Production of FAME from Acid Oil Model Using Immobilized *Candida antarctica* Lipase

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ABSTRACT: Acid oil is a by-product in the neutralization step of vegetable oil refining and is an alternative source of biodiesel fuel. A model substrate of acid oil, which is composed of TAG and FFA, was used in experiments on the conversion to FAME by immobilized *Candida antarctica* lipase. FFA in the mixture of TAG/FFA were efficiently esterified with methanol (MeOH), but the water generated by the esterification significantly inhibited methanolysis of TAG. We thus attempted to convert a mixture of TAG/FFA to FAME by a two-step process comprising methyl esterification of FFA and methanolysis of TAG by immobilized *C. antarctica* lipase. The first reaction was conducted at 30°C in a mixture of TAG/FFA (1:1, wt/wt) and 10 wt% MeOH using 0.5 wt% immobilized lipase, resulting in efficient esterification of FFA. The reaction mixture after 24 h was composed of 49.1 wt% TAG, 1.3 wt% FFA, 49.1 wt% FAME, and negligible amounts of DAG and MAG (<0.5 wt%). The reaction mixture was then dehydrated and used as a substrate for the second reaction, which was conducted at 30°C in a solution of the dehydrated mixture and 5.5 wt% MeOH using 6 wt% immobilized lipase. The activity of the lipase increased gradually when the reaction was repeated by transferring the enzyme to a fresh substrate mixture. The activity reached a maximum after 6 cycles, and the content of FAME achieved was >98.5 wt% after a 24-h reaction. The immobilized lipase was very stable in the first- and second-step reactions and could be used for >100 d without significant loss of activity.

Paper no. J11126 in *JAOCS 82*, 825–831 (November 2005).

KEY WORDS: Acid oil, biodiesel fuel, *Candida antarctica*, FAME, immobilized enzyme, lipase, methanolysis, methyl esterification.

The use of FAME, produced from agricultural fats and oils, as a fuel in diesel engines has been proposed as a means to help reduce air pollution. Initial studies on the production of these methyl esters, termed biodiesel fuel, were carried out using refined vegetable oils as starting materials (1). Many research groups have studied the production of biodiesel fuel from sources such as used frying oils (2,3), restaurant greases (4,5), and oils produced as by-products from industrial processes (6–11), with the aim of using these sources as renewable feedstocks and reducing the cost of production. One of these industrial by-products is the acid oil produced by the acidulation of soapstock generated in the neutralization step of vegetable oil refining. The amount of acid oil recovered from soapstock reaches approximately 3 wt% of degummed oil; in Japan this amounts to about 60,000 t/yr. Although acid oil has been used as a source of industrial FFA, the oversupply of industrial FA has caused a decrease in the market price. An alternative usage is therefore desired to adjust to the fluctuation of the demand in a market.

Acid oil is composed of acylglycerols, FFA, and small amounts of lipophilic compounds. Conventional alkaline catalysts are not applicable in the conversion of a mixture of TAG and FFA because FFA forms alkaline soaps. Recently, Haas *et al.* (11) reported a chemical process for converting acid oil to FAME with an acid catalyst. A sufficient conversion was achieved by using excess amounts of methanol (MeOH) or by complete hydrolysis of acylglycerols to FFA prior to production of FAME.

Another possibility for converting a mixture of acylglycerols/FFA to FAME is to use an enzyme catalyst. Ghosh and Bhattacharyya (6) converted acid oil to esters of alcohols having >4 carbons by using microbial lipases. Tueter *et al.* (7) also converted it to various alcohol esters in *n*-hexane using immobilized *Candida antarctica* lipase. Most of the TAG remained in the reaction mixture, although the content of FAME reached 64 wt%. In addition, Hsu *et al.* (5) reported efficient conversion of restaurant grease to FA ethyl ester (FAEE) by lipases immobilized in a sol-gel matrix: the content of FAEE reached about 90 wt% when the grease, including <35 wt% FFA, was treated in the presence of 4 mol ethanol relative to total FA in the reaction mixture. There has not been any previous report of >95 wt% FAME being produced from an acid oil that contains >50 wt% FFA. This paper deals with an efficient reaction system for conversion of an acid oil model (a mixture of equal amounts of TAG and FFA) to FAME using immobilized *C. antarctica* lipase.

MATERIALS AND METHODS

Materials. Rapeseed oil rich in oleic acid and FFA originating from the oil were products of the Nisshin OilliO Group Ltd. (Tokyo, Japan). The FA composition of rapeseed oil was 4.3 wt% palmitic acid, 2.0 wt% stearic acid, 62.4 wt% oleic acid

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(OA), 20.1 wt% linoleic acid (LnA), 8.3 wt% α-linolenic acid, and 2.9 wt% other FA, and that of FFA was almost the same as that of the rapeseed oil. LnA was a gift from Yashiro Co. Ltd. (Osaka, Japan), and it contained 0.2 wt% palmitic acid, 3.8 wt% OA, 95.3 wt% LnA, 0.4 wt% α-linolenic acid, and 0.3 wt% other FA. Triolein was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan), and the FA composition was 3.6 wt% palmitic acid, 2.0 wt% stearic acid, 79.8 wt% OA, 4.5 wt% LnA, and 10.1 wt% other FA. The immobilized *C. antarctica* lipase was obtained from Novozymes (Bagsværd, Denmark). MeOH and 2′,7′-dichlorofluorescein was purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemicals Industries Co. Ltd. (Tokyo, Japan), respectively. Other chemicals were of analytical grade.

Preparation of FAME. FFA originating from rapeseed oil rich in OA were esterified with MeOH as described previously (12). In brief, a mixture of 70 g FFA and 8 g MeOH was shaken at 30°C for 24 h in a 100-mL screw-capped vessel using 0.78 g *C. antarctica* lipase (89.3% esterification). To the reaction mixture was added 300 mL of 0.5 N KOH/20% ethanol and 700 mL *n*-hexane, and FAME were recovered in the hexane layer. The extracts were then washed again with 300 mL of 0.5 N KOH/20% ethanol, and *n*-hexane was evaporated (yield of FAME, 59.1 g). The FA composition of FAME was the same as that of FFA.

Reactions. A small-scale reaction was performed at 30°C in a 20- or 50-mL screw-capped vessel with shaking at 130 oscillations/min. The first-step reaction mixtures were 10 or 30 g in total weights and contained predetermined amounts of TAG, FFA, MeOH, and immobilized *C. antarctica* lipase. A large-scale first-step reaction was conducted at 30°C in a 2-L two-necked, round-bottomed flask with agitation at 100 rpm. The reaction mixture consisted of 810 g FFA, 810 g TAG, 180 g MeOH, and 9 g of the immobilized lipase. After the reaction, the mixture separated into water and oil layers. The oil layer was then dehydrated at 90°C and 5 mm Hg for 15 min, and was used as a substrate for the second-step reaction. A 10- or 30-g mixture of dehydrated substrate and MeOH was shaken in a 20- or 50-mL vessel using immobilized *C. antarctica* lipase under similar conditions to the first-step reaction.

Analyses. About 0.8 g of the reaction mixture was taken at predetermined time intervals and analyzed for TAG, DAG, MAG, FFA, and FAME by a TLC/FID analyzer (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo, Japan). The reaction mixture was dissolved in *n*-hexane at a concentration of 1 vol%. The solution (5 μL) was spotted on a silica gel rod, and the components were developed first with *n*-hexane/ethyl acetate/acetic acid (90:10:1, by vol) and then with *n*-hexane/diethyl ether (65:5, vol/vol). Acid value was determined by neutralization of FFA in the sample with 0.1 or 1.0 N KOH solution using phenolphthalein as an indicator.

Components in the sample were fractionated by TLC after developing with *n*-hexane/diethyl ether (65:5, vol/vol). They were visualized by UV light after spraying with an EtOH solution containing 0.5% 2′,7′-dichlorofluorescein. TAG and FAME fractionated by TLC were scraped off the plate. The TAG were converted to FAME by adding 3 mL MeOH solution containing 5% sodium methylate, followed by heating at 75°C for 15 min. The FAME were extracted three times with 1 mL *n*-hexane, and the extracts were then combined. Methyl esterification of FFA was performed at 75°C for 10 min in 3 mL MeOH solution containing 3% BF_3. FAME were analyzed with a gas chromatograph 6890 N (Agilent Technologies, Palo Alto, CA) equipped with a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA). Temperature was controlled at 150°C for 0.5 min, increased to 170°C at 4°C/min, then to 195°C at the rate of 5°C/min, and further increased to 215°C at the rate of 5°C/min. It was then held at 215°C for 5 min. Injector and detector temperatures were 245 and 250°C, respectively.

All analyses were done in triplicate and the mean values are presented. SD were <0.6% for average values of <20%, and <1.2% for average values of >20%.

RESULTS AND DISCUSSION

When an immobilized lipase is used for the production of FAME from TAG or FFA, the useful life of the enzyme has a large effect on the production cost. The studies reported to date show that only immobilized *C. antarctica* lipase is usable for >100 d (13). Hence, this lipase was selected as a catalyst in this study.

Conversion of a mixture of TAG/FFA to FAME using immobilized C. antarctica lipase. Unless otherwise specified, the TAG and FFA used in this study were rapeseed oil rich in oleic acid and FFA originating from the oil, respectively.

Acid oil prepared by acidulation of soapstock consists of almost equal amounts of TAG and FFA. In contrast, acid oil prepared by hydrolysis (saponification) of soapstock under alkaline conditions followed by acidulation of the resulting mixture includes 80–90 wt% FFA. TAG and FFA were therefore mixed at the ratio of 50:50 to 10:90 (wt/wt), and the mixtures were used as substrates.

MeOH was added to the TAG/FFA mixture at a concentration of 10 wt% (about an equimolar amount of MeOH to the total FA in the oil mixture), and the mixture was shaken at 30°C with 4 wt% immobilized *C. antarctica* lipase (Table 1). Following 1 h of reaction with TAG/FFA (50:50, wt/wt), the composition in the oil fraction was 49.2 wt% TAG, 5.8 wt% FFA, and 44.4 wt% FAME. The reaction was continued to 24 h, but most of the TAG were unreacted: The content of TAG decreased from 49.2 to 44.5 wt%. The content of TAG in the initial reaction mixture was then reduced to 35, 20, and 10 wt%. In all reactions, 90% of the FFA was converted to FAME after 1 h, but the content of TAG decreased only about 10% of the initial content even after 24 h. These results suggest that the lipase catalyzes mainly methyl esterification of FFA in the mixture of TAG, FFA, and MeOH.

To show that most of the FAME originate from FFA, triolein and LnA were selected as substrates. A commercially available triolein included 79.8 wt% OA, 4.5 wt% LnA, and 15.7 wt% other FA. The commercial preparation of LnA contained 3.8

TABLE 1
Reaction of Immobilized *Candida antarctica* Lipase on a Mixture of TAG, FFA, and Methanol (MeOH)[a]

TAG/FFA (wt/wt)	Reaction time (h)	Composition in oil fraction (wt%)[b]			
		DAG	TAG	FFA	FAME
50:50	0	0	50.0	50.0	0
	1	0.6	49.2	5.8	44.4
	24	0.7	44.5	1.7	53.1
35:65	0	0	35.0	65.0	0
	1	0.5	34.9	5.9	58.7
	24	0.5	33.2	2.9	63.4
20:80	0	0	20.0	80.0	0
	1	ND[c]	19.4	5.6	75.0
	24	ND	18.0	4.8	77.2
10:90	0	0	10.0	90.0	0
	1	ND	9.9	8.0	81.6
	24	ND	9.1	8.0	82.9

[a]A 10-g mixture of TAG/FFA/MeOH was shaken with 4 wt% immobilized lipase.
[b]MAG were not detected (<0.5 wt%).
[c]ND, not detected (<0.5 wt%).

wt% OA, 95.3 wt% LnA, and 0.9 wt% other FA. The reaction of triolein/LnA (1:1, wt/wt), 10 wt% MeOH, and 4 wt% immobilized lipase was conducted at 30°C (Table 2). The FFA content decreased from 50 to 2.4 wt% after 1 h and that of FAME increased to 48.3 wt%, while the content of TAG decreased very slowly and reached 47.5 wt% after 10 h.

Samples of the reaction mixture were periodically withdrawn, and FAME and TAG were fractionated by TLC. FA compositions in the FAME and TAG fractions were then analyzed (Table 2). FA composition of TAG was almost the same during the reaction, showing that TAG did not undergo methanolysis, acidolysis with FFA, and interesterification with FAME. If only FFA converts to FAME, the FA composition of FAME should be the same as that of FFA. Actually, little change was observed in FA composition of FAME compared with that of LnA preparation: the content of LnA methyl ester decreased only 3.5 wt%, and that of OA methyl ester increased 3.7 wt%. This phenomenon can be explained by 5% methanolysis of triolein after 10 h, which produces 4 wt% (=79.8 wt% × 5%) OA methyl ester. Therefore, the result in Table 2 showed that FFA is converted efficiently to its methyl ester and TAG is converted with difficulty to its corresponding methyl ester in the reaction of TAG/FFA/MeOH with immobilized *C. antarctica* lipase.

Effect of water on lipase-catalyzed reaction in a mixture of TAG, FFA, and MeOH. We reported previously that immobilized *C. antarctica* lipase efficiently catalyzes methanolysis of TAG in a system without addition of water (water content, <250 ppm) (13). In this study, however, the lipase catalyzed hardly any methanolysis of TAG in a mixture of TAG/FFA/MeOH, even though water was not added to the mixture (Table 2). This inconsistency may be due to the water generated by methyl esterification of FFA.

A mixture of TAG/FAME/MeOH (47.4:47.4:5.2, by wt; the amount of MeOH was the same on an equimolar basis to FA in TAG) was shaken at 30°C in the presence of 0–5 wt% water with 4 wt% immobilized *C. antarctica* lipase (Fig. 1). The lipase efficiently catalyzed the methanolysis of TAG when water

TABLE 2
Contents of Oleic Acid (OA) and Linoleic Acid (LnA) in FAME and TAG Fractions Obtained from Reaction of Triolein/LnA/MeOH with Immobilized *C. antarctica* Lipase[a]

Time (h)	FA composition (wt%)[b]						
	Composition (wt%)			FAME fraction		TAG fraction	
	FFA	FAME	TAG	OA	LnA	OA	LnA
0	50.0	0	50.0	—[c]	—[c]	79.8	4.5
1	2.4	48.3	49.3	4.1	95.2	79.5	4.3
3	1.9	49.1	49	3.9	95.7	80.0	4.5
7	1.7	49.8	48.5	5.8	93.6	80.3	4.6
10	1.8	50.7	47.5	7.8	91.7	79.5	4.7

[a]The reaction was performed at 30°C with shaking in a 30-g mixture of triolein/LnA/MeOH using 4 wt% immobilized lipase.
[b]FAME and TAG fractions were separated by TLC, and FA compositions in their fractions were analyzed.
[c]Contents of OA and LnA in FFA used as a substrate were 3.8 and 95.3 wt%, respectively. See Table 1 for abbreviations.

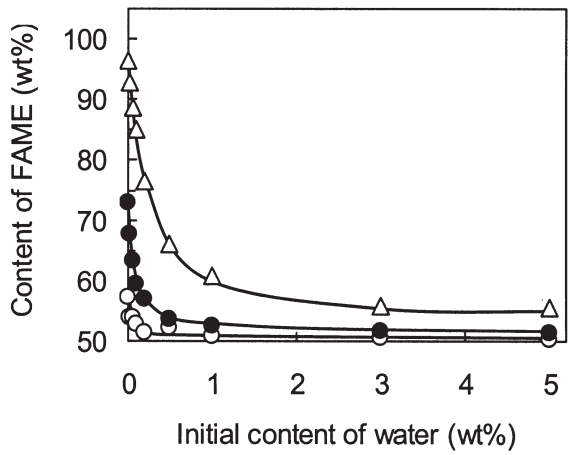

FIG. 1. Effect of water on *Candida antarctica* lipase-catalyzed reaction in a mixture of TAG, FAME, and methanol (MeOH). A 10-g mixture of TAG/FAME/MeOH (47.4:47.4:5.2, by wt) and different amounts of water was shaken at 30°C with 4 wt% immobilized lipase. ○, Content of FAME at 2 h; ●, at 7 h; △, at 24 h.

was not added to the mixture (water content in the reaction mixture, 240 ppm), and the content of FAME reached 96.5 wt% after 24 h. The methanolysis was, however, inhibited slightly by the addition of 0.02 wt% water (water content in the reaction mixture, 450 ppm), and was inhibited significantly by addition of 3 wt% water; the content of FAME increased only slightly from 50 to 56 wt% even after 24 h. The reactions with >1 wt% water showed further that the content of FFA generated by hydrolysis of TAG and/or FAME was <2 wt%. When 90% of FFA in a mixture of FFA/TAG (1:1, wt/wt) is esterified with MeOH, the content of water generated is calculated to be 2.6 wt%. It was therefore concluded that the water generated by the esterification inhibits the methanolysis of TAG.

After the reactions with 1, 3, and 5 wt% water had been allowed to proceed for 24 h, the lipase was transferred to a fresh substrate mixture without addition of water, and the reaction was repeated under similar conditions. Methanolysis of TAG recovered gradually as the reaction was repeated, and the content of FAME reached >98 wt% after 8 cycles (total 9 cycles). This result indicated that the inhibition of methanolysis by water is reversible, and that the lipase activity can be recovered by removing water from the reaction system. This observation was consistent with the observation that a small amount of water inhibited reversibly the stepwise methanolysis of TAG by immobilized *C. antarctica* lipase (13).

Effect of the amount of MeOH on lipase-catalyzed reaction in a mixture of FFA, TAG, and MeOH. The reaction systems described thus far contained a stoichiometric amount of MeOH, which was required for the complete conversion of TAG to FAME (13). In this study, however, most of the TAG in the mixture were unreacted. Because the equilibrium of reaction generally depends on the concentration of substrate, large amounts of MeOH may eliminate the inhibition of the methanolysis by water and may achieve a high conversion of TAG to FAME.

When a mixture of TAG/FFA (1:1, wt/wt) was shaken at 30°C for 24 h in the presence of 10, 20, 30, and 50 wt% MeOH using 4 wt% immobilized *C. antarctica* lipase, the content of TAG in the oil layer decreased to 45.0, 34.8, 32.6, and 15.1 wt%, respectively. In these reactions, the contents of FAME in the oil layer were 53.6, 61.3, 62.4, and 79.3%, respectively. Conversion of a mixture of TAG/FFA (1:9, wt/wt) to FAME was also conducted at 30°C for 24 h in the presence of 50 wt% MeOH using 4 wt% of the same lipase. The content of the remaining TAG was 2.5 wt%, and the contents of FAME, FFA, and DAG were 89.3, 6.4, and 1.8 wt%, respectively. These results showed that a large amount of MeOH increased the degree

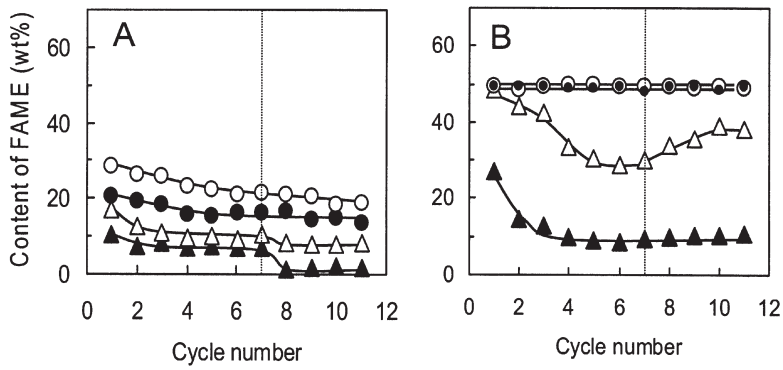

FIG. 2. Effect of MeOH on the first-step reaction using *C. antarctica* lipase. A 30-g mixture of TAG/FFA (1:1, wt/wt) and different amounts of MeOH was shaken at 30°C for 24 h with 0.5 wt% immobilized lipase. The reactions were repeated for 7 cycles by transferring the lipase to a fresh substrate mixture every 24 h. Each reaction was then repeated 4 more cycles in a substrate mixture of TAG/FFA (1:1, wt/wt) and 10 wt% MeOH. (A) The content of FAME at 2 h. (B) The content of FAME at 24 h. ○, Reaction with 10 wt% MeOH until the 11th cycle; ●, 20 wt% MeOH for the first 7 cycles and thereafter 10 wt% MeOH; △, 30 wt% MeOH for the first 7 cycles and thereafter 10 wt% MeOH; ▲, 50 wt% MeOH for the first 7 cycles and thereafter 10 wt% MeOH. See Figure 1 for abbreviations.

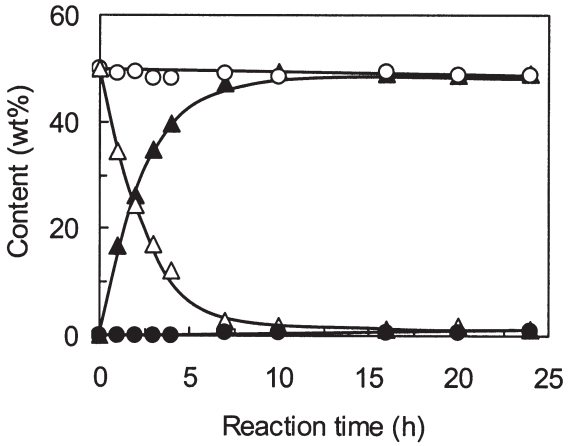

FIG. 3. Time course of the first-step reaction with *C. antarctica* lipase. A 30-g mixture of TAG/FFA/MeOH (13.5:13.5:3, by wt) was shaken at 30°C using 0.5 wt% immobilized lipase. ○, Content of TAG; ●, DAG; △, FFA; ▲, FAME. See Figure 1 for abbreviations.

of methanolysis but complete conversion of a mixture of TAG/FFA to FAME was not achieved.

Strategy for conversion of a mixture of TAG/FFA to FAME. When a mixture of TAG, FFA, and MeOH was treated with immobilized *C. antarctica* lipase, FFA were esterified efficiently with MeOH. But methanolysis of TAG proceeded with difficulty because water generated by the esterification inhibited the methanolysis. We thus planned a two-step process. The first step is methyl esterification of FFA in the mixture of TAG and FFA. The resulting product is composed of mainly FAME and TAG. Because the mixture contains water generated by the esterification, the water is removed by evaporation. The second step is methanolysis of TAG in the mixture of TAG and FAME. According to this strategy, we attempted to convert a mixture of TAG/FFA (1:1, wt/wt) to FAME.

Effect of the amounts of MeOH on the first step. A mixture of TAG/FFA (1:1, wt/wt) was shaken at 30°C in the presence of 10, 20, 30, and 50 wt% MeOH using 0.5 wt% immobilized *C. antarctica* lipase (a stoichiometric amount of MeOH to FFA is 5.4 wt%) (Fig. 2). The content of FAME in the oil layer at 2 h decreased with increasing amount of MeOH (Fig. 2A) and at 24 h reached 49.4, 49.5, 48.5, and 27.0 wt% in the reactions with 10, 20, 30, and 50 wt% MeOH, respectively (Fig. 2B). To study the stability of the lipase, each reaction was repeated for a total of 7 cycles by transferring the enzyme to a fresh substrate mixture every 24 h. Reactions with 10 and 20 wt% MeOH maintained about 50 wt% of the content of FAME at 24 h. In contrast, the content of FAME at 24 h decreased significantly by repeating the reactions in the presence of 30 wt% or more amount of MeOH. Each reaction was then repeated 4 more cycles (total 11 cycles) in a substrate mixture of TAG/FFA (1:1, wt/wt) and 10 wt% MeOH using the same lipase. The activity of the lipase that had been used in the presence of 30 wt% MeOH recovered somewhat but not to the original level. In addition, the activity of the lipase that had been used in the presence of 50 wt% MeOH did not recover. These results showed that the lipase inactivates irreversibly in the presence of large amounts of MeOH. Based on these findings, the amount of MeOH was fixed at 10 wt%.

Time course of the first-step reaction. The reaction conditions were fixed as follows: TAG/FFA, 1:1 (wt/wt); MeOH amount, 10 wt%; reaction temperature, 30°C. In addition, the amount of immobilized lipase was fixed at 0.5 wt% on the assumption that the reaction is recycled at 24-h intervals using the same enzyme. A typical time course is shown in Figure 3. The content of FAME increased efficiently with a decrease of the content of FFA, and the reaction reached nearly a steady state after 10 h. During the reaction, there was very little methanolysis of TAG. The contents of TAG, FAME, FFA, and DAG at 24 h were 49.1, 48.8, 1.3, and 0.8 wt%, respectively. The contents of MAG were negligible (<0.5 wt%) through 24 h.

Effect of the amount of MeOH on the second-step reaction. The first-step reaction was conducted on a 2-L scale as described in Materials and Methods section. The resulting oil layer was then dehydrated and used as a substrate for the second-step reaction; its composition was 48.5 wt% TAG, 49.7 wt% FAME, 1.3 wt% FFA, and 0.5 wt% DAG (the content of water, 270 ppm). The substrate was referred to as dehydrated first-step product.

A 10-g mixture of dehydrated first-step product and 5.5, 10, and 20 wt% MeOH was shaken at 30°C with 4 wt% immobilized *C. antarctica* lipase (the stoichiometric amount of MeOH for complete conversion of acylglycerols and FFA is 5.2 wt%). The reaction with 5.5 wt% MeOH was repeated for 4 cycles by transferring the lipase to a fresh substrate mixture every 24 h. The first-cycle reaction with 5.5 wt% MeOH increased the content of FAME to 53.1, 58.5, and 81.8 wt% after 2, 7, and 24 h, respectively. The repetition of the reaction increased the initial rate of production of FAME but did not decrease its content at 24 h (described in the next section).

The first-cycle reaction with 10 wt% MeOH increased the content of FAME to 57.5, 70.1, and 92.3 wt% after 2, 7, and 24 h, respectively. This result showed that a large amount (10 wt%) of MeOH increased the initial velocity to approximately 2 times of that in the reaction with 5.5 wt% MeOH. The activity of the lipase, however, decreased significantly when the reaction was repeated using the same lipase: Methanolysis of TAG was scarcely detected in the third-cycle reaction. In addition, methanolysis of TAG with 20 wt% MeOH did not proceed even in the first-cycle reaction. The lipases, which had been inactivated in the reactions with 10 and 20 wt% MeOH, were used as catalysts for the reactions with 5.5 wt% MeOH. But the recovery of the activity was not observed; thus, the inactivation was irreversible. These results determined the amount of MeOH at 5.5 wt%.

Time course of the second-step reaction. A mixture of dehydrated first-step product and 5.5 wt% MeOH was shaken at 30°C using immobilized 6 wt% *C. antarctica* lipase, an amount chosen on the assumption that the reaction is repeated at 24-h intervals using the same enzyme. A typical time course is shown in Figure 4. The content of FAME increased gradually with a decrease of the content of TAG, and reached 95.1 wt%

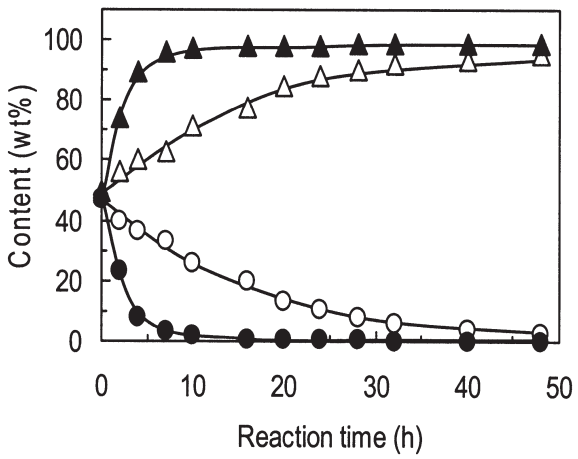

FIG. 4. Time course of the second-step reaction with *C. antarctica* lipase. A 30-g mixture of dehydrated first-step product and 5.5 wt% MeOH was shaken at 30°C using 6 wt% immobilized lipase. The reaction was repeated for 5 cycles by transferring the enzyme to a fresh substrate mixture every 48 h. ○, The content of TAG in the first-cycle reaction; △, the content of FAME in the first-cycle reaction; ●, the content TAG in the fifth-cycle reaction; ▲, the content of FAME in the fifth-cycle reaction. See Figure 1 for abbreviations.

after 48 h. The reaction was repeated by transferring immobilized lipase to a fresh substrate mixture every 48 h, resulting in a gradual acceleration of the reaction. The fifth-cycle reaction reached a nearly steady state after 10 h, and the content of FAME reached >98.5 wt% at 24 h (Fig. 4). The contents of other components, TAG, DAG, MAG and FFA, were negligible (<0.5 wt%). Other research groups observed similar phenomena: The pretreatment of the lipase with TAG or FAME accelerated the subsequent methanolysis of TAG (14,15). This may be explained by an increase of the rate of transfer of substrate to the lipase molecule on the immobilized carrier and/or by removal of a small amount of water contained in the immobilized lipase. Therefore, pretreatment of the lipase with TAG or FAME may help the full expression of its activity from the first cycle of the second-step reaction.

Long-term repetition of the first- and second-step reactions. The first reaction was conducted at 30°C in a 30-g mixture of TAG/FFA (1:1, wt/wt) and 10 wt% MeOH using 0.5 wt% immobilized *C. antarctica* lipase. The reaction was repeated by transferring the lipase to a fresh substrate mixture every 24 h (Fig. 5A). The first-step reaction mixtures were collected every 10 cycles and then dehydrated for 30 min at 90°C and 5 mm Hg. The second-step reaction was conducted at 30°C in a 10-g mixture of dehydrated first-step product and 5.5 wt% MeOH using 6 wt% immobilized *C. antarctica* lipase. The reaction was repeated by transferring the lipase to a fresh substrate mixture every 48 h for the first 5 cycles and every 24 h thereafter (Fig. 5B).

The first step-reaction converted FFA to FAME. The contents of FAME and FFA of the first cycle were 49.8, and 1.3 wt%, respectively. Even after 100 cycles, the content of FAME was 47.5 wt%, although 4.1 wt% FFA remained. The content of FAME at 1 h decreased gradually from 17 to 9 wt% during the first 30 d but thereafter remained steady at about 9 wt% till 100 d. The result indicated that the decrease during the early cycles may be due to the release of lipase under the chosen conditions and that the lipase is stable for >100 d.

The lipase did not express full activity at the first cycle in the second-step reaction. In the sixth cycle, the content of FAME at 24 h reached 98.8 wt%; thus, the reaction was conducted for 24 h thereafter. The content of FAME at 2 h decreased only a little with an increase of cycle number, but that at 24 h maintained *ca.* 98.5 wt% even after 100 d. The result showed that the lipase is extremely stable under the conditions determined. The reaction mixtures of all cycles were combined, and the composition was analyzed. The content of FAME was 98.6 wt%, and those of acylglycerols and FFA were negligible (<0.5 wt%).

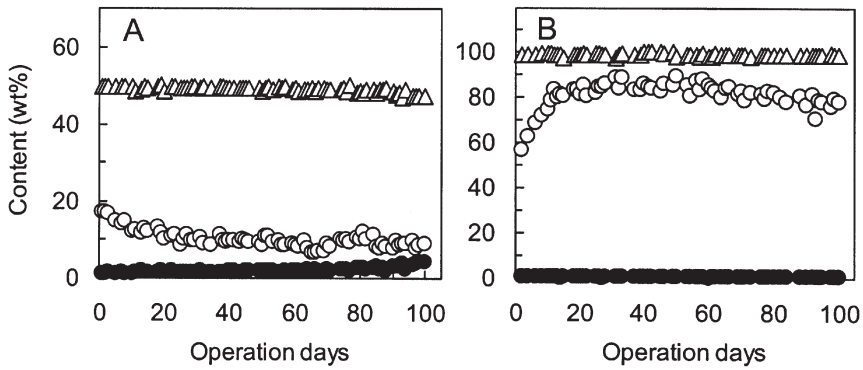

FIG. 5. Repetition of the first- and second-step reactions. (A) First-step reaction: a 30-g mixture of TAG/FFA (1:1, wt/wt) and 10 wt% MeOH was shaken at 30°C using 0.5 wt% immobilized *C. antarctica* lipase. The reaction was repeated by transferring the enzyme to a fresh substrate mixture every 24 h. ○, The content of FAME at 1 h; △, the content of FAME at 24 h; ●, the content of FFA at 24 h. (B) Second-step reaction: a 10-g mixture of dehydrated first-step product and 5.5 wt% MeOH using 6 wt% immobilized lipase. The reaction was repeated every 48 h for the first 5 cycles and every 24 h thereafter. ○, The content of FAME at 2 h; △, the content of FAME at 24 or 48 h; ▲, the content of TAG at 24 or 48 h. See Figure 1 for abbreviations.

Advantages of the two-step process for converting the acid oil model to FAME. We have described a two-step system comprising of methyl esterification of FFA and methanolysis of TAG using immobilized *C. antarctica* lipase that is effective for converting a mixture of TAG/FFA to FAME. It was previously reported that the conversion of acid oil (FFA content, 55 wt%) in *n*-hexane using the same immobilized lipase achieved 64 wt% FAME content at 40°C, although TAG in acid oil were unreacted (7). To obtain 90 wt% FAEE from restaurant grease (FFA content, <35 wt%) with immobilized *Burkholderia cepacia* lipase, ethanol in amounts of 4 molar excess were required (5). In addition, none of these reports studied the reuse of immobilized lipases. In contrast, the system presented in this paper has the following advantages: (i) >98% conversion of TAG/FFA to FAME is achieved; (ii) the amount of MeOH required is only 1.5 times the stoichiometric amount of MeOH; (iii) the lipase is very stable for a long term; and (iv) organic solvent is not required. This system may therefore be applicable to an enzymatic production of biodiesel fuel from acid oil prepared by acidulation of soapstock.

Kinetics of reactions catalyzed by immobilized C. antarctica lipase. The immobilized lipase catalyzed hydrolysis, esterification, and transesterification in this study. Kinetics of the reactions are estimated approximately.

Immobilized *C. antarctica* lipase catalyzed methyl esterification of FFA, and 0.5 wt% of the enzyme achieved >90% esterification after 10 h (Fig. 3). Meanwhile, methanolysis of TAG required 6 wt% enzyme to achieve >90% conversion after 10 h (Fig. 4). These facts showed that this immobilized lipase catalyzes methyl esterification of FFA more efficiently than methanolysis of TAG. In addition, methanolysis of TAG is inhibited strongly in the presence of only a small amount of water (Figs. 1, 2; Table 2), although methyl esterification of FFA achieves nearly 90% esterification even in the presence of 10 wt% water (9).

When a mixture of TAG/FAME (1:1, wt/wt) was shaken with immobilized *C. antarctica* lipase in the presence of 5 wt% water, the content of FFA was <2 wt% (Fig. 1). This result showed that hydrolyses of TAG and FAME proceeds very weakly. In addition, this immobilized lipase converted FFA/TAG to FAME at >90% conversion with only a stoichiometric amount of MeOH (Table 1, Fig. 5; also see Ref. 9), indicating that reverse reactions hardly occur either in methyl esterification of FFA or in methanolysis of TAG.

ACKNOWLEDGMENTS

The authors cordially thank Dr. Hideki Fukuda of Kobe University, Dr. Hideo Noda, and Nobuyuki Kuratani of Kansai Chemical Machinery Co. Ltd., and Dr. Asao Yamauchi of Osaka Municipal Technical Research Institute for their valuable discussions.

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[Received May 11, 2005; accepted August 31, 2005]