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Biodiesel production from jatropha oil catalyzed by immobilized *Burkholderia cepacia* lipase on modified attapulgite

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HIGHLIGHTS

- Lipase from *Pseudomonas cepacia* was firstly immobilized onto modified ATP.
- The important reaction parameters were studied by single-factor experiment.
- More than 94% biodiesel yield was obtained by immobilized lipase.
- Immobilized lipase was stable and retained 94.7% relative activity after 10 cycles.

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ABSTRACT

Lipase from *Burkholderia cepacia* was immobilized on modified attapulgite by cross-linking reaction for biodiesel production with jatropha oil as feedstock. Effects of various factors on biodiesel production were studied by single-factor experiment. Results indicated that the best conditions for biodiesel preparation were: 10 g jatropha oil, 2.4 g methanol (molar ratio of oil to methanol is 1:6.6) being added at 3 h intervals, 7 wt% water, 10 wt% immobilized lipase, temperature 35 °C, and time 24 h. Under these conditions, the maximum biodiesel yield reached 94%. The immobilized lipase retained 95% of its relative activity during the ten repeated batch reactions. The half-life time of the immobilized lipase is 731 h. Kinetics was studied and the V_{max} of the immobilized lipases were 6.823 mmol L^{-1}. This immobilized lipase catalyzed process has potential industrial use for biodiesel production to replace chemical-catalyzed method.

1. Introduction

Biodiesel is made from renewable biological sources and it does not produce sulfur oxide and may reduce soot discharge by one-third that of existing petroleum-based products (Ranganathan et al., 2008). Biodiesel in industrial applications may be produced by chemical-catalyzed or enzyme-catalyzed methods. The biodiesel produced by chemical catalyst has several drawbacks such as difficulty in removal of acid or base catalysts from product, high energy requirements, difficulties in the recovery of the catalyst and potential pollution to the environment (Winayanuwattikun et al., 2011; Tan et al., 2010). Enzyme-catalyzed biodiesel production has received more attention because of its advantages, such as low energy consumption, mild operating conditions, nontoxicity, and environment friendly processes, as compared with the chemical-catalyzed method (Dwiarti et al., 2010; Lee et al., 2011). However, the enzyme-catalyzed method is not favored for industrial use because the high cost and low stability of lipases limit its potential application (Chen and Wu, 2003; Soumanou and Bornscheuer, 2003).

Immobilization may lower biodiesel cost and increase lipase stability. The appropriate type of support is a key factor in enzyme immobilization. Enzyme supports are composed of macroporous and microporous polymers (Dizge et al., 2009; Hernandez-Martin and Otero, 2008), silica sol–gel matrix or aerogels (Orçaire et al., 2006; Noureddini et al., 2005), ordered mesoporous silica (Blanco et al., 2007; Macario et al., 2009), and other porous ceramics (Zaidan et al., 2010). Attapulgite (ATP) is a fibrillar type of hydrated magnesium aluminum silicate present in nature (Fan et al., 2008; Zhang et al., 2007). It is a kind of inexpensive, natural, and adsorbent clay mineral. It has reactive –OH groups on its surface, a fibrous morphology, high temperature stability, and a large specific

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surface area (Zhang et al., 2010). The use of attapulgite as an immobilized enzyme support presents numerous advantages, such as good mechanical strength, high specific surface area, and ease of water dispersion/recuperation. Its structure contains water bound in different forms, namely, water of constitution, water of crystallization, and adsorbed water. This implies that attapulgite can be used to supply water for the immobilized lipase, which is crucial for retaining the structure of the lipase. In additional, its natural origin and low cost make it feasible for commercial applications. The specific properties of attapulgite has stimulated studies on the material and its use as absorbent, catalyst support, food additive, immobilization material, and so on (Zhao et al., 2010; Cao et al., 2008; You et al., 2011). However, to the best of our knowledge, lipase immobilization on modified attapulgite has not been previously reported.

In this study, *Burkholderia cepacia* (*B. cepacia*) lipase was immobilized on modified ATP by cross-linking reaction for biodiesel production with jatropha oil as feedstock. The water content, method of methanol addition, amount of immobilized lipase, reaction temperature, and reaction time were examined to determine the best biodiesel reaction conditions.

2. Methods

2.1. Materials

Lipase from *B. cepacia* was purchased from Sigma–Aldrich. ATP was acquired from the Key Laboratory for Attapulgite Science and Applied Technology of Jiangsu Province, China. 3-Aminopropyltriethoxysilane and the 25% glutaraldehyde solutions were purchased from the Sinopharm Chemical Reagent Co., Ltd. The crude jatropha oil was purchased locally. The characteristic of the crude jatropha oil is given in Table 1. The acid value, peroxide value, saponification value were 9.30 mg KOH/g, 2.52 mmol/kg and 188.50 mg/g, respectively. Other chemicals and solvents used in this study were of analytical grade and procured from the Shanghai Chemical Reagent Co., Ltd.

2.2. Preparation of support (modified ATP) and immobilization of lipase

The support was prepared according the process that had been previously described by us (You et al., 2011). The ATP was first added to dilute hydrochloric acid at 80 °C and stirred 12 h. Excess acid was removed by repeated rinsing with deionized water. The acid-treated ATP was dried at temperature of 50 °C and was further modified by 3-aminopropyltriethoxysilane (3-APTES). The modification was performed by adding 20 g of acid-treated ATP to a 50 mL aqueous solution of 10% 3-APTES (w/v). The pH was adjusted to 3.5 using concentrated HCl. The mixture was stirred slowly for 12 h at room temperature and then filtered. The modified ATP was rinsed with deionized water and subsequently dried at 50 °C.

Table 1
Characteristic of the crude jatropha oil.

Fatty acid	Structure	% (w/w)
Palmitic acid	C16:0	14.3
Palmitoleic acid	C16:1	1.1
Stearic acid	C18:0	6.8
Oleic acid	C18:1	45.3
Linoleic acid	C18:2	31.6
Linolenic acid	C18:3	0.1
Arachidic acid	C20:0	0.2

The modified ATP (1 g) was suspended in 20 mL glutaraldehyde solution with the concentration of 0.4% (v/v). The suspension was mechanically agitated at room temperature for 1 h and suction filtered. The residual glutaraldehyde was removed by repeated rinsing with deionized water, and the ATP was finally dried. ATP is a class of clay mineral with a 2:1 layer composition usually with a lath or fibrous morphology (Huang et al., 2008a).

2 g of lipase was dissolved in 100 mL of the sodium phosphate buffer solution (pH, 6.0) with sufficient stirring. Subsequently, 10 g of the glutaraldehyde-activated ATP support was added to 100 mL of the lipase solution. The mixture was shaken at 150 rpm for 24 h at 25 °C and filtered. The immobilized lipase was dried in a vacuum oven at 30 °C for 24 h.

The activity of immobilized lipase was measured using 0.2 mL of glycerol trioleate dissolved in 3.8 mL of *n*-hexane as the substrate. 4 mL of glycerol trioleate and 30 μL of methanol were subject to react with 0.2 g immobilized lipase at 30 °C. After 1 h of reaction, 0.4 mL of the solution was taken and diluted with 0.6 mL of *n*-hexane. One enzyme unit (U) is the amount of biocatalyst necessary to liberate 1.0 μmol of fatty acid methyl esters (FAMEs) per minute under the assay conditions. The relative activity was represented as a percentage of the initial activity.

2.3. Gas chromatography analysis of biodiesel

Samples were collected from the reaction mixture at specified time points for fatty acid methyl ester (FAME) determination by gas chromatography (GC). Methanol was completely distilled off using a rotary evaporator at 50 °C under vacuum. The samples were then placed in a separatory funnel for phase separation. The FAME-containing upper phase was separated and dehydrated using anhydrous sodium sulfate (25 wt% of the weight of the ester product). Finally, the samples were dissolved in 10 mL of *n*-hexane. A standard FAME mixture (Nu-Chek Prep, Inc.) was used as an external standard to quantify the FAME concentration (Yin et al., 2012).

The biodiesel product was determined by a HP5890A gas chromatography equipped with a flame-ionization detector and a HP-5 capillary column (30 m × 0.25 mm, 0.25 μm film thickness). A sample volume of 1.0 μL was injected into the column using the split mode (split ratio 1:30). The carrier gas used was high purity nitrogen at a flow rate of 1.0 mL min^{-1}. The temperatures of the injector and detector were maintained at 250 °C. The column temperature was increased by 5 °C from 210 °C to 230 °C and maintained for 15 min.

The biodiesel yield (biodiesel conversion) was calculated as follows:

$$\text{Biodiesel yield } (\%) = \frac{W_{\text{Fame}} \times M_{\text{Oil}}}{3 \times W_{\text{Oil}} \times M_{\text{Fame}}} \times 100\% \quad (1)$$

In Eq. (1), W_{Fame} and W_{Oil} is the weight of fatty acid methyl esters (FAME) in the FAME phase and the weight of oil used, respectively, M_{Fame} and M_{Oil} are the average molecular weights of the FAME and the oil, respectively, and the factor 3 indicates that one mole of triglyceride yields three moles of FAME (Yin et al., 2012).

2.4. General procedure for biodiesel production

Immobilized lipase was used as catalyst for biodiesel production via the transesterification of jatropha oil. The reaction mixture containing 10 g of jatropha oil, water and immobilized lipase was put in 25 mL screw-capped glass vials and then were incubated in a shaking table with the rotation speed of 150 rpm at certain temperature. Methanol of 2.4 g (molar ratio of oil to methanol is 1:6.6) was added to the reaction mixture in a certain method.

Effects of various methanol treatments, water contents, reaction temperatures, and methods of methanol addition on the biodiesel production were studied by single-factor experiment design. In the experiments, when one variable range was studied the other variables were kept constant.

2.5. Determination of kinetic parameters

The kinetics parameters were analyzed according to the previously reported method with slight modifications (Al-Zuhair et al., 2006). The substrate concentrations of jatropha oil solution were varied from 0.1 mol L^{-1} to 0.8 mol L^{-1}, while the methanol concentrations were 2 mol L^{-1} and 3 mol L^{-1}. The chosen conditions for biodiesel preparation were: 10 wt% immobilized lipase (both based on the oil weight), at a reaction temperature of 35 °C. The initial rate of reaction was determined by analyzing the concentration of fatty acid methyl esters after 15 min.

All the experiments data were an average of three parallel replicates.

3. Results and discussion

3.1. Immobilization of lipase

ATP was functionalized with organosilane 3-APTES and glutaraldehyde to form a layer of aldehyde groups which could react with the amino groups of lipase. Due to the presence of the amino functional groups in the ATP, the lipase can be immobilized on modified ATP through the amine-aldehyde Schiff linkage with glutaraldehyde (Huang et al., 2008b). Glutaraldehyde is a bifunctional cross-linking agent and it was a cross-linking reaction for the lipase being immobilized on it.

3.2. Studies of reaction conditions

3.2.1. Effect of water content

The appropriate water content is a crucial parameter in biodiesel production that could affect the reaction rate and final yield greatly. Lipases function at the interface between the aqueous and organic phases. Its activity is generally affected by the available interfacial area. The addition of the appropriate water content is necessary to maintain lipase activity and to increase the available interfacial area between water and oil, thereby enhancing the reaction. However, excess water may dilute the amount of methanol in the reaction mixture and redirect the transesterification reaction into the hydrolysis reaction instead.

The effect of different water contents from 1% to 12% (v/w, based on jatropha oil) on biodiesel production was investigated while other variables were set as follows: 2.4 g methanol was added at 8 h intervals and the total reaction time was 30 h, temperature 30 °C and lipase amounts 8% (w/w) and the results are shown in Fig. 1. These results indicate that the biodiesel yield is markedly increased when 2% water was added to the reaction solutions. Therefore, relatively less water is necessary to activate the lipase. The results likewise show that the maximum biodiesel yield was 82% at 7% water content, after which the biodiesel yield slightly decreased. The excess water content probably increased the flexibility of lipase and caused side reactions such as hydrolysis. Excess water facilitates lipase aggregation, thereby decreasing lipase activity. Previous reports have suggested that the addition of the appropriate amount of water to the enzyme-catalyzed reaction mixture may increase the synthesis rate of the fatty acid ester; the best water content depends on the lipase type, feedstock oil, and immobilized support (Royon et al., 2007; Antczak et al., 2009). In our study, the support may influence the water content

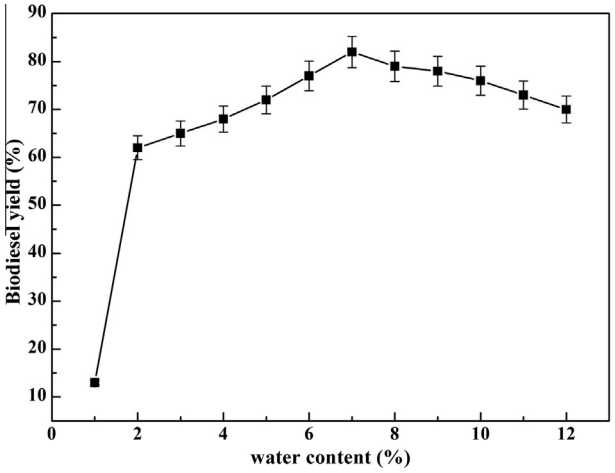

Fig. 1. Effect of water content on yield of biodiesel (%) (the reaction parameters: 10 g jatropha oil; 2.4 g methanol (methanol is added at an intervals of 8 h), 8% (w/w, based on oil weight) immobilized lipase, 30 °C, 150 rpm for 30 h).

in that the structure of ATP facilitates various forms of water bonding, namely, the water of crystallization, water of constitution, and adsorbed water. This makes it need lower water content compared with some reports that the optimum water content required to maintain the highest transesterification activity is 10–20% based on the oil weight (Tan et al., 2010). In our study, the best water content is 7%.

3.2.2. Effect of methanol addition

High methanol concentrations are generally toxic to lipase, thereby decreasing its enzyme activity (Salis et al., 2005). Thus, the method of methanol addition may affect the biodiesel yield. The influences of four different methanol addition methods on the biodiesel yield were studied while the other variables were set as follows: the total reaction time was 30 h, temperature 30 °C, lipase amounts 8% (w/w) and water content 7% (v/w). These methods were that methanol was added in one step (Method I), methanol was added at 8 h intervals (Method II), methanol was added at 5 h intervals (Method III), and methanol was added at 3 h intervals (Method IV). The effects of the methanol addition method on the methanolysis of jatropha oil by immobilized lipase for biodiesel production are presented in Fig. 2. The highest biodiesel yield was obtained when methanol was added at 3 h intervals. This phenomenon suggested that the addition of a small quantity of methanol into the reaction mixture during the early stages of transesterification enhances the biodiesel yield. Approximately 89% biodiesel yield was acquired after 30 h of the reaction. However, the results obtained by Methods III and IV were not significantly different. This result indicates that when methanol was added at 5 h intervals or at 3 h intervals, the methanol concentration in the reaction medium was not toxic to lipase and did not have negative effects. In our results, we prefer 3 h intervals as the best condition.

3.2.3. Effect of immobilized lipase

The amount of immobilized lipase is a key factor that influences biodiesel production. The effects on biodiesel yield caused by the amount of lipase were studied while the other variables were set as follows: 2.4 g methanol was added at 3 h intervals and the total reaction time was 30 h, temperature 30 °C and water content 7% (v/w). Results are shown in Fig. 3. The biodiesel yield increased with the increasing of immobilized lipase amount. The biodiesel yield peaked at approximately 92% with 10% immobilized lipase at reaction time of 30 h; this value remained constant as the

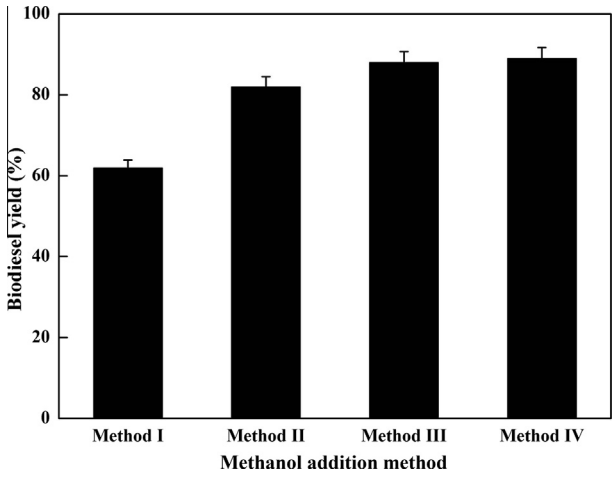

Fig. 2. Effect of the methanol addition method on yield of biodiesel (%) (the reaction parameters: 10 g jatropha oil; 2.4 g methanol, 7% water content (w/w, based on oil weight); 8% (w/w, based on oil weight) immobilized lipase; 30 °C, 150 rpm for 30 h, Method I: methanol was added in one step; Method II: methanol was added at 8 h intervals; Method III: methanol was added at 5 h intervals; Method IV: methanol was added at 3 h intervals).

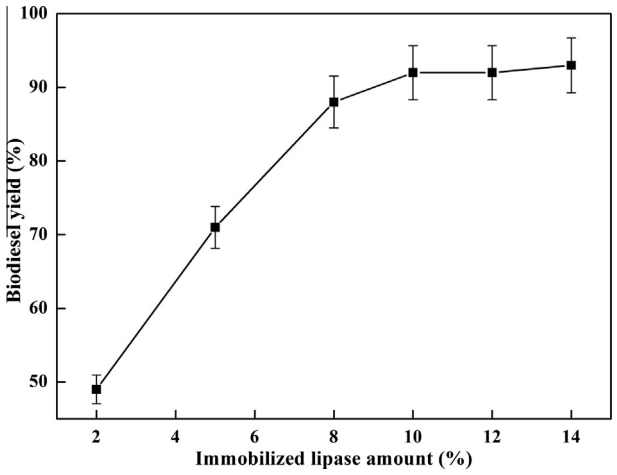

Fig. 3. Effect of the immobilized lipase amount on yield of biodiesel (%) (the reaction parameters: 10 g jatropha oil; 2.4 g methanol, methanol was added at intervals of 3 h; 7% water content (w/w, based on oil weight); 30 °C, 150 rpm for 30 h).

amount of immobilized lipase was increased. The higher amounts of available immobilized lipase allowed for more substrate molecules to be absorbed by the active center of the lipase. However, the rate of biodiesel content declined as the immobilized lipase content rising from 10% to 14%. This phenomenon indicated that excess jatropha oil was presented when the amount of immobilized lipase was below 10%. As the amount of lipase increased from 10% to 14%, the biodiesel yield was increased by only 1%. Thus, 10% is the best immobilized lipase amount for biodiesel synthesis from jatropha oil in terms of the production cost.

3.2.4. Effect of reaction temperature

Each enzyme has its own appropriate optimum temperature. The effects of temperatures from 25 °C to 50 °C on the methanolysis of jatropha oil for biodiesel production using immobilized lipase were studied with the other variables were set as follows: 2.4 g methanol was added at 3 h intervals and the total reaction time was 30 h, lipase amounts 10% (w/w) and water content 7% (v/w). Results are shown in Fig. 4a. The results showed that the maximum biodiesel yield (94%) was observed when the reaction temperature was 35 °C. The biodiesel yield increased when the reaction temperature gradually increased from 25 °C to 35 °C. When the reaction temperature increased from 35 °C to 50 °C, the biodiesel yield rapidly decreased. Therefore, 35 °C was hypothesized to be the best reaction temperature in this paper. Higher reaction temperatures may inactivate the immobilized lipase and favor hydrolysis reaction, which agrees with the trends in previous reports (Li and Yan, 2010). Other studies have revealed that the optimum temperature for enzymatic transesterification is the effect of the interplay between the operational stability of the biocatalyst and the transesterification rate (Antczak et al., 2009; Liu et al., 2010). The optimum temperature likewise depends on the molar ratio of alcohol to oil and the thermostability of the enzyme preparation. These results show that the optimum temperature of the transesterification reaction is 35 °C.

3.2.5. Effect of reaction time

Longer reaction time usually has a positive effect on the biodiesel yield. The effects of reaction times of 1–36 h on the biodiesel yield were studied while the other variables were set as follows: 2.4 g methanol was added at 3 h intervals and reaction temperature 35 °C, lipase amounts 10% (w/w) and water content 7% (v/w). Results are shown in Fig. 4b. The biodiesel yield increased as the reaction time increased from 0 h to 36 h. The maximum yield

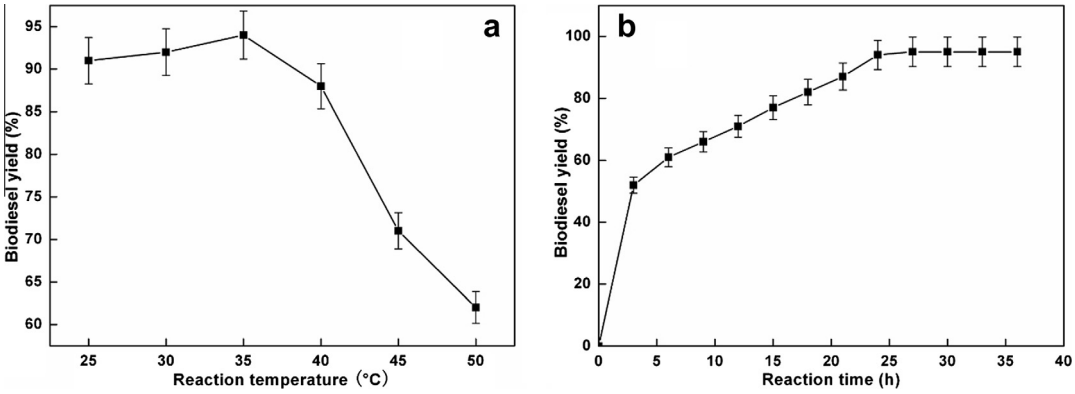

Fig. 4. (a) Effect of the reaction temperature on yield of biodiesel (%) (the reaction parameters: 10 g jatropha oil; 2.4 g methanol, methanol was added at intervals of 3 h; 7% water content (w/w, based on oil weight); 10% (w/w, based on oil weight) immobilized lipase; 150 rpm for 30 h). (b) Effect of the reaction time on yield of biodiesel (%) (the reaction parameters: 10 g jatropha oil; 2.4 g methanol, methanol was added at intervals of 3 h; 7% water content (w/w, based on oil weight); 10% (w/w, based on oil weight) immobilized lipase, 35 °C, 150 rpm).

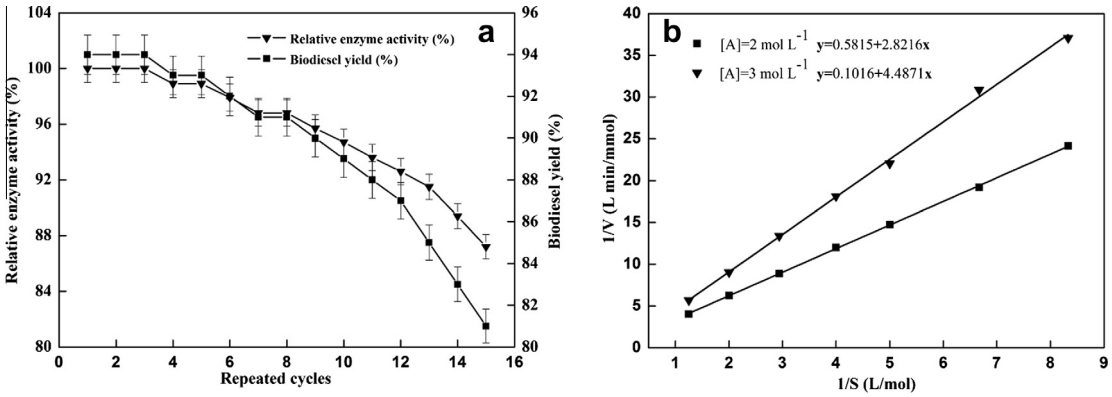

Fig. 5. (a) Operational stability of immobilized lipase (the reaction parameters: 10 g jatropha oil; 2.4 g methanol, methanol was added at intervals of 3 h; 7% water content (w/w, based on oil weight); 10% (w/w, based on oil weight) immobilized lipase, 35 °C, 150 rpm for 24 h). (b) Lineweaver–Burk plots of the immobilized lipase.

(94%) was observed with a reaction time of 24 h. Subsequently, the biodiesel yield maintained a dynamic equilibrium with the increasing reaction time and did not significantly increase when the reaction time exceeded 24 h. This phenomenon may be attributed to the increasing water content as the transesterification reaction progressed and the higher water content induced biodiesel hydrolysis (Li and Yan, 2010). The free fatty acid content of jatropha oil was high. The free fatty acid and methanol could react and produce fatty acid methyl esters and water. So the water content increases during transesterification. Thus, the optimum reaction time in this study is 24 h.

3.3. Reusability of immobilized lipase

The reuse of immobilized enzymes could reduce production costs. Thus, the immobilized enzyme preparation should have sufficiently high stability to permit its reuse. The reusability of the immobilized lipase is demonstrated in Fig. 5a. No significant loss of biodiesel yield was observed when the immobilized lipase was reused for 15 cycles. Jatropha oil was used as the feedstock, and the reaction time of each cycle was 24 h. The biodiesel yield gradually decreased with the increasing number of times reused. The original biodiesel yield with the immobilized lipase was 94%, and the biodiesel yield was retained at 89% after 10 times of reuse. This phenomenon indicated that the immobilized lipase could retain 94.7% of its relative activity after 10 cycles. The reusability of the immobilized lipase is due to the high mechanical strength and the specific surface area of ATP. The 5.3% loss of biodiesel yield suggests that ATP could be easily reactivated and reused even after losing some of its activity. The high reusability of the immobilized lipase may significantly reduce the operation costs of industrial biodiesel production. This result indicated that the immobilized lipase on the modified ATP enhances lipase stability. The improved reusability of immobilized lipase would make its future application more economical. Additionally, Fig. 5a also indicated that the activity of the immobilized lipase gradually decreased with the increase of the reuse times. The immobilized lipase retained 94.7% of its initial relative activity after 10 cycles of reuse. One cause of the loss of activity is the inactivation of enzyme and the leakage of protein from the supports upon reuse (Zhao et al., 2010). The high reusability of the immobilized lipase may markedly reduce the operation costs in industrial production. The inactivation constant (K_d) and half-life time ($t_{1/2}$) were analyzed according to the reported methods with modifications (Henley and Sadana, 1985; Costa e Silva et al., 2013). K_d and $t_{1/2}$ for the immobilized lipase were calculated using Eq. (2):

$$A_{\text{residual }(\%)} = 100 - 50\,K_d * n^2 \tag{2}$$

where $A_{\text{residual }(\%)}$ is the residual activity of the immobilized lipase. The results were that the inactivation constant (K_d) was $1.1 \times 10^{-3}\ h^{-1}$ and the half-life time of the immobilized lipase ($t_{1/2}$) was 731 h.

3.4. Kinetics analysis of immobilized lipase

The reaction kinetic studies of immobilized lipase-catalysed esterifications were described by a Ping-Pong Bi–Bi kinetic model (Al-Zuhair et al., 2006) with competitive inhibition by the methanol. The model can be used to describe our system within the range of operating conditions considered and is shown in the following Eq. (3):

$$\frac{1}{V} = \frac{1}{V_{\max}}\left(1 + \frac{K_A}{[A]}\right) + \frac{K_{JO}}{V_{\max}}\left(1 + \frac{[A]}{K_{IA}}\right)\frac{1}{[JO]} \tag{3}$$

Since the methanol concentration was kept constant in all experimental runs, Eq. (3) could be simplified to suggest a linear plot:

$$\frac{1}{V} = a + b\frac{1}{[JO]} \tag{4}$$

where $a = \frac{[A]+K_A}{[A]V_{\max}}$ and $b = \frac{K_{JO}}{K_{IA}V_{\max}}(K_{IA} + [A])$ where V is the initial rate of reaction, $V_{\max}$ is the maximum reaction rate, reflects the intrinsic characteristics of the immobilized lipase; K_A and K_{JO} are the binding constants for the methanol and the jatropha oil, [JO] is the concentration of jatropha oil, [A] is the concentration of methanol, and K_{IA} is the inhibition constant for the methanol.

The kinetic parameters of the immobilized lipase were investigated from the Lineweaver–Burk graph acquired by plotting the inverse values of the jatropha oil concentrations against the inverse of values of the initial reaction rates at methanol concentration of 2 mol L^{-1} and 3 mol L^{-1}, respectively. The kinetic parameters of the immobilized *B. cepacia* lipase were investigated using the Eq. (4). The nonlinear fitting of the experimental data were carried out by mathematics software of Origin 7.5. Two fitting equations were obtained from double-reciprocal plots (Fig. 5b). The values of the kinetics parameters of the immobilized *B. cepacia* lipases were calculated according to the coefficient of two fitting equation. The values of the kinetics parameters were: $V_{\max}$ is 6.823 mmol L^{-1} min^{-1}, K_A is 0.159 mol L^{-1}, K_{JO} is 0.0837 mol L^{-1}, K_{IA} is 0.00821 mol L^{-1}, respectively.

The study showed that jatropha oil can be efficiently converted to biodiesel using immobilized *B. cepacia* lipase as catalyst. Compared with the other studies presented in the literatures (Da Rós et al., 2010; Li et al., 2011; Li and Yan, 2010), the cost of enzymatic

process was lower in that the APT is cheaper than the other supports such as polystyrene macroporous resin, niobium oxide, polysiloxane–polyvinyl alcohol and the immobilized enzyme could catalyze the methanolysis of low quality feedstock such as jatropha oil that has high acid value for biodiesel production. In addition, the enzyme preparation was more stable and the immobilized lipase retained its high activity even after 15 cycles. This research suggests the applicability of immobilized *B. cepacia* lipase to biodiesel production.

4. Conclusions

Lipase from *B. cepacia* was immobilized on modified ATP by cross-linking reaction for biodiesel production with jatropha oil as feedstock. The best conditions for biodiesel production were: 10 g jatropha oil, 2.4 g methanol being added at 3 h intervals, 7 wt% water, 10 wt% immobilized lipase, temperature 35 °C, and time 24 h. Under these reaction conditions, the biodiesel yield reached 94% after 24 h. After 10 times of reuse, the biodiesel yield was around 89%, corresponding to 95% of that obtained in the first batch, which suggests its potential for industrial applications.

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