

Production of Biodiesel from Acid Oil via a Two-Step Enzymatic Transesterification

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Abstract: A two-step enzymatic transesterification process in a solvent-free system has been developed as a novel approach to the production of biodiesel using acid oil from rice bran oil soapstock. The acid oil consisted of 53.7 wt% fatty acids, 2.4 wt% monoacylglycerols, 9.1 wt% diacylglycerols, 28.8 wt% triacylglycerols, and 6.0 wt% others. Three immobilized lipases were evaluated as potential biocatalysts, including Novozym 435 from *Candida antarctica*, Lipozyme RM IM from *Rhizomucor miehei*, and Lipozyme TL IM from *Thermomyces lanuginosus*. The effects of molar ratio of acid oil to ethanol, temperature, and enzyme loading were investigated to determine the optimum conditions for the transesterification with the three immobilized lipases. The optimum conditions of the three immobilized lipases were a molar ratio of 1:5 (acid oil to ethanol), the temperature range of 30-40°C, and the enzyme loading range of 5-10%. The two-step transesterification was then conducted under the optimum conditions of each lipase. The stepwise use of Novozym 435 and Lipozyme TL IM or Lipozyme RM IM and Lipozyme TL IM resulted in similar or higher levels of yield to the individual lipases. The maximum yields obtained in both stepwise uses were ca. 92%.

Key words: acid oil, biodiesel, by-product, lipase, transesterification

1 INTRODUCTION

Biodiesel is an alternative fuel for diesel and is becoming increasingly important because of diminishing petroleum reserves and the adverse environmental impact of the green-house gases derived from fossil fuels. There has also been considerable interest in the use of biodiesel as a non-toxic, biodegradable, and renewable source of fuel and energy because it produces low exhaust emissions of particulate matter and green-house gases such as CO, CO₂, and SO_x¹⁻³.

Biodiesel is synthesized by the transesterification of fats and oils with short-chain alcohols⁴. The synthesis of biodiesel can be classified as a chemical or enzymatic production process depending on the type of catalyst used to facilitate the transformation. Although biodiesel has been successfully produced using a variety of chemical catalysts, such as sodium and potassium hydroxide^{5,6}, there are several drawbacks to these methods, including the requirement for an additional work-up step to remove the catalyst, difficulties associated with the recovery of glycerol, and

the energy intensive nature of these processes⁷. The use of lipase as a catalyst for the production of biodiesel allows for most of these issues to be avoided, as well as allowing for the transesterification reaction to be conducted under mild reaction conditions in the presence of water, which avoids any issues associated with soap formation⁸⁻¹².

In the biodiesel industry, methanol is most widely used because of its economic feasibility. However, for enzymatic transesterification, methanol has a critical problem that it has a stronger denaturing activity compared with longer aliphatic alcohols^{3,4}. In contrast, ethanol is not only a renewable resource that can be obtained from agricultural feedstocks but also affects less enzyme deactivation.

Refined oils are used as a major feedstock for the production of biodiesel, and account for ca. 70% of the total cost of the biodiesel production process¹³⁻¹⁵. Because of the expensive nature of the refined oil feedstock, it is difficult for the resulting biodiesel product to compete economically with petroleum-derived diesel fuel^{16,17}. Numerous studies have been conducted towards investigating the

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production of biodiesel using lower value lipids, namely animal fats, and waste cooking oils as feedstocks^{18–20}.

Acid oil, which is generated as the major by-product during the acidulation of the soapstock formed in oil refining processes, represents a low value feedstock candidate for the preparation of biodiesel^{21, 22}. Acid oil can be obtained largely from rice bran oil soapstock during the refining process because of the high lipase activity of rice bran. Several studies concerning the production of biodiesel from acid oil have been reported in the literature. Tüter *et al.*²¹ reported the production of biodiesel via the transesterification reactions of sunflower acid oil and corn acid oil with a variety of alcohols in *n*-hexane. Novozym 435 was used as a biocatalyst in their study, and gave a maximum yield of ca. 72% when the sunflower acid oil was reacted with *n*-octanol. Watanabe *et al.*²² reported that the acylglycerols present in rapeseed acid oil could be completely converted to the free fatty acids by the lipase activity of *Candida rugosa*. The resulting fatty acid was then converted to the corresponding fatty acid methyl ester using Novozym 435. Watanabe *et al.*²³ also succeeded in producing biodiesel from acid oil and methanol via the repeated enzymatic transesterification of these materials in the presence of Novozym 435. It is noteworthy, however, that the use of Novozym 435 as a biocatalyst for the large-scale production of biodiesel would not be economically viable, because this material is one of the most expensive immobilized lipases currently available.

In the current study, we have developed a two-step enzymatic transesterification process as a novel approach to the production of biodiesel using acid oil from rice bran oil soapstock under solvent-free conditions. The effects of the molar ratio (acid oil to ethanol), temperature, and enzyme loading on the production of biodiesel have been investigated using three immobilized lipases, including Novozym 435 from *Candida antarctica*, Lipozyme RM IM from *Rhizomucor miehei*, and Lipozyme TL IM from *Thermomyces lanuginosus*. In this way, we have successfully developed a two-step enzymatic transesterification process for the production of biodiesel with a high level of economic efficiency.

2 EXPERIMENTAL

2.1 Materials

Soapstock from a refining process for the production of rice bran oil was donated by CJ LTE. (Seoul, Korea). Novozym 435 from *Candida antarctica*, Lipozyme RM IM from *Rhizomucor miehei*, and Lipozyme TL IM from *Thermomyces lanuginosus* were purchased from Novozymes (Seoul, Korea). All of the other chemicals used in the current study were purchased as the analytical grade unless otherwise noted.

2.2 Preparation of acid oil from rice bran oil soapstock

The acidification reaction for preparation of acid oil from soapstock was conducted in a 3 L three-necked flask equipped with an RZR 2051 impeller mixer (Heidolph, Schwabach, Germany). Soapstock (100 g) was mixed with isopropanol (300 mL), *n*-hexane (300 mL), and distilled water (100 mL) in the flask. The reaction was started by stirring and then sulfuric acid with concentration of 50 wt% was added drop wise by drop funnel until the pH reached the range of 2–3. The reaction was finished after 1 h and the liquid was allowed to settle into two layers. The bottom layer containing salt water, isopropanol, and other water soluble matters was discharged using a separate funnel. The upper phase containing acid oil and *n*-hexane was washed several times with hot water until the pH of waste water was neutral. The upper phase was then dried over anhydrous sodium sulfate before being distilled to dryness *in vacuo*. A stream of nitrogen was passed over the resulting product to remove any residual solvent. The acid oil consisted of 53.7 wt% fatty acids, 2.4 wt% monoacylglycerols, 9.1 wt% diacylglycerols, 28.8 wt% triacylglycerols, and 6.0 wt% others. The fatty acids in acid oil were composed of 21.6 wt% palmitic acid, 1.6 wt% stearic acid, 38.8 wt% oleic acid, 36.5 wt% linoleic acid, and 1.5 wt% linolenic acid. The yield of acid oil obtained from 100 g soapstock was approximately 54 g.

2.3 Enzymatic transesterification

The enzymatic transesterification of the acid oil with ethanol was performed in a 25 mL screw-capped Erlenmeyer flask. Acid oil (1.65 g; 5.86 mmole) was mixed with ethanol (1.35 g; 29.30 mmole) at a molar ratio of 1:5 in a 25 mL screw-capped Erlenmeyer flask. The average molecular weight of acid oil was calculated as follows:

$$Mn = \frac{\sum (Mi \times Ni)}{\sum Ni}$$

Mn: Average molecular weight

Mi: Molecular weight of fatty acids

Ni: Number of moles of fatty acids

Then, the molar ratio of acid oil to ethanol was calculated based on 3 g total weight.

The lipase of interest (2.5 to 15% of the reactant weight) was then added. The mixture was agitated in an orbital shaker water bath (Model G76; New Brunswick Scientific Co. Inc., New Brunswick, NJ) at 300 rpm and temperatures ranging from 10 to 60°C. Sample aliquots were removed at selected times and filtered through a 0.45 µm nylon microfilter (Pall Corporation, Port Washington, NY, USA) to remove the lipases. All of the trials were conducted in duplicate. In this study, the moles of acid oil were calculated by converting the moles of acylglycerols in the acid oil to moles of fatty acid bound to the acylglycerols, and the molar ratio of the substrates was then calculated based on the moles of the fatty acid.

2.4 Two-step enzymatic process

The two-step reaction was carried out with a stepwise use of a high-cost lipase and a low-cost lipase. For the first step, a high-cost lipase such as Novozym 435 or Lipozyme RM IM was used for a short time; 5, 15, 30, and 60 min. Subsequently, the lipase was removed by filtration through a 0.45 µm nylon microfilter (Pall Corporation, Port Washington, NY, USA). For the second step, a low-cost lipase, which is Lipozyme TL IM, was applied to the reaction mixture from the first step for the remainder of the reaction; 23 h 55 min, 23 h 45 min, 23 h 30 min, and 23 h. All trials were carried out equally up to 24 h. The reaction was carried out under the different optimized conditions, depending on lipases.

2.5 Analytical methods

To determine the amount (yield, %) of biodiesel produced by the enzymatic transesterification, the composition and the content of free fatty acid were analyzed by gas chromatography and acid value, respectively.

To determine the yield in the reaction mixture, 20 µL samples corresponding to the different reaction conditions were dissolved in 1 mL of chloroform. A gas chromatograph (Model 3800; Varian, Palo Alto, CA, USA) equipped with a DB-1ht column (15 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA, USA) and a flame ionization detector (FID) was used to analyze the different samples. The column was initially held at 120°C for 3 min, and then heated to rise 370°C at a rate of 20°C/min. The column was then held at 370°C for 3 min. Helium was used as the carrier gas, and the total gas flow rate was set at 75 mL/min. The injector and detector temperatures were set at 370°C. The acid value was measured by the neutralization of the fatty acid in the sample with a 0.1 N solution of KOH using alkali blue (Merck Chemical, Darmstadt, Germany) as an indicator (AOCS Cd 3d-63).

The yield (%) of biodiesel was calculated using the weights of the free fatty acid and acylglycerols as follows:

$$\text{Yield (\%)} = \frac{a}{b} \times 100$$

a: the weight of fatty acid ethyl esters in the reaction product

b: the total weight of fatty acid ethyl esters, free fatty acids, and acylglycerols in the reaction mixture.

To convert area% to weight%, response factor was considered. Area% was adjusted by response factors which were calculated by oleic acid ethyl ester, oleic acid, monoolein, diolein, and triolein. Oleic acid was chosen in that it was the most composition of fatty acids in the acid oil.

The response factor (f) was expressed as follows:

$$f = \frac{A_i}{A_{st}}$$

A_i: the peak area of component

A_{st}: the peak area of standard

To determine the composition of fatty acid, the oil was methylated with 14% boron trifluoride in methanol. Acid oil of 30 mg was transferred into a 50 mL test tube and 3 mL of 0.5 M methanolic sodium hydroxide solution was added. It was incubated at 80–90°C for 10 min. Subsequently, 3 mL of 14% methanolic boron trifluoride was added and the mixture was incubated at 80–90°C for 10 min. Then, 5 mL of saturated sodium chloride and 2 mL of *n*-hexane were added and the *n*-hexane layer was separated. It was analyzed by a Varian 3800 gas chromatograph equipped with a Supelcowax 10 fused-silica capillary column (30 m × 0.25 mm i.d.; Supelco, Bellefonte, PA, USA) and flame-ionization detector. The column was held at 180°C for 1 min and then heated to 210°C for 10 min at a rate of 1.5°C/min. Helium was used as the carrier gas with a flow rate of 1 mL/min and split ratio was 1/50. The injector and detector temperatures were set at 240 and 250°C, respectively. The FAMES were identified by comparison with the retention times of the standards. Heptadecanoic acid was used as an internal standard.

3 RESULTS AND DISCUSSION

3.1 Molar ratio

The effect of the molar ratio of the substrate on the synthesis of biodiesel via the enzymatic transesterification of acid oil with ethanol is shown in Fig. 1. Molar ratios of acid oil to ethanol in the range of 1:2–1:6 were evaluated for the transesterification reaction. For these trials, the reaction temperature and enzyme loading were kept constant at 30°C and 5% of the total substrate weight, respectively.

A significant increase in yield was observed during the first 2 h of the trial reactions involving Novozym 435 for all of the different molar ratios tested. For the remaining 22 h of these reactions, however, there was only a steady increase in the yield. The yield increased significantly during the same time frame when the molar ratio of acid oil to ethanol was increased from 1:2 to 1:5 (acid oil to ethanol). There were no significant differences, however, between the levels of yield achieved at molar ratios of 1:5 and 1:6 (acid oil to ethanol). Similar trends in the yield were also observed throughout the entire reaction profile when Lipozyme RM IM was used as the catalyst.

Compared with the results of the trials involving Novozym 435 and Lipozyme RM IM, the use of Lipozyme TL IM as the catalyst led to much lower levels of yield throughout the entire reaction. For example, the maximum yield achieved with Lipozyme TL IM was only 67%, whereas those from Novozym 435 and Lipozyme RM IM were 88 and 90%, respectively. This result can be explained in terms of the deactivation of Lipozyme TL IM by short chain alcohols. Short chain alcohol can hinder not only its ability to catalyze the alcoholysis reaction, but also

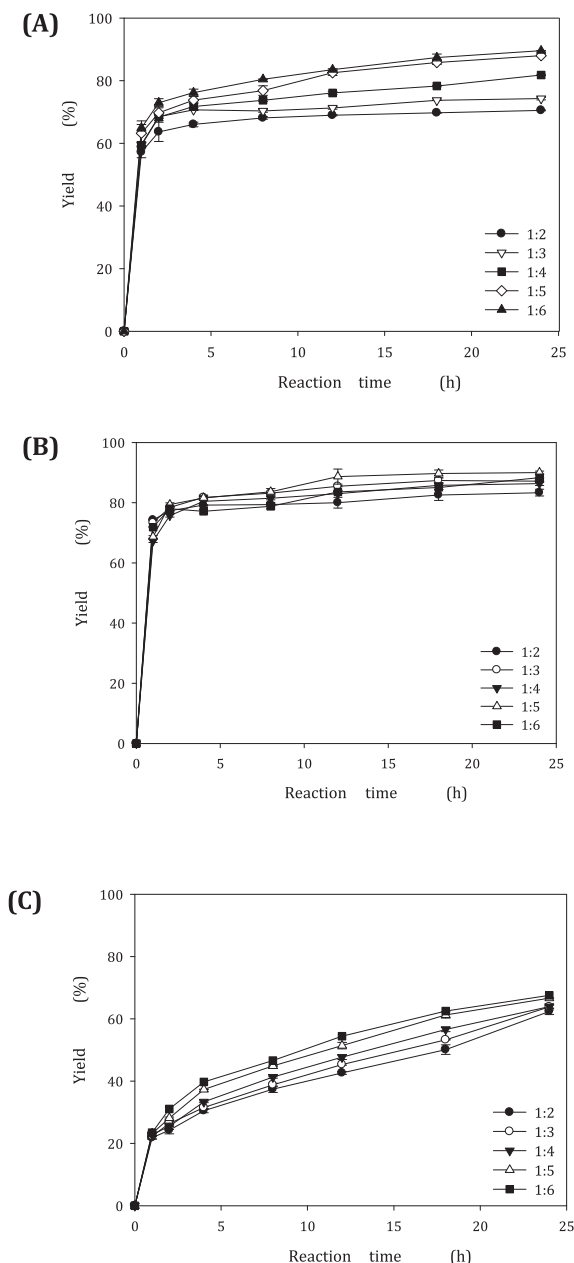


Fig. 1 Effect of the molar ratio (acid oil to ethanol) on the yield for the enzymatic transesterification of acid oil with ethanol as a function of reaction time. (A) Novozym 435 (B) Lipozyme RM IM (C) Lipozyme TL IM. The reactions were performed at a temperature of 30°C and an enzyme loading of 5% of the total substrate weight.

its capacity to approach to maximum equilibrium²⁴). Lipozyme TL IM has been reported to be much more vulnerable to short chain alcohols than Novozym 435 and Lipozyme RM IM²⁴). Also, Hernández *et al.* reported that the maximum yield obtained with Lipozyme TL IM was much lower than that with Novozym 435 after the esterification of soybean oil with ethanol²⁴). The use of an excess of the

alcohol substrate led to a reduction in the activity of the enzyme and a decrease in the yield²⁵). However, increasing the amount of alcohol in the transesterification reaction, up to a certain amount, could enhance the solubility of the oil in the alcohol, and lead to a higher yield. Several different studies have reported different optimum molar ratios for similar reaction systems, which were dependent on the type of lipase and substrate^{25–27}). Our results were consistent with those of Watanabe *et al.*²²) for the synthesis of biodiesel by the enzymatic transesterification of rapeseed acid oil with methanol.

Overall, these results indicated that a molar ratio of 1:5 (acid oil to ethanol) was optimum for the transesterification of the acid oil with ethanol in the presence of the three different lipases. This ratio was then used to study the other parameters.

3.2 Temperature

The reaction temperature is one of the most important parameters for an enzymatic reaction. High temperatures can lead to a reduction in the viscosity of the substrate mixture, and enhance the efficiency of the transfer of the substrate and product materials from the surface and inner regions of the enzyme particles¹⁶). Furthermore, high temperatures can lead to the irreversible denaturation of the enzyme protein and a reduction in its activity^{28, 29}). The effect of temperature on the synthesis of biodiesel by enzymatic transesterification of acid oil with ethanol as a function of reaction time is depicted in Fig. 2. For these trials, the molar ratio of acid oil to ethanol and the enzyme loading were held constant at 1:5 and 5% of total substrate weight, respectively. The range of temperature tested in the current study was 10–60°C.

For the trials involving Novozym 435, the yield increased rapidly during the early stages of the reaction for all of the temperature tested. The yield then increased at a much slower rate for the rest of the reaction. An increase in the temperature from 10 to 40°C led to an increase in the yield. Further increases in the temperature, however, did not lead to further increases in the yield, and the yield of ca. 90% was obtained after 24 h at 40°C.

For the trials involving Lipozyme RM IM, the yield increased as the temperature was increased from 10 to 30°C. There was a significant decrease in the yield, however, when the temperature was further increased to 60°C. In particular, a significant decrease in the yield was observed as the temperature was increased from 50 to 60°C, and the yield after 24 h at 60 °C was only 54 %. In contrast, the use of a temperature of 30°C gave a yield of ca. 89% after only 12 h. Correa *et al.*²⁵) reported that Lipozyme RM IM behaved as a stable and active lipase in anhydrous media but underwent a significant reduction in its activity towards esterification at temperatures above 40°C in the presence of low-molecular-weight alcohols. Similar results

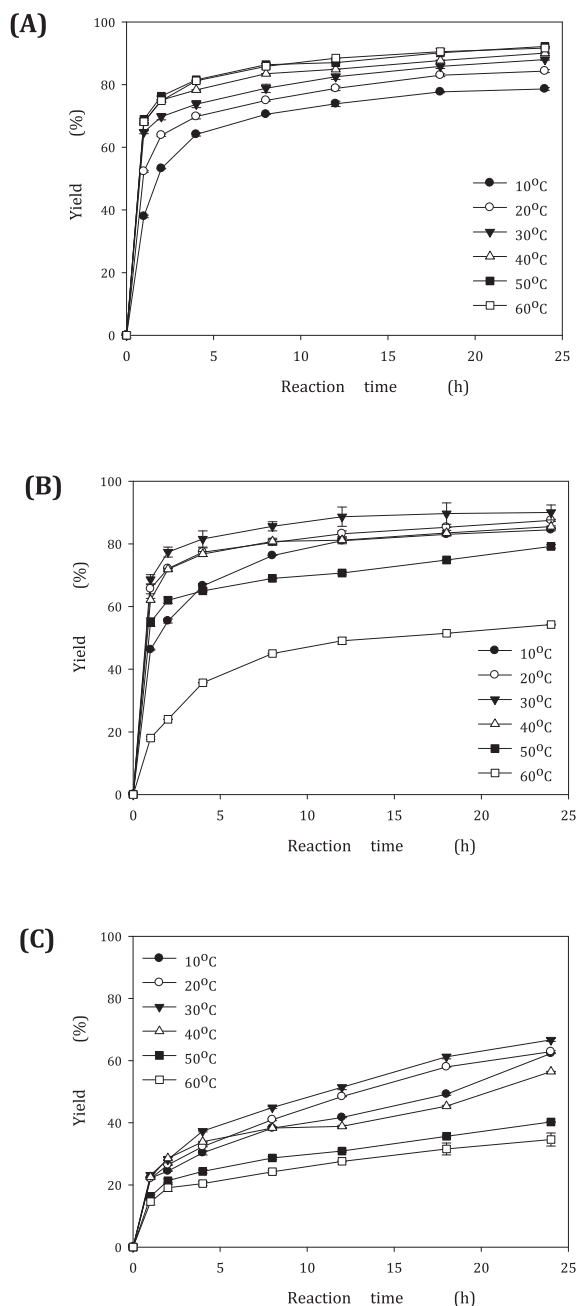


Fig. 2 Effect of temperature on the yield for the enzymatic transesterification of acid oil with ethanol as a function of reaction time. (A) Novozym 435 (B) Lipozyme RM IM (C) Lipozyme TL IM. The reactions were performed at a molar ratio of 1:5 (acid oil to ethanol) and an enzyme loading of 5% of the total substrate weight.

to these were also obtained in the current study.

For the trials with Lipozyme TL IM, a significant decrease was observed in the yield at temperatures greater than 30°C, and the overall yield was much lower than that of Lipozyme RM IM. Dizge *et al.*³⁰⁾ reported that the yield

decreased significantly from 85 to 25% as the temperature increased from 40 to 70°C for the production of biodiesel by the Lipozyme TL IM-catalyzed transesterification of cottonseed oil with methanol.

Based on these results, 40°C was selected as an optimum temperature for the transesterification using Novozym 435, whereas 30°C was selected as the optimum temperature for Lipozyme RM IM and Lipozyme TL IM.

3.3 Enzyme loading

The number of active sites available for a reaction is proportional to the enzyme loading and is one of many important factors affecting the performance of an enzymatic reaction²⁴⁾. For this reason, it is crucially important to identify the optimum enzyme loading for an enzymatic reaction to allow for the reaction to reach its highest level of efficiency.

The effect of enzyme loading on the synthesis of biodiesel by the enzymatic transesterification of acid oil with ethanol as a function of reaction time is depicted in Fig. 3. For these trials, the reactions were conducted using the optimum molar ratio and temperature conditions identified above for each lipase. Thus, the reactions were performed at a molar ratio of 1:5 (acid oil to ethanol) for all three of the lipases, and the reaction temperature was set at 40°C for Novozym 435, and 30°C for Lipozyme RM IM and Lipozyme TL IM. The enzyme loading was tested over the range of 2.5–15% of the total substrate weight. For the trials involving Novozym 435, there was an obvious increase in the yield as the enzyme loading was increased from 2.5 to 10%. Further increases in the enzyme loading, however, had no significant impact on the yield. The yield in the presence of Novozym 435 was ca. 93%, which was achieved after 24 h with an enzyme loading of 10%.

For the trials with Lipozyme RM IM, no significant differences were observed in the yield levels for enzyme loadings in the range of 5–15%. The use of an enzyme loading of 2.5%, however, did result in a much lower yield.

For the trials with Lipozyme TL IM, a significant increase in the yield was observed during the first 12 h of the reaction when the enzyme loading was increased from 2.5 to 15%. However, after 12 h, even though the yield obtained with an enzyme loading of 15% was higher than that obtained with a loading of 10%, the difference between these two scenarios was small. The yield of ca. 88% was achieved at a reaction time of 24 h with an enzyme loading of 10%.

Based on these results, the optimum enzyme loadings were determined to be 5% for Lipozyme RM IM, and 10% for Novozym 435 and Lipozyme TL IM.

3.4 Two-step enzymatic transesterification

Novozym 435 and Lipozyme RM IM have been used extensively for the synthesis of biodiesel and exhibit high levels of activity as well as residual activity. The use of

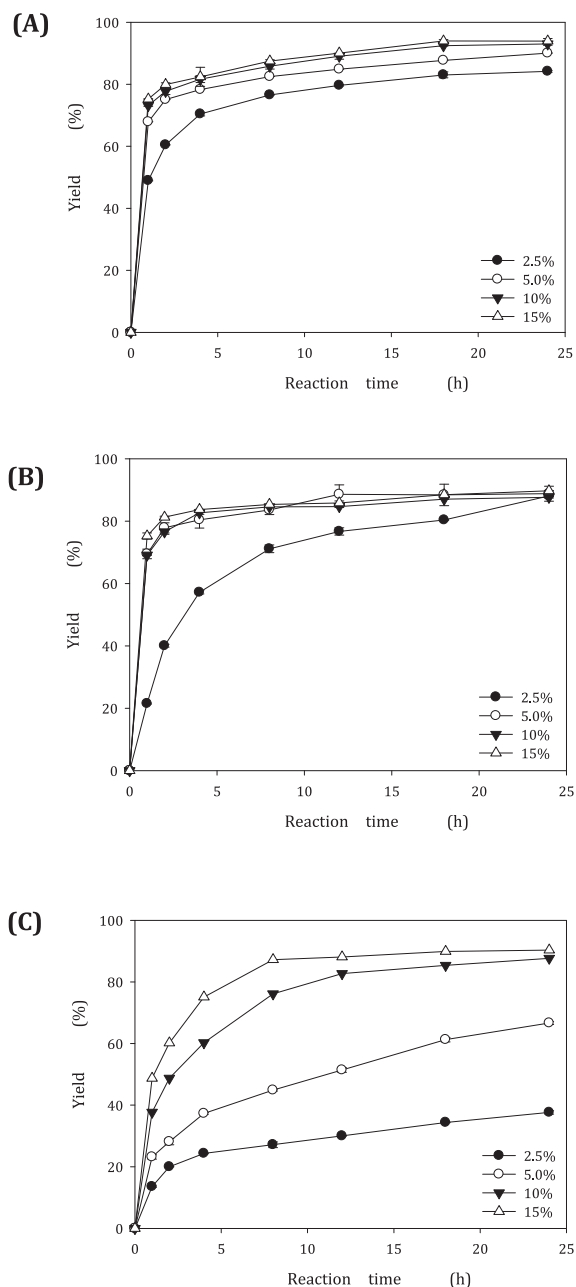


Fig. 3 Effect of enzyme loading on the yield for the enzymatic transesterification of acid oil with ethanol as a function of reaction time. (A) Novozym 435 (B) Lipozyme RM IM (C) Lipozyme TL IM. The reactions were performed at a molar ratio of 1:5 (acid oil to ethanol) and temperature of 40°C using Novozym 435. The reactions were performed at a molar ratio of 1:5 (acid oil based on fatty acid to ethanol) and a temperature of 30°C using Lipozyme RM IM and Lipozyme TL IM.

Novozym 435 or Lipozyme RM IM for the synthesis of biodiesel on the industrial scale, however, is not economically viable because of the high cost of these lipases. With this in

mind, we investigated the use of Lipozyme TL IM as a potential alternative to allow for a reduction in the reaction time as well as the cost of the transesterification³¹⁾.

The main goals of the two-step enzymatic transesterification were to achieve the yield as high as the yield obtained through one-step reaction using the high-cost lipase and to reduce the process cost by using the high-cost lipase for a short amount of reaction time. There have been a few reports in the literature pertaining to the production of biodiesel in a solvent system using mixed two lipases^{13, 14, 32)}. Li *et al.*¹⁴⁾ reported that the efficiency of a transesterification reaction could be increased significantly using mixed two lipases (i.e., Novozym 435 and Lipozyme TL IM) instead of the individual lipases in isolation. However, there could be some major drawbacks to this method because the optimum reaction conditions and the residual activities of the two lipases could be different. To overcome these issues, we devised and operated a two-stage reaction strategy in the current study, where each reaction was conducted in a separate reaction system with a stepwise use of different lipases.

Novozym 435 and Lipozyme RM IM have been reported to have higher activity for the esterification of free fatty acid than Lipozyme TL IM^{24, 33)}. The acid oil, which was used as a feedstock in this study contained over 50 wt% of free fatty acid. In these respects, Novozym 435 or Lipozyme RM IM was employed in the first reaction for a short time in order to esterify rapidly the free fatty acid in reaction mixture and then Lipozyme TL IM was used in the second reaction for the remainder of the reaction to hydrolyze triacylglycerol and subsequently esterify the free fatty acid. The content of free fatty acid in the original acid oil was 52.67 wt %, whereas that in the reaction mixtures obtained by using Novozym 435, Lipozyme RM IM, and Lipozyme TL IM for 15 min were 20.49 wt%, 30.72 wt%, and 43.4 wt%, respectively. Therefore, this result demonstrates that Novozym 435 and Lipozyme RM IM have higher activity than Lipozyme TL IM in the esterification toward free fatty acid.

In addition, we employed the two-step reaction process to increase half-life of the high-cost lipases by reducing the duration of their usage at the first step. Every enzyme loses its activity depending on its own life span. Even though the amount of enzyme used in two-step reaction was bigger than that used in one-step reaction, two-step reaction process can increase the number of use with the high-cost lipase since the high-cost lipases are employed for only a short time. Hence, in a long-term point, the two-step reaction can ensure the reduction in the process cost by increasing the high-cost lipase reusability. In these aspects, two-step enzymatic transesterification can be beneficial in reducing process cost.

In the present study, different stepwise use of the lipases were investigated to the two-stage process, including the

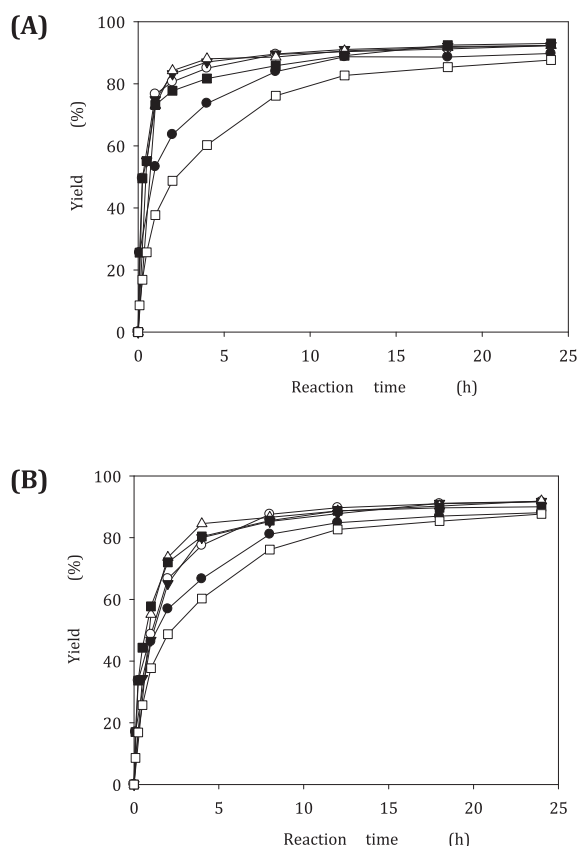


Fig. 4 Effect of the two-step enzymatic reaction on the yield for the enzymatic transesterification of acid oil with ethanol as a function of reaction time. The reactions were performed under the optimum conditions for each lipase. Novozym 435 and Lipozyme RM IM were used for the first step and Lipozyme TL IM was used for the second step. (A) Reaction with Novozym 435 only (■); reaction with Lipozyme TL IM only (□); and combinations of reactions where a first reaction of Novozym 435 for initial 5 min (●), 15 min (○), 30 min (▼), and 60 min (△) was followed by a second reaction with Lipozyme TL IM for the rest of the 24 h reaction. (B) Reaction with Lipozyme RM IM only (■); reaction with Lipozyme TL IM only (□); and combinations of reactions where a first reaction of Lipozyme RM IM for initial 5 min (●), 15 min (○), 30 min (▼), and 60 min (△) was followed by a second reaction with Lipozyme TL IM for the rest of the 24 h reaction.

use of Novozym 435 and Lipozyme TL IM or Lipozyme RM IM and Lipozyme TL IM. The reactions were carried out under the optimum conditions identified above for each lipase. The effect of the two-step enzymatic transesterification reaction on the synthesis of biodiesel as a function of reaction time is depicted in Fig. 4.

For the first combination, the initial step of the two-step

reaction process was carried out in the presence of Novozym 435 for 5, 15, 30, and 60 min from the initiation of reaction, and the second step of the reaction was then carried out with Lipozyme TL IM in such a way as to obtain a constant total reaction time of 24 h. The initial reaction rates of all of these two-step reactions were faster than that of the reaction with Novozym 435 alone, except for the two-step reaction performed with Novozym 435 for 5 min. For the trials involving the use of Novozym 435 for 15, 30, and 60 min after the initiation of the reaction, the yields reached 90% after a reaction time of only 8 h. The overall yield levels obtained from the two-step reactions involving a combination of Novozym 435 and Lipozyme TL IM were significantly higher than those obtained from the reaction with Lipozyme TL IM. Consequently, the best enzyme combination in this trial was Novozym 435 for 15 min and Lipozyme TL IM for 23 h 45 min. The final product obtained with this combination consisted of 92.30 wt% fatty acid ethyl esters, 4.21 wt% fatty acids, 3.35 wt% monoacylglycerols, and 0.15 wt% diacylglycerols.

For the second combination, the first stage of the two-step reaction process was carried out with Lipozyme RM IM for 5, 15, 30, and 60 min after the initiation of the reaction, and the second stage of the reaction process was carried out with Lipozyme TL IM to obtain a constant total reaction time of 24 h. These two-step reactions showed similar trends to those observed for the first combination of Novozym 435 and Lipozyme TL IM. Consequently, the best enzyme combination in this trial was Lipozyme RM IM for 15 min and Lipozyme TL IM for 23 h 45 min. The final product obtained with this combination was composed of 91.66 wt% fatty acid ethyl esters, 4.57 wt% fatty acids, 0.31 wt% diacylglycerols, and 3.46 wt% triacylglycerols.

During the first 4 h of these reactions, the initial reaction rate of the two-step reaction involving a combination of Novozym 435 and Lipozyme TL IM was slightly faster than that of the two-step reactions involving a combination of Lipozyme RM IM and Lipozyme TL IM. However, at reaction times greater than 4 h, there were no discernible differences between the yield levels achieved by the two different combinations. Although most time of the two-step reaction was carried out with the low-cost lipase, Lipozyme TL IM, the maximum yield achieved at two-step reaction was almost same as the one-step reaction with the high-cost lipase. In addition, the reaction time to approach the equilibrium at the two-step reaction was shorter than that at the one-step reaction with the high-cost lipase. Meanwhile, the maximum yield and reaction rate of the one-step reaction with Lipozyme TL IM was much lower than the two-step reaction.

Taken together, these results demonstrate that the two-step reaction strategy provided a reduction in the reaction time and process cost.

4 CONCLUSIONS

Biodiesel has been effectively synthesized by the enzymatic transesterification of acid oil and ethanol under solvent-free conditions. We have developed a two-step transesterification process involving the use of two different lipases, which had a significant synergistic effect in terms of enhancing the yield and the reaction rate. The best enzyme combinations for two-step reaction were Novozym 435 or Lipozyme RM IM for 15 min and Lipozyme TL IM for the rest of reaction time. The maximum yields of ca. 92% were obtained via two-step reactions. Based on these results, we have proposed a novel process for the production of biodiesel via a two-step enzymatic transesterification process.

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