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Conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel by immobilized *Candida antarctica* lipase

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Abstract

Acid oil, which is a by-product in vegetable oil refining, mainly contains free fatty acids (FFAs) and acylglycerols, and is a candidate of materials for production of biodiesel fuel. A mixture (acid oil model) of refined FFAs and vegetable oil was recently reported to be converted to fatty acid methyl esters (FAMEs) at >98% conversion by a two-step reaction system comprising methyl esterification of FFAs and methanolysis of acylglycerols using immobilized *Candida antarctica* lipase. The two-step system was thus applied to conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel. Under similar conditions that were determined by using acid oil model, however, the lipase was unstable and was not durable for repeated use. The inactivation of the lipase was successfully avoided by addition of excess amounts of methanol (MeOH) in the first-step reaction, and by addition of vegetable oil and glycerol in the second-step reaction. Hence, the first-step reaction was conducted by shaking a mixture of 66 wt% acid oil (77.9 wt% FFAs, 10.8 wt% acylglycerols) and 34 wt% MeOH with 1 wt% immobilized lipase, to convert FFAs to their methyl esters. The second-step reaction was performed by shaking a mixture of 52.3 wt% dehydrated first-step product (79.7 wt% FAMEs, 9.7 wt% acylglycerols), 42.2 wt% rapeseed oil, and 5.5 wt% MeOH using 6 wt% immobilized lipase in the presence of additional 10 wt% glycerol, to convert acylglycerols to FAMEs. The resulting product was composed of 91.1 wt% FAMEs, 0.6 wt% FFAs, 0.8 wt% triacylglycerols, 2.3 wt% diacylglycerols, and 5.2 wt% other compounds. Even though each step of reaction was repeated every 24 h by transferring the immobilized lipase to the fresh substrate mixture, the composition was maintained for >100 cycles.

Keywords: Acid oil; Biodiesel fuel; *Candida antarctica*; Fatty acid methyl esters; Immobilized enzyme; Lipase; Methanolysis; Methyl esterification

1. Introduction

Growing concerns on environmental conservation and on a remarkable rise of petroleum price have demanded the use of fatty acid methyl esters (FAMEs) as a substitute of petroleum fuel. FAMEs derived from vegetable oil, are often called biodiesel fuel, have following advantages: (i) low concentration of unburned hydrocarbons, CO, small particulate matter, and SO_x in exhaust gas; (ii) no increase of carbon amount in total environment, unlike petroleum fuel. In order to provide FAMEs at a reasonable price, production of FAMEs not only from refined vegetable oils, but also from crude or waste materials and from by-products of oil processing has been attempted [1–7]. One of the materials is acid oil. Alkali deacidification, one of the steps in vegetable oil refining, by-produces soapstock that mainly contains soap and water. Acid oil is obtained by acidulation of the soapstock, and contains free fatty acids (FFAs), acylglycerols, and other lipophylic compounds. It is reproduced currently as industrial FFAs, although their demand is almost in saturation. Conversion of acid oil to biodiesel fuel is thus expected to avoid oversupply of the industrial FFAs and their price down.

There have been several reports on chemical and enzymatic conversion of acid oil to FAMEs [8,9]. However, two drawbacks were indicated to attain high degree of conversion in chemical process [8]. One was the addition of overexcess amounts of methanol (MeOH) and acid catalyst. The other was that the

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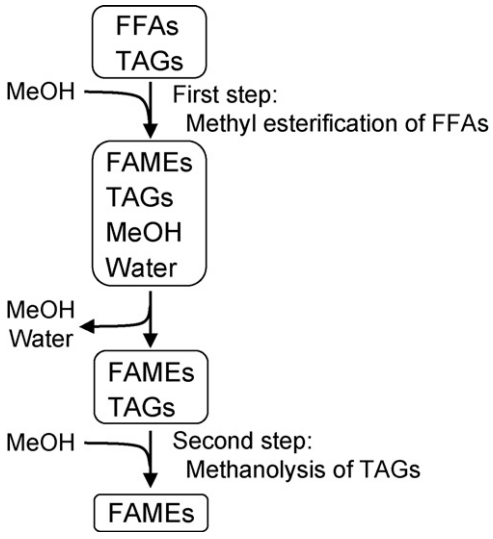

Scheme 1. Two-step reaction system to convert acid oil model to fatty acid methyl esters using immobilized *Candida antarctica* lipase. The amount of lipase for the first step was 0.5 wt%, and that for the second step was 6 wt%. FFAs, free fatty acids; TAGs, triacylglycerols; FAMEs, fatty acid methyl esters; MeOH, methanol.

complete hydrolysis of acylglycerols in acid oil to FFAs prior to FAME production was required to avoid the use of overexcess amounts of MeOH and acid. Meanwhile, enzymatic method in *n*-hexane converted only FFAs in acid oil to FAMEs, and acylglycerols remained as they were [9]. Furthermore, the lipase was not used repeatedly, though it was the immobilized one. We therefore attempted to construct a solvent-free system to achieve high degree of conversion of fatty acids (FAs) in acid oil to FAMEs, as well as repeated use of immobilized lipase for long term. Consequently, it was found that a two-step reaction system comprising methyl esterification of FFAs and methanolysis of triacylglycerols (TAGs) with immobilized *Candida antarctica* lipase converted an acid oil model (a mixture of refined rapeseed oil and FFAs) to FAMEs (Scheme 1) [10]. The first-step reaction is methyl esterification of FFAs in the acid oil with 2 mol of MeOH for FFAs. After removal of MeOH and water from the first-step product, the second-step methanolysis of TAGs was conducted with a stoichiometric amount of MeOH. The two-step reaction system converted >98% FAs in acid oil model to their methyl esters. In addition, when each step of reaction was repeated every 24 h, the lipase was reused for >100 cycles without significant decrease in the degrees of conversion. Because high conversion and long life of the lipase are advantageous for production of biodiesel fuel at low cost, the two-step reaction system was applied to the production of FAMEs from acid oil that was obtained in the vegetable oil refining. In this paper, we show how a modified two-step reaction is effective for conversion of acid oil.

2. Materials and methods

2.1. Materials

Acid oil, rapeseed oil rich in oleic acid, and FFAs of the rapeseed oil are products of the Nisshin OilliO Group Ltd. (Tokyo, Japan). The acid oil was centrifuged at 10,000 × *g* for 15 min to remove precipitates. The composition of the resulting supernatant, referred to as acid oil supernatant, is shown in Table 1. Its acid value and water content was 156 mg KOH/g and 1.1 wt%, respectively. FA composition of acid oil supernatant was 5.6 wt% palmitic acid, 2.0 wt% stearic acid, 39.3 wt% oleic acid, 44.9 wt% linoleic acid, 7.6 wt% α-linolenic acid, and 0.6 wt% other FAs. FA composition of rapeseed oil was 4.3 wt% palmitic acid, 2.0 wt% stearic acid, 62.4 wt% oleic acid, 20.1 wt% linoleic acid, 8.3 wt% α-linolenic acid, and 2.9 wt% other FAs. FA composition of FFAs from rapeseed oil was the same as that of rapeseed oil. Immobilized *C. antarctica* lipase was obtained from Novozymes (Bagsvaerd, Denmark). MeOH and glycerol were purchased from Wako Pure Chemicals Industries Co. Ltd. (Tokyo, Japan). Oleic acid methyl ester and tricaprylin were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

Table 1
Composition of materials

Components	Composition (wt%)	
	Acid oil supernatant[a]	Dehydrated first-step product[b]
Fatty acid methyl esters	0	79.7
Free fatty acids	77.9	2.1
Triacylglycerols	8.1	8.3
Diacylglycerols	2.7	1.4
Phytosterols	1.0	0.9
Fatty acid phytosteryl esters	2.0	2.0
Unknown compounds	8.3	5.6

[a] A supernatant recovered by centrifugation of acid oil at 10,000 × *g* for 15 min.
[b] An oil layer, recovered after the first-step reaction, was evaporated at 50 °C and 15 mmHg.

2.2. First-step reaction: methyl esterification of FFAs

A small-scale reaction was performed at 30 °C with shaking at 130 oscillations/min in a 50 mL screw-capped vessel containing a 30 g mixture of acid oil supernatant, MeOH, and immobilized *C. antarctica* lipase.

A large-scale reaction was conducted to prepare the substrate to be used for the second-step reaction. The reaction was performed at 30 °C in a 2 L two-necked round-bottomed flask containing 1056 g acid oil supernatant, 544 g MeOH, and 16 g immobilized *C. antarctica* lipase, with agitating at 100 rpm for 24 h. After the reaction, immobilized enzyme was separated by filtration. The recovered reaction mixture was separated to MeOH and oil layers by standing. Additionally, a small amount of middle layer existed at the interface and contained solid materials. The MeOH and middle layers were removed. The oil layer (lower layer) was then subjected to evaporation at 50 °C and 15 mmHg to remove remaining MeOH and water. The composition of the resulting preparation (1100 g; referred to as dehydrated first-step product) is shown in Table 1. The content of water in the preparation was 0.03%.

2.3. Second-step reaction: methanolysis of TAGs

When immobilized *C. antarctica* lipase was used as a catalyst for conversion of a mixture of TAGs/FAMEs (1:1, w/w) to FAMEs, the activity was increased by repeating the reaction [10]. Therefore, pretreatment of the immobilized lipase was performed in a 50 mL screw-capped vessel with shaking at 130 oscillations/min prior to the second-step reaction. A mixture of 14.2 g rapeseed oil, 14.2 g oleic acid methyl ester, 1.6 g MeOH (an equimolar amount for total FAs in the substrates) was shaken at 30 °C for 48 h with 1.8 g immobilized lipase. The pretreatment was repeated five cycles by transferring the lipase to the fresh substrate mixture. The activated lipase was used as a catalyst for the second-step reaction.

The second-step reaction was conducted at 30 °C in a 50 mL vessel with shaking. A 30 g mixture of dehydrated first-step product, rapeseed oil, and MeOH was shaken with 1.8 g immobilized *C. antarctica* lipase, which was activated previously, in the presence of 3.0 g glycerol. The second-step reaction by-produces glycerol. In order to reuse the glycerol, reaction mixture including glycerol was taken out from the reaction vessel. Glycerol layer was separated completely from the enzyme-free reaction mixture by standing overnight. In this study, however, glycerol layer was recovered by centrifugation at 10,000 × *g* for 5 min to save operation time.

2.4. Analyses

About 0.8 g of the reaction mixture was taken at time intervals and was used for analyses of the composition of TAGs, DAGs, FFAs and FAMEs by a TLC/flame ionization detector (FID) analyzer (Iatroscan MK-5, Iatron Laboratories Inc., Tokyo, Japan). A reaction mixture was dissolved in *n*-hexane at the concentration of 1 vol%. The solution (5 μL) was spotted on a silica gel rod, and the components were developed with a mixture of toluene/chloroform/acetic acid (50:20:1, v/v/v) and then with a mixture of *n*-hexane/diethyl ether (65:5, v/v). Acid value was determined by neutralization of FFAs in the sample with 0.1 or 1.0 M KOH solution.

Chemical conversion of FFAs and acylglycerols to FAMEs was performed at 75 °C for 10 min in 3 mL MeOH solution containing 3% BF_3 and NaOMe, respectively. The FAMEs were analyzed with a gas chromatograph (GC) 6890N (Agilent Technologies, CA, USA) equipped with a DB-23 capillary column (0.25 mm × 30 m, Agilent Technologies). The column temperature was kept at 150 °C for 0.5 min, and was increased to 170 °C at the rate of 4 °C/min, to 195 °C at the rate of 5 °C/min, and to 215 °C at the rate of 5 °C/min. It was then kept at 215 °C for 5 min. Injector and detector (FID) temperatures were set at 245 and 250 °C, respectively.

The contents of FAMEs, FFAs, phytosterols, FA phytosteryl esters, acylglycerols in acid oil supernatant and in the final product were determined with a GC equipped with a DB-1ht capillary column (0.25 mm × 5 m, Agilent Technologies) using tricaprylin as an internal standard. The column temperature was controlled at 120 °C for 0.5 min, and was increased to 280 °C at the rate of 15 °C/min, to 370 °C at the rate of 10 °C/min. It was then kept at 370 °C for 1 min. Injector and detector (FID) temperatures were set at 370 and 390 °C, respectively.

Table 2
Methyl esterification of acid oil supernatant using immobilized *Candida antarctica* lipase[a]

Cycle number	FAME content (wt%)		
	1 h	6 h	24 h
1	44.3	71.3	73.4
5	5.7	32.9	67.1
10	5.6	20.7	55.8

[a] A mixture of 27.2 g acid oil supernatant and 2.8 g MeOH was shaken at 30 °C for 24 h with 0.3 g immobilized *C. antarctica* lipase. The reaction was repeated 10 cycles every 24 h by transferring the lipase to a fresh substrate mixture.

3. Results

3.1. Effect of MeOH on the first-step reaction using immobilized C. antarctica lipase

The first-step reaction is methyl esterification of FFAs in acid oil supernatant prepared according to Section 2. A 30 g mixture of acid oil supernatant and an equimolar amount of MeOH for total FAs was shaken at 30 °C with 1 wt% immobilized *C. antarctica* lipase by weight of the reaction mixture. The contents of FAMEs at 1 and 24 h were 44 wt% (55% esterification) and 73 wt% (91% esterification), respectively (Table 2). The reaction was repeated by transferring the immobilized lipase to a fresh substrate mixture every 24 h. The contents of FAMEs decreased with repeating the reaction; the contents at 1 and 24 h in the l0th-cycle reaction were 6 and 56 wt%, respectively. On the other hand, a similar reaction was repeated using refined FFAs instead of acid oil supernatant: the reaction mixture was composed of 27.2 g FFAs from rapeseed oil, 2.8 g MeOH, and 0.3 g of the immobilized lipase. The contents of FAMEs at 1 and 24 h in the first cycle were 45 wt% (55% esterification) and 72 wt% (89% esterification), respectively. Even in the l0th-cycle reaction, the contents at 1 and 24 h decreased scarcely: 38 and 71 wt%, respectively. These results indicated that the lipase was unstable in a mixture of 27.2 g acid oil supernatant and 2.8 g MeOH, although it was stable in esterification of refined FFAs with MeOH.

In order to find conditions under which the immobilized lipase is stable, the first-step reaction was conducted in the presence of different amounts of MeOH (Fig. 1). The content of FAMEs in 1 h reaction mixture decreased with increasing the amount of MeOH, and the content in 24 h reaction mixture was >78 wt% (esterification of FFAs, >96%) when using 3–7.5 molar amounts of MeOH for total FAs (Fig. 1A). TAGs and DAGs were detected, but monoacylglycerols were not. The total content of acylglycerols slightly decreased with increasing the amount of MeOH; the content decreased from 11 to 7 wt% at 7.5 mol MeOH, and decreased to 6 wt% at 10 mol MeOH.

Each reaction with a different amount of MeOH was repeated by transferring the lipase to a fresh substrate mixture every 24 h. The half life of the lipase was calculated based on an increase in the content of FAMEs during the first 1 h. The

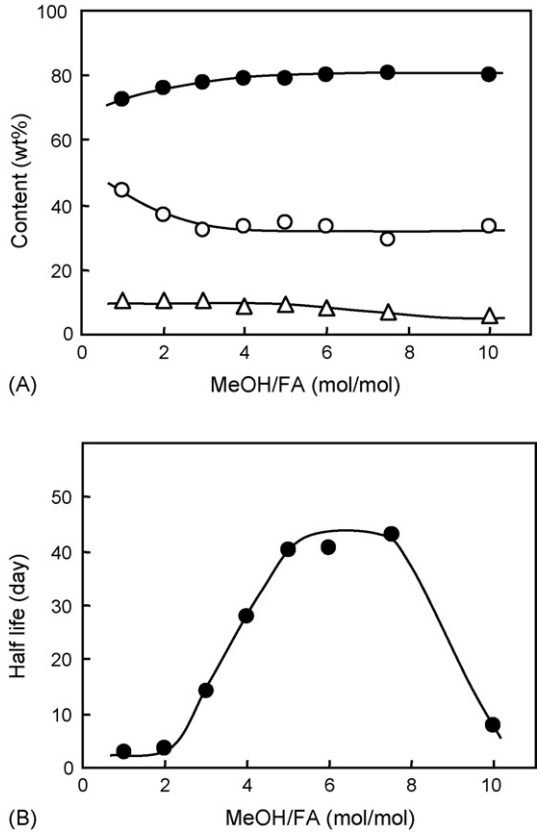

Fig. 1. Effect of MeOH amount on the first-step conversion of acid oil to FAMEs. A 30 g mixture of acid oil supernatant and MeOH (1:1–1:10, mol/mol) were shaken at 30 °C for 24 h with 0.3 g immobilized *C. antarctica* lipase. The reaction was repeated every 24 h by transferring the lipase to a fresh substrate mixture. (A) Contents of FAMEs and acylglycerols in the first-cycle reaction. The content of acylglycerols before the reaction was 10.8 wt% (8.1 wt% TAGs and 2.7 wt% DAGs). Open circles, content of FAMEs at 1 h; closed circles, at 24 h; open triangles, content of acylglycerols at 24 h. (B) Half life of immobilized lipase. The half life was calculated based on an increase in the content of FAMEs during the first 1 h.

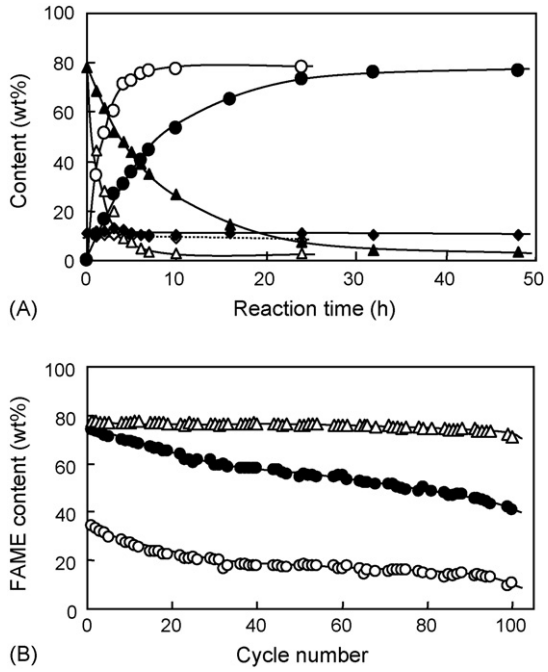

Fig. 2. Continual first-step reaction of acid oil supernatant. A mixture containing 19.8 g acid oil supernatant and 10.2 g MeOH was shaken at 30 °C for 24 h with 0.3 g immobilized *C. antarctica* lipase. The reaction was repeated every 24 h by transferring the lipase to a fresh substrate mixture. (A) Time course of the reaction. Open circles, content of FAMEs in the first-cycle reaction; closed circles, in the l00th-cycle reaction; open triangles, content of FFAs in the first-cycle reaction; closed triangles, in the l00th-cycle; open diamonds, content of acylglycerols in the first-cycle reaction; closed diamonds, in the l00th-cycle. (B) Content of FAMEs in the repeated reaction. Open circles, content of FAMEs at 1 h; closed circles, at 6 h; open triangles, at 24 h.

half life reached a maximum value (*ca*. 40 days) in the presence of 5–7.5 molar amounts of MeOH for total FAs (Fig. 1B). Even though the reactions with these amounts of MeOH were repeated 20 cycles, the content of FAMEs at 24 h maintained >76%. These results showed that the inactivation of the lipase by an equimolar amount of MeOH (Table 2) was disappeared by adding 5–7.5 mol MeOH to the reaction mixture. Hence, the amount of MeOH in the first-step reaction was determined at five molar amounts for total FAs, which is 34 wt% of the reaction mixture.

3.2. Continual first-step reaction of acid oil using immobilized C. antarctica lipase

The first-step reaction was conducted using 0.5–4 wt% immobilized *C. antarctica* lipase in the presence of five molar amounts of MeOH. The reaction velocity depended on the amount of lipase, and the content of FAMEs after 24 h in all reactions reached >76 wt% (esterification of FFAs, >95%). Here, the amount of the lipase was set at 1 wt%. The time course is shown in Fig. 2A. The content of FAMEs reached a constant value (77 wt%) after 10 h.

The first-step reaction was conducted repeatedly in a 30 g mixture of acid oil supernatant and MeOH (66:34, w/w) using 0.3 g immobilized *C. antarctica* lipase (Fig. 2B). The contents of FAMEs in 1 and 6 h reaction mixtures were gradually decreased with increasing the cycle number. However, the content in 24 h reaction mixture maintained >76 wt% (esterification of FFAs, >94%) for the first 60 cycles (60 days) and was 71 wt% (88%) at the 100th cycle. The time course in the 100th-cycle reaction is shown in Fig. 2A. The use of immobilized lipase for 100 cycles decreased the activity to 1/3 of the original activity. The content of acylglycerols after the reaction did not change during 100 cycles: 11 wt%.

3.3. Addition of refined oil to the second-step reaction

Dehydrated first-step product was prepared according to Section 2 and was used as a substrate for the second-step reaction (methanolysis of acylglycerols).

A mixture of 28.35 g dehydrated first-step product and 1.65 g MeOH was shaken at 30 °C with 1.8 g (6 wt%) immobilized *C. antarctica* lipase, which was preactivated according to Section 2. After 24 h, the content of FAMEs increased from 80 to 91 wt%, and the content of acylglycerols decreased from 10 to 0.9 wt%

Table 3
Effect of TAGs on stability of immobilized *C. antarctica* lipase in the second-step reaction

Composition of reaction mixture (g)			Acylglycerol content[a] (wt%)	Half life[b] (day)
Dehydrated first-step product[c]	Rapeseed oil	MeOH		
28.35	0	1.65	9.7[c]	5
23.55	4.80	1.65	25	8
18.84	9.51	1.65	40	16
15.70	12.65	1.65[d]	50	32
12.40	15.60	2.00[d]	60	44

[a] Content of acylglycerols in a mixture of rapeseed oil and dehydrated first-step product.
[b] Reaction mixture was shaken at 30 °C for 24 h with 1.8 g immobilized *C. antarctica* lipase. The reactions containing <40 wt% acylglycerols were repeated 10 cycles by transferring the lipase to the fresh substrate mixtures every 24 h, and those containing >50 wt% acylglycerols were repeated 15 cycles. Half life was calculated based on a decrease in the amount of acylglycerols during the first 2 h.
[c] Composition of dehydrated first-step product is given in Table 1.
[d] Required amount for FAs in the reaction mixture.

(the degree of conversion of acylglycerols to FAMEs, 91%). The reaction was repeated every 24 h by transferring the lipase to a fresh substrate mixture. In the l0th-cycle reaction, the contents of FAMEs and acylglycerols after 24 h were 84 and 7 wt%, respectively. The contents corresponded to only 25% conversion of acylglycerols to FAMEs. The half life of the lipase was 5 days, which was calculated from a decrease in the amount of acylglycerols during the first 2 h (Table 3).

The stability of the lipase in the first-step reaction was increased by addition of MeOH to the reaction mixture (Fig. 1B). MeOH was therefore added to the reaction mixture. But the lipase was inactivated in the presence of 10–20 wt% MeOH. A difference between acid oil and its model (a mixture of refined rapeseed oil and FFAs) was the content of acylglycerols. The content in acid oil supernatant was 11 wt%, whereas the content of TAGs in acid oil model was 50 wt% [10]. Rapeseed oil was therefore added to dehydrated first-step product (Table 3). The half life of the lipase became long with increasing the amount of rapeseed oil, and reached 32 days when a mixture of 12.65 g oil and 15.70 g dehydrated first-step product was used as a substrate. Further addition of the oil prolonged the half life: When the oil was added to give acylglycerol content of 60 wt%, the half life of the lipase was 44 days. An increase in the addition of oil decreases the amount of dehydrated first-step product in the unit weight of reaction mixture. Hence, the second-step reaction was conducted in a mixture of 15.70 g dehydrated first-step product, 12.65 g rapeseed oil, and 1.65 g MeOH (referred to as second-step substrate; the amount of MeOH is stoicheometric for unreacted FAs in the substrate) with 1.8 g immobilized *C. antarctica* lipase. This reaction was repeated every 24 h using the same lipase (Fig. 3). The content of FAMEs at 24 h maintained 91 wt% during 27 cycles. The content, however, decreased rapidly after 30 cycles, and the lipase activity was scarcely observed after 40 cycles.

3.4. Effect of glycerol on lipase stability in the second-step reaction

The 30-days durability of immobilized lipase (Fig. 3) is not enough for industrial application of this system. To further increase the stability of the lipase, we focused on glycerol, which is generally known to stabilize enzymes and also is a product in methanolysis of TAGs.

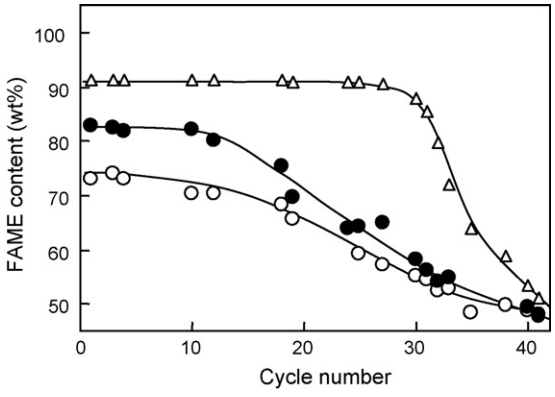

Fig. 3. Continual second-step reaction in a mixture of dehydrated first-step product, rapeseed oil, and MeOH. A mixture containing 15.70 g dehydrated first-step product, 12.65 g rapeseed oil, and 1.65 g MeOH was shaken at 30 °C with 1.8 g immobilized *C. antarctica* lipase, which was preactivated as described in Section 2. The reaction was repeated every 24 h by transferring the lipase to a fresh substrate mixture. Open circles, content of FAMEs at 2 h; closed circles, at 4 h; and open triangles, at 24 h.

The second-step reaction was repeated every 24 h in a mixture of 30 g second-step substrate and different amounts of glycerol using 1.8 g activated immobilized lipase (Table 4). The half life

Table 4
Effect of glycerol on stability of immobilized *C. antarctica* lipase in the second-step reaction

Glycerol amount[a] (g)	Half life[b] (day)
0	32
1.5	46
3.0	>50[c]
6.0	>50[c]
9.0	>50[c]

[a] Given amount of glycerol was added to a mixture of 15.70 g dehydrated first-step product, 12.65 g rapeseed oil, and 1.65 g MeOH.
[b] Reaction mixture was shaken at 30 °C with 1.8 g immobilized *C. antarctica* lipase. The reaction was repeated eight cycles by transferring the lipase to a fresh substrate every 24 h. Half life was calculated based on a decrease in the amount of acylglycerols during the first 2 h in the repeated reactions.
[c] Significant loss of the activity was not observed.

of the lipase became long depending on an increase in the amount of glycerol. When >3 g glycerol was added to 30 g second-step substrate, any loss of lipase activity was not observed during 8 cycles. Based on these results, the amount of glycerol was fixed at 3 g for 30 g second-step substrate.

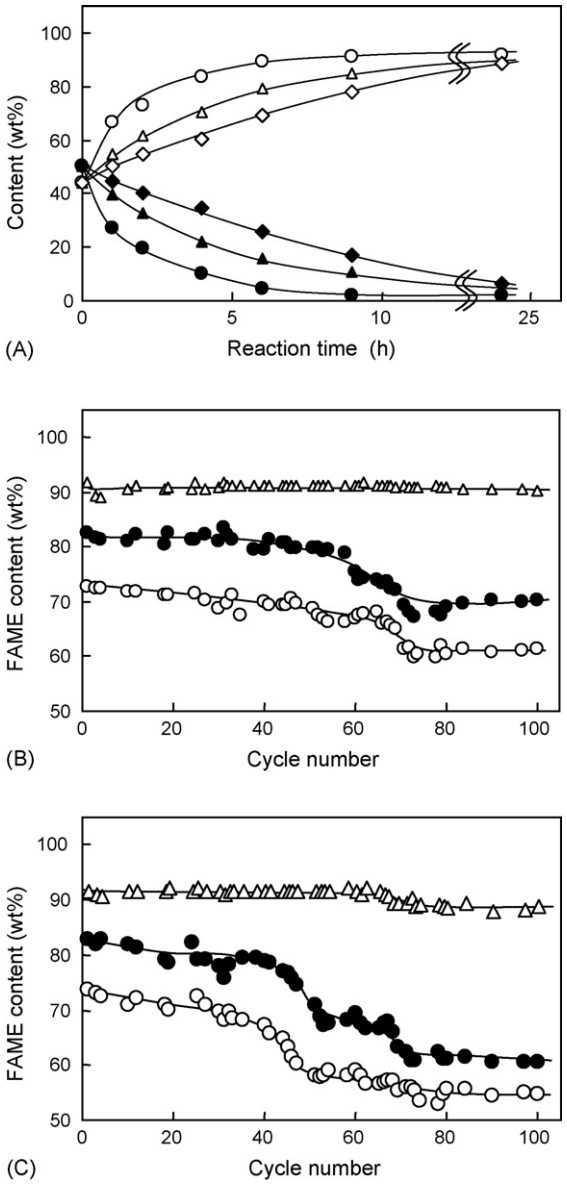

Fig. 4. Continual second-step reaction in the mixture of dehydrated first-step product, rapeseed oil, MeOH, and glycerol. Reaction was conducted at 30 °C in a mixture of 15.70 g dehydrated first-step product, 12.65 g rapeseed oil, 1.65 g MeOH, and 3.0 g glycerol, and were repeated every 24 h by transferring the lipases to fresh substrate mixtures and glycerol. (A) Time course of the reaction. Open circles, content of FAMEs in the first-cycle reaction; closed circles, content of acylglycerols in the first-cycle reaction; open triangles, content of FAMEs in the l00th-cycle reaction with fresh glycerol; closed triangles, content of acylglycerols in the l00th-cycle reaction with fresh glycerol; open diamonds, content of FAMEs in the l00th-cycle reaction with recovered glycerol; closed diamonds, content of acylglycerols in the l00th-cycle reaction with recovered glycerol. (B) Repeated reaction with fresh glycerol. (C) Repeated reaction with glycerol recovered from the previous cycle. When the amount of recovered glycerol was less than 3.0 g, fresh glycerol was added to give 3.0 g. Open circles, content of FAMEs at 2 h; closed circles, at 4 h; and open triangles, at 24 h.

3.5. Continual second-step reaction using immobilized C. antarctica lipase

The second-step reaction was conducted in a mixture of 30 g second-step substrate and 3 g glycerol with 1.8 g activated immobilized *C. antarctica* lipase. The time course of the first-cycle reaction is shown in Fig. 4A. The content of acylglycerols decreased from 50 to 2 wt% after 9 h, and the content of FAMEs increased from 44 to 91 wt%. The reaction was repeated every 24 h using the same lipase (Fig. 4B). The contents of FAMEs at 2 and 4 h decreased with increasing the cycle number, showing that immobilized lipase was inactivated slightly. However, the content of FAMEs at 24 h kept >91 wt% for 80 cycles (80 days), and the content of acylglycerols was maintained <3 wt%. Even after the reaction was repeated 100 cycles, the contents of FAMEs and acylglycerols were 90 and 4 wt%, respectively. The time course of the l00th-cycle reaction is shown in Fig. 4A. The use of immobilized lipase for 100 cycles decreased the activity to 1/2 of the original activity. Rapid inactivation of the lipase observed after 30 days in a reaction system without glycerol (Fig. 3) was suppressed efficiently by addition of glycerol.

Here, glycerol was discarded after the reaction, and fresh one was added every cycle. This procedure means that the cost of glycerol must be added to the production cost of biodiesel fuel. On the other hand, 50 wt% acylglycerols contained in the second-step substrate produce about 5 wt% glycerol in addition to FAMEs, and the by-produced glycerol was recovered in the lower layer which was obtained easily by centrifugation of the enzyme-free reaction mixture. Hence, the by-produced glycerol was used instead of fresh one, and the second-step reaction was repeated (Fig. 4C). The lipase activity, which is expressed in an increase of the amount of FAMEs during the first 2 h, decreased fast compared with that in the reaction using fresh glycerol. However, the content of FAMEs after 24 h kept >91 wt% for 60 cycles (60 days), and the content of acylglycerols was <3 wt%. Even after the reaction was repeated 100 cycles, the contents of FAMEs and acylglycerols were 88 and 6 wt%, respectively. The time course of the l00th-cycle reaction (Fig. 4A) showed that the use of immobilized lipase for 100 cycles decreased the activity to 1/4 of the original activity. These results showed that by-produced glycerol can substitute fresh glycerol, and that the periodical change of the recovered glycerol to fresh one may enhance the lipase stability.

The composition of oil layer produced by 50 cycles of the second-step reaction using recovered glycerol (Fig. 4B) was 91.1 wt% FAMEs, 0.6 wt% FFAs, 0.8 wt% TAGs, 2.3 wt% DAGs, 0.6 wt% phytosterols, 1.3 wt% FA steryl esters, and 3.3 wt% unknown compounds. Its acid value was 1.2 mg KOH/g.

4. Discussion

4.1. Comparison of this enzymatic process with a chemical process

We have described that the two-step enzymatic process comprising methyl esterification of FFAs and methanolysis of TAGs

achieved an efficient conversion of FFAs and acylglycerols in acid oil, which was a by-product in vegetable oil refining, to FAMEs. The final product contained 0.6 wt% FFAs, 0.8 wt% TAGs, and 2.3 wt% DAGs. On the other hand, a chemical process for conversion of acid oil to FAMEs was also reported so far [8]. The process required 15 mol MeOH and 1.5 mol sulfuric acid for total FAs, and the reaction should be conducted at 65 °C. In addition, the final product contained 3.2 wt% FFAs, 1.3 wt% TAGs, and 0.2 wt% DAGs. Although the composition of the acid oil (59.3 wt% FFAs, 38.0 wt% TAGs, and 4.4 wt% DAGs) was different from that of the acid oil used in this study, the contents of contaminants in the product obtained in this study were lower than those in the product obtained by the chemical process.

Compared with the chemical process, the two-step enzymatic process may have the following advantages: (i) the degree of conversion of acid oil to FAMEs is equivalent or slightly higher compared with the chemical process, (ii) the enzymatic process requires smaller amounts of MeOH; five molar amount for total FAs in the first-step reaction and an equimolar amount for the unreacted FAs in the second-step reaction. Excess amount of MeOH in the first-step reaction can be easily recovered by distillation and can be reused in the following reactions, (iii) a process for removing sulfuric acid is not necessary, (iv) glycerol can be recovered easily and be reused, (v) reaction temperature is moderate. Therefore, energy for heating can be saved, (vi) immobilized lipase can be used for long period; thus, the production cost is reduced, and (vii) refined rapeseed oil used in the second step reaction might be replaced by waste oil or surplus vegetable oil. The use of these oils could reduce the load of environment. These advantages of the two-step enzymatic process may be applicable to the industrial process for production of biodiesel fuel from acid oil.

4.2. Stabilization of lipase in the first-step reaction

When FFAs was esterified with 1–10 mol MeOH using immobilized *C. antarctica* lipase, an increase in the amount of MeOH promoted the irreversible inactivation of the lipase [11]. An increase in the amount of MeOH in the first-step reaction (methyl esterification of FFAs) for conversion of acid oil model to FAMEs also promoted the irreversible inactivation [10]. These results showed that the amount of MeOH should be fixed at 1–2 molar amounts for total FAs in the reaction mixture for a long-term continual methyl esterification of FFAs using immobilized *C. antarctica* lipase.

On the other hand, the lipase was stable in the presence of 5–7.5 mol MeOH for total FAs, in the first-step reaction (methyl esterification of FFAs) for conversion of acid oil supernatant to biodiesel fuel (Fig. 1B). This result, which seems to be contradictory, may be explained by assuming the existence of unknown compound(s) in acid oil supernatant: (i) immobilized *C. antarctica* lipase inactivates in the presence of large amounts of MeOH; (ii) acid oil supernatant includes unknown compound(s) which inactivates the lipase; (iii) addition of large amounts of MeOH suppresses the inactivation by reducing interaction of the lipase and unknown compound(s). Meanwhile, the inactivation of the lipase in the presence of >8 mol MeOH is supposed to be caused by MeOH itself.

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