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Review

Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing

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Abstract

Biodiesel fuel (fatty acid methyl esters; FAMEs) can be produced by methanolysis of waste edible oil with a lipase. The degree of methanolysis was low in reaction systems so far reported, and the lipase catalyst could not be reused in spite of using immobilized enzyme. We clarified this problem was due to the irreversible inactivation of the lipase by contact with insoluble methanol (MeOH). Based on this result, we developed a stepwise methanolysis system with immobilized *Candida antarctica* lipase. Two-step batch methanolysis was most effective for the production of biodiesel fuel from waste oil: the first-step reaction was conducted in the presence of 1/3 molar equivalent of MeOH for the stoichiometric amount, and the second-step reaction was performed by adding 2/3 molar equivalent of MeOH. If the immobilized carrier is destroyed by agitation in a reactor with impeller, three-step flow reaction will be available: the first-step substrates were waste oil and 1/3 molar equivalent of MeOH; the second-step, the first-step eluate and 1/3 molar equivalent of MeOH; the third-step, the second-step eluate and 1/3 molar equivalent of MeOH. The conversion of waste oil to biodiesel fuel reached >90% in the two reaction systems, and the lipase catalyst could be used for >100 days without decrease of the activity. The stepwise alcoholysis could successfully be applied to ethanolysis of tuna oil.

Keywords: Biodiesel fuel; Alcoholysis; *Candida antarctica* lipase; Fixed-bed bioreactor; Docosahexaenoic acid ethyl ester

1. Introduction

Increase in the consciousness for environmental pollution creates the concept of zero emission, and complete recycling of waste edible oil recently attracts considerable attention as a world-wide social problem. In Japan, 400,000 t of waste edible oils are discharged yearly [1]. Half of this amount is estimated to be recycled as animal feed or row materials for lubricant and paint. The remainder, however, is discharged into the environment. Hence, production of a biodiesel fuel (fatty acid methyl esters, FAMEs) from waste edible oil is considered an important step of reducing and recycling waste oil. In this regard, several local governments in Japan have started collecting used flying oils from households and have converted them to biodiesel fuel for public transportation.

Presently, the industrial production of biodiesel fuel is performed by methanolysis of waste oil using alkaline catalysts. A by-product, glycerol, thus contains the alkali, and have to treated as a waste material. In addition, because waste oils contain a small amounts of water and free fatty acids (FFAs), the reaction generates fatty acid alkaline salts (soaps). The soaps are removed by washing water, which also removes glycerol, methanol (MeOH), and catalyst. Hence, disposal

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of the resulting alkaline water creates other environmental concerns. On the other hand, since enzymatic methanolysis of waste oil does not generate any waste materials, production of biodiesel fuel with a lipase is strongly desired.

2. Enzymatic alcoholysis so far reported

Lipases catalyze not only hydrolysis, but also esterification and transesterification in nonaqueous medium. Methanolysis of triacylglycerols (TAGs) with a lipase thus is considered one of the effective reactions for production of biodiesel fuel from waste edible oil. Before we started the study of enzymatic biodiesel production, the following enzymatic alcoholysis systems had been reported.

Immobilized *Rhizopus delemar* and *Rhizomucor miehei* lipases efficiently catalyzed alcoholysis with long-chain fatty alcohols even in the presence of 20% water [2–4]. Because the lipases acted on polyunsaturated fatty acids (PUFAs) weakly, alcoholysis of fatty acid ethyl esters (FAEEs) originating from tuna oil with lauryl alcohol (LauOH) converted FAEEs except PUFA ethyl esters (PUFAEEs) to their corresponding lauryl esters, resulting in enrichment of PUFAEEs in the ethyl ester fraction. Ethanolysis of fish oil was also conducted to enrich PUFAs [5–7]. When sardine and tuna oils underwent alcoholysis with a stoichiometric amount of ethanol (EtOH) using free *Pseudomonas* lipase, fatty acids except PUFAs were preferentially converted to their ethyl esters. Hence, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were enriched in the acylglycerol fraction.

In addition to the above selective alcoholysis, non-selective ethanolyses of TAGs had been reported. Immobilized *Candida antarctica* lipase was used for alcoholysis of PUFA-rich oil with 1.7 mol equivalents of EtOH for the stoichiometric amount. Because the lipase acted on PUFAs as strongly as the other constituent fatty acids, the oil was converted to its corresponding FAEEs [6]. Even a lipase which possesses weak activity on PUFAs can be used as a catalyst for efficient ethanolysis of fish oils, if the reaction is conducted in an organic solvent system. Alcoholysis of sardine and tuna oils with 1.3 molar equivalents of EtOH using immobilized *R. miehei* lipase in the presence of *n*-hexane allowed to convert >90% of the oils to their FAEEs [8].

Nelson et al. [9] first carried out enzymatic alcoholysis of TAGs with the aim of biodiesel production. When alcoholyses of several oils and fats with MeOH and EtOH were conducted using immobilized *R. miehei* lipase in the presence of *n*-hexane, >95% of the TAGs were converted to their methyl (ethyl) esters. Methanolysis of beef tallow reached 65% under the similar reaction conditions but in the absence of organic solvent. Meanwhile, the use of longer-chain alcohols as substrates increased alcoholysis of beef tallow even in an organic solvent-free system: 90% conversion was obtained in the reaction with isopropanol; 95% conversion in the reactions with *iso*-butanol and 2-butanol. In addition, they reported that immobilized *C. antarctica* lipase was suitable as a catalyst for alcoholysis of TAGs with MeOH and EtOH.

The above reports can be summarized as follows. (i) Lipases catalyze alcoholysis of TAGs, and the reactions with longer-chain fatty alcohols proceeded more efficiently than those with MeOH and EtOH; (ii) alcoholyses of TAGs with MeOH and EtOH efficiently proceeded in the presence of organic solvent, but not in the absence; (iii) though immobilized lipase was used as a catalyst, the enzyme preparation could not be reused. An organic solvent system is not suitable for the production of biodiesel fuel from waste oil because of the risk of explosion and requirement of the solvent removal. We thus aimed to develop an organic solvent-free system, in which >90% of TAGs can be converted to their methyl esters and the lipase catalyst can be used for a long period.

3. Enzymatic production of biodiesel fuel

3.1. Inactivation of lipase by insoluble MeOH

In general, lipases efficiently catalyze the reactions, when the substrates dissolve each other. Our first experiment was to investigate the solubility of fatty alcohols in a vegetable oil (a mixture of soybean and rapeseed oils). Fatty alcohols, of which the carbon lengths are >3, completely dissolved in the oil in a stoichiometric amount, but the solubility of MeOH and EtOH was 1/2 and 2/3 of the stoichiometric amount, respectively. Disregarding the low solubility, all

alcoholyses of TAGs so far reported were conducted with more than the stoichiometric amount of MeOH or EtOH. We were aware that proteins generally are unstable in short-chain alcohols, such as MeOH and EtOH. We thus hypothesized that low methanolysis (ethanolysis) is due to the inactivation of lipases by contact with insoluble MeOH (EtOH) which exists as drops in the oil. Actually, MeOH was completely consumed in methanolysis of vegetable oil with <1/3 molar equivalent of MeOH for the stoichiometric amount using immobilized *C. antarctica* lipase, but the methanolysis was decreased significantly by adding >1/2 molar equivalent of MeOH (Fig. 1). In addition, the decreased activity did not restore in subsequent reaction with 1/3 molar equivalent of MeOH, showing that the immobilized lipase was irreversibly inactivated by contact with insoluble MeOH in the oil.

3.2. Stepwise batch methanolysis of vegetable oil

At least a stoichiometric amount of MeOH is required for the complete conversion of TAGs to their corresponding FAMEs. Immobilized *C. antarctica* lipase, however, was inactivated by adding >1/2 molar equivalent of MeOH for the stoichiometric amount, and methanolysis ceased. Hence, we attempted the methanolysis of vegetable oil by three successive additions of 1/3 molar equivalent of MeOH [10] (Fig. 2). The first-step methanolysis was conducted at 30 °C in a mixture of the oil, 1/3 molar equivalent of MeOH, and 4% of the immobilized lipase by weight of the reaction mixture. The conversion of the oil reached 33.1% at 7 h. The addition of a second 1/3 molar equivalent of MeOH at 10 h converted 66.4% of the oil to its corresponding FAMEs after 10 h (total, 20 h). A third 1/3 molar equivalent of MeOH was added again after a total of 24 h, and the reaction was continued. After 24 h (48 h in total), the conversion reached 97.3%, showing that three-step methanolysis was effective for nearly complete conversion of the oil.

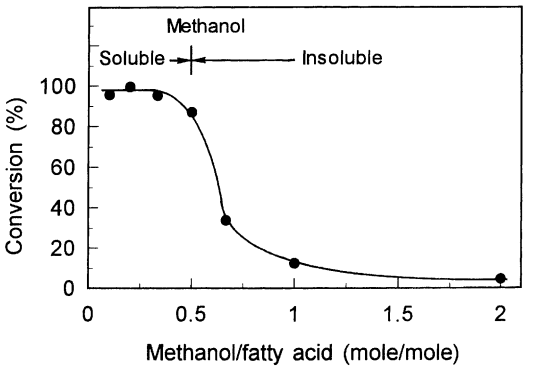

Fig. 1. Methanolysis of vegetable oil with different amounts of MeOH using immobilized *C. antarctica* lipase (Novozym 435; Novozymes, Bagsvaerd, Denmark). A mixture of 10 g vegetable oil/MeOH and 0.4 g immobilized lipase was shaken at 30 °C for 24 h. The conversion is expressed as the amount of MeOH consumed for the ester conversion of the oil (when the molar ratio of MeOH/oil is less than 1.0), and as the ratio of FAMEs to oil (more than 1.0).

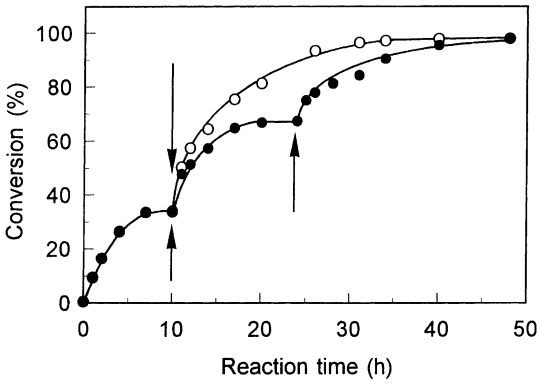

Fig. 2. Time courses of stepwise methanolysis of vegetable oil. Three-step reaction (●): a mixture of 28.95 g vegetable oil, 1.05 g MeOH (1/3 molar equivalent for the stoichiometric amount), and 4 wt.% immobilized *C. antarctica* lipase was incubated at 30 °C with shaking at 130 oscillations/min, and 1.05 g MeOH was added at 10 and 24 h. Two-step reaction (○): the first-step reaction was conducted under the same conditions as those of three-step reaction. After 10 h reaction, 2.10 g MeOH (2/3 molar equivalent) was added in the reaction mixture. Upward and downward arrows indicate the addition of 1/3 and 2/3 molar equivalent of MeOH, respectively.

Solubility of MeOH in TAG is low, but high in FAME. The first-step reaction product was composed of acylglycerols and 33% FAMEs, and 2/3 molar equivalent of MeOH against total fatty acids was completely soluble in the reaction mixture. Immobilized *C. antarctica* lipase was found not to inactivate even in the mixture of acylglycerols/FAMEs and the amount of MeOH [11]. This finding led to success in a two-step methanolysis of the oil TAG. Fig. 2 shows a typical time course. The first-step methanolysis was started at 30 °C with shaking in a mixture of the oil,

1/3 molar equivalent of MeOH, and 4 wt.% immobilized lipase. The conversion of the oil reached 33.2% at 7 h. The addition of a second 2/3 molar equivalent of MeOH at 10 h converted 96.8% of the oil to its corresponding FAMEs after 24 h (total, 34 h).

To investigate the stability of immobilized lipase preparation, two- and three-step methanolyses were repeated [10,11]. The three-step methanolysis was performed as follows: the first-step reaction was conducted with shaking at 30 °C for 10 h in a mixture of vegetable oil, 1/3 molar equivalent of MeOH, and 4 wt.% immobilized lipase, the second and third 1/3 molar equivalent of MeOH were respectively added at 10 and 24 h, and the third-step reaction was continued for 24 h (total, 48 h). Meanwhile, the two-step reaction was started under the same conditions as those of the three-step reaction. After the addition of 2/3 molar equivalent of MeOH at 12 h, the reaction was continued for 24 h (total, 36 h). The two reactions were recycled by transferring the lipase to the fresh first-step oil/MeOH mixture. More than 95% conversion was maintained during 52 cycles in three-step methanolysis (104 days) or during 70 cycles in two-step methanolysis (105 days). The remaining acylglycerols were not TAG but partial acylglycerols. In addition, the FFA content was less than 0.25%, showing that simultaneous hydrolysis was negligible. These results indicated that *C. antarctica* lipase can be used for more than 100 days without significant loss of activity. In addition, the two-step methanolysis increased the productivity of 25% with the same lipase durability.

3.3. Continuous flow methanolysis of vegetable oil

When biodiesel fuel is industrially produced by the two-step batch reaction, a reactor with impeller can be used. However, disruption of the enzyme carrier by the physical agitation force may not promise the use of immobilized lipase for a long period. We thus attempted a two-step continuous flow reaction using a fixed-bed bioreactor with immobilized *C. antarctica* lipase. The lipase was not inactivated by feeding a mixture of vegetable oil and 1/3 molar equivalent of MeOH, but was inactivated significantly by feeding a mixture of first-step eluate and 2/3 molar equivalent of MeOH [11]. As stated previously, the addition of 2/3 molar equivalent of MeOH did not inactivate the lipase in the second step of two-step batch reaction. This discrepancy may be explained by the effect of by-product of the methanolysis, glycerol. In the flow reaction with fixed-bed reactor, quite a lot of glycerol remained in the bottom of the reactor because of its high viscosity. The glycerol disturbs the diffusion of substrates to lipase molecule, and the decrease in reaction efficiency gradually increases unreacted MeOH. The MeOH migrates from reaction mixture to glycerol layer, and the lipase is inactivated by higher concentration of MeOH in glycerol layer. Meanwhile, in two-step batch reaction, the amount of MeOH is fixed, and the immobilized carrier is not covered with glycerol by vigorous shaking. Therefore, the lipase in two-step batch reaction may not be inactivated by MeOH.

Because immobilized *C. antarctica* lipase was not inactivated at the lower concentration of MeOH, we attempted methanolysis of vegetable oil by a three-step flow reaction [11]. The reaction was conducted at 30 °C with three columns (15 mm × 80 mm) in which 3 g of immobilized lipase was packed. A mixture of vegetable oil and 1/3 molar equivalent of MeOH was fed into the first column at a constant flow rate of 6.0 ml/h (the conversion, 33%). The eluate was allowed to stand overnight to remove glycerol. The second 1/3 molar equivalent of MeOH was added in the glycerol-free eluate, and the substrate mixture was then fed into the second column at the same flow rate, 6.0 ml/h (conversion, 66%). The final step reaction was conducted by feeding a mixture of glycerol-free eluate and 1/3 molar equivalent of MeOH at the same flow rate (conversion, 93%). The three-step reaction was continued for 100 days, but the lipase activity scarcely decreased. The FAME content in the final product of flow reaction was slightly lower than that in the product of batch reaction, but the FFA content was only 0.2% and the remaining acylglycerols mainly were partial acylglycerols. These results show that immobilized *C. antarctica* lipase works efficiently not only in stepwise batch reaction but in three-step flow methanolysis.

3.4. Production of biodiesel fuel from waste edible oil

Our aim is enzymatic conversion of waste edible oil to biodiesel fuel. Because stepwise methanolysis was effective for conversion of vegetable oil to its

corresponding FAMEs, we next attempted to produce biodiesel fuel from waste edible oil [12].

3.4.1. Effect of water in waste oil on its methanolysis

A fresh vegetable oil and its waste differ significantly in water and FFA contents. The waste edible oil used here contained 1980 ppm water, 2.5% FFA, and 4.6% partial acylglycerols. We clarified that >500 ppm water decreased the rate of TAG methanolysis but did not affect the equilibrium of the reaction [10]. Hence, we first studied the effect of water on waste oil methanolysis. When vegetable oil underwent methanolysis with 1/3 molar equivalent of MeOH for the stoichiometric amount, the conversion after 1 and 7 h were 9.0 and 33.2%, respectively (Fig. 2). The conversion rate did not change even though the methanolysis was repeated by transferring the lipase into a fresh substrate.

Methanolysis of waste oil was similarly repeated (Fig. 3). The conversion after 1 h in the first-cycle reaction was slower than the vegetable oil methanolysis, and the conversion increased with repeating the reaction; the conversions after 1 h in the first-, third-, and fifth-cycle reactions were 3.0, 8.3, and 10.7%, respectively. These results show that the inhibition of methanolysis by a small amount of water present in the oil is eliminated by performing the reaction in cycles.

The increase in velocity of waste oil methanolysis can be explained as follows. Water initially present in the waste oil transfers into the glycerol layer generated by methanolysis. Because the water goes out of the methanolysis system, the reaction velocity gradually increased. Actually, the water content in the FAMEs/acylglycerols layer (oil layer) decreased to 500 ppm and that of the glycerol layer was 4.1% after five cycles. When 33% waste oil is converted to its FAMEs, the glycerol content in the reaction mixture is calculated to be 3.9%. Water content in the oil layer decreased 1480 ppm (=1980 − 500). If the water transferred to glycerol layer, the content would become 4.2%. This value well coincided with the observed one. These results show that the content of water adsorbed on immobilized lipase reaches a constant value by repeating the reaction, and that the water content does not disturb the methanolysis reaction.

3.4.2. Three-step batch methanolysis of waste oil

Immobilized *C. antarctica* lipase that had been used for five cycles was employed for three-step methanolysis of waste and vegetable oils. As shown in Fig. 4, no differences were observed between the time courses of the first- and second-step methanolyses of the two oils, and the FAME contents reached 34 and 66% after the first- and second-step reactions, respectively. However, the conversion of waste oil reached 90.4% after

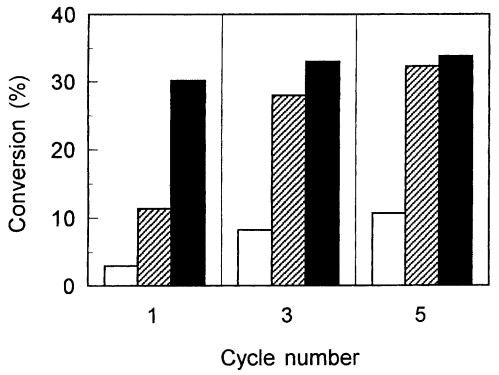

Fig. 3. Effect of a small amount of water on methanolysis of waste oil (water content, 1980 ppm) by immobilized *C. antarctica* lipase. The reaction was performed at 30 °C with shaking (130 oscillations/min) in a 30 g mixture of the oil, 1/3 molar equivalent of methanol, and 4 wt.% immobilized lipase. The methanolysis was repeated by transferring the lipase to a fresh substrate mixture every 24 h. □, FAME content in the reaction after 1 h; ▨, FAME content after 7 h; ■, FAME content after 24 h.

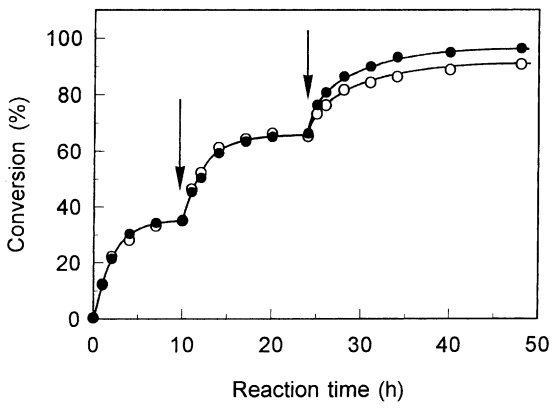

Fig. 4. Three-step batch methanolyses of waste oil (●) and vegetable oil (○) with *C. antarctica* lipase. The reactions were started at 30 °C with shaking (130 oscillations/min) in a 30 g mixture of oil, 1/3 molar equivalent of MeOH for the stoichiometric amount, and 4 wt.% immobilized lipase. Arrows indicate the addition of 1/3 molar equivalent of MeOH.

the three-step methanolysis, although that of vegetable oil was 95.9%. The difference may be attributed to the oxidized fatty acid compounds in waste oil. In general, when a vegetable oil is used for frying, some fatty acids are converted to epoxides, aldehydes, polymers, etc. by oxidation or thermal polymerization [13,14]. Because the lipase did not recognize these oxidized compounds, the conversion of waste oil decreased a little. In addition, the FFA content in the product after the three-step reaction (0.3%) was lower than that in the waste oil (2.5%), indicating that methylation of FFA occurred along with methanolysis of the oil.

To investigate the lipase stability, the three-step batch methanolysis was repeated by transferring the lipase to a fresh substrate: first-step, 10 h; second-step, 14 h; and third-step, 24 h. The conversion did not significantly decrease even after 50 cycles (100 days), showing that contaminants in waste oil did not affect the stability of the lipase preparation.

3.4.3. Three-step flow methanolysis of waste oil

Water and FFA present in waste oil had little effect on the methanolysis. We thus studied three-step flow methanolysis in a fixed-bed bioreactor packed with *C. antarctica* lipase [12]. The effect of flow rate on methanolysis of waste oil was first examined, and the amount of lipase packed in three reactors was fixed as follows: the first and second reactors, 3 g; the third reactor, 4.5 g. Continuous flow methanolysis of waste oil using the three fixed-bed bioreactors in series is schematically depicted in Fig. 5. A mixture of waste oil and 1/3 molar equivalent of MeOH was fed into the first reactor. The eluate was collected in a receiver, in which acylglycerols/FAMEs mixture and glycerol were separated completely by allowing to stand. The glycerol-free oil layer was mixed with 1/3 molar equivalent of MeOH, and the resulting substrate mixture was fed into the second reactor. The third-step reactor was similarly operated by feeding a mixture of the second-step eluate and MeOH. Each substrate was fed into the first, second, and third reactors at the same rate, 6 ml/h, and the flow reaction system was operated at 30 °C during 100 days. The composition of the three-step reaction product is shown in Table 1. After the reaction, the immobilized lipase was removed from each reactor, and the remaining activity was measured from the batch reaction of which reaction conditions were the same as those of the first-step methanolysis

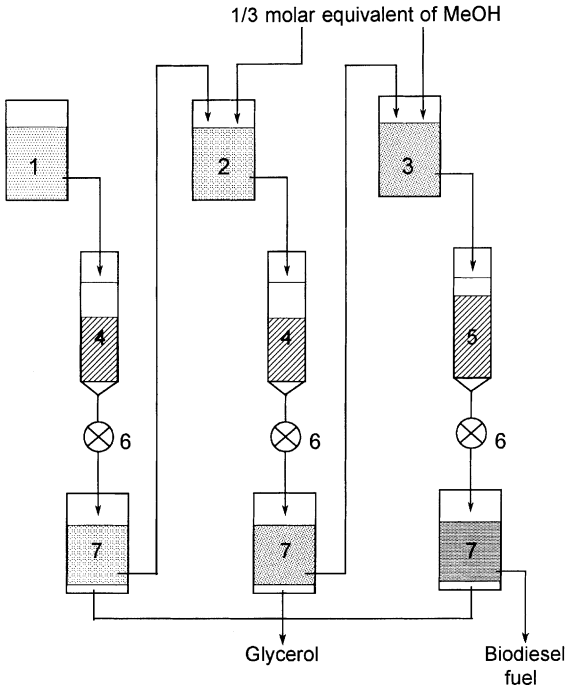

Fig. 5. Continuous flow production of biodiesel fuel with three fixed-bed bioreactor in series. 1, Storage vessel of first-step substrate (waste oil and 1/3 molar equivalent of MeOH); 2, storage vessel of second-step substrate (oil layer of first-step eluate and 1/3 molar equivalent of MeOH); 3, storage vessel of second-step substrate (oil layer of second-step eluate and 1/3 molar equivalent of MeOH); 4, fixed-bed bioreactor packed with 3 g immobilized *C. antarctica* lipase; 5, fixed-bed bioreactor packed with 4.5 g immobilized *C. antarctica* lipase; 6, peristaltic pump; 7, receiver of eluted reaction mixture.

of waste oil. The activity of the used lipase was the same as that of the fresh enzyme preparation, showing that the lipase preparation was very stable under the conditions described here.

Methylation product was prepared only by removing glycerol after allowing the third-step eluate to stand overnight, and any post-treatment was not performed. Some fuel properties of the product were investigated according to international organization standardization (ISO) method, and the results are shown in Table 2. Contamination of MeOH in the methylation product decreased the flash point, which was increased to >110 °C by evaporating the MeOH. These properties showed that the methylation product can be used as biodiesel fuel. Indeed, we could drive

Table 1
Composition of product obtained by three-step flow methanolysis of waste oil

Operation (day)	FAME content (wt.%)		Composition of third step reaction product (wt.%)				
	First step	Second step	FAME	MAG[a]	DAG[b]	TAG	FFA
1	32.6	65.3	89.9	3.6	4.3	2.0	0.2
10	33.8	66.1	90.5	3.3	4.1	1.9	0.2
30	32.0	65.7	90.1	3.4	4.5	1.8	0.2
50	33.4	66.9	89.0	3.5	4.8	2.4	0.3
75	33.7	65.4	89.7	3.3	4.7	2.1	0.2
100	33.5	65.8	90.3	3.1	4.5	1.9	0.2

[a] Monoacylglycerol.
[b] Diacylglycerol.

a diesel car without any trouble using the product as a fuel.

3.5. Production of biodiesel fuel from crude oil

Waste edible oil is a suitable material for production of biodiesel fuel, and surplus edible oils are also the candidates. We thus attempted to produce biodiesel fuel from crude soybean oil (details are reported in this issue). The crude oil, however, was not converted to its corresponding FAMEs by methanolysis with immobilized *C. antarctica* lipase. In the refining process of edible oil, the next step of extraction is degumming. Hence, we performed methanolysis of degummed soybean oil in the three-step batch system. While the reaction velocity in each step was approximately half compared with that of the refined oil methanolysis, the conversion in the third step reached >90% and the immobilized lipase could be used for >25 cycles (50 days). The main components of soybean gum are phospholipids (PLs), and addition of 1% soybean PLs in refined soybean oil significantly inhibited the methanolysis. In addition, PLs were detected in chloroform/MeOH (2:1, vol/vol) extracts from the immobilized lipase which had been used for methanolysis of crude soybean oil. These results showed that PLs bound around the immobilized carrier and interfered with interaction of the lipase molecule with substrates. The degummed oils therefore have to be used as substrates for methanolysis of surplus edible oils.

Table 2
Fuel properties of enzymatically produced biodiesel fuel from waste edible oil

Property		Method
Specific gravity (15/4 °C)	0.893 g/cm^3	ISO3675
Kinematic viscosity (at 30 °C)	9.7 mm^2/s	ISO3104
Flash point	75 °C	ISO2719
Cetan number	50	ISO5165

4. Methanolysis of TAGs using free lipase

As stated previously, Dr. Haraldsson et al. reported that fish oil underwent alcoholysis with more than the stoichiometric amount of EtOH using a large amount of *Pseudomonas* lipase (10% by weight of reaction mixture) [5,7]. We assumed the necessity of a large amount of lipase was due to the inactivation by EtOH. Hence, if the inactivation was eliminated, the ethanolysis should proceed efficiently with a small amount of enzyme. Laboratory of Prof. Fukuda, Kobe University, found that 80–90% of soybean oil was converted to its corresponding FAMEs by a three-step methanolysis with free *Rhizopus oryzae* lipase when 10–30% water was present in the reaction mixture [15]. This result indicated that the inactivation of lipase was eliminated by further dilution of a small amount of MeOH with water.

The reaction mixture contained a large amount of water, but TLC analysis showed that the content of FFA was very low at the end of reaction although it was detected in the early stage of reaction [15]. It was shown from this fact that hydrolysis of soybean oil occured along with its methanolysis. However, lipases catalyze esterification of FFA with MeOH strongly and

hydrolysis of FAME very weakly [16], resulting in a small content of FFA in the product at the end of reaction. Under the influence of this study, we conducted methanolysis of TAGs with immobilized *C. antarctica* lipase in the presence of water. But >1% water significantly inhibited the methanolysis. This may be due to the poor hydrolysis activity of the immobilized preparation.

5. Application of stepwise alcoholysis to oil processing

5.1. Necessity of enzymatic ethanolysis of fish oils

PUFAs have various physiological functions, and are used in various areas. Ethyl ester of EPA has been used for the treatment of arteriosclerosis and hyperlipemia since 1991 in Japan [17]. DHA possesses the same physiological activities as those of EPA, and has been reported to exhibit an important function in the brain [18] and retina [19]. In addition, DHA accelerates the growth of preterm infants as does arachidonic acid [20,21]. For these reasons, ethyl ester of DHA (DHAEE) is currently expected to be used as a pharmaceutical agents, and the development of the purification methods is strongly desired.

Enzymatic process is one of the effective techniques for purification of DHAEE. When FAEEs containing DHAEE underwent alcoholysis with LauOH using a lipase which acted on DHA weakly, FAEEs except DHAEE were preferentially converted to their lauryl esters. The selective alcoholysis increased the DHAEE content in the ethyl ester fraction to >90% [2,3,22]. Furthermore, the reaction products easily were separated to LauOH, FAEEs rich in DHAEE, and fatty acid lauryl esters (FALEs) by molecular distillation [4,22]. Meanwhile, FAEEs containing DHAEE can be prepared by chemical ethanolysis of tuna oil with an alkaline catalyst, but enzymatic process under mild conditions are preferable because heating under alkaline conditions often results in the isomerization of DHA. Hence, in order to develop a new process of purifying DHAEE from tuna oil only by a combination of enzyme reaction and distillation, we applied the stepwise alcoholysis system developed for biodiesel production to ethanolysis of tuna oil.

5.2. Stepwise ethanolysis of tuna oil

We first studied the inactivation of immobilized *C. antarctica* lipase by EtOH. The lipase also was irreversibly inactivated in the presence of 2/3 molar equivalent of EtOH for the stoichiometric amount [23]. To avoid such inactivation, the first-step ethanolysis was conducted at 40 °C in a mixture of tuna oil and 1/3 molar equivalent of EtOH using 4 wt.% immobilized lipase. The reaction completely consumed EtOH after 10 h, and 33% of tuna oil was converted to its corresponding ethyl esters. The lipase was not inactivated in the presence of 2/3 molar equivalent of EtOH against the total fatty acids in the first-step reaction product. Based on these results, The reaction conditions for two- and three-step ethanolyses of tuna oil were determined as follows. Two-step reaction: the first step was carried out at 40 °C for 12 h in a 10 g mixture of tuna oil, 1/3 molar equivalent of EtOH, and 4 wt.% immobilized lipase; the second step was performed for 36 h after adding 2/3 molar equivalent of EtOH (total reaction period, 48 h). Three-step reaction: the first-step was carried out under the same conditions as those in the two-step reaction; the second and third 1/3 molar equivalents of EtOH were added after 12 and 24 h, respectively, and the third step was continued for 24 h (total, 48 h). To investigate the lipase stability, the two- and three-step reactions were repeated by transferring the enzyme to the fresh first-step substrate mixture [23]. The results are shown in Fig. 6. The conversion in the two-step ethanolysis did not decrease up to the 37th cycle, and then decreased rapidly. However, the

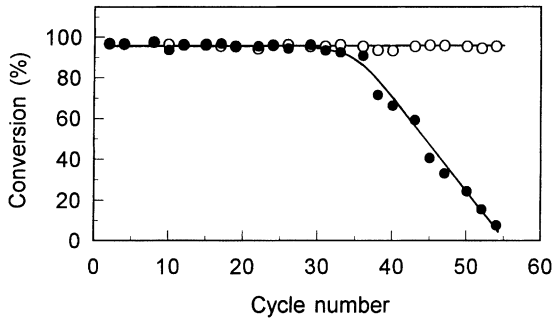

Fig. 6. Stability of immobilized *C. antarctica* lipase in two- and three-step ethanolyses of tuna oil. The reaction conditions were described in the text. (○) Three-step ethanolysis; (●) two-step ethanolysis.

three-step reaction maintained a conversion of over 95% up to the 54th cycle (108 days).

5.3. Inactivation of lipase in two-step ethanolysis

In the three-step reaction described in Fig. 6, the activity of the immobilized lipase used for 54 cycles decreased to 32% of its initial activity (half-life of the lipase preparation, 68 days). It was also observed that some EtOH still remained in the reaction mixture at the end of the first step. From these results, the significant decrease in enzymatic activity in the two-step reaction could be explained as follows: (i) the lipase was gradually inactivated after a long period use; (ii) some EtOH remained in the reaction mixture at the end of the first step; (iii) when 2/3 molar equivalent of EtOH was added for starting the second-step reaction, the amount of EtOH exceeded the permitted limits for maintaining the lipase stability; (iv) the high concentration of EtOH inactivated the lipase, thus, decreasing the conversion. On the other hand, because the amount of EtOH added in the three-step reaction was 1/3 molar equivalent, the amount of EtOH in the reaction mixture did not exceed 2/3 molar equivalent. Thus, the lipase was not inactivated by EtOH. These results suggest that the lipase can be used for a long period in the two-step reaction by adding the second 2/3 molar equivalent of EtOH after the complete consumption of the first 1/3 molar equivalent of EtOH.

Production of biodiesel fuel from waste edible oil was conducted at 30 °C. Meanwhile, ethanolysis of tuna oil was carried out at 40 °C, because some of the component(s) was crystallized in the early stage of reaction and the viscosity of the reaction mixture increased. The activity of the lipase preparation scarcely decreased after 100 days in the former reaction, but decreased to 1/3 of the initial activity in the latter reaction. These results shows that lower reaction temperature is preferable for using the lipase for a long period.

5.4. Purification of DHAEE from tuna oil by enzymatic process

Development of an effective ethanolysis system of tuna oil allowed to purify DHAEE from tuna oil only by a combination of enzymatic reactions and distillation. Outline of the total purification process is shown in Fig. 7. Tuna oil is converted to its corresponding ethyl esters by stepwise ethanolysis with immobilized *C. antarctica* lipase which acts on PUFAs as strongly as the other constituent fatty acids. The reaction mixture is applied to molecular distillation. Tuna oil is constituted of 14–22 carbon-number fatty acids, and their ethyl esters can roughly be separated into fractions of different carbon-number fatty acids even by molecular distillation. FAEEs rich in DHAEE are alcoholyzed with LauOH using immobilized *R. miehei* lipase which acts on DHA weakly. FAEEs except DHAEE easily are converted to their lauryl esters, and the resulting reaction mixture contains LauOH (molecular weight, 186), FAEEs (DHAEE, 328), and FALEs (>396). These components are easily separated by molecular distillation, and the purity of DHAEE can be increased to more than 90 wt.% [4].

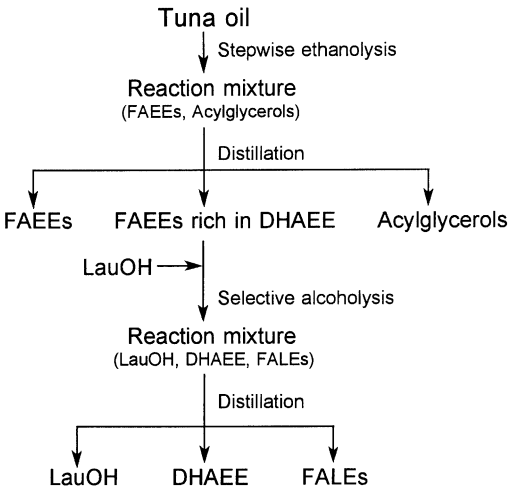

Fig. 7. Outline of DHAEE purification from tuna oil by a combination of enzymatic reactions and distillation.

6. Conclusion

We have shown that stepwise alcoholysis system is effective for production of biodiesel fuel from waste edible oil. Based on these studies, we take it for granted that two-step batch methanolysis at 30 °C is most effective for production of biodiesel fuel from waste edible oil: the daily amount of waste oil converted to biodiesel fuel is 16.7 g/g lipase. If

immobilized carrier is destroyed by the mechanical agitation, three-step flow reaction will be available for the purpose although it decreases the daily conversion to 13.7 ml (11.9 g)/g lipase. Either system can be selected according to circumstances.

We have also shown stepwise alcoholysis was effective for conversion of fish oil to its FAEEs. In addition, this reaction system is applicable not only to the continuous reaction with immobilized lipase but to the batch reaction with free enzyme. Batch reaction is generally useful for the industrial production of a small amount of high value-added substances. Recently, a great deal of attention is focused on the improvement of oils and fats and purification of useful oil- and fat-related materials using lipase reactions. We hope that application of new lipase reactions to oil processing will expand further.

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