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Co-immobilization of *Candida rugosa* and *Rhyzopus oryzae* lipases and biodiesel production

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Abstract−*Candida rugosa* lipase and *Ryzopus oryzae* lipase were simultaneously immobilized on silica gel following enzyme pretreatment. The factors affecting the co-immobilization process, such as reaction time and enzyme ratio, were investigated. Biodiesel was then produced by using the co-immobilized enzyme matrix. A batch system was employed with stepwise methanol feeding, and the continuous process involved a packed-bed reactor. Under optimal immobilization conditions, the activity was approximately 16,000 U/g·matrix. When co-immobilized enzyme was used with optimized stepwise methanol feeding, conversion of biodiesel reached about 99% at 3 h and was maintained at a level of over 90% for about 30 reuses.

Key words: Biodiesel, Enzymatic Process, Co-immobilization, Lipase

INTRODUCTION

Recent increases in crude oil prices combined with environmental concerns have brought attention to biodiesel production [1,2]. In most countries, biodiesel is produced through a chemical catalytic process. However, several problems are associated with this process, including removal of the catalyst and inorganic materials from the product, recovery of glycerol, and excessive energy consumption. Due to these drawbacks, alternative and more sustainable routes for biodiesel production are being studied [3-5].

An enzymatic process offers an environmentally more attractive option due to its shorter purification step, high selectivity, and mild operative conditions. Lipases (triacylglycerol acyl hydrolases - EC3.1.1.3), one of the most important industrial enzymes, are very often used in various biotechnological applications, including enzymatic processes, detergent industries, food and pharmaceutical industries, and so on. However, the high cost of lipase often makes such processes economically unattractive. Efficient immobilization greatly enhances recovery and reuse of lipase [6-9]. In our previous work, we successfully developed a new process for biodiesel production using a mixture of *Rhizopus oryzae* and *Candida rugosa* lipases and investigated the optimal conditions [10]. Mixture of 1, 3-specific lipase and non-specific lipase removed the acyl-migration step, which is the rate-determining step of biodiesel production, and enzyme activity was notably enhanced. Further, a buffer mixture system with improved activity and stability was investigated and reported [11].

The objectives of the present study were the co-immobilization of lipases from *Rhizopus oryzae* and *Candida rugosa* on silica-gel matrix after enzyme pretreatment and optimization of the enzyme ratio and immobilization time. Co-immobilized lipase was also applied to biodiesel production by using a batch reactor.

MATERIALS AND METHODS

1. Materials and Enzyme Preparation

3-Aminopropyltriethoxysilane (3-APTES), *Candida rugosa* lipase (700 U/mg), and *Rhizopus oryzae* lipase (41.6 U/mg) were purchased from Sigma (USA). Glutaraldehyde was purchased from Fluka (Switzerland) and MOPS-free acid was supplied by Bio Basic Inc (Canada). Silica gel was obtained from Grace Davison (USA). All other chemicals used were of reagent grade.

Candida rugosa lipase (1 g) and *Rhizopus oryzae* lipase (1 g) were each suspended in 100 ml of 0.25 M MOPS-Sodium phosphate buffer mixture (pH 6.5). Each solution was centrifuged at 4 °C, 4,000 rpm for 15 min. The supernatants were then stored separately at 4 °C prior to pretreatment.

Pretreatment was necessary to protect the active site of the enzyme. The supernatant (20 ml) was pretreated with 0.1% soybean oil. The mixture solution was then incubated at 40 °C with stirring at 200 rpm for 45 min [10,11].

2. Silica Gel Activation and Enzyme Immobilization

Washed and dried silica gel (1 g) was mixed and activated using 10% of 3-APTES and 25% of glutaraldehyde, subsequently. Activated silica gel (500 mg) was mixed with 10 ml of the pretreated *Candida rugosa* lipase solution, followed by incubation at 20 °C with stirring at 200 rpm for 24 h. *Rhizopus oryzae* lipase was immobilized the same as mentioned above [9,11]. For co-immobilization, 8 g of each lipase powder was dissolved in the buffer mixture of phosphate and MOPS. Two hundred mg of soybean oil was added to 20 ml of *C. rugosa* and *R. oryzae* lipase solution. The mixtures were then incubated at 40 °C with stirring at 200 rpm for 45

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min. Next, 10 ml of the lipase solutions produced was subjected to immobilization.

A total of 10 ml of isooctane containing 10% (*w/v*) soybean oil was added to 10 ml of 50 mM phosphate buffer (pH 7) containing 200 mg of immobilized lipase. The reaction mixture was then incubated in a shaking water bath at 37 ºC and 150 rpm for 30 min. The upper layer (2 ml) was then transferred to a test tube, after which a cupric acetate-pyridine reagent (0.5 ml) was added. The free fatty acids liberated and dissolved by isooctane were quantified using a UV spectrophotometer at 715 nm [9-11]. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 μmol of free fatty acids per min.

3. Biodiesel Production and Analysis

In the batch process, co-immobilized *C. rugosa* and *R. oryzae* lipases, a small amount of water (10% (*w/w*) of co-immobilized enzyme), methanol, and soybean oil were used for biodiesel production. Exactly 3 mmol of soybean oil was mixed with 3-6 mmol of methanol, followed by the addition of 20% (*w/w*) of co-immobilized lipase. The mixture solution was incubated at 45 ºC and stirred at 250 rpm for 4 h. An equivalent amount of methanol was added once or twice during the biodiesel production process [9,11].

The fatty acid methyl ester (FAME) contents in the reaction were quantified by gas chromatography (GC) M6000D (Younglin. Co. Ltd., Korea) equipped with an HP-INNOWAX 1909IN-133 column (30 m×25 μm, Agilent, USA). Analysis condition of GC followed that of our previous work [11]. Samples were collected from the reaction mixture and then centrifuged to obtain the upper layer. One micro-liter of the treated sample was injected into the GC and the column temperature was increased from 150 ºC to 180 ºC at a rate of 15 ºC min^{-1}, and then from 180 ºC to 240 ºC by increasing the temperature at a rate of 5 ºC min^{-1}, after which the temperature was maintained at 240 ºC for 1 min. The injector and the detector temperature were both set at 260 ºC, respectively.

The equation of conversion yield was defined.

$$\text{Conversion yield} = \frac{\text{moles of FAME}}{\text{moles of triglyceride} \times 3} \times 100$$

RESULTS AND DISCUSSION

1. Optimization of Immobilization Condition

In our previous work, a novel process for biodiesel production using a mixture of *Rhizopus oryzae* lipase (ROL) and *Candida rugosa* lipase (CRL) was successfully developed and the optimal conditions were investigated [10]. Moreover, the mixture of CRL and ROL was employed in a buffer mixture system. As a result, the acyl-migration step of the trans-esterification mechanism was eliminated, resulting in higher enzyme activity compared to before [9]. In this process, since both enzymes were immobilized on silica gel individually, there is the limitation of the mass transfer of reactants. Silica gel is a good enzyme supporter because of its cheap price and good mechanical characteristics. It provides an abundant OH group on its surface. Silica-gel used was different from the general silica-gels for adsorption and dehumidification agent. Then its porosity caused the enhancement of immobilization yield [10]. Further, after reaction with 1, 3-specific enzyme, hydrolysates move to the non-specific enzyme. On the other hand, if the mass transfer conditions are improved, enzyme activity is enhanced and the pretreated enzyme solution can be immobilized on one matrix. In the immobilization process, hydrogen peroxide was employed to activate silica-gel surface. 3-APTES was used for silanization to generate amine group on surface, and glutaraldehyde was used for coupling of both amine

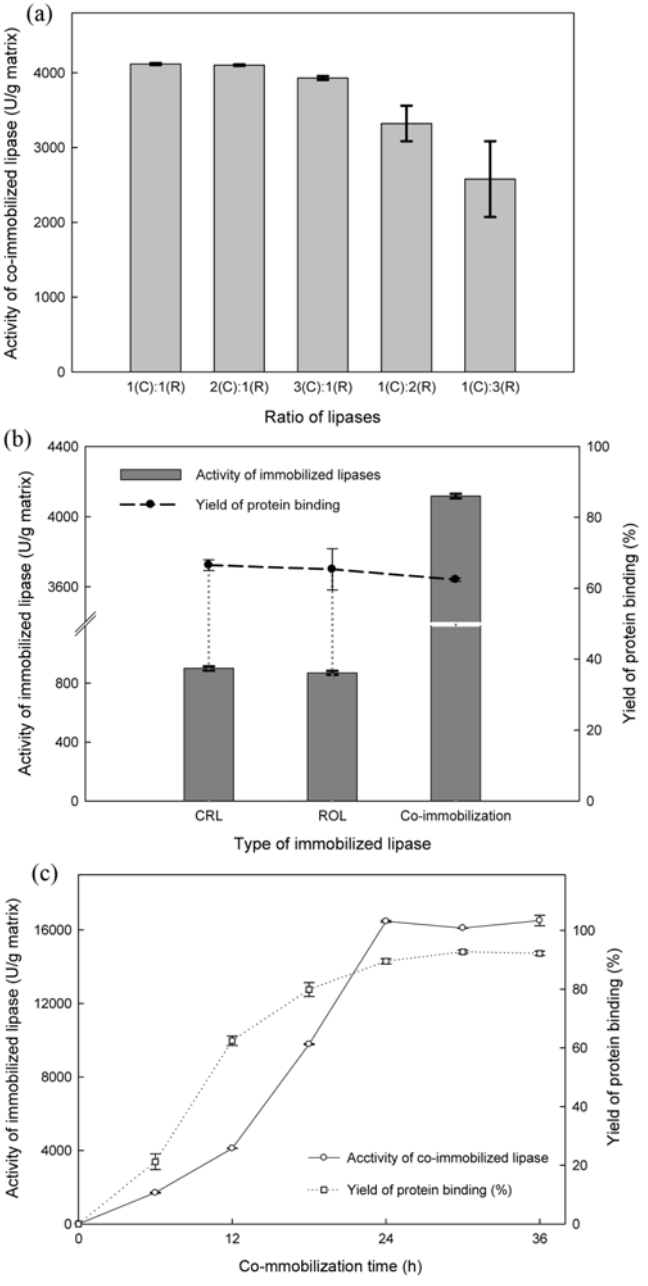

Fig. 1. The results of co-immobilization. (a) Enzyme activities of various ratios of co-immobilized *Candida rugosa* and *Rhizopus oryzae* lipases. (C): *Candida rugosa* lipase, (R): *Rhizopus oryzae* lipase. (b) Enzyme activities of immobilized lipases and protein binding yield at an immobilization time of 12 h. CRL: *Candida rugosa* lipase, ROL: *Rhizopus oryzae* lipase. Co-immobilization: Immobilized lipase (CRL : ROL=1 : 1). (c) Effect of co-immobilized lipase on the activity and yield of protein binding by CRL and ROL (1 : 1). Lipase solutions (20 ml) were pretreated with 20 mg of soybean oil at 40 ºC.

group (-NH_2) of aminosilane and protein (enzyme) [10].

To determine the ideal conditions for co-immobilization, the optimal immobilization time was investigated. The ratio of pretreated lipases was optimized to improve the efficiency of immobilization and biodiesel production. Pretreated CRL and ROL solutions were mixed at ratios of 1 : 1, 2 : 1, 3 : 1, 1 : 2, and 1 : 3 (*v* : *v*) in 10 ml test tubes. Activated silica gel (500 mg) was added into a water jacket flask, followed by 10 ml of each mixed solution and immobilized [9]. Fig. 1(a) shows the effect of the lipase ratio on the activity of the co-immobilized lipases. When CRL and ROL were mixed at an equal ratio, the activity was approximately 4,100 U/g·matrix. The activities were over 4,000 U/g·matrix when the CRL dosage was increased to 2 : 1 or 3 : 1. When the ROL dosage was increased to a ratio of 1 : 2 or 1 : 3, the enzyme activities decreased steeply. Further, the optimal ratio of CRL to ROL was found to be 1 : 1. When immobilization was performed with mixed enzyme solution (1 : 1 ratio of CRL to ROL), the protein binding yields were found to be 67.59%, 69.46%, and 62.7% at 12 h, respectively (Fig. 1(b)). Thus, single lipase was slightly more effective than that of the enzyme mixture for immobilization, although the difference was negligible. After immobilization, enzyme activity was found to be 910 U/g·matrix for CRL, 880 U/g·matrix for ROL, and 4,130 U/g·matrix for the co-immobilized lipases. This result shows that mass transfer between the enzymes during the reaction was notably improved (Fig. 1(b)).

The optimal ratio of CRL to ROL (1 : 1) was used, and the mixed solution was immobilized to activated silica gel for 6, 12, 18, 24, 30, and 36 h (Fig. 1(c)). After each reaction time, the protein binding yield and enzyme activities were measured. Protein binding yield was calculated by measuring the amount of remaining unbound protein after the immobilization. Specifically, the protein binding yield appeared to be a phenomenon of mass transfer since the yield increased with the reduced mass transfer gradient. The protein binding yield increased with the increasing reaction time and reached maximum at 12 h, after which it decreased gradually and became approximately 90% after 24 h. Thus, the optimal enzyme concentration for efficient co-immobilization was determined to be approximately 20 g/*l*. Further, enzyme activity gradually increased according to reaction time. In detail, enzyme activity was approximately 4,000 U/g·matrix at 12 h and reached about 16,000 U/g·matrix at 24 h. These results are about 4.5-fold higher than those of the buffer mixture system, which is the most often employed enzyme mixture system, and 20-fold higher than those of the mono enzyme immobilization system. Mass transfer of 1, 3-hydrolyzed reactants to nonspecific lipase might also be enhanced. The immobilization time for efficient co-immobilization of CRL and ROL was found to be 24 h.

2. Batch Production of Biodiesel Using Co-immobilized Enzyme

Co-immobilized lipase was utilized to produce biodiesel, while the stepwise methanol feeding method was employed to prevent the accumulation of methanol during the enzymatic reaction. Initially, 3, 4.5, and 6 mmol of methanol were added to the reactor. After the reaction began, methanol was added into reactor with three times for the 3 mmol, two times for the 4.5 mmol, and just one time for the 6 mmol [11]. Biodiesel production was performed at 45 °C with shaking at 300 rpm for 4 h. Fig. 2(a) shows the results of biodiesel production using co-immobilized enzyme. When 4.5 mmol of methanol was added every 1.5 h, the biodiesel conversion rate reached 98.32% at 4 h. When stepwise methanol feeding was performed

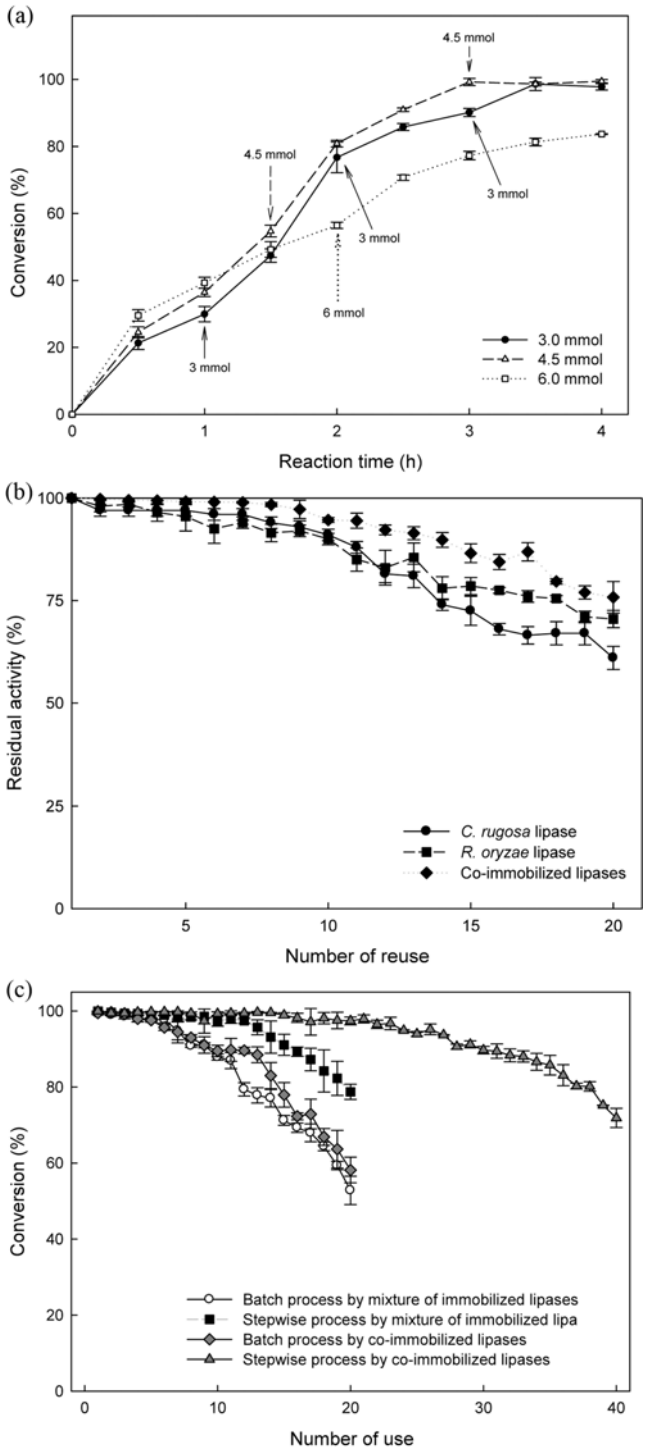

Fig. 2. Biodiesel production by using co-immobilized lipases and reuse. (a) Biodiesel production by co-immobilized lipase using various stepwise reaction methods. (b) Enzyme activities of reused immobilized *Cancida rugosa* lipase, *Ryzopus oryzae* lipase, and co-immobilized lipase. (c) Reusing of immobilized *Cancida rugosa* lipase, immobilized *Ryzopus oryzae* lipase, and co-immobilized lipase in a batch reactor with stepwise methanol feeding.

every hour with 3 mmol of methanol, approximately 99% of the biodiesel was converted at 3 h. However, when 6 mmol of methanol was added at 2 h, the biodiesel conversion rate was only 83.79%. In this case, the immobilized lipases were deactivated by accumulated methanol in the reactor. Enzymatic biodiesel production system consists of three steps. The first step is the heterogeneous system and rate-determining step because lipid and methanol are not soluble with each other and interfacial reaction occurs. The reaction is relatively slow, and fatty acid methyl ester (FAME) conversion production rate could be low. As FAME concentration increases, intermediate and final products such as FAME and partial glyceride could be an agent of emulsifier and the interface disappears. At this second step, the system is homogeneous and the reaction rate increases. Finally, the last step would occur when glycerol concentration is increased late in the reaction. At this step, the remaining methanol moves into the glycerol layer, and reaction rate is decreased.

The ability to reuse immobilized lipase is very important for industrial applications, and it is the ultimate goal of lipase immobilization. After the enzymatic reaction, the immobilized lipase was isolated and washed with water and isopropyl alcohol. The reaction was performed using the immobilized lipase (CRL, ROL) mixture and co-immobilized enzyme, and enzyme stability was compared between the two. The methanol feeding methods were then compared. Fig. 2(b) shows the relative residual activity of the co-immobilized and individual immobilized lipases. Immobilized CRL and ROL decreased in activity by about 73% and 60%, respectively, after 20 reuses. The activity of the co-immobilized lipase was about 75% after 20 reuses and maintained higher than 98% activity after 7-8 reuses. Fig. 2(c) shows biodiesel production using the reused immobilized lipase mixture and co-immobilized lipase. Biodiesel conversion by the stepwise methanol feeding method was maintained at higher than 95% after about 25 reuses, followed by a decrease to 85% after about 35 reuses. In the reaction without stepwise methanol feeding, the conversion rate was 85% after 13 uses. Therefore, stepwise methanol feeding is critical to enzymatic biodiesel production. Moreover, when stepwise methanol feeding was combined with the co-immobilization system, synergic effects were observed. Compared to the immobilized lipase mixture process, the co-immobilization system had superior enzyme activity, biodiesel production, and stability. These results might also enhance mass transfer during biodiesel reaction.

CONCLUSION

Biodiesel was produced using co-immobilized lipase and by stepwise methanol feeding. Under optimal immobilization conditions, the activity of co-immobilized enzyme was approximately 16,000 U/g·matrix. The biodiesel conversion rate for the co-immobilized enzyme reached about 99% after 3 h upon stepwise addition of 3 mmol of methanol every hour, and the rate was maintained at higher than 90% after about 30 reuses.

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