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Biodiesel Production from Various Oils Under Supercritical Fluid Conditions by *Candida antartica* Lipase B Using a Stepwise Reaction Method

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Abstract In this study, we evaluate the effects of various reaction factors, including pressure, temperature, agitation speed, enzyme concentration, and water content to increase biodiesel production. In addition, biodiesel was produced from various oils to establish the optimal enzymatic process of biodiesel production. Optimal conditions were determined to be as follows: pressure 130 bar, temperature 45 °C, agitation speed 200 rpm, enzyme concentration 20%, and water contents 10%. Among the various oils used for production, olive oil showed the highest yield (65.18%) upon transesterification. However, when biodiesel was produced using a batch system, biodiesel conversion yield was not increased over 65%; therefore, a stepwise reaction was conducted to increase biodiesel production. When a reaction medium with an initial concentration of methanol of 60 mmol was used and adjusted to maintain this concentration of methanol every 1.5 h during biodiesel production, the conversion yield of biodiesel was 98.92% at 6 h. Finally, reusability was evaluated using immobilized lipase to determine if this method was applicable for industrial biodiesel production. When biodiesel was produced repeatedly, the conversion rate was maintained at over 85% after eight reuses.

Keywords Biodiesel · Initial reaction rate · Lipase activity · Optimization · Solubility · Supercritical fluid condition

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Introduction

Biodiesel, which has been defined as the alkyl esters of long-chain fatty acids, is derived from regenerable sources of feedstock such as vegetable and animal oil by transesterification with alcohol [1]. Biodiesel has several environmental advantages, including being biodegradable and nontoxic; therefore, it is anticipated that these substances will be used as a substitute for common fossil fuels [2, 3]. Enzymatic production processes of biodiesel can overcome problems associated with chemical production such as being energy intensive and difficult in separating the glycerol from the catalyst, but they have not yet been industrialized because of its high price, the instability of the enzymes used and the slow reaction rate [4–9].

The use of supercritical CO_2 in the production of biodiesel has several advantages, such as being nontoxic, nonflammable, environmental benign, and economical. Although the products and enzymes used for the production of biodiesel do not dissolve under atmospheric conditions, they can be easily separated by decreasing the pressure. In addition, the enzymes used in this system are stable under supercritical fluid conditions [10, 11]. The utilization of supercritical CO_2 can overcome problems that are often associated with the enzymatic process during biodiesel production, but the conversion yields obtained have been very low when supercritical carbon dioxide has been used.

To our knowledge, no investigations have been conducted to optimize the supercritical reaction conditions under which enzymatic biodiesel production is conducted. Therefore, in this study, the supercritical reaction conditions under which the enzymatic production of biodiesel was conducted were optimized using various oils and a commercial enzyme, and a stepwise reaction method was investigated for increment of biodiesel production under supercritical carbon dioxide condition.

Materials and Methods

Materials

Candida antartica lipase B was obtained from NOVO Nordisk (Denmark). Soybean, olive, palm, rapeseed, and sunflower oils were purchased from Sigma (USA). All other chemicals used in this study were of reagent grade and analytical grade.

Assay of Immobilized Lipase Activity

Ten milliliters of isoocatane containing 10% (*w*/*v*) soybean oil was added to 10 ml of a 50 mM phosphate buffer (pH 7) containing 200 mg of the immobilized lipase. The reaction mixture was incubated in a shaking water bath at 37 °C and 150 rpm for 30 min. Two milliliters of the upper layer was then transferred to a test tube and a cupric acetate-pyridine reagent (0.5 ml) was added. The free fatty acids liberated and dissolved by the isooctane were quantified using a UV spectrophotometer at 715 nm [12]. One unit of lipase activity was defined as the amount of the enzyme required to liberate 1 μmol of the free fatty acid per minute.

Biodiesel Production in Supercritical Fluid Condition by Immobilized Lipase

Biodiesel production was conducted in a 100 ml supercritical carbon dioxide (SC-CO_2) reactor unit (Fig. 1). The reactants, which included soybean, rapeseed, olive, sunflower, and

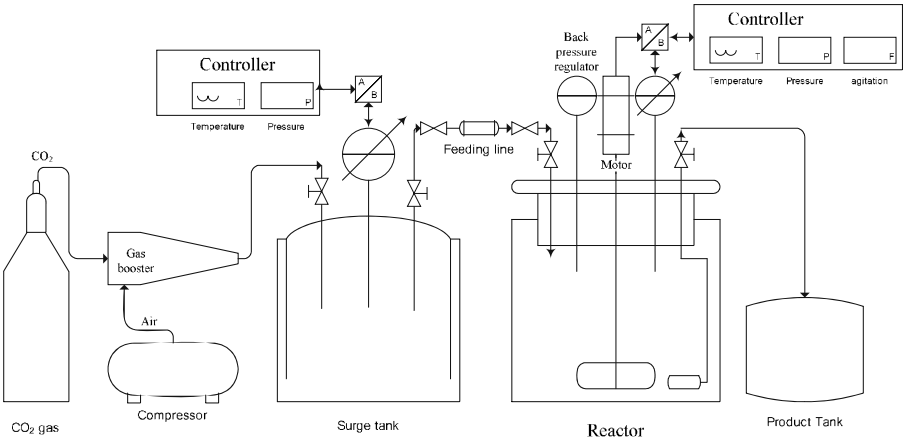

Fig. 1 Schematic diagram of the supercritical carbon dioxide-enzyme reactor unit

palm oil with methanol, were loaded into the reactor and mixed with enzyme, and the temperature of reactor was then increased from 20 to 50 °C. As the temperature of the reactor was increased, the pressure of the reactor was also increased from 75 to 150 bar. Additionally, in the stepwise reaction, the methanol concentration of the reaction medium, which contained 60 mmol of soybean oil and 15 g of the immobilized lipase, was adjusted to 60∼90 mmol of methanol. An equivalent amount of methanol was then added 1, 2, or 3 times during biodiesel production. After the reaction completion, the reactor was depressurized and the products were eluted in hexane. Next, the enzyme was deactivated by heat (80 °C, 15 min) and was removed by centrifugation and the samples were then analyzed by gas chromatography.

Analytical Methods

Biodiesel was analyzed using a GC M600D (Younglin. Co. Ltd., Korea) with an HP-innowax 1909IN-133 column (30 m×25 μm, Agilent, USA). Samples were collected from the reaction mixture and then centrifuged to obtain the upper layer. One microliter of the treated sample was then injected into the GC, and the column temperature was then raised from 150 to 180 °C at a rate of 15 °C min^{-1} and then from 180 to 240 °C by increasing the temperature at a rate of 5 °C min^{-1}; after which, the temperature was maintained at 240 °C for 1 min. The injector and the detector temperatures were both set at 260 °C.

The equations of conversion yield and initial reaction rate were defined.

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

$$\text{Initial reaction rate} = \frac{\text{concentration of FAME (mmol/l)}}{\text{reaction time (s)}}$$

Results and Discussion

Effect of Reaction Conditions on Biodiesel Production

Several studies have investigated the effect of reaction conditions on biodiesel production using various methods [13–15]. Giridhar et al. reported that the reaction time (6 h) required to produce biodiesel from sunflower oil was very fast but that the conversion yield was very low (about 27%) when the reaction was conducted using supercritical carbon dioxide [14]. In this study, the pressure, temperature, substrate molar ratio, and enzyme concentration in the reaction were optimized to increase the conversion yield of biodiesel. Reaction medium containing 180 mmol of methanol and 20% (weight of the mixture of immobilized lipases/weight of soybean oil) of immobilized *Candida antartica* lipase B were blended with soybean oil (60 mmol). Biodiesel production was then conducted at various temperatures at 200 rpm for 6 h. As shown in Fig. 2, the optimal temperature for lipase activity from soybean oil under supercritical fluid conditions was 45 °C, which resulted in conversion yield of 56.74%. In addition, when the temperature was increased, initial reaction rate also increased; however, once the temperature reached 60 °C, it decreased to 1.18×10^{-2} ($mol{\cdot}L^{-1}{\cdot}s^{-1}$). Particularly, at 55 and 60 °C, initial activity of lipase was high. However, lipase activity was decreased markedly with time.

Generally, the reaction rate of the enzymatic system for biodiesel production using lipase was evaluated later than chemical process. Therefore, the enzymatic process has not yet been commercialized. However, the use of supercritical fluid was developed to overcome the slow reaction rate that occurs in enzymatic system. This method allows the mass and thermal transfer to be increased, thereby, allowing a faster reaction rate than can occur at atmosphere pressure. Therefore, in this study, we determined the optimal pressure by

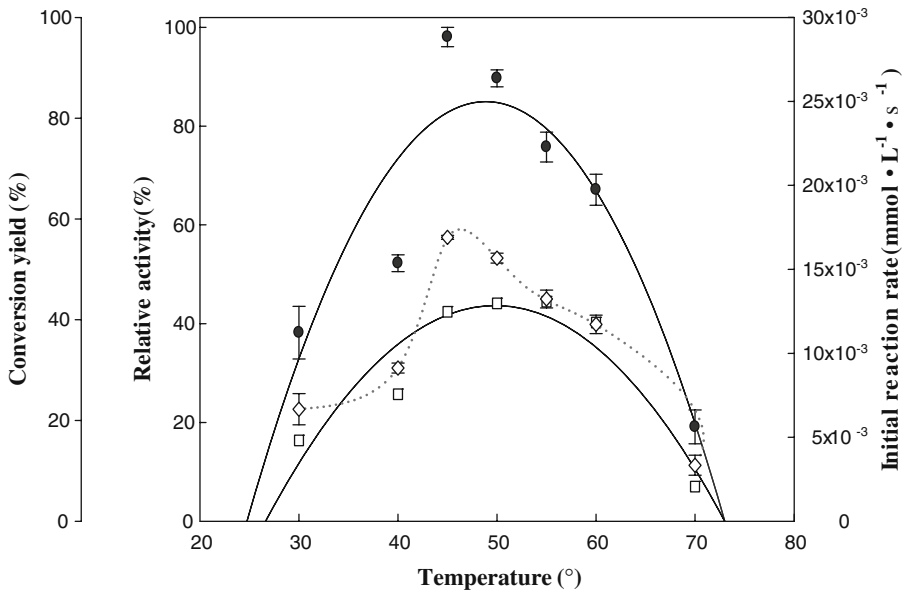

Fig. 2 Effect of temperature on biodiesel production under supercritical fluid conditions by *Candida antartica* lipase B: relative activity (*filled circle*), initial reaction rate (*empty square*), conversion yield (*empty diamond*)

relative activity at 45 °C with various pressures (Fig. 3). The results showed that relative activity and initial reaction rate increased as pressure increased, at which maximum initial reaction rate and point the conversion of biodiesel were 1.18×10^{-2} (mmol·L^{-1}·s^{-1}) and 58.95%.

In enzymatic process for biodiesel production, because substrates (oil, methanol and water) were formed to two phases such as polar and nonpolar region, mass transfer could be inhibited [16, 17]. Therefore, to increase the amount of mass transfer between the substrate and the enzyme in the reactor, the agitation speed was optimized. Relative activity increased as the agitation speed was increased (Fig. 4). In addition, the initial reaction rate increased as the agitation speed increased to 400 rpm; however, the conversion yield (58.23%) did not increased further more. This phenomenon occurred by increment of mass transfer using supercritical carbon dioxide and mechanical agitation; however, as the level of mass transfer increased, lipase activity can be decreased as a result of the accumulation of methanol [13]. Taken together, these results provide valuable information that is applicable to industrial biodiesel production using supercritical carbon dioxide.

Enzyme concentration is also known to be an important factor in the enzymatic process. High diffusion limitation of the intraparticle may cause a decrease of enzyme activity by high enzyme loading in biodiesel production processes using immobilized lipase. Actually, Gao et al. reported that inhibition of biodiesel production was induced by a high enzyme concentration. Therefore, the optimal enzyme concentration was investigated for establishment of efficient biodiesel production [13]. To accomplish this, biodiesel was produced using various amounts of lipases (5~50%, weight of the mixture of immobilized lipases/weight of soybean oil), which were added to reaction mediums containing 60 mmol of soybean oil and 180 mmol of methanol. Biodiesel production was then performed at 45 °C and 200 rpm for 6 h. As shown in Fig. 5, when 35% immobilized lipases were used, biodiesel conversion reached 61.88% at 6 h. Moreover, initial reaction rate increased

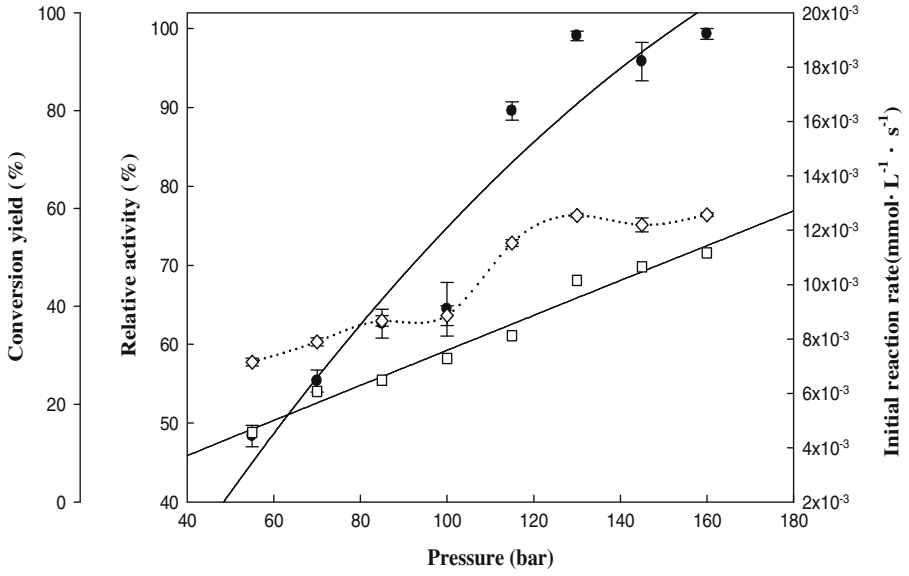

Fig. 3 Effect of pressure on biodiesel production under supercritical fluid conditions by *Candida antartica* lipase B: relative activity (*filled circle*), initial reaction rate (*empty square*), conversion yield (*empty diamond*)

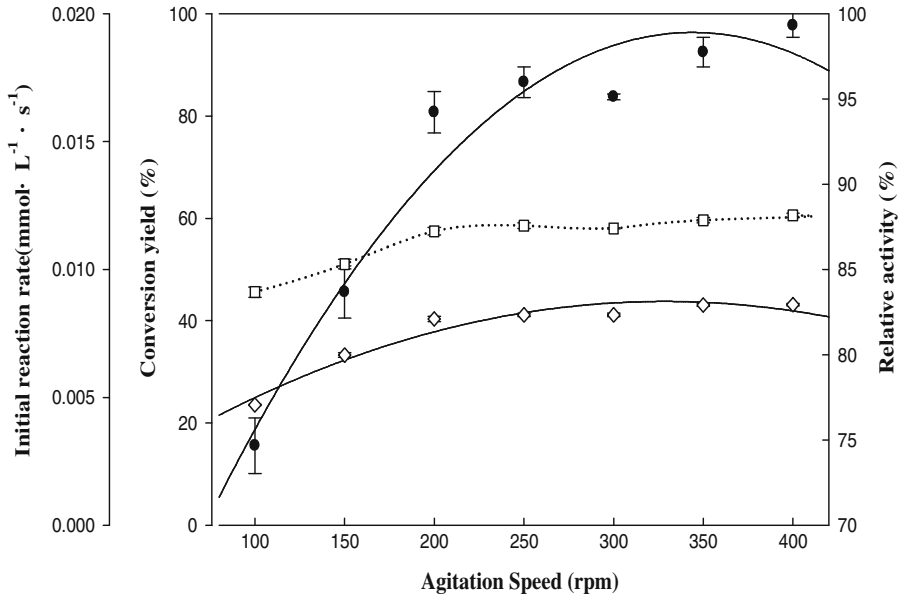

Fig. 4 Effect of agitation speed on biodiesel production under supercritical fluid conditions by *Candida antartica* lipase B: relative activity (*filled circle*), initial reaction rate (*empty square*), conversion yield (*empty diamond*)

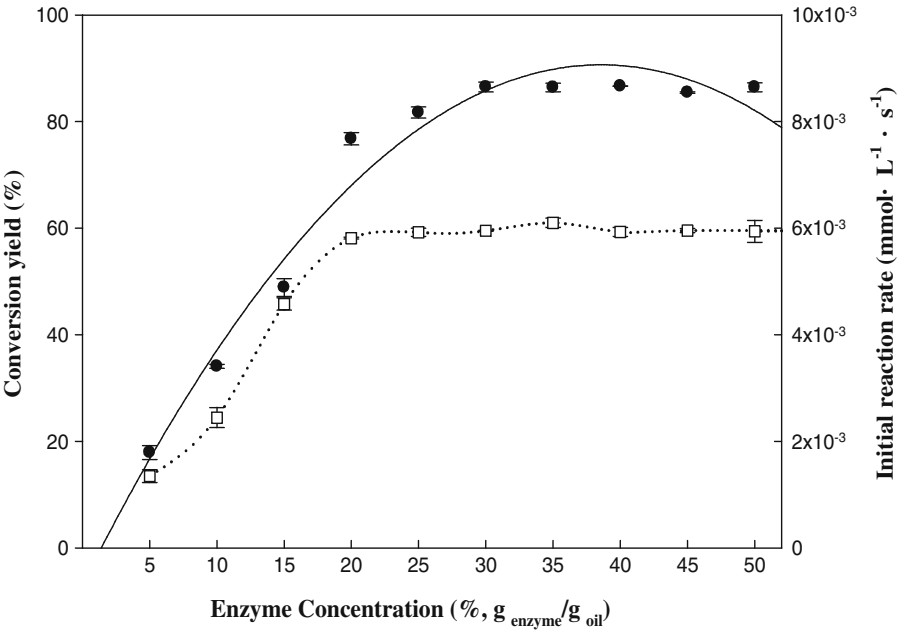

Fig. 5 Effect of enzyme concentration on biodiesel production under supercritical fluid conditions by *Candida antartica* lipase B: relative activity (*filled circle*), initial reaction rate (*empty square*)

quickly over 20% enzyme concentration. However, when 20%, 25%, and 30% enzyme concentrations were compared no significant differences in conversion yields and initial reaction rate were observed. Particularly, when less than 20% immobilized lipase was used, the biodiesel conversion yield decreased markedly. These results indicate that mass transfer between the enzyme and the substrate was prevented when the concentration of enzyme used was less than 20%; therefore, this concentration was taken to be the optimal concentration of the immobilized lipase.

Lipases have the special characteristics at the interface between an aqueous and an organic phase. Moreover, lipases show their maximal activity with felicitous water content in low aqueous systems. This requires that the water interacts with hydrophilic groups located on the enzyme molecule surface to alter the hydrogen bond interaction inside the enzyme, thereby inducing an open conformation of the enzyme [16]. Therefore, water content should be considered when optimizing the lipase activity. To accomplish this, biodiesel was produced using various amounts of water (0~25%, weight of water/weight of soybean oil) in reaction medium that contained 60 mmol of soybean oil and 180 mmol of methanol. Biodiesel production was then performed at 45 °C, 200 rpm, and 20% enzyme concentration for 6 h. When the water content was 10%, the biodiesel conversion and initial reaction rate reached 64.23% and 9.29×10^{-3} ($mol \cdot L^{-1} \cdot s^{-1}$) at 6 h (Fig. 6), which was higher than the other water contents evaluated.

Biodiesel Production from Various Oils in Supercritical Fluid and Its Analysis Using Solubility of Fatty Acids

Vegetable oils form fatty acids such as palmitic acid, linoleic acid, oleic acid, stearic acid, and linolenic acid when they are combined with glycerol. Therefore, when a trans-

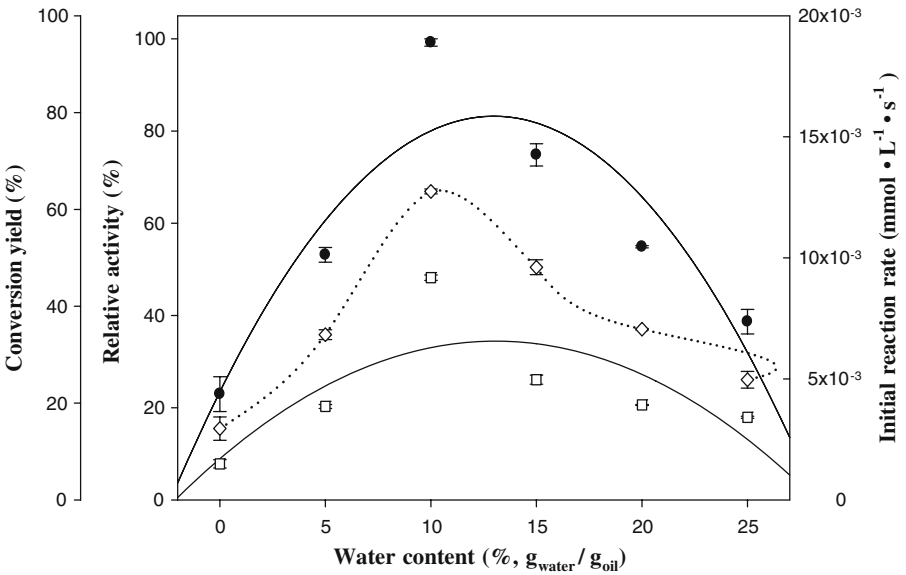

Fig. 6 Effect of water contents on biodiesel production under supercritical fluid condition by *Candida antartica* lipase B: relative activity (*filled circle*), initial reaction rate (*empty square*), conversion yield (*empty diamond*)

esterification batch process using lipase is employed, vegetable oils may be converted to biodiesel differently based on the fatty acids that are produced. In addition, because of high mass transfer, the solubility of vegetable oils under supercritical fluid conditions can be an important factor. Thus, in this study, the biodiesel conversion of palm, soybean, sunflower, olive, and rapeseed oil was investigated to establish of the reaction conditions under supercritical fluid conditions. As shown in Fig. 7, when olive oil was used, maximum conversion yield and initial reaction rate were 65.18% and 12.7×10^{-3} ($mol{\cdot}L^{-1}{\cdot}s^{-1}$), respectively. The solubility of dense carbon dioxide in substrates is known to be increased [18, 19]. The mass transfer may be increased under supercritical fluid conditions as a result of increased substrate solubility. Therefore, increment of solubility can be induced fast reaction rate. The primary component of olive oil was oleic acid, and this produced the highest solubility at 45 °C and 130 bar [20]. These results indicate that olive oil could produce the highest value of biodiesel and that reaction rate can be increased by increasing the solubility of the fatty acids.

The Effects of the Initial Concentration of Methanol and Stepwise Reaction on Biodiesel Production in Supercritical Fluid Condition

In previous studies, some researchers reported that lipase can be deactivated by using short chain alcohols because they have intense polarity and hydrophilic property. In addition, accumulated methanol can induce the deactivation of lipase under atmospheric conditions [8, 13]. Especially, we found that the effect of methanol on lipase deactivation in high mass transfer systems, such as those that operate under supercritical fluid conditions, was higher than the effects that are induced under atmospheric conditions. Therefore, to prevent methanol accumulation, the optimal initial concentration of methanol was evaluated. From 60 to 120 mmol of methanol was initially added to the reaction medium, and then the same

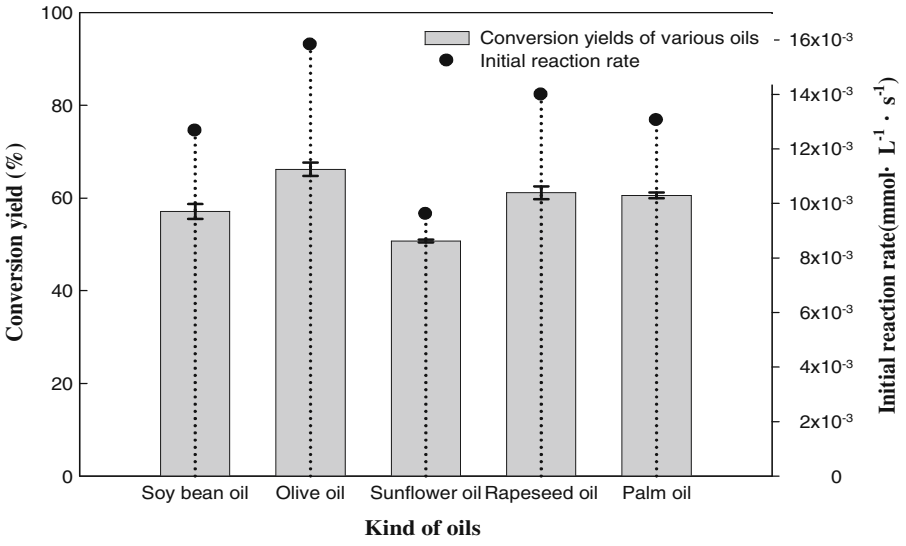

Fig. 7 Biodiesel production using various oils by *Candida antartica* lipase B; Reaction conditions were as follow: pressure 130 bar, temperature 45 °C, enzyme concentration 20%, water content 10%

amount of initial methanol concentration was added at some intervals during reaction. Biodiesel production was then conducted at 45 °C, 200 rpm, and 20% enzyme concentration for 6 h. As shown in Fig. 8, when the initial concentration of methanol was 90 mmol, the biodiesel conversion was 98.92% at 6 h. However, when the initial concentration of methanol was 60 and 120 mmol, biodiesel conversion yield were only 85% and 65%, respectively. Base on these results, biodiesel conversion was increased using a stepwise reaction method; however, this resulted in the denaturation of lipase by accumulated methanol in the reaction medium. Therefore, the best method of methanol addition was determined to be initially adjusting the concentration of methanol to 90 mmol and then adjusting the concentration to 90 mmol every 2 h during biodiesel production.

Reusing the Immobilized Lipases for Repeated Biodiesel Production

The reuse of immobilized lipase is of key importance for industrial applications. Twenty percent of immobilized *C. antartica* lipase was used consecutively in a series of biodiesel productions for 6 h. After a batch and stepwise reaction, the immobilized lipase was filtered, washed with water and isopropyl alcohol, and then reused for the next batch and stepwise reaction. As shown in Fig. 9, the biodiesel conversion was 85% after eight reuses by the stepwise reaction method. However, when a batch reaction was performed, the relative activity of immobilized *C. antartica* lipase was decreased to 35.2% after six reuses. These results indicate that lipase activity was improved as a result of the reduced concentration of accumulated methanol. Thus, the stepwise reaction method under

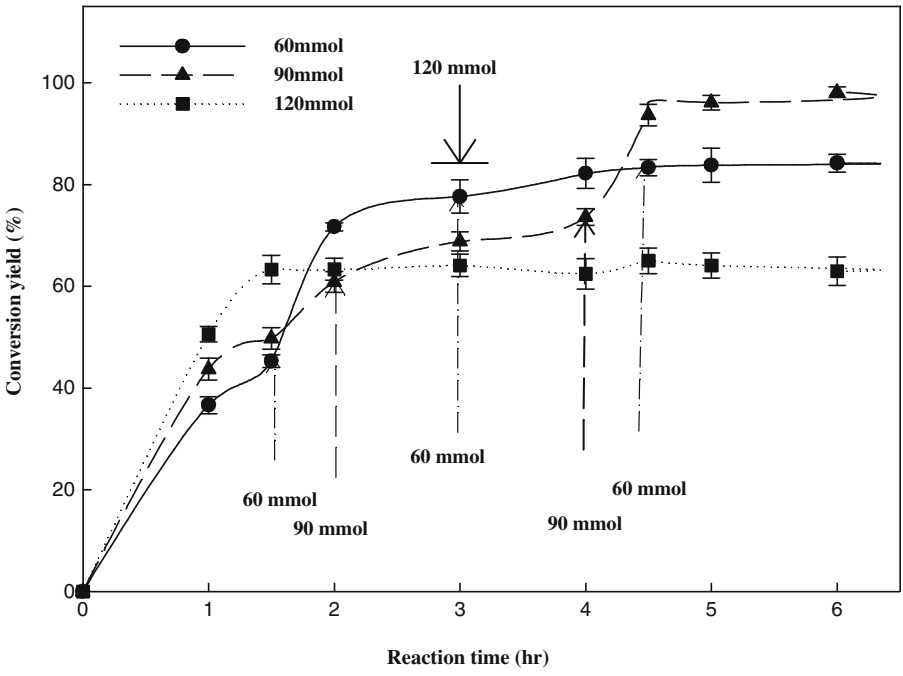

Fig. 8 Biodiesel production using various stepwise reaction methods under supercritical fluid conditions; Reaction conditions were as follow: pressure 130 bar, temperature 45 °C, enzyme concentration 20%, water contents 10%

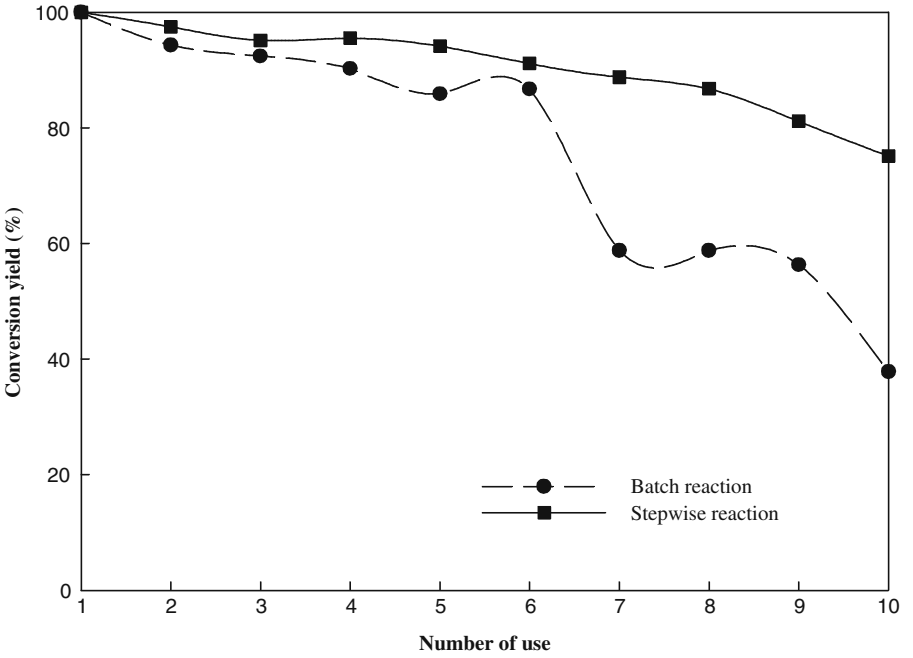

Fig. 9 Biodiesel production attained by reusing *Candida antartica* lipase B using batch and stepwise reaction methods

supercritical fluid conditions was highly effective for biodiesel production from vegetable oil and methanol. Finally, these results suggest that this process has the potential for application to a scale-up study.

Conclusions

It has been suggested that efficient production of biodiesel is possible via immobilized lipase catalysis under supercritical fluid conditions. By optimizing reaction factors, we were able to increase the biodiesel production and the reaction rate. The optimal conditions determined were as follows: pressure 130 bar, temperature 45 °C, agitation speed 200 rpm, enzyme concentration 20%, and water content 10%. Among the various oils, olive oil showed the highest yield and initial reaction rate after transesterification (65.18% and 12.7×10^{-3} mol·L^{-1}·s^{-1}). However, in the batch system, the conversion yield of the biodiesel could not be increased above 65%. Therefore, a stepwise reaction was conducted to increase the biodiesel production. When the initial concentration of methanol in the reaction medium was 90 mmol and this concentration was maintained throughout the reaction by the addition of methanol every 1.5 h, conversion yield of biodiesel was 98.92% at 6 h. Finally, reusability was evaluated using immobilized lipase to be determined if this process was applicable for use in industry. When repeated biodiesel production was evaluated, the biodiesel conversion was maintained at over 85% after eight reuses. Further studies will be conducted to evaluate the development of biodiesel production using various enzymes to induce a reduction in the reaction rate.

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