2000]