Enzymatic Conversion of Waste Edible Oil to Biodiesel Fuel in a Fixed-Bed Bioreactor

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ABSTRACT: The conversion of waste edible oil to biodiesel fuel in a fixed-bed bioreactor was investigated. Three-step methanolysis of waste oil was conducted using three columns packed with 3 g of immobilized *Candida antarctica* lipase. A mixture of waste oil and 1/3 molar equivalent of methanol against total fatty acids in the oil was used as substrate for the first-step reaction, and mixtures of the first- and second-step eluates and 1/3 molar equivalent of methanol were used for the second- and third-step reactions, respectively. Ninety percent of waste oil was converted to the corresponding methyl esters (ME) by feeding substrate mixtures into the first, second, and third reactors at flow rates of 6, 6, and 4 mL/h, respectively. We also attempted one-step methanolysis of waste oil. When a mixture of waste oil and 90% ME-containing eluate (1:3, wt/wt) and an equimolar amount of methanol against total fatty acids in the waste oil was fed into a reactor packed with 3 g of immobilized *C. antarctica* lipase at a flow rate of 4 mL/h, the ME content in the eluate reached 90%.The immobilized biocatalyst could be used for 100 d in the two reaction systems without significant decrease in its activity. Waste oil contained 1980 ppm water and 2.5% free fatty acids, but these contaminants had little influence on enzymatic production of biodiesel fuel.

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KEY WORDS: Acylglycerol specificity, biodiesel fuel, *Candida antarctica*, fixed-bed bioreactor, lipase, methanolysis, waste oil.

In Japan, four hundred thousand tons of edible oils are discharged yearly (1). Half of this amount is estimated to be recycled as animal feed or as raw material for lubricant and paint. The remainder, however, is discharged into the environment. Hence, production of a biodiesel fuel from waste edible oil is considered an important step for reducing and recycling waste oil. In this regard, several local governments in Japan have started collecting used frying oils from households and have converted them to biodiesel fuel for public transportation.

Presently, the industrial-scale production of biodiesel fuel from waste edible oils is performed by a chemical process using alkaline catalysts. Waste oils, however, contain small amounts of water and free fatty acids (FFA) in addition to oxidized compounds, such as aldehydes, epoxides, and polymers (2,3). Accordingly, fatty acid alkaline salts (soaps) are generated as by-products. The soaps are removed by washing with water, which also removes glycerol, methanol, and catalyst. Hence, disposal of the resulting alkaline wash water creates other environmental concerns. On the other hand, enzymatic methanolysis of triacylglycerols (TAG) does not generate any waste materials. Enzymatic production of biodiesel fuel from waste oils therefore is strongly desirable.

Enzymatic alcoholysis of TAG with or without the use of organic solvent has been reported (4–13). Alcoholysis with relatively long-chain and branched alcohols proceeds efficiently even in organic solvent-free systems, but the organic solvent-free methanolysis typically does not give high conversion. This phenomenon may be due to the inactivation of enzyme by methanol. Indeed, in our methanolysis system of vegetable oil with immobilized *Candida antarctica* lipase, soluble methanol (1/2 molar equivalent against total fatty acids in the oil) did not inactivate, but the presence of insoluble methanol inactivated the enzyme (14). Based on this fact, we achieved 95% transmethylation of vegetable oil to its corresponding methyl esters (ME) by a stepwise process using immobilized *C. antarctica* lipase. The stepwise addition of methanol prevented the inactivation of the lipase, which allowed its continued usability. We now have applied this stepwise methanolysis procedure to the production of biodiesel fuel from waste oil. This paper shows that contaminants in the waste oil have little or no effect on the efficiency of stepwise methanolysis and the reusability of the immobilized biolcatalyst.

MATERIALS AND METHODS

Materials. Waste edible oil was provided by Yashiro Co. Ltd. (Osaka, Japan). The oil contained 1980 ppm water, 2.5% FFA (acid value, 4.8 mg KOH/g), and 4.6% partial acylglycerols. The saponification value was 191 mg KOH/g and the peroxide value (POV) was 9.5 milliequivalents/kg. Unsaponified matter in the oil was not detected. Vegetable oil (mixture of soybean and rapeseed oils; saponification value, 192 mg KOH/g; Showa Sangyo Co. Ltd., Tokyo, Japan) and diacylglycerol (DAG)-rich oil (Econa Cooking Oil; TAG/DAG/monoacylglycerol (MAG) = 6.6:89.1:4.3, by weight; Kao Corp., Tokyo, Japan) were purchased from a local supermarket. Monoolein (MO) and tricaprylin were obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Methanol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Immobilized *C. antarctica* lipase (Novozym 435) was donated by Novozymes (Bagsvaerd, Denmark). Other chemicals were of reagent grade.

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Preparation of DAG and MAG. DAG were purified from 15 g of Econa Cooking Oil by silica gel 60 column chromatography (100 g; column size, 30 × 325 mm; Merck, Darmstadt, Germany). DAG were recovered by eluting with 400 mL *n*-hexane/ethyl acetate (80:20, vol/vol) after eluting TAG with 500 mL *n*-hexane/ethyl acetate (98:2, vol/vol). The purity of the resulting DAG (11.5 g) was 97.2%. The MO purchased from Tokyo Kasei Kogyo contained 5.2% TAG and 29.5% DAG. Crude MO (20 g) was purified using the same silica gel column as that used for the purification of DAG. MO was eluted with 400 mL of *n*-hexane/ethyl acetate (60:40, vol/vol) after eluting DAG with 500 mL of *n*-hexane/ethyl acetate (80:20, vol/vol). The purity of the resulting MO (10.7 g) was 98.1%.

Reaction. Batch reactions were performed at 30°C in a 50-mL screw-capped vessel by shaking at 130 oscillations/min using 4% immobilized *C. antarctica* lipase. Three-step batch methanolysis of waste or vegetable oil was conducted as follows. The first-step reaction contained 30 g of oil and 1/3 molar equivalent of methanol against total fatty acids in the oil. The second- and third-step reactions were performed by adding 1/3 molar equivalent of methanol after consumption of methanol. Batch reactions were repeated by transferring the lipase to fresh substrate mixture.

Three-step flow methanolysis was conducted at 30°C using three columns each packed with 3 g immobilized *C. antarctica* lipase (15 × 80 mm)for 100 d. A mixture of waste oil and 1/3 molar equivalent of methanol against total fatty acids in the oil was fed into the first-step reactor with a peristaltic pump. The eluate was left to stand overnight to allow glycerol to separate (lower layer). A mixture of the resulting first-step eluate and 1/3 molar equivalent of methanol was then fed into the second-step reactor. The third-step methanolysis was similarly performed by feeding a mixture of the second-step eluate and 1/3 molar equivalent of methanol to the third-step third reactor. The flow rates in the first-, second-, and third step reactions were 6, 6, and 4 mL/h, respectively.

One-step flow methanolysis of waste oil was performed at 30°C using a single column packed with 3 g of immoblized *C. antarctica* lipase. The substrate was prepared by diluting waste oil with three times its weight of 90% ME-containing eluate, followed by addition of equimolar methanol against total fatty acids in the waste oil. The mixture was fed into the reactor, and the reaction was continued by recycling 3/4 of the eluate.

Analysis. The ME content in the reaction mixture was quantified by a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph equipped with a DB-5 capillary column (0.15 mm × 10 m; J&W Scientific, Folsom, CA) using tricaprylin as an internal standard. The analysis was performed under the conditions described previously (13). The ME, MAG, DAG, TAG, and FFA contents were measured by a thin-layer chromatograph (TLC)/flame-ionization detector analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after double development. The first and second developments were performed using solvent mixtures of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol) and *n*-hexane/ethyl acetate (65:5, vol/vol), respectively. Water content was analyzed by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan). Peroxide value was determined according to the American Oil Chemists' Society official methods (14).

RESULTS

Effect of water in waste oil on its methanolysis. Vegetable and waste oils differ significantly in water and FFA content. The waste edible oil used in this study contained 1980 ppm water. It was previously reported that >500 ppm of water decreased the initial rate of TAG methanolysis but did not affect the equilibrium of the reaction (15). We thus studied the effect of water (*ca.* 2000 ppm) on waste oil methanolysis. A mixture of vegetable oil (water content, 250 ppm) and 1/3 molar equivalent of methanol against total fatty acids in the oil was shaken at 30°C for 24 h with 4 wt% immobilized *C. antarctica* lipase. The reaction was repeated by transferring the lipase to a fresh substrate mixture every 24 h (Fig. 1A). The conversions after 1 h in the first-, third-, and fifth-cycle methanolysis were 9.3, 10.5, and 11.2%, respectively. The initial rate increased slightly with increasing number of cycles. Methanolysis of waste oil was similarly repeated (Fig. 1B). The conversion after 1 h in the first-cycle reaction was slower than the vegetable oil methanolysis, and the conversion increased with increasing number of cycles; the ME content after 1 h in the first-, third-, and fifth-cycle reactions was 3.0, 8.3, and 10.7%, respectively. These results show that the inhibition of methanolysis by the small amount of water present in the oil is eliminated by performing the reaction in cycles. To confirm the effect of water, vegetable oil methanolysis was repeated in the presence of 2010 ppm water (Fig. 1C). The initial rate was slow in the first reaction but increased with a number of cycles. As in waste oil methanolysis, the ME content after 1 h in the first-, third-, and fifth-cycle reaction was 6.4, 10.1, and 11.5%, respectively. The increase in velocity can be explained as follows. Water initially present in the waste oil transfers into

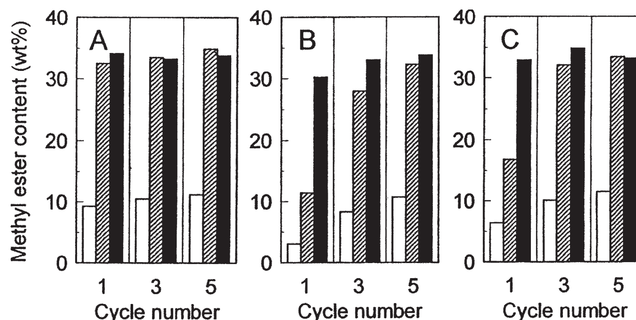

FIG. 1. Effect of a small amount of water on the methanolysis of waste and vegetable oils by immobilized *Candida antarctica* lipase. (A) Methanolysis of vegetable oil (water content, 250 ppm). (B) Methanolysis of waste oil (water content, 1980 ppm). (C) Methanolysis of vegetable oil (water content, 2010 ppm). The reaction was performed in a 30-g mixture of oil, 1/3 molar equivalent of methanol, and 4 wt% immobilized lipase. The methanolysis was repeated by transferring the lipase to a fresh substrate mixture every 24 h. ▭, Methyl ester (ME) content in the reaction mixture after 1 h; ▨, ME content after ■, ME content after 24 h.

the glycerol layer generated by methanolysis. Because the water goes out of the methanolysis system, the reaction velocity gradually increases. Actually, the water content in the ME/acylglycerol layer (oil layer) decreased to 700 ppm, and that in the glycerol layer was 3.7%, after five cycles. When 33% waste oil is converted to its ME, the glycerol content in the reaction mixture is calculated to be 3.37%. Water content in the oil layer decreased from 1980 to 700 ppm. If the water transferred to the glycerol layer, the content would become 3.70%. This value coincided well with the observed one. These results show that the content of water adsorbed on immobilized lipase reaches a constant value and that the water content does not disturb the methanolysis reaction.

Three-step batch methanolysis of waste oil. Immobilized *C. antarctica* lipase that had been used for five cycles (Fig. 1) was employed for three-step methanolysis of waste and vegetable oils. The reaction was started at 30°C in a mixture of oil, 1/3 molar equivalent of methanol against total fatty acids in the oil, and the lipase. Another 1/3 molar equivalent of methanol was added after 10 and 24 h. As shown in Figure 2, no differences were observed between the reaction time courses of the first- and second-step methanolysis of the two oils, and the ME content reached 34 and 66% after the first- and second-step reactions, respectively. The conversion of waste oil reached 90.4% after the third-step methanolysis, although that of vegetable oil was 95.9%. The acid value after the third-step reaction of waste and vegetable oils was 1.4 and 0.4 mg KOH/g, respectively. The initial acid value of the waste oil was 4.8 mg KOH/g, indicating that methylation of FFA occurred along with methanolysis of the waste oil.

Three-step flow methanolysis of waste oil with three fixed-bed bioreactors. Water and FFA present in waste oil had little effect on the methanolysis. We thus studied flow methanolysis using three fixed-bed bioreactors in series packed with 3 g of immobilized *C. antarctica* lipase. Table 1 shows the effect of flow rate on three-step methanolysis of waste oil. A mixture of waste oil and 1/3 molar equivalent of methanol was used as substrate for the first-step methanolysis. The ME content in the eluate increased with decreasing flow rate and reached 33.5% at a flow rate of 6.1 mL/h. The glycerol generated was removed after allowing the reaction eluate to stand overnight. A mixture of 33.7% ME-containing oil layer and 1/3 molar equivalent of methanol was used as substrate for the second-step reaction. The conversion to ME in the second-step reaction was 65.7% after the flow rate was reduced to 6.3 mL/h. The third-step reaction was conducted similarly using 65.9% ME-containing eluate and 1/3 molar equivalent of methanol as substrate. The ME content was 85.1% at a flow rate of 6.1 mL/h but was increased to 90.9% by reducing the flow rate to 4.2 mL/h.

TABLE 1
Effect of Flow Rate on Three-Step Methanolysis of Waste Oil Using Fixed-Bed Bioreactors

First step[a]		Second step[b]		Third step[c]	
Flow rate (mL/h)	ME content (%)	Flow rate (mL/h)	ME content (%)	Flow rate (mL/h)	ME content (%)
6.1	33.5	6.3	65.7	4.2	90.9
10.9	27.2	9.6	64.8	6.1	85.1
19.6	20.0	19.5	60.7	10.4	75.4
38.4	12.6	38.8	53.4	18.3	70.7

[a]Substrate: waste oil and 1/3 molar equivalent of methanol.
[b]Substrate: the first-step eluate and 1/3 molar equivalent of methanol; methyl ester (ME) content, 33.7%.
[c]Substrate: the second-step eluate and 1/3 molar equivalent of methanol; ME content, 65.9%.
[d]Methyl ester content in the eluate.

To investigate the durability of the immobilized lipase in the methanolysis of waste oil, a three-step flow reaction was performed by fixing the flow rate in the first-, second-, and third-step reactors at 6, 6, and 4 mL/h, respectively. The ME content in the first-, second-, and third-step eluates at the beginning of the reaction was 32.6, 65.3, and 89.9%, respectively. Even after the reaction was continued for 100 d, the ME content in the eluates from the three reactors remained at 33.5, 65.8, and 90.3%, respectively. The immobilized lipase was then removed from each reactor and shaken at 30°C in a 75-g mixture of waste oil and 1/3 molar equivalent of methanol. The reaction rate of the methanolysis reaction with used lipase was the same as that of methanolysis with the immobilized lipase that had been used for five cycles. These results showed that contaminants in the waste oil did not affect the stability of the immobilized lipase preparation.

One-step flow methanolysis of waste oil. The immobilized lipase inactivated when >1/2 molar equivalent of methanol against total fatty acids was present in a reaction mixture. Stepwise addition of methanol, however, avoids the inactivation, and a three-step methanolysis procedure has been developed (15,16). On the other hand, if waste oil is diluted with twice its weight of ME, the lipase will not inactivate despite the presence of equimolar methanol against total fatty acids in the waste oil. Waste oil can be converted to biodiesel fuel in one step by feeding a mixture of waste oil, methanol, and its methylation product containing 90% ME into a fixed-bed bioreactor.

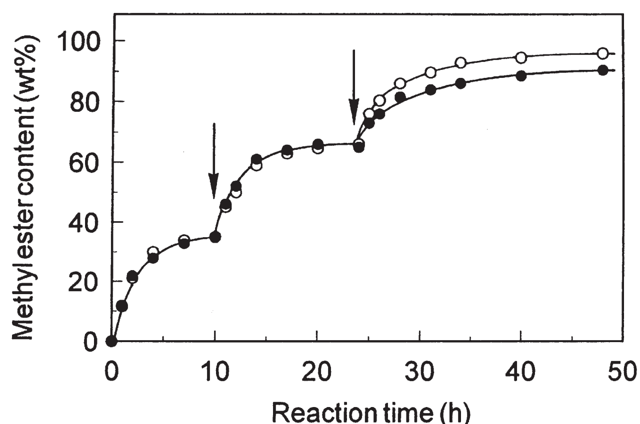

FIG. 2. Three-step batch methanolysis of waste and vegetable oils with immobilized *C. antarctica* lipase. The reaction was started in a 30-g mixture of oil, 1/3 molar equivalent of methanol, and 4 wt% immobilized lipase. Arrows indicate the addition of 1/3 molar equivalent of methanol. ○, Vegetable oil; ●, waste oil.

TABLE 2
Effect of Flow Rate on Methanolysis of the Third-Step Eluate and Waste Oil Mixture[a]

Flow rate (mL/h)	ME content (wt%)
3.9	90.2
6.3	80.0
10.7	71.3
22.3	68.9

[a]ME content of the substrate was 67.6%. See Table 1 for abbreviation.

TABLE 3
Compositions of the Second-Step Eluate and Mixture of the Third-Step Eluate and Waste Oil

	Composition[a] (wt%)				
Substrate	ME	MAG	DAG	TAG	FFA
Second-step eluate	64.8	9.6	7.4	18.0	0.2
Third-step eluate/waste oil	65.2	2.7	2.5	28.8	0.8

[a]MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.; FFA, free fatty acids. See Table 1 for other abbreviation.

Waste oil and methylation product (ME content, 90.7%) were mixed to final ME contents of 65, 75, and 8%. Each mixture (30 g) was shaken at 30°C with 4 wt% immobilized lipase in the presence of equimolar methanol against the total fatty acids in the waste oil. The reaction velocity was fast as the ME content was low. A significant difference, however, was not observed in the reaction time required to reach the steady state; the ME content reached 89–91% after 20–24 h in all reactions (data not shown).

From the above, the ratio of waste oil to methylation product was fixed at 3:1 (w/w), and the substrate was prepared by adding equimolar methanol against the total fatty acids in the waste oil. The mixture (ME content, 67.6%) was fed at 30°C into a column packed with 3 g of immobilized lipase at different flow rates. As shown in Table 2, the ME content in the eluate reached 90.2% at a flow rate of 3.9 mL/h. The flow rate was thus fixed at 4 mL/h, and the reaction was continued by returning 3/4 of the eluate onto the column, together with additional waste oil and equimolar methanol. In this one-step methanolysis of waste oil, the immobilized lipase could be used for 100 d without significant decrease in its activity.

Acylglycerol specificity of C. antarctica *lipase.* The second-step eluate in the three-step methanolysis procedure contained 65.9% ME, and the mixture of the third-step eluate and waste oil (3:1, w/w) contained 67.6% ME. The effects of flow rate on methanolysis of these two substrates were compared (Tables 1 and 2). Methanolysis of the second-step eluate was more efficient than methanolysis of the third-step eluate/waste oil, although ME content in the two substrates was similar. For an exact comparison of the efficiency of methanolysis, a 30-g mixture of each substrate and 1/3 molar equivalent of methanol against total fatty acids in the reaction mixture was shaken at 30°C with 4 wt% immobilized lipase. As shown in Figure 3, the methanolysis rate of the third-step eluate/waste oil mixture was half that of the second-step eluate. Table 3 shows the compositions of the two substrates. The main acylglycerols in the third-step eluate/waste oil mixture were TAG, whereas the second-step eluate contained a larger amount of partial acylglycerols. We thus investigated the activity of the *C. antarctica* lipase on acylglycerols. Either MAG, DAG, or TAG were individually added to a third-step eluate to a final 65% ME content. The resulting substrate mixtures were methanolyzed at 30°C with 1/3 molar equivalent of methanol and 4 wt% immobilized lipase (Fig. 4). The reaction velocities were in the order of MAG > DAG > TAG, indicating that the difference between the methanolysis velocities of the third-step eluate/waste oil and the

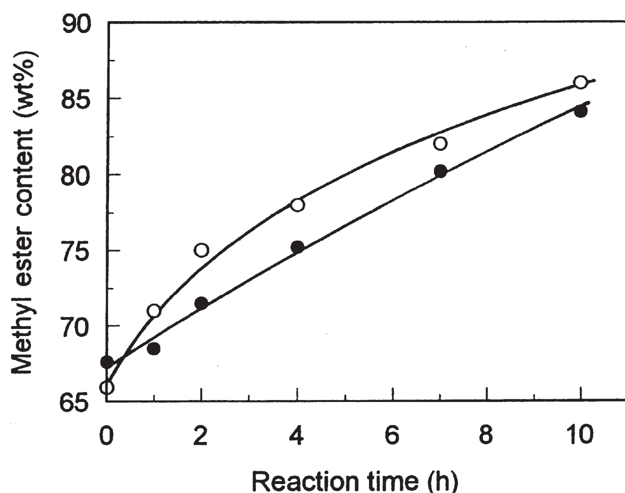

FIG. 3. Methanolysis of the second-step eluate and the mixture of third-step eluate and waste oil with immobilized *C. antarctica* lipase. A 30-g mixture of each substrate and 1/3 molar equivalent of methanol was shaken at 30°C with 4 wt% immobilized lipase. ○, Second-step eluate; ●, third-step eluate/waste oil.

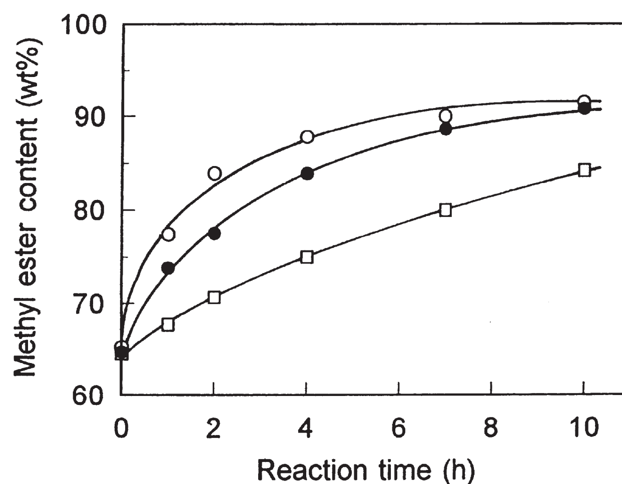

FIG. 4. Methanolysis of mono-, di-, and triacylglycerols with immobilized *C. antarctica* lipase. A 30-g mixture of the third-step eluate, acylglycerol, and 1/3 molar equivalent of methanol was shaken at 30°C with 4 wt% immobilized *C. antarctica* lipase. ○, Monoacylglycerols (ME content, 65.2%); ●, diacylglycerols (64.7%); □, triacylglycerols (64.5%). See Figure 1 for abbreviation.

second-step eluate is due to the activities of the lipase on acylglycerols (acylglycerol specificity).

DISCUSSION

We have shown that waste edible oil can be efficiently converted to its corresponding ME (biodiesel fuel) in a three-step fixed-bed methanolysis process. When the immobilized *C. antarctica* lipase is packed into the first, second, and third bioreactors at a ratio of 2:2:3, the reaction in each reactor can be conducted at the same flow rate. The three-step reaction also can be performed with one reactor by fixing the flow rates of the first, second, and third reactions at a ratio of 3:3:2. The amount of waste oil converted daily to biodiesel fuel by this three-step methanolysis process was 13.7 mL (11.9 g)/g lipase. Alternatively, a one-step methanolysis process is available for the production of biodiesel fuel from waste oil. When a mixture of the eluate and waste oil (3:1, w/w) is fed into the reactor together with methanol, one-quarter of the reaction eluate could be taken out as product. The daily production of biodiesel fuel from waste oil by this one-step process was 8 mL (7.0 g)/g lipase. These results show that the three-step flow methanolysis process is more effective for biodiesel fuel production from waste oil than the one-step reaction process.

Three-step batch methanolysis of vegetable and waste oils gave 95.9 and 90.4% conversions to ME, respectively (Fig. 2). After the reactions, a 1/3 molar equivalent of methanol against total fatty acids in the reaction mixtures was added, and the reactions were continued for 24 h under the same conditions. The conversion of vegetable and waste oils to ME was 97.9 and 92.7%, respectively. In general, when a vegetable oil is used for frying, some fatty acids are converted to polymers, epoxides, aldehydes, etc. by oxidation or thermal polymerization (2,3). The low conversion of waste oil to its corresponding MEs may be due to the oxidized fatty acid compounds that are not recognized as substrates by the lipase. Assuming that the content of these compounds in the used oil is about 5%, the methanolysis efficiency of waste oil will coincide with that of vegetable oil (92.7/95 = 97.6%). In addition, it is suggested that these compounds do not inhibit methanolysis of acylglycerols by *C. antarctica* lipase.

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