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Pseudomonas cepacia lipase immobilized onto the electrospun PAN nanofibrous membranes for biodiesel production from soybean oil

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ABSTRACT

Lipase from *Pseudomonas cepacia* has good transesterification activity and can be employed for biodiesel production. For recycled uses, polyacrylonitrile nanofibrous membrane was fabricated by electrospinning method and activated by amidination reaction for immobilizing *P. cepacia* lipase. Fourier transform infrared spectroscopy (FTIR) spectra confirmed that the lipases were covalently attached to the nanofibers. After enzyme immobilization, the immobilized *P. cepacia* lipase was used as the catalyst for transesterification reaction to convert the soybean oil to biodiesel by adding methanol as the reactant. The effects of methanol concentration, methanol-to-water ratio, reaction temperature, fatty acid content and the reusability were investigated by using the immobilized *P. cepacia* lipase. Under the optimal reaction conditions, the biodiesel conversion of soybean oil achieved 90% after reacting 24 h. In examining the reusability, the immobilized *P. cepacia* lipase still retained 91% of its initial conversion capability after 10 times reused.

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1. Introduction

Biodiesel, also known as fatty acid methyl esters (FAMEs), is made from renewable biological sources like vegetable oils and animal fats. It is an alternative diesel fuel because of its environmental benefits such as being biodegradable, nontoxic and low carbon dioxide emission profiles [1]. The cetane number, energy content, viscosity and other physical properties of biodiesel are also similar to those of petroleum diesel (petro-diesel). For these reasons, biodiesel is considered an excellent replacement for common petroleum diesel [2].

In general, there are two methods to produce biodiesel in industrial application: (1) chemical-catalyzed method, and (2) enzymatic-catalyzed method. In the chemical-catalyzed method, basic and acidic solutions are employed to be the catalysts for biodiesel production. Homogeneous basic catalysts, such as sodium or potassium methoxide and hydroxide, are used for transesterification reaction of oil and methanol to produce FAMEs and glycerol [1,3]. The unpurified and waste oil are usually used as the inexpensive feedstocks. However, these feedstocks often contain more than 0.5% free fatty acids, which leads to low catalytic performance because of neutralization and contributes to increase in process cost [4,5]. In order to improve the economic competitiveness of producing biodiesel using the materials with low cost, homogeneous acidic catalysts, such as hydrochloric acid or sulfuric acid, are used to pre-esterify the free fatty acids prior to transesterification of oil by homogeneous basic catalysts [6,7]. Whether it is homogeneous acidic or basic catalyst, high energy cost, complicated separation and purification process, and the waste disposal problems of chemical-catalyzed method must be addressed carefully [8].

Enzymatic-catalyzed method can circumvent the above disadvantages and offer attractive alternatives. For example, lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) can be used to transesterify oil with methanol to produce FAMEs [9]. However, the enzymatic-catalyzed method is not favored in industrial development because the high cost and low stability of lipase limit its potential application [10,11]. To lower the cost and increase the stability of lipase, several enzyme immobilization methods have been studied and reported [9,12–16]. In the enzyme immobilization process, the appropriate type of support is the key factor for immobilization. Nanofibrous membranes, which were made by electrospinning method, have gained popularity in recent years because of the larger surface area and porous structure that can lower the diffusion resistance of substrate and increase enzyme loading for efficient immobilization [17–19].

The amidination reaction was firstly used to activate the nitrile groups (CN functional groups) of the PAN for glucoamylase immobilization in 1982 [20,21]. To our knowledge, this activation method is only applied for *Candida rogusa* lipase immobilization in our

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pervious study [18] and still not use for the other kinds of lipase immobilization systems. In this present study, polyacrylonitrile (PAN), with the advantages of cheaper, better mechanical strength and high thermal resistance, was used to manufacture the nanofibrous membrane by electrospinning method. The PAN nanofibrous membrane was activated by using the amidination reaction and then employed to immobilize *Pseudomonas cepacia* (*P. cepacia*) lipase with covalent binding. After enzyme immobilization, the immobilized *P. cepacia* lipase was used in the transesterification reaction for converting soybean oil to biodiesel. The important factors which included methanol concentration, methanol-to-water ratio, reaction temperature, and fatty acid content were examined to determine the optimal reaction conditions for biodiesel conversion.

2. Materials and methods

2.1. The fabrication of electrospun PAN nanofibrous membrane

The apparatus for the electrospinning process includes a glass syringe, a stainless-steel needle, a syringe pump (KD Scientific Corp.), a high-voltage power supply (GLASSMAN, EL40P1, USA), and aluminum foil as the collector. First, polyacrylonitrile (PAN) with an average molecular weight of 1.5×10^5 g/mol and a density (ρ_p) of 1.18 g/cm^3, purchased from Scientific Polymer Products (Ontario, NY, USA), was dissolved in N,N-dimethylformamide (DMF) (99.8%, TEDIA, USA) at 60 °C with gentle stirring to manufacture the homogeneous polymer solution. The homogeneous PAN polymer solution was drawn vertically from the tip of stainless-steel needle, driven by the electrostatic force which produced from the high voltage between the tip and the grounded collector. The PAN polymer solution formed a pyramid which was called "Taylor cone" in the needle tip and jetted through the needle tip to the aluminum foil collector. The important operation parameters of electrospinning, including polymer concentration, flow rate of polymer solution, applied voltage, distance between needle tip and collector, and size of the needle tip were controlled at 8 wt% (w/w), 1.5 ml/h, 20.0 kV, 20.0 cm, and 18 gauge, respectively. Once the solvent of the polymer solution evaporated, the PAN nanofibers were solidified and deposited on the collector to form the nonwoven mats. The eletrospun nanofibrous membrane was dehydrated under vacuum before it was removed from the collector.

2.2. Lipase immobilization

The nitrile groups (CN functional groups) of the PAN nanofibrous membrane were activated by an amidination reaction followed by reaction with lipase solution (from *Pseudomonas cepacia*, Amano Enzyme, Nagoya, Japan) in 50 mM phosphate buffer solution (pH 6.0) (Fig. 1). The PAN nanofibrous membrane was first immersed in the absolute ethanol and hydrogen chloride gas was bubbled through the mixture to produce the corresponding imidoester derivatives. After 5 min of activation reaction, the PAN nanofibrous membrane was removed from the absolute ethanol solution and washed several times with distilled water. Then, the activated nanofibrous membranes were transferred into a 0.5% (w/w) *P. cepacia* lipase solution for immobilization process at 50 °C under gentle shaking (100 rpm). After 30 min of immobilization, the nanofibrous membrane was removed and washed with 50 mM phosphate buffer solution (pH 6.0) several times to remove the unbound lipase molecules. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) (Varian 2000 FT-IR Scimitar Series) was used to check whether or not the lipase had become immobilized onto the surface of the electrospun PAN nanofibrous membranes.

2.3. Assays of protein loading and lipase activity

The protein content of enzyme was estimated by using the Bradford method with the Bio-Rad protein dye reagent concentrate and bovine serum albumin was used as the standard reactant [22]. The activity of *P. cepacia* lipase was measured using 0.1 ml of triolein (C18:1) dissolved in 1.9 ml of n-hexane as the substrate. 2 ml of triolein solution and 15 μl of methanol were subject to react with either 0.1 ml of free lipase or immobilized *P. cepacia* lipase onto the PAN nanofibrous membranes (4 cm^2) at 30 °C. After 1 h of reaction, 0.2 ml of the solution was taken and diluted with 0.3 ml of n-hexane. 0.5 ml of methyl pentadecanoate (2 g/l) as the standard was added to the product samples and analyzed by gas chromatograph (GC). Thermo Finnigan Fcous GC, which is equipped with FID and a compatible standard capillary column (THERMO TR-FAME part No.260M142P), was used for FAMEs detection. Temperature was programmed from 150 °C to 180 °C at 10 °C/min and thereafter to 200 °C at 1.5 °C/min, finally to 230 °C at 30 °C/min and was kept constant at 230 °C for 5 min. Injector and detector were maintained at 250 °C and 280 °C, respectively. The conversion was determined with dividing their peak areas by peak areas of methyl pentadecanoate, which was used as an internal standard [23]. One enzyme unit (U) is the amount of biocatalyst necessary to liberate 1.0 μmol of FAMEs per minute under the assay conditions. Specific activity (SA) is defined as the amount of enzyme per milligram of protein.

2.4. Kinetic parameters determination

The initial substrate concentrations of triolein solution were varied from 12.5 mM to 200 mM. Firstly, 2 ml of triolein solution and 15 μl of methanol were mixed to react with either 0.1 ml of free lipase (or 4 cm^2 of immobilized *P. cepacia* lipase onto the PAN nanofibrous membranes) at 30 °C under gentle shaking (100 rpm). After 1 h of reaction, 0.2 ml of the solution was taken and diluted with 0.3 ml of n-hexane. 0.5 ml of methyl pentadecanoate (2 g/l) as the standard was added and analyzed the enzymatic activity by GC. The operation parameters of GC were described in Section 2.3.

2.5. Hydrolysis of soybean oil

In order to investigate the effect of different fatty acid content, oil of 100% fatty acids must to be prepared. Soybean oil was obtained from Uni-President Corporation, Taiwan and the molecular weight is assumed to be 850 g/mol. The soybean oil was thoroughly mixed with distilled water at room temperature under constant stirring to obtain an oil–water emulsion. The initial molar ratio of water to oil was 6:1. The solution was hydrolyzed by lipase solution (type VII from *Candida rugosa*, Sigma, St. Louis, MO, USA) dissolved in distilled water (50 mg free lipase per milliliter of distilled water). The hydrolysis reaction was carried out in 24 h and fatty acids were derived from enzymatic hydrolysis of soybean oil [24]. The supernatant containing high level of fatty acids (almost 100%) was collected by centrifugation at 10,000 rpm for 10 min at room temperature. The conversion of hydrolyzed soybean oil was determined by the amount of triacylglycerol reduction in the oil and analyzed by using high performance liquid chromatography (HPLC) equipped with a Lichro CART RP-18e column (Merck, Germany) at 35 °C [25].

First, the HPLC analyzed samples were diluted 50 times in n-hexane before injection. The mobile phase consisted of methanol and a mixture of hexane and isopropanol (4/5, v/v) and was flowed at 1 ml/min. Gradient elution of the mobile phase was performed by varying the ratio of n-hexane and isopropanol from 0% to 50% (v/v) for the first 30 min after sample injection. The ratio was maintained at 50% for 5 min and was then decreased from 50% to 0% in 0.1 min.

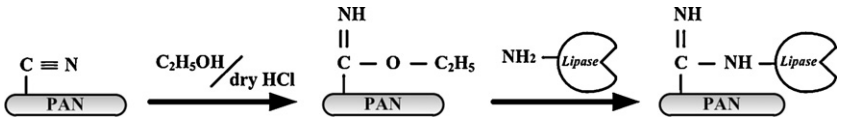

Fig. 1. Schematic illustration of *P. cepacia* lipase immobilized onto the PAN nanofibrous membrane by activating with the amidination reaction.

The products of oil hydrolysis were detected at 205 nm in a UV/VIS detector (SPD-10A; Shimadzu, Japan).

2.6. Biodiesel production

In the process of biodiesel production, 10 g of soybean oil was transesterified by *P. cepacia* lipase immobilized onto the PAN nanofibrous membrane. Various methanol concentrations, methanol-to-water ratios, reaction temperatures, and fatty acid contents were employed to determine the optimal reaction conditions. The reaction was carried out with 50 cm^2 of immobilized *P. cepacia* lipase (0.035 g) inside the thoroughly mixed reaction solution. Once the sampling time was reached, the methanol of reaction solution was removed by evaporation at 70 °C for 2 h and glycerol was separated from the reaction mixture with centrifugation at 10,000 rpm for 5 min. 10 μl of oil phase solution was taken and diluted with 490 μl of n-hexane. 500 μl of methyl pentadecanoate (2 g/l), used as the standard, was added into the product samples and analyzed by GC and operation parameters of GC were set the same as the activity measurement method.

3. Results and discussion

3.1. Characterization of PAN nanofibrous membrane

To investigate the binding between the lipase and the nanofibrous membrane, FTIR spectra for the original PAN nanofibrous membrane, the activated PAN nanofibrous membrane and the nanofibrous membrane with immobilized *P. cepacia* lipase were measured. The peaks from the original PAN nanofibrous membrane and the activated PAN nanofibrous membrane were 2241–2243 cm^{-1}, indicating the presence of CN groups (Fig. 2). Moreover, the absorption peaks at 2870–2931, 1450–1460, 1350–1380 and 1220–1270 cm^{-1} were signatures of aliphatic CH group vibrations, as well as the similar results from the PAN nanofibers [26]. It was evident that the nanofibrous membrane with the immobilized lipase demonstrated a new absorption peak at 3000–3500 cm^{-1}. This peak corresponded to the NH vibration in the protein backbone, suggesting that the amine groups of lipase was covalent bound with nitrile groups of the PAN and was successfully immobilized onto the nanofibrous membrane.

3.2. Kinetic parameters and enzymatic activities of free and immobilized P. cepacia lipases

The apparent K_m and V_{max} are important kinetic parameters of an enzyme which can be used for determining the enzymatic reaction and diffusion effects. K_m indicates the effective diffusion characteristics of the enzyme. V_{max}, the maximum reaction rate, reflects the intrinsic characteristics of the enzyme. The kinetic parameters, K_m and V_{max}, of the free and immobilized *P. cepacia* lipases were investigated using the following equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

The Lineweaver–Burk plots of the free and immobilized *P. cepacia* lipases were shown in Fig. 3 and the resulting parameters were listed in Table 1, respectively. From Table 1, the K_m value for the immobilized *P. cepacia* lipase was 88.4 mM, which was higher than that of free lipase (56.7 mM). The K_m value of enzyme usually increases after the immobilization process. The increase in K_m is caused by the conformational changes of the enzyme, resulting in a lower affinity for substrate contact. This may also be caused by the lower accessibility of the substrate to the active site of the immobilized enzyme by the increased diffusion limitations [27]. As shown in Table 1, the V_{max} value of the immobilized *P. cepacia* lipase was found to be 18.3 U/mg, which was lower than that of free lipase (22.5 U/mg). In general, the V_{max} value of enzyme usually becomes lower after enzyme immobilization [28,29]. However, the V_{max} value of the immobilized *P. cepacia* lipase still maintained 81% of that of free lipase, suggesting that the present immobiliza-

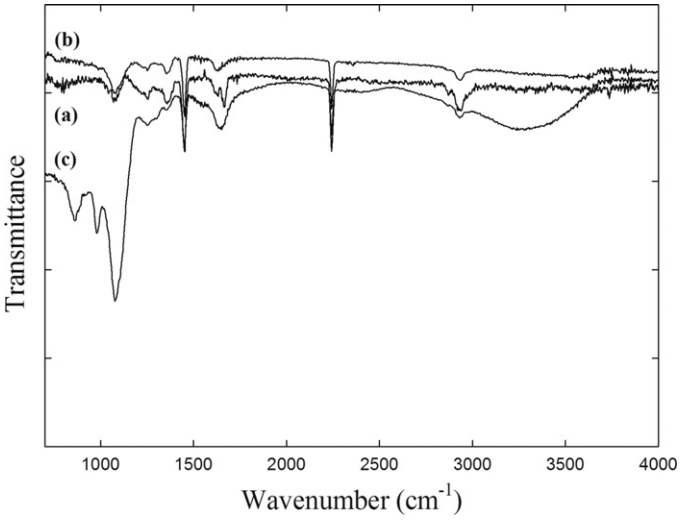

Fig. 2. FTIR spectra of PAN nanofibrous membrane, (a) original; (b) activated with amidination reaction; (c) with immobilized *P. cepacia* lipase.

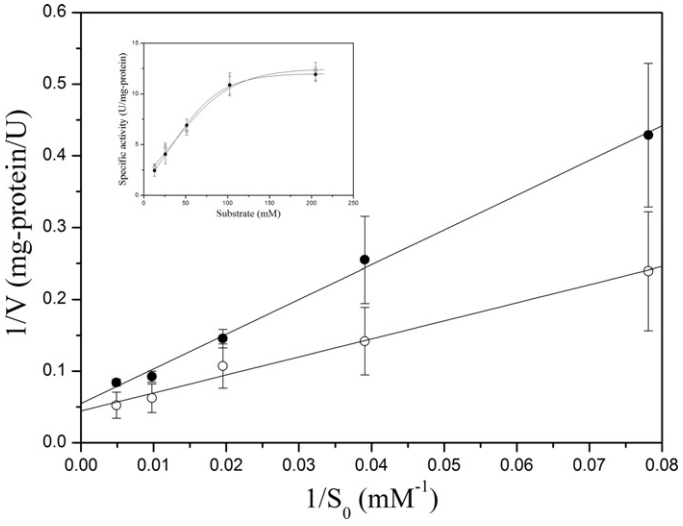

Fig. 3. Lineweaver–Burk plot (double reciprocal plot) with (○) free *P. cepacia* lipase and (●) immobilized *P. cepacia* lipase. The substrate saturation curves for free and immobilized *P. cepacia* lipases are shown as inert.

Table 1
Kinetic parameters, protein loading and specific activity of the free and immobilized lipases (lipase from *Pseudomonas cepacia*).

Type	K_m (mM)	V_{max} (U/mg-protein)	Protein loading (mg/g-material)	Specific activity (U/mg-protein)
Free	56.7	22.5	NA[a]	11.2 ± 2.7
PAN nanofibrous membrane	88.4	18.3	43 ± 4.0	8.9 ± 1.8

[a] NA: not available.

Table 2
The conversion of biodiesel using the immobilized *P. cepacia* lipase under different methanol concentrations in an orthogonal experiment (soybean oil: 10 g; reaction time: 1 h; reaction temperature: 30 °C).

Run	Molar ratio of methanol to oil	Methanol (g)	Water (g)	Methanol concentration (%)	Conversion of biodiesel (%)
1	6.6:1	2.5	1.6	61.0	52.3
2	8.0:1	3	1.6	65.2	47.5
3	9.3:1	3.5	1.6	68.6	33.3
4	6.6:1	2.5	0.8	75.8	13.2
5	6.6:1	2.5	2.4	51.0	52.9
6	6.6:1	2.5	3.2	43.9	52.5
7	6.6:1	2.5	4	38.5	47.7

tion system can provide an efficient catalytic ability similar to that of free enzyme.

The enzymatic activity of immobilized *P. cepacia* lipase onto the PAN nanofibrous membrane was investigated by using triolein solution as the substrate and listed in Table 1. The protein loading and specific activity were 43 ± 4.0 mg/g-material and 8.9 ± 1.8 U/mg, respectively. The specific activity of immobilized lipase was equivalent to 79.5% of that of free lipase (11.2 ± 2.7 U/mg). From Table 1, the enzymatic activity of immobilized enzyme decreased after the immobilization process. It can be explained by the variation of active center caused by the three dimensional structure of the enzyme and the similar result has been reported for lipase from *Candida rugosa* immobilized onto the PAN nanofibrous membrane [18].

3.3. Immobilized lipase for biodiesel production

Since the *P. cepacia* lipase was successfully immobilized onto the PAN nanofibrous membrane, the immobilized biocatalyst was tested on the biodiesel production system. The following is a discussion of operation parameters for analyzing the effects in the biodiesel production.

3.3.1. Effect of methanol concentration

For the transesterification reaction of biodiesel production, the triglyceride and methanol usually were employed to be the reactants. According to Le Chatelier's Principle, when the concentration of the reactant is altered, the equilibrium of chemical reaction will drive to the opposite side to reduce the change in concentration. Subsequently, the rate of reaction will change and the yield of products will be altered in response to the impact on the system. Accordingly, the amount of biodiesel produced can usually be improved by increasing the amount of the methanol added. However, high content of methanol could be toxic to lipase and result in low enzyme activity [30,31]. In addition, the amount of water added is also very important in the enzyme reaction. The proper amount of water is necessary to maintain the enzyme activity as well as produce many emulsion interfaces between water and oil phases to promote the reaction [32].

As mentioned above, methanol and water are important factors in transesterification reaction. Methanol has high solubility in water so they are easily mixed. However, excess water would dilute the amount of methanol in the reaction solution and drive the transesterification reaction to the hydrolysis reaction instead [23,33]. In the present study, the factors of methanol and water are combined to create the new factor "methanol concentration", which will be the subject of our investigation. The effect of different methanol concentration which was used for converting soybean oil to biodiesel was investigated and the results were listed in Table 2.

As shown in Table 2, the conversion of biodiesel can be maintained and reached the highest with the methanol concentration ranging from 43.9% to 61.0%. The result indicates that an appropriate amount of methanol can enhance the conversion of biodiesel significantly and avoid the toxicity situation of the lipase below the methanol concentration of 61.0%. In the present study, the methanol concentration of 51% is used to investigate the following reaction conditions.

3.3.2. Effect of methanol-to-water ratio

In transesterification reaction, the appropriate methanol-to-water ratio is a very important parameter that influences the reaction rate and final conversion of reaction. Based on the appropriate methanol concentration of 51%, the methanol-to-water ratio of 2.5/2.4, 3/2.88, 4/3.84 and 5/4.8 (g/g) were applied to investigate the optimal methanol-to-water ratio and the results were shown in Fig. 4.

The methanol-to-water ratio of 4/3.84 and 5/4.8 had higher conversion rates than that of low methanol-to-water ratio (2.5/2.4 and 3/2.88). It also shows that the conversion rates between the methanol-to-water ratio of 4/3.84 and 5/4.8 had no significant

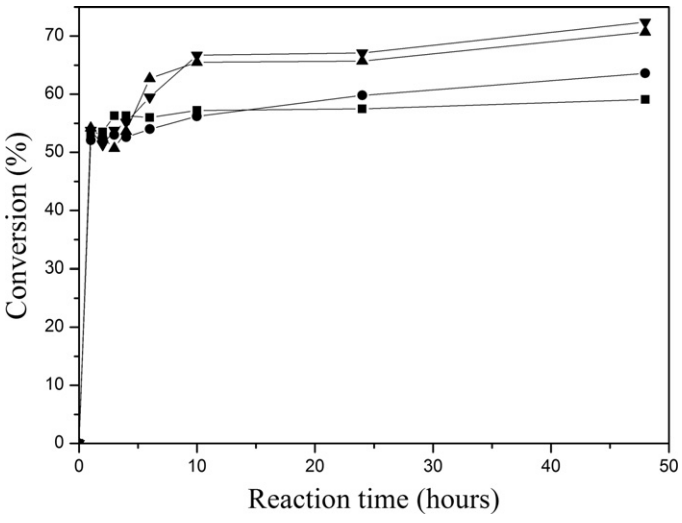

Fig. 4. Time course of biodiesel conversion by using immobilized *P. cepacia* lipase for various methanol-to-water ratios: (■)2.5/2.4, (●) 3/2.88, (▲) 4/3.84, and (▼) 5/4.8.

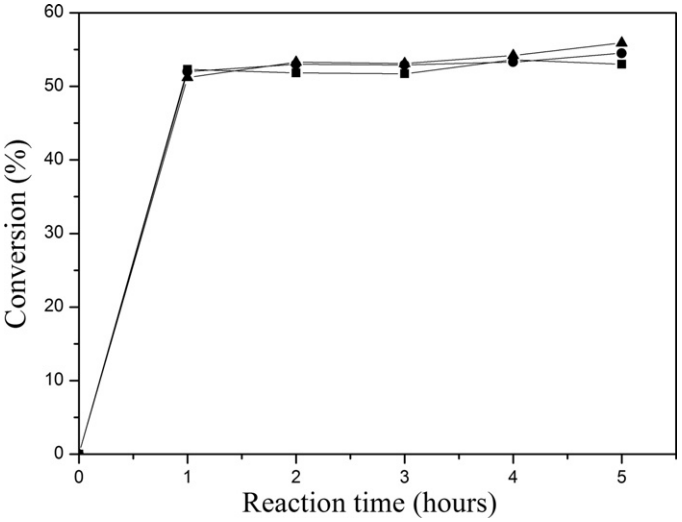

Fig. 5. Time course of biodiesel conversion by using immobilized *P. cepacia* lipase at various reaction temperatures: (■) 30 °C, (●) 40 °C, and (▲) 50 °C.

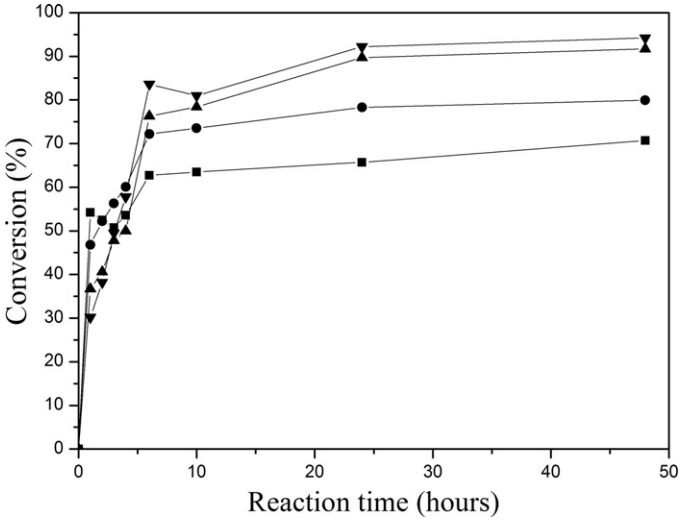

Fig. 6. Time course of biodiesel conversion by using immobilized *P. cepacia* lipase for various fatty acid contents: (■) 0%, (●) 25%, (▲) 50%, and (▼) 75%.

difference. For economic consideration, the optimal fraction of methanol to water is determined to be 4:3.84.

3.3.3. Effect of reaction temperature

The reaction temperature is an important factor in the transesterification reaction. The reaction rate usually can be improved by increasing the reaction temperature [34–36]. However, the structure of enzyme is easily denatured at higher temperature, resulting in lower activity. In order to identify the optimal reaction temperature for converting soybean oil to biodiesel, the effect of reaction temperature on the biodiesel conversion of soybean oil by using the immobilized *P. cepacia* lipase was investigated under temperature conditions of 30, 40 and 50 °C. The time courses of biodiesel conversion at various temperatures were shown in Fig. 5. The result suggests that there is no obvious difference in conversion rate with the temperature range from 30 °C to 50 °C. For the energy consumption consideration, 30 °C is chosen to be the optimal reaction temperature of transesterification reaction by using the immobilized *P. cepacia* lipase.

3.3.4. Effect of fatty acids content

Pure soybean oil is used to be the feedstock and the final conversion of biodiesel is about 70%. The reaction sample is also analyzed to determine the contents of monoglyceride, diglyceride and triglyceride by HPLC. The contents of monoglyceride and diglyceride are about 22% and triglyceride is still about 5% after 24 h reaction. The reason may be that the catalysis ability of *P. cepacia* lipase was changed after immobilization. Therefore, there are still some monoglyceride, diglyceride and triglyceride that cannot be catalyzed to FAMEs. For raising the conversion of biodiesel, 100% fatty acids were mixed with original soybean oil to prepare the feedstocks with different fatty acid contents and were used to investigate the effect of fatty acids content. 10 g of oil feedstock with fatty acid content from 0% to 75% was converted to biodiesel by using the immobilized *P. cepacia* lipase. The time courses of biodiesel conversion at different fatty acid contents were shown in Fig. 6. The equilibrium conversion of biodiesel increased with increasing fatty acid contents of the feedstock. Both the fatty acid content of 50% and 75% can induce the highest conversion of 90% after 24 h reaction. The result demonstrates that appropriate fatty acid content can enhance the biodiesel conversion.

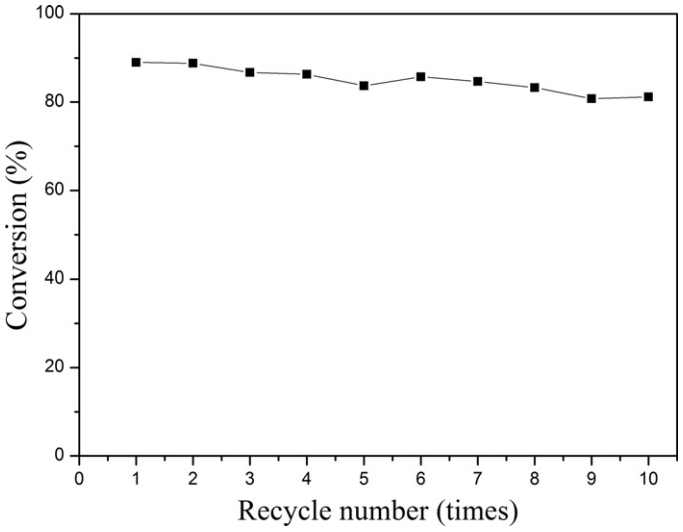

Fig. 7. The reusability of immobilized *P. cepacia* lipase in the biodiesel conversion.

3.3.5. Reusability of the immobilized lipase

In general, the reusability is the major advantage of immobilized enzyme. The cost of production process can be reduced by enhancing the reusability of immobilized enzyme. The effect of recycle usage on the biodiesel conversion of soybean oil by using the immobilized *P. cepacia* lipase was shown in Fig. 7. The oil with 50% of fatty acid was used as the feedstock and the reaction time of each recycle use was 24 h. The original biodiesel conversion of soybean oil using the immobilized *P. cepacia* lipase was 90% and the conversion still retained 91% of the original conversion after being reused for 10 times. This result suggests that the immobilized *P. cepacia* lipase onto the PAN nanofibrous membrane enhances the stability of enzyme. The improved reusability of immobilized enzyme would make its application more economical.

4. Conclusions

In the present study, polyacrylonitrile nanofibrous membrane was manufactured by electrospinning method and used as the support for *P. cepacia* lipase immobilization. After enzyme immobilization, protein loading of immobilized *P. cepacia* lipase reached 43 ± 4.0 mg/g-material and retained 79.5% activity retention of

that of free lipase. The kinetic parameters, K_m and V_{max}, of the immobilized *P. cepacia* lipase were 88.4 mM and 18.3 U/mg-protein, respectively. For biodiesel production, the immobilized *P. cepacia* lipase showed good biocatalytic activity. Under optimal reaction conditions, the conversion of biodiesel reached 90% after 24 h and the residual conversion after 10 times reused still kept 91% of its original conversion.

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