

Optimization of the Process for Biodiesel Production Using a Mixture of Immobilized *Rhizopus oryzae* and *Candida rugosa* Lipases

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In this study, the enzymatic process for biodiesel production was optimized using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. The optimal temperature and agitation speed for biodiesel production were 45°C and 300 rpm, respectively. The optimal ratio of *R. oryzae* and *C. rugosa* lipases in the mixture was 3:1 (w:w). When 3 mmol of methanol was the initial reaction medium and 3 mmol of methanol was added every 1.5 h during biodiesel production, biodiesel conversion was over 98% at 4 h. In addition, when the immobilized lipase mixture was reused, biodiesel conversion exceeded 80% after 5 reuses.

Keywords: Biodiesel, immobilization, *Rhizopus oryzae* lipase, *Candida rugosa* lipase

Biodiesel is mixture of alkyl esters of fatty acids derived from the transesterification of vegetable or animal oils with alcohol [10]. Generally, alkyl esters are obtained by chemical transesterification. However, the chemical process has some disadvantages, such as the environmental problems caused by using organic solvents and the high operating costs related to the severe reaction conditions [4, 11–14]. Thus, many investigators have intensively studied enzymatic processes for biodiesel production to solve those problems [1, 13–15]. Lipases are enzymes that can produce biodiesel from vegetable oils and alcohols [5, 16]. The use of lipases as biocatalysts in enzymatic processes for biodiesel production offers some advantages. Because lipases catalyze the transesterification under mild conditions, they may reduce the process cost in terms of energy consumption and capital equipment requirements. Furthermore, biodiesel can be produced by lipases without any organic

solvents; thus, the enzymatic process is environmentally benign [2, 4–7].

In previous work, a new process for biodiesel production using a mixture of *Rhizopus oryzae* and *Candida rugosa* lipases was successfully developed [5]. In this process, *R. oryzae* lipase, a 1,3-specific lipase, mainly converts fatty acids from oils into biodiesel. Many investigators have reported that it takes over 40 h for this 1,3-specific lipase to finish biodiesel production [2, 9]. However, the new process with *C. rugosa* lipase does not require acyl migration; thus, biodiesel production can be completed within 21 h.

In this study, the optimal temperature and agitation speed for biodiesel production were determined, and the molar ratio (methanol/oil) and methanol feeding method were optimized. Moreover, the reusability of immobilized lipases was investigated.

MATERIALS AND METHODS

Materials

3-Aminopropyltriethoxysilane (3-APTES) was purchased from Sigma (U.S.A.), glutaraldehyde from Fluka (Switzerland), and silica gel from Grace Davison (U.S.A.). *R. oryzae* lipase and *C. rugosa* lipase were purchased from Sigma (U.S.A.) and Fluka (Switzerland), respectively. All other chemicals were of reagent grade.

Lipase Preparation

R. oryzae lipase (1 g) was suspended in 100 ml of 1.0 M Tris-HCl buffer (pH 7.0) and *C. rugosa* lipase (1 g) was suspended in 100 ml of 0.25 M sodium phosphate buffer (pH 6.5). The lipase solutions were centrifuged at 4,000 rpm for 15 min at 4°C. The supernatant was stored at 4°C prior to immobilization.

Lipase Immobilization

One of dry silica gel was mixed with 10% 3-APTES in 20 ml of acetone and incubated at 50°C for 2 h with constant mixing. The silica gel was then washed with water and dried at 60°C for 2 h. The

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dried silica gel was then suspended in 20 ml of 1 mM phosphate buffer solution (pH 7). Two ml of 25% (w/v) glutaraldehyde was added to this solution and incubated at 20°C for 2 h to activate the silica gel. The activated gel was then washed with water and dried at 60°C for 2 h. Prior to immobilization, *R. oryzae* lipase was pretreated with 0.1% soybean oil at 40°C with stirring at 150 rpm for 45 min, and *C. rugosa* lipase was pretreated with 0.5% soybean oil at 37°C with stirring at 150 rpm for 45 min. Activated silica gel (500 mg) was mixed with 10 ml of pretreated lipase solution and then incubated at 20°C to immobilize the lipase. The immobilized lipase was recovered by filtration, washed with water, and then dried overnight at room temperature.

Biodiesel Production

Biodiesel was produced using a mixture of immobilized pretreated *R. oryzae* and *C. rugosa* lipases at ratios of 1:3, 1:1, and 3:1 (w:w), respectively. Three mmol or 4 mmol of methanol was initially added to the reaction medium containing 2 mmol of soybean oil and 0.5 g of the immobilized lipases mixture. An equivalent amount of methanol was then added once or twice during biodiesel production to investigate the effect of methanol concentration on biodiesel production.

Analysis

The methyl ester content in the reaction mixture was analyzed with a gas chromatograph M600D (Younglin Co., Korea) connected to a HP-INNOWAX column (0.25 mm, 30 m; HP, U.S.A.). The sample injection volume was 1 μ l, the injector temperature was 260°C, and the oven temperature was increased from 150°C to 180°C at the rate of 15°C/min and increased up to 240°C at the rate of 5°C/min, and then maintained for 1 min. A flame ionization detector (FID) was used with its temperature set at 260°C.

RESULTS AND DISCUSSION

Effects of Temperature and Agitation Speed on Biodiesel Production

In our previous work, we developed an immobilization process to produce biodiesel using immobilized *R. oryzae* and *C. rugosa* lipases [6, 7]. However, the reaction rate was later than another study on biodiesel production [3]. If optimization studies for biodiesel production were performed, the reaction time for biodiesel production could be reduced even further. Therefore, we considered to optimize the reaction conditions for the reduction of reaction time. By comparison with other studies, mixing systems were attempted to increase the mass transfer. In fact mass transfer was increased markedly in a high viscosity system [8, 13]. Lifka and Ondruschka [9] reported that temperature and agitation were important factors for biodiesel production. Therefore, in this study, these factors were investigated to improve the biodiesel productivity.

Methanol (3 mmol) was added to the reaction medium containing 2 mmol of soybean oil and 0.5 g of a mixture of immobilized *R. oryzae* and *C. rugosa* lipases at a ratio of 1:1 (w:w). Biodiesel production was performed at various temperatures with stirring at 200 rpm for 3 h. As shown in

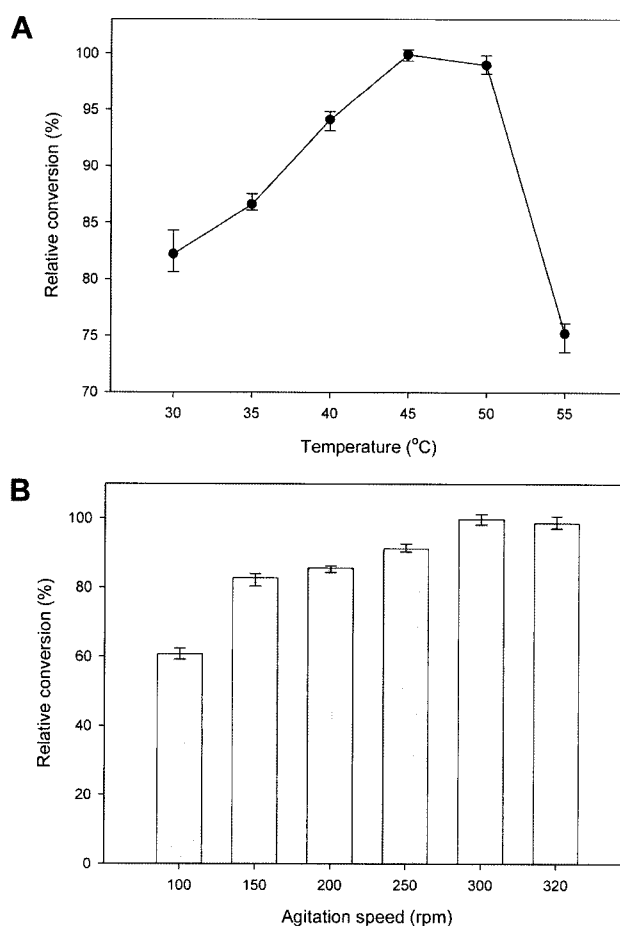


Fig. 1. Effects of temperature and agitation speed on biodiesel production using a mixture of immobilized *R. oryzae* and *C. rugosa* lipases.

A. Effect of temperature; B. Effect of agitation speed.

Fig. 1A, the optimal temperature for biodiesel production was 45°C and the conversion yield was 98%. At 40°C and 50°C, conversion yields were decreased by 6% and 3%, respectively, compared with 45°C.

To investigate the optimal agitation speed, biodiesel production was performed at 45°C with various agitation speeds. Biodiesel conversion increased in proportion to agitation speed up to 300 rpm, but did not increase further (Fig. 1B). Moreover, the reaction time was also decreased to 4 h. This result implies that mass transfer was not improved over 300 rpm, which suggests the potential information for application to scale-up studies. The conversion yield of biodiesel was 99.2% at 300 rpm. Thus, the optimal agitation speed for biodiesel production was chosen at 300 rpm.

Effects of Molar Ratio of Methanol and Soybean Oil, and the Ratio of Immobilized *R. oryzae* and *C. rugosa* Lipases on Biodiesel Production

In the enzymatic process, because methanol was insoluble over 0.5 molar ratios of fatty acids, the initial molar ratio

of methanol and soybean oil is an important factor. In fact, lipases were inactivated by adding over 0.5 molar equivalent of methanol for the stoichiometric amount, and methanolysis was stop [14]. Therefore, in this process, the reaction should be carried out as stepwise fed-batch for the maintenance of high enzyme activity. Moreover, the *R. oryzae* lipase that has 1,3-positional specificity performs a

reaction of acyl migration during the biodiesel production process. Therefore, it takes over 40 h for this 1,3-specific lipase to complete biodiesel production [9]. To eliminate the acyl migration, *C. rugosa* lipase, a nonspecific lipase, was applied. In this study, biodiesel was produced using mixtures of immobilized *R. oryzae* and *C. rugosa* lipases at ratios of 1:3, 1:1, and 3:1 (w:w), respectively. In order to investigate the molar ratio (methanol/soybean oil) on biodiesel production, various amounts of methanol ranging from 0.5 mmol to 4 mmol were added to the reaction medium containing 0.5 g of the mixture of immobilized lipases and 2 mmol of soybean oil. Biodiesel production was performed at 45°C with stirring at 300 rpm for 3 h. The biodiesel production using mixtures of immobilized *R. oryzae* and *C. rugosa* lipases at ratios of 1:3, 1:1, and 3:1 (w:w) are shown in Figs. 2A, 2B, and 2C. When the molar ratio (methanol/soybean oil) is 1.5 or 2, theoretical biodiesel conversions are 50% or 66.6%, respectively. As shown in Figs. 2B and 2C, when the molar ratio was 1.5 and 2, biodiesel conversions were 48% and 64%, respectively. However, when the 1:3 (w:w) mixture of immobilized *R. oryzae* and *C. rugosa* lipases was used for biodiesel production, biodiesel conversions were only 40% and 53%, respectively. Thus, this mixture is not appropriate for biodiesel production. Moreover, when the 1:1 (w:w) mixture of immobilized *R. oryzae* and *C. rugosa* lipases was used for biodiesel production with 1 molar ratio of methanol and soybean oil, biodiesel conversion did not reach 30%, the theoretical conversion. Thus, the optimal ratio of immobilized *R. oryzae* and *C. rugosa* lipases was 3:1 (w:w). In Fig. 2C, the slope indicates the reaction rate. The rate of biodiesel production increased in proportion to molar ratio (methanol/soybean oil). However, when biodiesel production was performed with molar ratios of 1.5 and 2 (methanol/soybean oil), each reaction rate was similar for biodiesel production.

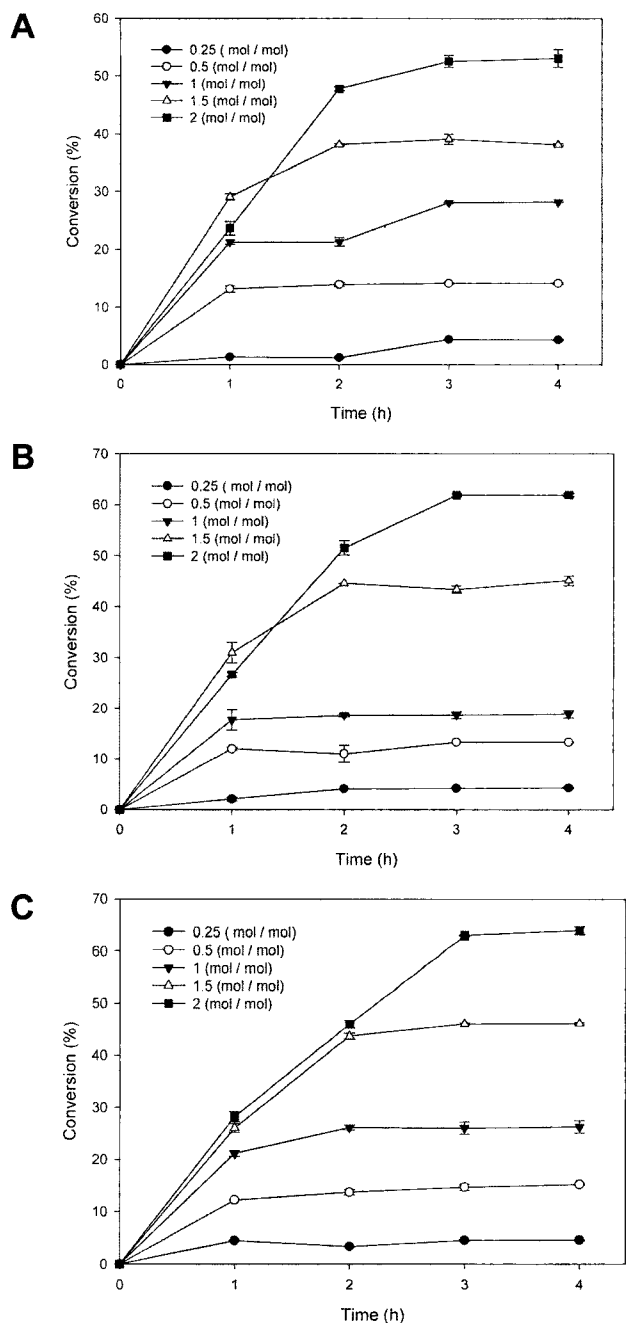


Fig. 2. Effects of molar ratio (methanol/soybean oil) on biodiesel production using a mixture of immobilized *R. oryzae* and *C. rugosa* lipases at various ratios.

A. Ratio of 1:3 (w:w); B. Ratio of 1:1 (w:w); C. Ratio of 3:1 (w:w).

Biodiesel Production Using a Mixture of Immobilized *R. oryzae* and *C. rugosa* Lipases at Ratio of 3:1 (w:w)

Biodiesel was produced using a 3:1 (w:w) mixture of immobilized *R. oryzae* and *C. rugosa* lipases. In order to prevent lipase denaturation caused by high methanol concentration, 3 mmol or 4 mmol of methanol was initially added to the reaction medium and then added twice more during biodiesel production [14].

Three mmol of methanol was added to a reaction medium containing 2 mmol of soybean oil and 0.5 g of the mixture of immobilized lipases. Biodiesel production was performed at 45°C and 300 rpm for 4 h. Three mmol of methanol was then added to the reaction medium every 1 h or 1.5 h during the biodiesel production. As shown in Fig. 3A, when methanol was added every 1.5 h, biodiesel conversion was over 98% at 4 h. However, when methanol was added every 1 h, biodiesel conversion was only 80%. When the methanol was added every 1 h, methanol accumulated in the reaction medium

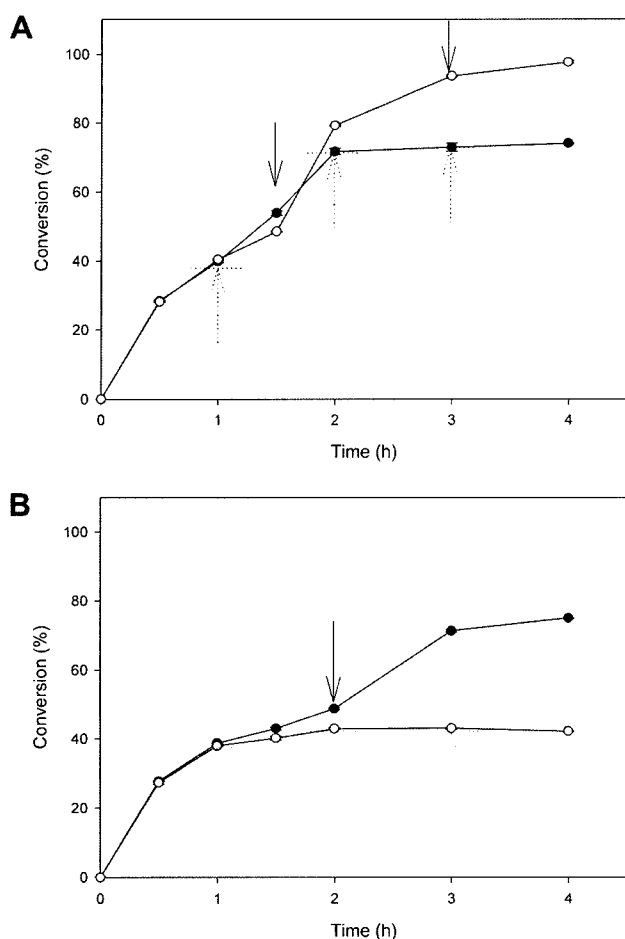


Fig. 3. Biodiesel production using a 3:1 (w:w) mixture of *R. oryzae* and *C. rugosa* lipases.

A. Three mmol of methanol was initially added to the reaction medium and 3 mmol of methanol was added every 1 h (●) or 1.5 h (○) during biodiesel production B. Four mmol of methanol was initially added to the reaction medium and 4 mmol of methanol was added every 1 h (●) or 2 h (○) during biodiesel production.

and denatured the lipases. In this case, biodiesel conversion stopped at 2 h when the second aliquot of methanol was added to the reaction medium.

Four mmol of methanol was added to the reaction medium and biodiesel production was performed at 45°C and 300 rpm for 4 h. Four mmol of methanol was then added to the reaction medium every 1 h or 2 h during biodiesel production. The results are shown in Fig. 3B. When methanol was added every 1 h, biodiesel conversion was 40% at 1 h and did not increase further. Again, methanol accumulated in the reaction medium and the lipases were denatured. When methanol was added every 2 h, biodiesel conversion reached 80% at 4 h. This result implies that the initial concentration of methanol is an important factor for the maintenance of lipase activity [12]. Moreover, the methanol accumulated in the reaction medium should be reduced for high activity of lipase.

According to these results, the best method for methanol addition is to initially add 3 mmol of methanol to the reaction medium and then to add 3 mmol of methanol every 1.5 h during biodiesel production.

Effect of Concentration of Immobilized Lipase on Biodiesel Production

The activity and concentration of enzyme are important factors in the enzymatic process. In our previous work, immobilized lipases were modified for biodiesel production by pretreatment of the enzyme [6, 7]. Through this method, lipases activities were increased markedly. However, the optimal concentration of lipases was not chosen for biodiesel production. Therefore, in this study, we considered to investigate the enzyme concentration for high conversion yield. Biodiesel was produced using a mixture of immobilized *R. oryzae* and *C. rugosa* lipases at the ratio of 3:1 (w:w). Various amounts of the mixtures of immobilized lipases (10%–30%, weight of the mixture of immobilized lipases/ weight of soybean oil) were added to reaction media containing 2 mmol of soybean oil and 3 mmol of methanol. Biodiesel production was performed at 45°C and 300 rpm for 4 h. Methanol (3 mmol) was added to the reaction medium at every 1.5 h during the biodiesel production. As shown in Fig. 4, when 25% immobilized lipases was used, biodiesel conversion reached 98% at 4 h. This is very similar to the conversion of when 30% immobilized lipases was used. These results proved that the 25% immobilized lipases were saturated with substrate [12–14]. Thus, the conversion yield and reaction rate were not increased over 25% enzyme concentration. However, when less than 20% immobilized lipases was used, biodiesel conversion decreased markedly. When 10% immobilized lipases was used, biodiesel conversion reached only 30%. According to these results, it is thought that mass transfer was prevented between the enzyme and substrate below a 20%

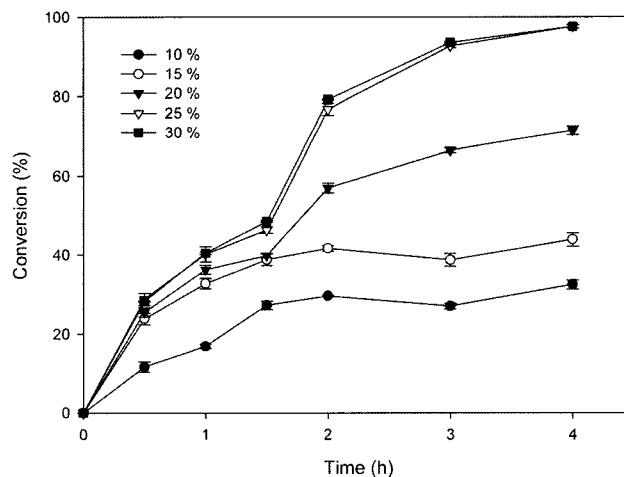


Fig. 4. Effect of concentration of the immobilized lipases on biodiesel production.

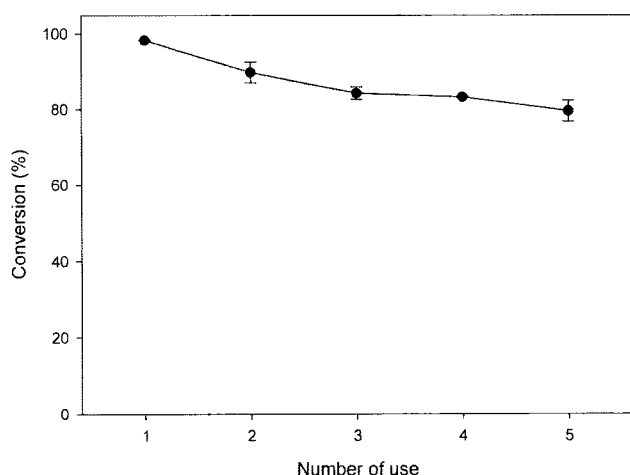


Fig. 5. Reusing the mixture of immobilized *R. oryzae* and *C. rugosa* lipases for repeated biodiesel production.

enzyme concentration [8]. As a result, the optimal concentration of immobilized lipases was determined to be 25%.

Reusing the Mixture of Immobilized Lipases for Repeated Biodiesel Production

The reusability of immobilized lipase is of key importance for industrial applications. The 3:1 (w:w) mixture of immobilized *R. oryzae* and *C. rugosa* lipases was used consecutively in a series of biodiesel productions for 4 h. After a single batch reaction, the immobilized lipases were filtered and washed with water and isopropyl alcohol, and then reused for the next batch reaction. Fig. 5 shows the biodiesel conversion of 80% after 5 reuses. Generally, immobilized lipase was used 5–10 times for maintenance of 80% conversion. This process was, therefore, highly effective for the biodiesel production from vegetable oil and methanol. In summary, based on the results of lipases reuse and high level of biodiesel production, it is suggested that this process of immobilized lipases has a potential for application to scale-up study.

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