Journal of Molecular Catalysis B: Enzymatic 73 (2011) 98–103

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of *Pseudomonas cepacia* lipase onto the electrospun PAN nanofibrous membranes for transesterification reaction

Sheng-Feng Li[a,1], Yi-Hsuan Fan[a], Jung-Feng Hu[a], Yu-Shan Huang[a], Wen-Teng Wu[a,b,*]

[a] Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan
[b] Research Center for Energy Technology and Strategy, National Cheng Kung University, Tainan 70101, Taiwan

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form 6 July 2011
Accepted 12 August 2011
Available online 22 August 2011

Keywords:
Pseudomonas cepacia lipase
Immobilized
Polyacrylonitrile
Nanofibrous membrane
Amidination reaction
Transesterification

ABSTRACT

Pseudomonas cepacia lipase was immobilized onto the electrospun polyacrylonitrile (PAN) nanofibrous membrane with covalent binding by activating the nitrile groups of PAN using the amidination reaction successfully. The fiber diameters of initial PAN nanofibers were between 150 and 300 nm. The lipase molecules (1–2 μm) were observed to be attached onto the nanofiber surfaces. After 5 min activation and 30 min reaction with lipase solution, the protein loading has reached saturation point and the immobilized lipase retained 79% activity compared to that of free lipase. The kinetic parameters, K_m and V_{max}, were determined to be 56.7 mM and 22.5 U/mg for free lipase and 88.4 mM and 18.3 U/mg for immobilized lipase. To compare with free lipase, the pH stability of immobilized lipase was improved significantly in the acidic conditions and the thermal stability of immobilized lipase still maintained 90% of initial specific activity when the temperature achieved 70 °C. After being stored at 30 °C for 20 days, the immobilized lipase kept almost 100% of its initial specific activity. For reusability test, the immobilized lipase still retained 98% of its original specific activity after 10 batch cycles. These results show that the proposed scheme for lipase immobilization has potential in industrial transesterification reaction such as biodiesel production.

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are common enzyme and have many industrial applications such as transesterification, esterification, aminolysis, acyl exchange and thiotransesterification [1]. Lipases can be used in a variety of commercial fields including food technology, detergents, beverages, cosmetics, biomedical uses, and chemical industry [2–7]. Otherwise, lipases can also be operated in organic and aqueous phases and retains its activity in organic solvents [8]; however, the poor stability and high cost of lipase restrict the potential applications in industrial development [9].

To increase the stability and lower the cost of lipase, various chemical and physical methods had been employed for enzyme immobilization [10–20]. In the immobilization process, the structure of support is a very important factor. The uses of supports constructed from non-porous materials, to which enzymes can be attached, minimize the diffusion resistance; however, such material has a lower enzyme loading capability [21]. In contrast, porous materials have high enzyme loading capabilities, but suffer from inadequate interaction between immobilized enzymes and substrates [22]. Recently, nonwoven nanofibrous membranes generated by electrospinning (ES) have attracted much attention as supports for enzyme immobilization. Electrospun nanofibrous membrane not only offers large specific areas and porous structures that has the lower diffusion resistance but also increases enzyme loading capabilities. These features, desirable in supports used for enzyme immobilization, represent an improvement over traditional immobilization materials [23–25].

Lipases are usually immobilized onto electrospun nanofibrous membranes by physical adsorption and rarely by covalent binding [26–30]. In our previous study, polyacrylonitrile (PAN), which has good mechanical strength and high thermal resistance, was used to manufacture the nanofibrous membranes by electrospinning method. The PAN nanofibrous membrane was used as the support to immobilize with *Candida rugosa* lipase for soybean oil hydrolysis [31]. In the present study, *Pseudomonas cepacia* (*P. cepacia*) lipase was used to immobilize onto the PAN nanofibrous membrane with covalent binding. *P. cepacia* lipase is a common enzyme because of its high transesterification activity and can be used in the biodiesel production [32–34]. The immobilization conditions, including the activation time and the immobilization time, were adjusted to

* Corresponding author at: Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan. Tel.: +886 6 275 7575x31451; fax: +886 6 209 5913.
E-mail address: wtwu@mail.ncku.edu.tw (W.-T. Wu).
[1] Biotechnology Experimental Center in Southern Taiwan, Academia Sinica, Tainan 74145, Taiwan.

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.08.005

determine the optimal conditions in order to make the immobilized *P. cepacia* lipase with highest catalysis activity. Subsequently, the operation stabilities of the free lipase and the immobilized *P. cepacia* lipases were investigated in detail. This immobilized *P. cepacia* lipase was expected to employ in the industrial application such as the transesterification reaction for biodiesel production.

2. Materials and methods

2.1. Preparation of PAN nanofibrous membranes by electrospinning

The average molecular weight and density (ρ_p) of polyacrylonitrile (PAN), purchased from Scientific Polymer Products (Ontario, NY, USA), are 1.5×10^5 g/mol and 1.18 g/cm^3, respectively. N, N-dimethylformamide (DMF) (99.8%, TEDIA, USA) is used as the solvent for dissolving the PAN powder to prepare polymer solution. The electrospinning apparatus 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. To manufacture PAN nanofibrous membrane, the PAN solution was first drawn vertically from the needle tip, driven by the electrostatic force generated from the high voltage between the needle tip and the grounded collector. The PAN polymer solution formed a pyramid (called "Taylor cone") in the needle tip and jetted through the needle tip to the collector. The spinning time, polymer concentration, applied voltage, flow rate of polymer solution, distance between needle tip and collector, and size of the needle tip were set at 80 min, 8 wt% (w/w), 20.0 kV, 1.5 ml/h, 20.0 cm, and 18 gauge, respectively. When the solvent evaporated, the PAN nanofibers deposited on the collector and formed the nonwoven mats. Before the eletrospun nanofibrous membrane was removed from the collector, it was dehydrated under vacuum overnight to ensure the complete evaporation of solvent. The morphology of the nanofibrous membranes was observed by using a field emission scanning electron microscope (FESEM) (JOEL, JSM-6700F). The thickness of electrospun membrane was measured using a Micrometer (TECLOCK, SM1201, Japan) and the apparent density (ρ_a) of the membrane was determined by measuring the weight of a 2 cm × 2 cm nanofibrous membrane sample. The porosity was calculated as $(1 - \rho_a/\rho_p) \times 100$.

2.2. Lipase immobilization

The nitrile groups (CN functional groups) of the PAN nanofibers were activated by an amidination reaction [35–37] followed by reacting with lipase solution (from *Pseudomonas cepacia*, Amano Enzyme, Nagoya, Japan) in 50 mM phosphate buffer solution (pH 7.0) (Fig. 1). The nanofibrous membranes were first immersed in the absolute ethanol and bubbled hydrogen chloride gas through the mixture to produce the corresponding imidoester derivatives. Upon the completion of activation, the membranes were removed from the solution and washed with distilled water. The membranes were then transferred into a 0.5 wt% (w/w) *P. cepacia* lipase solution for immobilization at 50 °C under gentle shaking (100 rpm). When the immobilization was completed, the membranes were removed from the lipase solution and washed with 50 mM phosphate buffer solution several times to remove the unbound lipases.

2.3. Assays of protein loading and lipase activity

The immobilized protein content 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 [38]. Lipase activity was measured using 0.1 ml of triolein (C18:1) dissolved in 1.9 ml of n-hexane as the reaction substrate. Briefly,

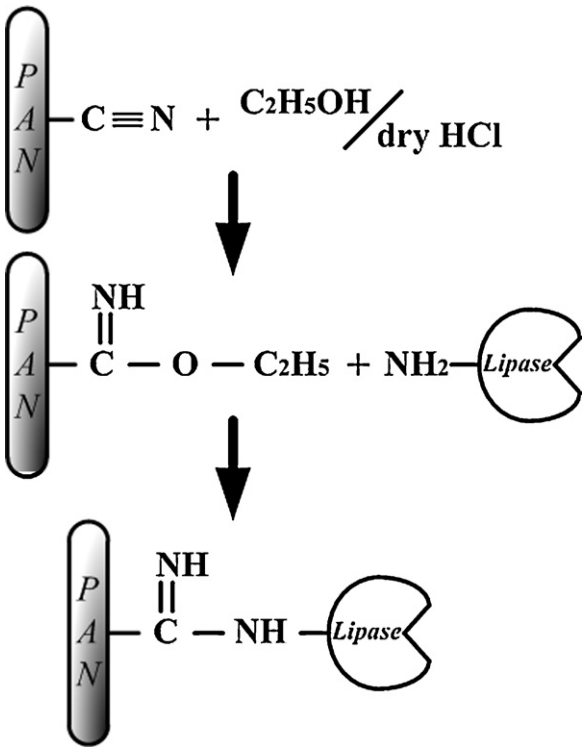

Fig. 1. Schematic illustration of lipase immobilized onto the PAN nanofibrous membrane by amidination reaction.

2 ml of triolein solution and 15 μl of methanol were reacted with 0.1 ml of free lipase or immobilized *P. cepacia* lipase onto the PAN nanofibrous membranes (2 cm × 2 cm) at 30 °C. After reacting for 1 h, 0.2 ml of reaction 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 into 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 methyl oleate 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 kept the temperature 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 peaks areas by peaks area of methyl pentadecanoate as an internal standard [39]. One enzyme unit (U) is the amount of biocatalyst necessary to liberate 1.0 μmol of methyl oleate per minute under the assay conditions. Specific activity is defined as the amount of enzyme units per milligram of enzyme protein.

2.4. Kinetic parameters determination

The apparent V_{max} and K_m are important kinetic parameters of an enzyme which can be used for determining the enzymatic reaction and diffusion effects. V_{max}, the maximum reaction rate, reflects the intrinsic characteristics of the enzyme. K_m indicates the effective diffusion characteristics of the enzyme. The kinetic parameters, K_m and V_{max}, of the free and immobilized *P. cepacia* lipases were determined using Eq. (1):

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

2.5. Storage stability and reusability of immobilized lipase

The storage stabilities of free and immobilized *P. cepacia* lipases were studied by incubating the free and immobilized *P. cepacia* lipases at 30 °C in 50 mM phosphate buffer solution (pH 7.0) and then determining the activity performance at the different incubation days. The initial activities of free and immobilized *P. cepacia* lipases were taken as 100%, respectively. For the reusability test, the immobilized *P. cepacia* lipase was subjected to a transestrification reaction with triolein solution for 1 h. After the specified reaction time, the immobilized *P. cepacia* lipase was removed from the solution and washed with n-hexane and phosphate buffer solution (50 mM, pH 7.0). Then, the immobilized *P. cepacia* lipase was introduced into the solution containing fresh substrate to repeat the experiment again. The initial activity of immobilized *P. cepacia* lipases was taken as 100%.

3. Results and discussion

3.1. The morphology of nanofibrous membranes

In the electrospinning process, several operation factors affect the morphology and structure of the electrospun nanofibrous membranes. These factors include: the concentration of the polymer solution, the power voltage, the flow rate from the syringe pump and the distance between the metal needle and collector. The key factor which affects the morphological change in the nanofiber is the concentration of the polymer solution. The appropriate concentration of polymer solution is characterized by uniform nanofibers. If the polymer concentration is too low or too high, it will form beads or cannot produce uniform fiber structure. In the present study, 8 wt% (w/w) of PAN solution in DMF was determined as the appropriate concentration to generate nanofibrous membranes [31]. The thickness, apparent density, and porosity of PAN nanofibrous membrane were 100 μm, 0.23 g/cm^3 and 81.3%, respectively. The fiber structure of PAN nanofibrous membrane was observed by FESEM image as shown in Fig. 2(a). The morphology of the nanofiber was very uniform, with the diameter of the nanofibers ranging from 150 to 300 nm. After amidination reaction, the nitrile groups of PAN were activated in ethanol by hydrogen chloride gas to produce an imidoester derivative. However, the diameter and the morphology of the nanofibers did not change substantially (Fig. 2(b)). After lipase immobilization, the small particles appeared and attached to the nanofiber surfaces as shown in Fig. 2(c). It could be explained that the nitrile groups of PAN nanofibrous membranes were activated by amidimation reaction and bound with amine groups of lipase protein. The bound protein acted as a nucleus and the other unbound lipase molecules were adsorbed onto the bound protein to form a protein aggregation by molecular interactions. The size of the attached proteins with FESEM observation were about 1–2 μm (as Fig. 2(c) shown). The similar result was also observed and reported for lipase from *C. rugosa* immobilized onto the PAN nanofibrous membrane [31].

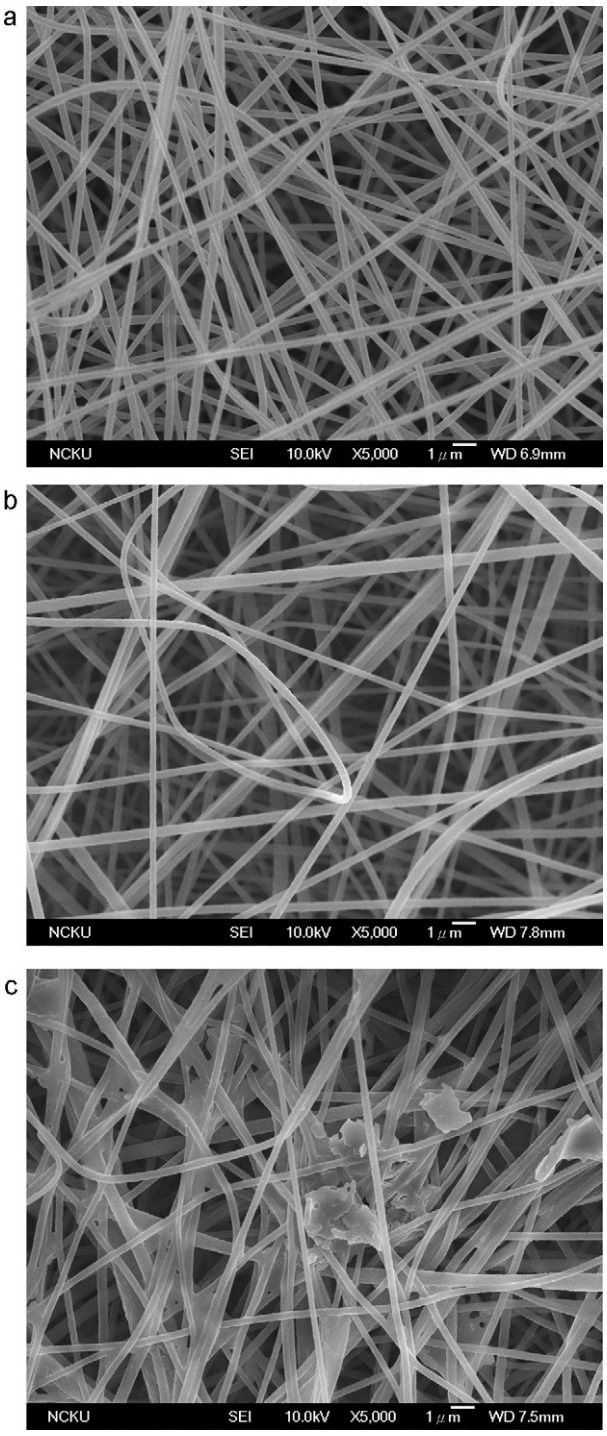

Fig. 2. Field emission scanning electron micrographs of PAN nanofibrous membrane: (a) original PAN nanofibrous membrane; (b) PAN nanofibrous membrane after activated with amidination reaction; and (c) PAN nanofibrous membrane with immobilized *P. cepacia* lipase.

3.2. Protein loading, activity, and kinetic parameters of the immobilized lipase on the PAN nanofibrous membranes

3.2.1. Protein loading and activity performance of immobilized lipase

For enzyme immobilization by amidination reaction, the nitrile groups of PAN were first activated in ethanol by hydrogen chloride gas to produce an imidoester derivative, followed by reacting with enzyme-containing solution for conjugation with the amino groups of enzyme [35,36]. However, imidoester is very active and easily hydrolyze to methylester or amidine by a trace amount of water [36]. For connecting lipases with imidoester groups on the PAN nanofibrous membrane, the optimum conditions for immobilization reaction must be determined. The protein loading and specific activity of immobilized *P. cepacia* lipase were studied by varying the activation time and the result is listed in Table 1. The amount of protein immobilized and the activity performance reached the highest level when the activation time was 5 min. Presumably, the activation process is a rather efficient method which can convert the nitrile group of PAN to the reactive imindoester

Table 1
Effect of varying activation time on the protein loading and specific activity of the immobilized *P. cepacia* lipase. The error bar at each data point represents the standard error ($n = 3$).

Activation time (min)	Protein loading (mg/g-matrix)	Specific activity (U/mg protein)
2.5	31.8 ± 2.4	8.7 ± 1.1
5	43.4 ± 4.0	8.9 ± 1.8
7.5	43.4 ± 4.8	8.6 ± 1.6
10	40.9 ± 1.9	7.9 ± 2.1

derivative completely in a short time. For the following enzyme immobilization step, the efficiency of protein loading and the specific activity were also investigated with different immobilization time (as Table 2 shown). The immobilized *P. cepacia* lipase reached a constant amount of immobilized protein and the highest specific activity within 30 min. It may be explained that the support surface for lipase binding achieved saturation after just 30 min of immobilization reaction. In previous studies with a PAN support the amidination reaction was used as the activation method, with 1 h for activation and 6 h for enzyme reaction [40]. In this study, the PAN nanofibrous membrane was activated for 5 min by amidination reaction, and reacted for 30 min with the enzyme. The results implied that the nanofibers with larger specific surface area can be easily activated, so the immobilization steps could be completed in a shorter time.

Under the appropriate activation time and immobilization time, the protein loading and specific activity of immobilized *P. cepacia* lipase were determined and listed in Table 3. The protein loading and specific activity of immobilized *P. cepacia* lipase were 43.4 ± 4.0 mg/g-matrix and 8.9 ± 1.8 U/mg, respectively, which was equivalent to 79% activity retention of free lipase. In general, the enzymatic activity of immobilized enzyme decreased after immobilization process due to variation of the active center caused by changing the three dimensional structure of the enzyme [41].

3.2.2. Kinetic parameters of immobilized lipase

The important kinetic parameters of the free and immobilized *P. cepacia* lipases, V_{max} and K_m, were investigated by using various initial substrate concentrations from 12.5 mM to 200 mM. The results are shown in Fig. 3(a). The Lineweaver–Burk plot from Eq. (1) and the resulting parameters are shown in Fig. 3(b) and listed in Table 3, respectively. As Table 3 shows, 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). In general, the K_m value usually increases via immobilization process. This phenomenon 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.

Generally, the V_{max} value usually becomes lower after enzyme immobilization. However, the V_{max} value of 18.3 U/mg exhibited by immobilized *P. cepacia* lipase on the PAN nanofibrous membrane was found to be lower than that of free lipase (22.5 U/mg). It still maintained 81% of V_{max} value of free lipase, suggesting that the present immobilization system can provide an efficient catalytic ability similar to that of free enzyme.

Table 2
Effect of varying immobilization time on the protein loading and specific activity of the immobilized *P. cepacia* lipase. The error bar at each data point represents the standard error ($n = 3$).

Immobilization time (min)	Protein loading (mg/g-matrix)	Specific activity (U/mg protein)
15	34.1 ± 5.4	8.0 ± 2.1
30	43.0 ± 4.0	8.9 ± 1.8
60	43.6 ± 6.8	8.7 ± 2.5
120	43.8 ± 4.3	8.4 ± 1.4

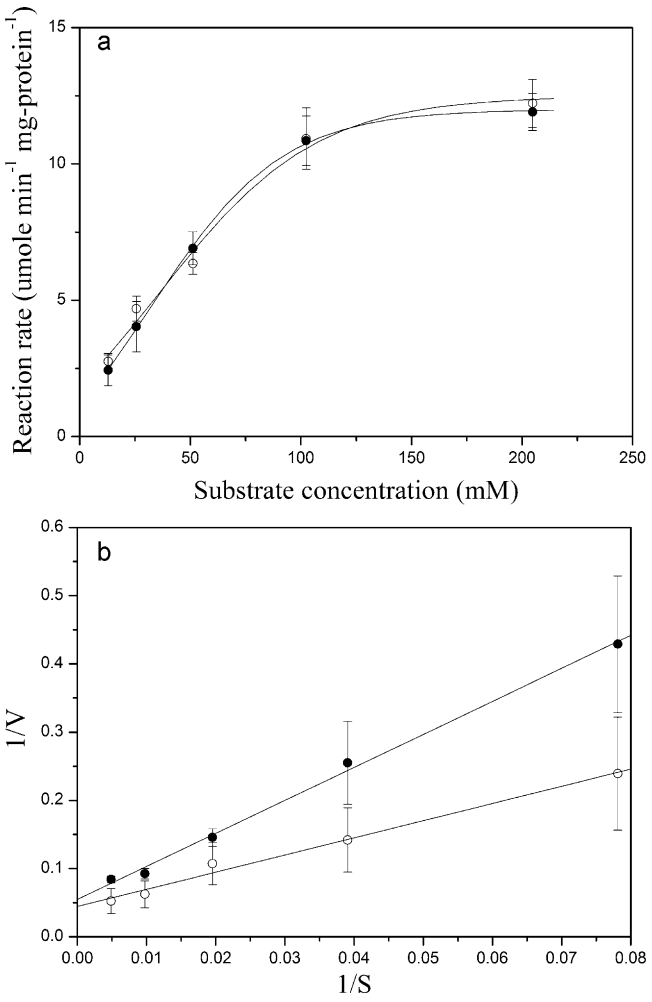

Fig. 3. Calculation of kinetic parameters for (○) free lipase; (●) immobilized *P. cepacia* lipase on the PAN nanofibrous membrane: (a) Michaelis–Menten plot and (b) Lineweaver–Burkplot (double reciprocal plot). The error bar at each data point represents the standard error ($n = 3$).

3.3. The operation stabilities of the immobilized lipase

3.3.1. The pH stability of immobilized lipase

The pH stabilities of free and immobilized lipases were compared by incubating in the pH range of 5–9 for 2 h at 30 °C and then measuring their activities. The results are shown in Fig. 4. Both free and immobilized *P. cepacia* lipase showed the highest specific activity at pH 7.0, suggesting that there are no significant changes in the electrostatic state of the amino acids at the active site and immobilized lipase [42]. Furthermore, the immobilized *P. cepacia* lipase remained stable in the pH ranging from 5 to 8. The results indicate that the *P. cepacia* lipase immobilized on the PAN nanofibrous membrane was more stable than free lipase in acidic reaction conditions. The present immobilized *P. cepacia* lipase can be employed in a broad pH range.

3.3.2. The thermal stability of immobilized lipase

Thermal stabilities were tested by incubating the free and immobilized *P. cepacia* lipases at temperatures ranging from 30 °C to 70 °C for 2 h and the results are shown in Fig. 5. There was no obvious activity loss for immobilized *P. cepacia* lipase when the temperature was increased from 30 °C to 70 °C. However, the free

Table 3
Protein loading, specific activity and kinetic parameters of free and immobilized lipase (lipase from *Pseudomonas cepacia*).

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

[a] NA, not available.

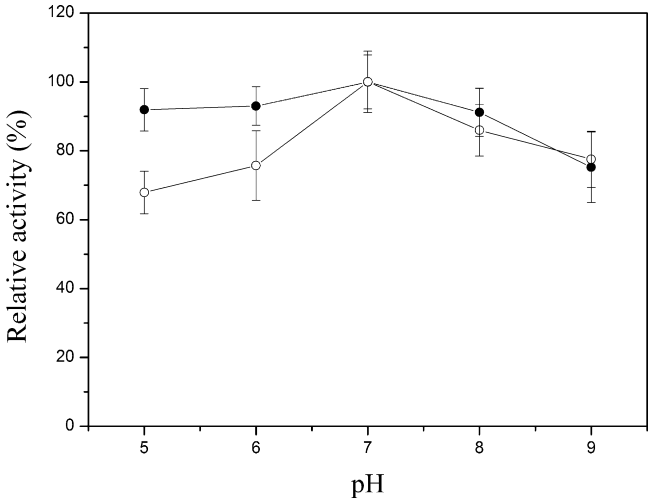

Fig. 4. The pH stabilities of free lipase and immobilized *P. cepacia* lipase onto the PAN nanofibrous membrane at 30 °C: (◯) free lipase; (●) immobilized lipase. The error bar at each data point represents the standard error ($n = 3$).

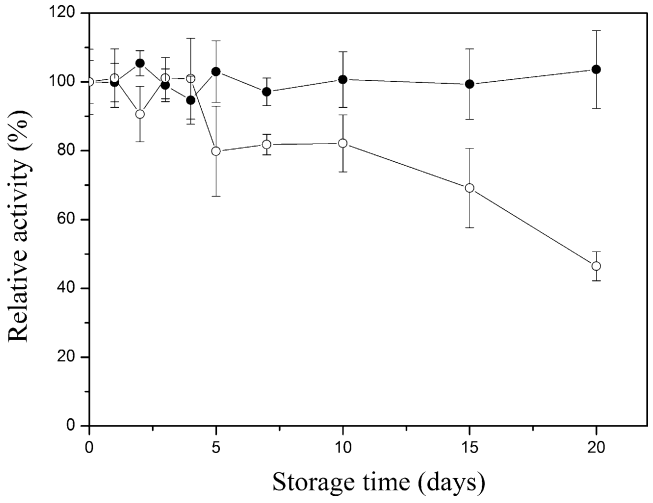

Fig. 6. The storage stabilities of free lipase and immobilized *P. cepacia* lipase onto the PAN nanofibrous membrane at 30 °C and pH 7.0: (◯) free lipase; (●) immobilized lipase. The error bar at each data point represents the standard error ($n = 3$).

lipase was only stable up to 60 °C. When the temperature was 70 °C, the free and immobilized *P. cepacia* lipase exhibited 5% and 90% of their initial specific activity, respectively. The improved thermal stability of the immobilized *P. cepacia* lipase probably could be explained by increasing stabilization of its active conformation by multipoint bond formation between the support and the enzyme [16].

3.3.3. The storage stability of immobilized lipase

For immobilized enzyme, the important advantages are that storage stability and the reusability can be enhanced via immobilization method. The storage stabilities of free and the immobilized *P. cepacia* lipase are shown in Fig. 6. From the figure, the specific activity of free lipase decreased dramatically in the first 5 days and showed only 46% of relative specific activity after 20 days. In contrast, the relative specific activity of immobilized *P. cepacia* lipase was kept close to 100% even after 20 days. It indicated that enzyme immobilization has considerably enhanced the storage stability of lipase.

3.3.4. The reusability of immobilized lipase

When comparing the performance of immobilized enzyme intended for industrial use, the characterization of reusability is one of the important key features. The effect of repeated use on the specific activity of immobilized *P. cepacia* lipase is shown in Fig. 7. After 10 repeated uses, the immobilized *P. cepacia* lipase retained about

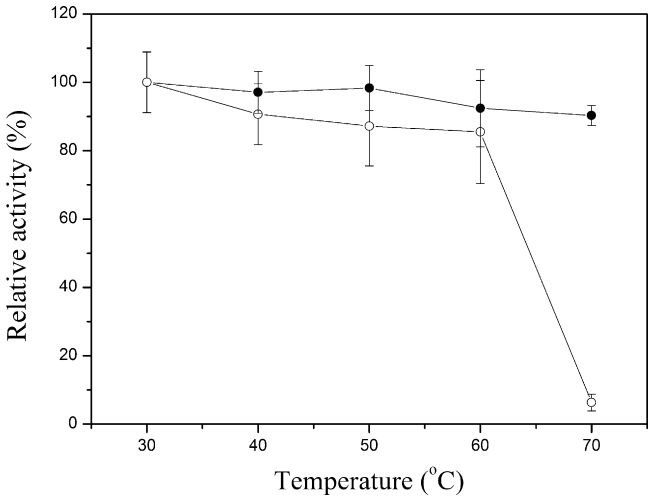

Fig. 5. The thermal stabilities of free lipase and immobilized *P. cepacia* lipase on the PAN nanofibrous membrane in pH 7.0: (◯) free lipase; (●) immobilized lipase. The error bar at each data point represents the standard error ($n = 3$).

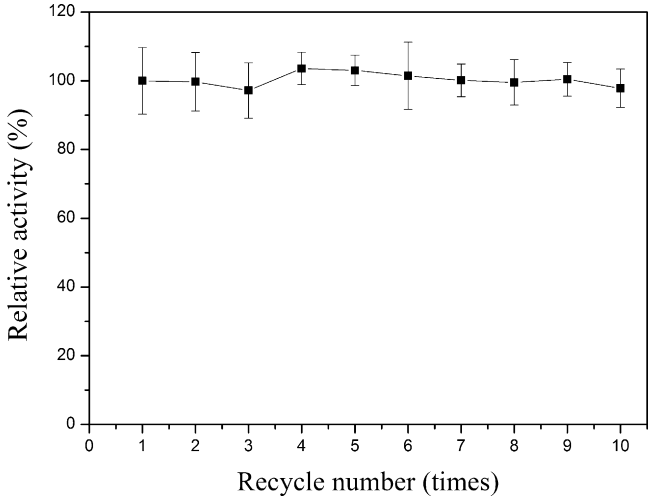

Fig. 7. The reusability of immobilized *P. cepacia* lipase onto the PAN nanofibrous membrane at 30 °C and pH 7.0. The error bar at each data point represents the standard error ($n = 3$).

Table 4
Comparison of the reusability of different immobilized lipases for use in transesterification reactions.

Support	Enzyme type	Binding type	Activity retention (reuse times)	Reference
Macro-porous resin (NW-ZT2)	*Candida antrarctica*	Adsorption/crosslinking	62.1%/10 times	[43]
Silica-PEG gel	*Candida antrarctica*	Modification-coupled	74.4%/10 times	[43]
PAN nanofibrous membrane	*Pseudomonas cepacia*	Adsorption	80%/10 times	[29]
PAN nanofibrous membrane	*Pseudomonas cepacia*	Covalent binding	98%/10 times	This work

98% of its original specific activity. A comparison of the reusability of immobilized *P. cepacia* lipase on the PAN nanofibrous membrane and that of the other studies is shown in Table 4. The activity retentions of lipase immobilized onto the macro-porous resin (NW-ZT2), silica-PEG gel and PAN nanofibrous membrane retained about 62.1% after 10 reuses, 74.4% after 10 reuses and 80% after 10 reuses, respectively [29,43]. In contrast, the immobilized *P. cepacia* lipase on the PAN nanofibrous membrane still retained 98% of the activity retention after being reused 10 times. These results clearly indicate that the immobilized *P. cepacia* lipase on the PAN nanofibrous membrane performs well in retaining enzyme activity and reusability.

4. Conclusions

Polyacrylonitrile (PAN) nanofibrous membranes was fabricated by electrospinning method with fiber diameter in the range of 150–300 nm and used for *P. cepacia* lipase immobilization. Lipase molecules were covalently bound to the nanofiber surface and formed small protein aggregates. The immobilized *P. cepacia* lipase still retained 79% of activity compared to that of free lipase. Furthermore, the immobilized *P. cepacia* lipase displayed superior pH and thermal stabilities than that of free lipase. After enzyme immobilization, the storage stability was improved significantly over that of free enzyme and the immobilized *P. cepacia* lipase still retained 98% of its original specific activity after 10 repeated batches of reaction. The reusability of immobilized *P. cepacia* lipase on the PAN nanofibrous membrane performed well overall. This simple but effective enzyme immobilization system has great potential for industrial applications.

References

[1] A. Zaks, A.M. Klibanov, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 3192–3196.
[2] G. Langrand, N. Rondot, C. Triantaphylides, J. Baratti, Biotechnol. Lett. 12 (1990) 581–586.
[3] R. Talon, M.C. Montel, J.L. Berdague, Enzyme Microb. Technol. 19 (1996) 620–622.
[4] S.W. Tsai, B.Y. Liu, C.S. Chang, J. Chem. Technol. Biotechnol. 65 (1996) 156–162.
[5] T. Izumi, F. Tamura, M. Akutsu, R. Katou, S. Murakami, J. Chem. Technol. Biotechnol. 68 (1997) 57–64.
[6] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627–662.
[7] S. Spisák, A. Guttman, Curr. Med. Chem. 16 (2009) 2806–2815.
[8] R.J. Kazlauskas, Trends Biotechnol. 12 (1994) 464–472.
[9] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, J. Mol. Catal. B: Enzym. 9 (2000) 113–148.
[10] E.A. Kulik, K. Kato, M.I. Ivanchenko, Y. Ikada, Biomaterials 14 (1993) 763–769.
[11] K. Michael, G. Janina, M. Peter, K. Ingo, Biotechnol. Bioeng. 72 (2001) 530–540.
[12] H.F. Jia, G.Y. Zhu, B. Vugrinovich, W. Kataphinan, D.H. Reneker, P. Wang, Biotechnol. Prog. 18 (2002) 1027–1032.
[13] K. Moeschel, M. Nouaimi, C. Steinbrenner, H. Bisswanger, Biotechnol. Bioeng. 82 (2003) 190–199.
[14] S. Piletsky, E. Pileska, A. Bossi, N. Turner, A. Turner, Biotechnol. Bioeng. 82 (2003) 86–92.
[15] J.M. Palomo, R.L. Segura, G. Fernández-Lorente, M. Pernas, M.L. Rua, J.M. Guisán, R. Fernández-Lafuente, Biotechnol. Prog. 20 (2004) 630–635.
[16] C. Mateo, J.M. Palomo, G. Fernández-Lorente, J.M. Guisán, R. Fernández-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[17] L. Betancor, H.R. Luckarift, Trends Biotechnol. 26 (2008) 566–572.
[18] P.V. Iyer, L. Ananthanarayan, Process Biochem. 43 (2008) 1019–1032.
[19] R. Fernández-Lafuente, Enzyme Microb. Technol. 45 (2009) 405–418.
[20] K. Hernandez, R. Fernández-Lafuente, Enzyme Microb. Technol. 48 (2011) 107–122.
[21] J.P. Chen, D.R. Su, Biotechnol. Prog. 17 (2001) 369–375.
[22] T. Hayashi, S.H. Hyon, W.I. Cha, Y. Ikada, J. Appl. Polym. Sci. 49 (1993) 2121–2127.
[23] D.H. Reneker, I. Chun, Nanotechnology 7 (1996) 216–223.
[24] M.M. Demir, I. Yilgor, E. Yilgor, B. Erman, Polymer 43 (2002) 3303–3309.
[25] Z.M. Huang, Y.Z. Zhang, M. Kotaki, S. Ramakrishna, Compos. Sci. Technol. 63 (2003) 2223–2253.
[26] Z.G. Wang, J.Q. Wang, Z.K. Xu, J. Mol. Catal. B: Enzym. 42 (2006) 45–51.
[27] J.P. Chen, K.H. Ho, Y.P. Chiang, K.W. Wu, J. Membr. Sci. 340 (2009) 9–15.
[28] Z. Wang, L. Wan, Z. Liu, X. Huand, Z. Xu, J. Mol. Catal. B: Enzym. 56 (2009) 189–195.
[29] S. Sakai, Y. Liu, T. Yamaguchi, R. Watanabe, M. Kawabe, K. Kawakami, Biotechnol. Lett. 32 (2010) 1059–1062.
[30] S. Sakai, Y. Liu, T. Yamaguchi, R. Watanabe, M. Kawabe, K. Kawakami, Bioresour. Technol. 101 (2010) 7344–7349.
[31] S.F. Li, W.T. Wu, Biochem. Eng. J. 45 (2009) 48–53.
[32] Q. Li, Y.J. Yan, Appl. Energy 87 (2010) 3148–3154.
[33] K. Tongboriboon, B. Cheirsilp, A. H-Kittikun, J. Mol. Catal. B: Enzym. 67 (2010) 52–59.
[34] Y. Liu, X. Zhang, H. Tan, Y. Yan, B.H. Hameed, Process Biochem. 45 (2010) 1176–1180.
[35] T. Handa, A. Hirose, S. Yoshida, H. Tsuchiya, Biotechnol. Bioeng. 24 (1982) 1639–1652.
[36] T. Handa, A. Hirose, T. Akino, K. Watanabe, H. Tsuchiya, Biotechnol. Bioeng. 25 (1983) 2957–2967.
[37] S.F. Li, J.P. Chen, W.T. Wu, J. Mol. Catal. B: Enzym. 47 (2007) 117–124.
[38] M.M. Bradford, Anal. Bichem. 72 (1976) 248–254.
[39] H. Noureddini, X. Gao, R.S. Philkana, Bioresour. Technol. 96 (2005) 769–777.
[40] H. Yajima, A. Hirose, T. Ishii, T. Ohsawa, R. Endo, Biotechnol. Bioeng. 33 (1989) 795–798.
[41] P. Ye, Z.-K. Xu, J. Wu, C. Innocent, P. Seta, Macromolecules 39 (2006) 1041–1045.
[42] G.K. Kouassi, J. Irudayaraj, G. McCarty, J. Nanobiotechnol. 3 (2005) 1–9.
[43] J.J. Yang, X.X. Ma, Z.S. Zhang, B. Chen, S.A. Li, G.J. Wang, Biotechnol. Adv. 28 (2010) 644–650.