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Biodiesel Production Using a Mixture of Immobilized *Rhizopus oryzae* and *Candida rugosa* Lipases

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Abstract Biodiesel conversion from soybean oil reached a maximum of 70% at 18 h using immobilized 1,3-specific *Rhizopus oryzae* lipase alone. Biodiesel conversion failed to reach 20% after 30 h when immobilized nonspecific *Candida rugosa* lipase alone was used. To increase the biodiesel production yield, a mixture of immobilized 1,3-specific *R. oryzae* lipase and nonspecific *C. rugosa* lipase was used. Using this mixture a conversion of greater than 99% at 21 h was attained. When the stability of the immobilized lipases mixture was tested, biodiesel conversion was maintained at over 80% of its original conversion after 10 cycles.

Keywords: biodiesel, *Rhizopus oryzae* lipase, *Candida rugosa* lipase, immobilization, reuse

INTRODUCTION

Biodiesel is an alternative fuel, and is receiving much attention because of its benefits, *i.e.*, it is non-toxic, biodegradable, and renewable [1]. Biodiesel consists of a mixture of alkyl esters of fatty acids derived from the transesterification of vegetable or animal oils with alcohol [2]. Generally, the synthesis of alkyl esters is accomplished by chemical transesterification. Because biodiesel production is expensive and involves a complicated separation process, enzymatic processes for biodiesel production have been extensively studied [3-7].

Lipase (triacyl glycerol ester hydrolase, EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of triacylglycerols to fatty acids, mono- and di-acylglycerols and glycerol [8-10]. Lipase also catalyzes the transesterification of lipids to biodiesel (fatty acid alkyl esters). The use of lipases as biocatalysts in enzymatic processes for biodiesel production offers several advantages. Because lipases catalyze transesterification under mild conditions they offer the possibility of reducing process cost in terms of energy consumption and capital equipment requirements [11]. Furthermore, biodiesel can be produced by lipases without any organic solvent, and thus the enzymatic process becomes environmentally benign. However, no enzymatic process has been used yet to commercially produce biodiesel because they require much more time to complete the reaction than competing chemical processes. It has been reported by several investigators that it takes 30~60 h to reach 80% biodiesel conversion without using an organic solvent [12-14].

In this study, *Rhizopus oryzae* lipase and *Candida rugosa* lipase were immobilized and co-mixtures of the two were used to increase the rate of biodiesel production and biodiesel conversion. In addition, the effect of reuse on conversion efficiency of these immobilized lipases was investigated by repeating batch reactions with used immobilized lipases.

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MATERIALS AND METHODS

Materials

3-Aminopropyltriethoxysilane (3-APTES) was purchased from Sigma (St. Louis, MO, USA), glutaraldehyde was purchased from Fluka (Buchs, Switzerland), and Lipase OF was purchased from Meito Sangyo (Nagoya, Japan). The ultrafiltration membrane (15659-00-1) was purchased from Sartorius (Göttingen, Germany). Silica gel was kindly provided by Chong Kun Dang Pharmaceutical Co. (Ansan, Korea).

Preparation of Lipase

R. oryzae lipase was produced using the strain of *R. oryzae* KCCM 11970. Seed culture was performed in PDB medium at 30°C while stirring at 200 rpm for 2 days. Production culture was performed at 28°C while stirring at 250 rpm for 5 days. The production medium consisted of 4% olive oil, 8% bactopeptone, 0.1% KH_2PO_4, 0.1% $NaNO_3$, and 0.05% $MgSO_4 \cdot 7H_2O$ and its pH was initially adjusted to pH 6. After cultivation, the culture broth of *R. oryzae* was centrifuged at 4,000 rpm for 15 min and the supernatant was prepared as a crude lipase solution. Ammonium sulfate was then added to this crude lipase solution (to give 60% saturation) and the resulting suspension was centrifuged at 4,000 rpm for 15 min to obtain the supernatant. The precipitate was then suspended in 1 mM phosphate buffer (pH 7) and this

solution was concentrated using an ultrafiltration membrane and stored at 4°C.

The purchased *C. rugosa* lipase (Lipase OF, Meito Sangyo, Nagoya, Japan) 0.5 g was dissolved in 50 mL of 1 mM phosphate buffer (pH 7) and stored at 4°C.

Lipase Immobilization

One gram of dry silica gel was mixed with a 10% 3-APTES in 20 mL 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 dried silica gel was then suspended in 20 mL of 1 mM phosphate buffer solution (pH 7). Two mL of glutaraldehyde (25%, w/v) was added to this solution followed by incubation at 20°C for 2 h to activate the silica gel which was then washed with water and dried at 60°C for 2 h. Activated silica gel (500 mg) was then mixed with 10 mL of lipase solution and 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 from a mixture of 2 mmol of soybean oil and 9 mmol of methanol in a shaking water bath at 200 rpm and 45°C for 30 h. Water was added to the mixture to give a final solution of 10% (w/w substrate). The reaction was carried out by adding 30% (w/w substrate) of immobilized lipase. During the reaction, 0.9 mmol of methanol was fed to the reaction mixture every 3 h to avoid lipase denaturation.

Analysis

Methyl ester contents in the reaction mixture were analyzed using a M600D gas chromatography (Younglin Co., Anyang, Korea) with a capillary column (id 0.25 mm, 30 m; HP-INNOWAX, Agilent, Santa Clara, CA, USA). The sample injection volume used was 1 μL, the injector temperature was 260°C, and the oven temperature was increased from 150 to 180°C at a rate of 15°C/min then increased to 240°C at a rate of 5°C/min, which was maintained for 1 min. Peaks were observed using a Flame Ionization Detector (FID) set to 260°C.

RESULTS AND DISCUSSION

Biodiesel was produced using immobilized *R. oryzae* lipase with activities of 40, 60, and 90 U/g matrix, respectively. Fig. 1 shows that conversion increased in proportion to the activity of the immobilized lipase. However, when the immobilized *R. oryzae* lipase with an activity of 90 U/g matrix was used, conversion reached about 70% at 18 h and did not increase further. When the immobilized *R. oryzae* lipase with an activity of 40 U/g matrix was used, conversion was increasing to 30 h, however, after 30 h no further conversion was observed and conversion slightly decreased at 36 h (data not shown).

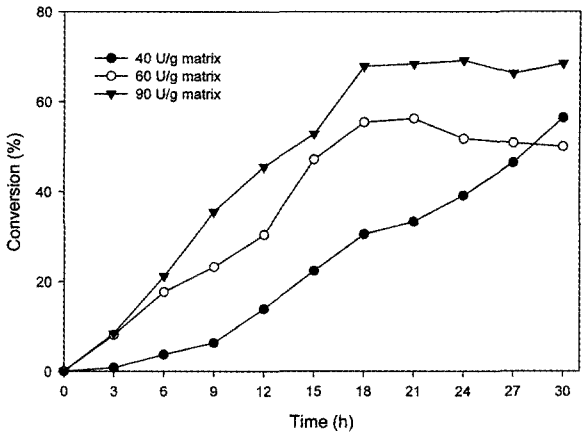

Fig. 1. Biodiesel production using immobilized *R. oryzae* lipase with activities of 40, 60, and 90 U/g matrix, respectively.

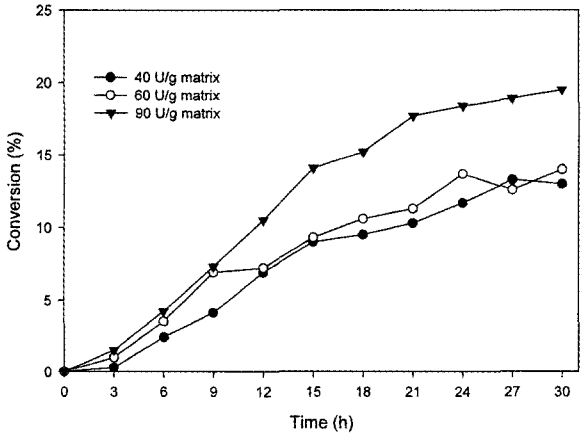

Fig. 2. Biodiesel production using immobilized *C. rugosa* lipase with activities of 40, 60, and 90 U/g matrix, respectively.

R. oryzae lipase is a 1,3-specific lipase [15]. Kaieda *et al.* [12] have suggested that *R. oryzae* lipase can not hydrolyze fatty acids on the second position of 1,2-diglyceride. However *R. oryzae* lipase has the ability to move a fatty acid from this position to the third position (acyl migration) which allows biodiesel conversion to reach greater than 80%. In the present study, we adopted a new approach to increase biodiesel conversion without relying on the acyl migration mechanism. In addition to the immobilized *R. oryzae* lipase, immobilized *C. rugosa* lipase, a nonspecific lipase, was used for biodiesel production. It has been reported by some investigators that *C. rugosa* lipase is inadequate for biodiesel production [16-18]. When *C. rugosa* lipase was used to produce biodiesel in this study, biodiesel conversion failed to reach 20% (Fig. 2). However, *C. rugosa* lipase does not have specificity and thus can efficiently hydrolyze 1,2-diglycerides. We presumed that *C. rugosa* lipase hydrolyzes oil to free fatty acid and this *R. oryzae* lipase then esterifies the free fatty acid and methanol. If the rationale of this process is correct, the rate of biodiesel production could be increased because it does not rely on the acyl

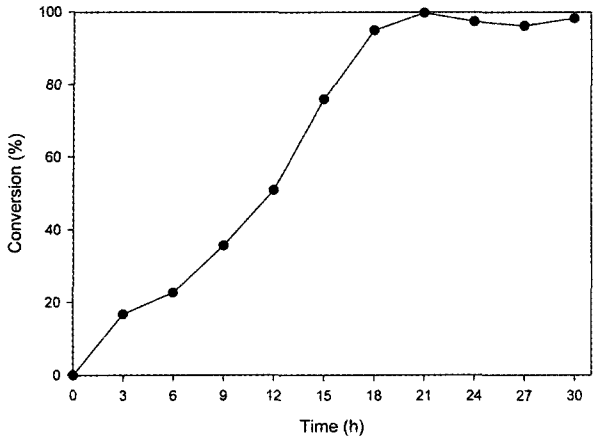

Fig. 3. Biodiesel production using a mixture of immobilized *R. oryzae* and *C. rugosa* lipases at a ratio of 1:1 (w:w). Both immobilized lipases had activities of 90 U/g matrix.

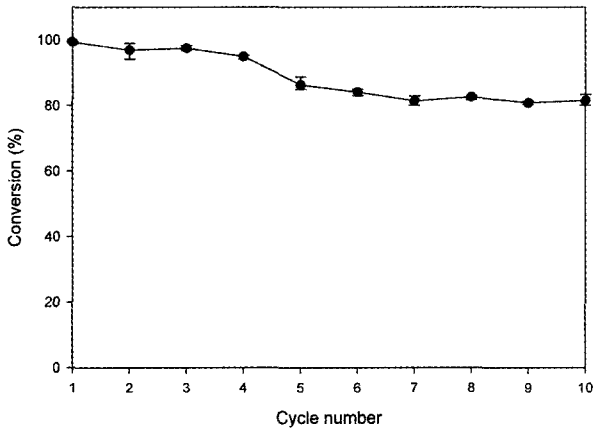

Fig. 4. Biodiesel production by reusing a mixture of immobilized *R. oryzae* and *C. rugosa* lipase at a ratio of 1:1 (w:w). The immobilized lipases each had activities of 90 U/g matrix. After batch reactions, immobilized lipases were filtered, washed with isopropyl alcohol and water and then reused for next batch reaction. The experiments were repeated 3 times.

migration mechanism.

Biodiesel was produced using a mixture of immobilized *R. oryzae* lipase and *C. rugosa* lipase at a ratio of 1:1 (w:w). Fig. 3 shows that biodiesel conversion of this mixture was greater than 90% at 18 h and 99% at 21 h. Compared with the result by Kaieda *et al.* [12] which showed a conversion of over 80% at 40 h, the rate of biodiesel production and conversion in the present study were greatly increased. Kaieda *et al.* [19] suggested a mechanism for transesterification of oil and methanol in which oil is first hydrolyzed to partial glyceride and free fatty acids and then methyl esters are produced by esterification of the free fatty acids with methanol. According to this mechanism and our results, it is believed that the first step, *i.e.*, the rate of liberating free fatty acids from soybean oil is increased by immobilized *C. rugosa* lipase, which importantly hydrolyzes 1,2-diglyceride without the need for acyl migration [15].

When the added amount of immobilized *R. oryzae* lipase in the reaction mixture was reduced, the rate of biodiesel production was actually similar to that observed when using a mixture of immobilized *R. oryzae* lipase and immobilized *C. rugosa* lipase at a ratio of 3:1 (w:w) (data not shown). These results could possibly be due to a low methanol feed rate and large amount of lipase in the reaction mixture, however the results require further study.

The reuse stability of immobilized lipase is important for industrial biodiesel production. In this study, a mixture of immobilizd *R. oryzae* and *C. rugosa* lipases in the ratio of 1:1 (w:w) was reused for biodiesel production 10 times to investigate this aspect. As shown in Fig. 4, biodiesel conversion was maintained at over 80% of its original conversion after being reused 10 times.

This study suggested that the mixture of two immobilized lipases was effective for biodiesel production. However, an optimization study of this process is needed in order to obtain higher productivity. If reaction conditions such as temperature, agitation speed, and molar ratio of methanol and oil are optimized and the method of methanol feeding is investigated, productivity of biodiesel could be greatly increased.

CONCLUSION

C. rugosa lipase has not been used to produce biodiesel in other studies. In this study, however, biodiesel conversion was greatly increased by adding the immobilized *C. rugosa* lipase to a reaction mixture containing immobilized *R. oryzae* lipase. This study suggests that nonspecific lipase can help 1,3-specific lipase to produce biodiesel by enhancing rate of oil hydrolysis by not relying on the acyl migration mechanism.

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