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Comparison of hydrolytic activities in aqueous and organic media for lipases immobilized on poly(acrylonitrile-*co*-maleic acid) ultrafiltration hollow fiber membrane

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Abstract

A novel matrix, poly(acrylonitrile-*co*-maleic acid) (PANCMA) ultrafiltration hollow fiber membrane, was used for enzyme immobilization. Lipase from *Candida rugosa* was covalently immobilized onto this membrane surface on which the carboxyl groups were activated with 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride/*N*-hydroxyl succinimide as coupling agent. Using the hydrolysis reaction of *p*-nitrophenyl palmitate in aqueous and organic media, the properties of the immobilized lipase were assayed and compared with those of the free ones. Compared to the free enzyme (at 37 °C), it was found that the maximum activity was observed at 45 °C for the immobilized enzyme in the aqueous medium. On the other hand, the amount of added water in the organic medium of heptane showed greater effect on the reaction rate for the free lipase than the immobilized ones. The kinetic constants of the free and immobilized lipases, K_m and V_{max}, were assayed in the two media. Results indicated that the enzyme activity in heptane increased due to the immobilization and the specific activity was 0.233 U/mg for the immobilized lipase, 0.204 U/mg for the free ones, respectively. The residual activities of the immobilized enzyme were 62% in aqueous media and 67% in organic media, after 10 reuses.

Keywords: Poly(acrylonitrile-*co*-maleic acid); Ultrafiltration hollow fiber membrane; Lipase; Enzyme immobilization; *p*-Nitrophenyl palmitate hydrolysis

1. Introduction

As biocatalyst, enzymes have been always studied and used in aqueous medium. However, the discovery that enzymes possess catalytic activity in media with low water content has made it possible to run enzymatic reactions in nonconventional media such as organic solvent, supercritical fluid or gas phase [1–3]. Among different systems potentially useful to run reactions in nonconventional media, the direct dispersion of enzyme in an organic medium has been widely explored. The applications of enzymes in organic media rather than aqueous media have several important advantages for the synthetic organic chemists, such as the shift in thermodynamic equilibrium in favor of the product over the hydrolysis reaction, the increased solubility of nonpolar substrates, the elimination of side reactions, and the increased thermal stability of the enzymes [4]. Among all enzymes, which show significant activity in organic media, lipases have attracted special interests, because they are widely used to catalyze the reactions of esterification, trans-esterification, alcoholysis or acidolysis and therefore, have considerable commercial potentials [5–10].

For industrial application, like most enzymes, lipases have often been immobilized onto insoluble or solid supports, which are regarded as a useful tool to increase their thermal and operational stability, and recoverability [11–16].

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On the other hand, immobilizing enzyme onto a support has proved to be a useful technique for improving enzymatic activity in organic media [17,18]. However, it is important to choose proper support materials and immobilization methods for enzyme immobilization [19,20]. Artificial membranes are ideal supports for enzyme immobilization, due to their high specific surface area and the possibility to combine separation with biochemical reaction [21]. Therefore, membrane-immobilized enzymes have great potentials in biosensors and enzyme reactors as less expensive, more stable and reusable alternatives to free enzymes. These interesting properties encouraged membrane scientists and biotechnologists to find new membranes as efficient supports for the immobilization of enzymes. There are various membrane materials reported in the literature [22–24]. Among them, polyacrylonitrile-based membranes were successfully applied as supports for the immobilization of a series of enzymes, such as urease [25], glucose oxidase [26,27], cellulase [28], amyloglucosidase and uwertase [29].

In our previous work [30], poly(acrylonitrile-*co*-maleic acid) (PANCMA) was synthesized by a water-phase precipitation copolymerization process and this copolymer was used to fabricate ultrafiltration hollow fiber membrane containing reactive carboxyl groups for further reaction. As a potential matrix for enzyme immobilization, the carboxyl groups on the membrane surface were activated with 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride/*N*-hydroxyl succinimide (EDC/NHS). Lipase from *Candida rugosa* was then covalently immobilized on this PANCMA membrane. Using the hydrolysis reaction of *p*-nitrophenyl palmitate (*p*-NPP), the properties of the free and immobilized lipase preparations were compared in aqueous and organic media respectively. Factors affecting lipases activity were investigated, such as water content, temperature and substrate concentration.

2. Experimental

2.1. Materials

Lipase (from *Candida rugosa*), Braford reagent, bovine serum albumin (BSA, molecular mass: 67,000 Da), EDC, NHS, 2-morpholinoethane sulfonic acid (MES) and *p*-NPP were purchased from Sigma and used as received. All other chemicals were of analytical grade and used without further purification.

Poly(acrylonitrile-*co*-maleic acid) ultrafiltration hollow fiber membrane was fabricated in our lab according to the reported process [30]. The outer and inner diameters of the ultrafiltration hollow fiber membrane were 850 and 545 μm, respectively, with water flux 146 L/(m^2 h atm), BSA rejection 96%, and breaking strengthen 135 N/cm^2. The molar fraction of maleic acid in the copolymer was 7.5%.

2.2. Immobilization of lipase on PANCMA membranes

Lipase was immobilized onto poly(acrylonitrile-*co*-maleic acid) membranes by an EDC/NHS activation procedure. Schematic representatives for the activation of PANCMA membrane and the enzyme immobilization are shown in Fig. 1. An appropriate amount of hollow fiber membranes were thoroughly washed with de-ionized water, and then rinsed with MES buffer (0.05 M, pH 6). After this, the pretreated membranes were submerged into a EDC/NHS solution (0.02 g/mL in MES buffer, 0.05 M, pH 6, the molar ratio of EDC to NHS = 1:1) and shaken gently for 6 h at room temperature. The activated membranes were taken out, washed several times with phosphate buffer (pH 5.5), and submerged into the enzyme solution (2 mg/mL in phosphate buffer, pH 5.5). Lipase immobilization was carried out

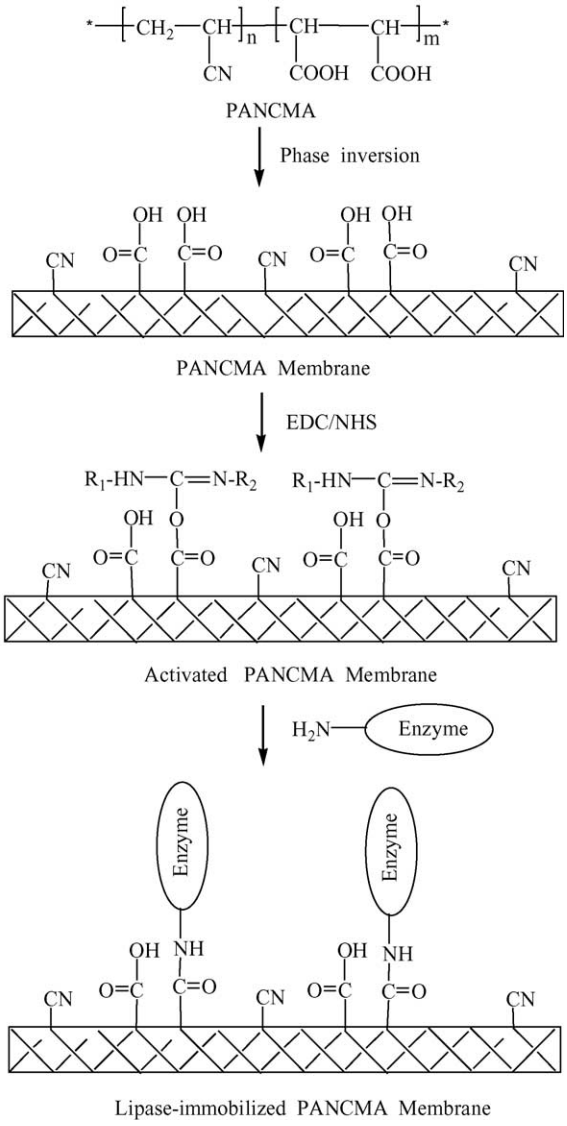

Fig. 1. Schematic representatives for the reactions of membrane activation and enzyme binding.

at 4 °C in a shaking water bath for 1 h. Finally, the membranes were taken out and thoroughly rinsed with de-ionized water.

2.3. Determination of the amount of immobilized enzyme

The amount of immobilized enzyme on the membrane was determined by measuring the initial and final concentrations of protein within the enzyme solutions and washings using Coomassie Brilliant Blue reagent following Bradford's method [31]. BSA was used as a standard to construct the calibration curve. The immobilization capacity of the enzyme on the membrane was defined as the amount of protein (mg) per gram of the hollow fiber membrane. The immobilization yield was defined as the ratio of the amount of protein coupled on the hollow fiber membrane to the amount of the protein added into the immobilization solution.

2.4. Activity assay of lipase in aqueous media

The reaction rate of the free and immobilized lipase preparations in aqueous medium was determined according to the method reported by Chiou and Wu with only minor modification [12]. In the standard conditions, the reaction mixture was composed of 1.0 mL ethanol containing 14.4 mM *p*-NPP and 1.0 mL 0.05 M PBS (phosphate buffer solution, pH 7.5) in an Erlenmeyer flask. The reaction was started by addition of 0.10 mL free lipase preparation (or 25 mg immobilized lipase preparation). The mixture was incubated at 37 °C under reciprocal agitation at 120 strokes per minute. After 5 min of reaction, agitation was stopped, and then the reaction was terminated by adding 2.0 mL of 0.5 N Na_2CO_3 followed by centrifuging for 10 min (8400 × *g*). The supernatant of 0.50 mL was diluted 10 folds with de-ionized water, and measured at 410 nm in an UV–vis spectrophotometer (UV-1601, Shimadzu, Japan) against a blank without enzyme and treated in parallel. The reaction rate was calculated from the slope of the absorbance versus the time curve. Molar extinction coefficient of $14.5 \times 10^3\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol (*p*-NP), which was determined from the absorbance of standard solutions of *p*-NP in the reaction medium, was used.

One enzyme unit was the amount of biocatalyst liberating 1.0 μmol *p*-NP/min in these conditions. Activity was defined as the number of lipase unit per milligram of protein. Activity yield was defined as the ratio of the activity of the amount of the enzyme coupled on the hollow fiber membrane to the activity of the same amount of free enzyme.

2.5. Activity assay of lipase in the organic medium

The reaction rate of the free and immobilized lipase preparations in heptane was determined according to the process described by Pencreac'h and Baratti [32,33]. In the standard conditions, the reaction mixture was composed of 2.0 mL of *n*-heptane containing 50 mM *p*-NPP in an Erlenmeyer flask. The reaction was started by the addition of 10 mg free lipase preparation (or 50 mg immobilized lipase preparation). The mixture was incubated at 37 °C under reciprocal agitation at 120 strokes per minute. After 5 min of reaction, agitation was stopped, the lipase powder was allowed to settle for 30 s, and the clear supernatant was withdrawn. Fifty microlitres of supernatant was immediately mixed with 1.0 mL, 10 mM NaOH, directly in 1.0 mL cuvette of the spectrophotometer. The *p*-NP was extracted by the aqueous alkaline phase. It displayed a yellow color because of the alkaline pH. The absorbance was read at 410 nm against a blank without enzyme and treated in parallel. When necessary (i.e. absorbance >1), the organic sample was diluted in *n*-heptane and 50 μL diluted solution was mixed with the alkaline aqueous phase for the extraction of *p*-NP. The reaction rate was calculated from the slope of the regression line of the data points obtained when plotting the absorbance versus the amount of biocatalyst put in the reaction mixture. Molar extinction coefficient of $17.4 \times 10^3\ M^{-1}\ cm^{-1}$ for *p*-NP was estimated using the standard solutions of *p*-NP in *n*-heptane and extraction as described above. Then, the activities were calculated as mentioned above.

3. Results and discussion

3.1. Determination of proper pH of immobilization solution

To covalently immobilize lipase on the membrane, a two-step process was employed. Firstly, the carboxyl groups on the PANCMA membrane surface were activated with EDC/NHS. Secondly, the condensation reaction of the amino group of the enzyme with the activated carboxyl group of the membrane was carried out. During the condensation reaction, amide bond formed between the amino group of the enzyme and the carboxyl group on the membrane surface.

To optimize the activity of the enzyme immobilized on the PANCMA membrane, various pH values were tried for lipase immobilization and the activity of the immobilized enzyme was assayed with the hydrolysis reaction of *p*-NPP in the aqueous medium. The variation of the relative activity for the immobilized lipase with the pH for immobilization is given in Fig. 2. Here, the relative activity was defined as the ratio of initial activity to the maximum initial activity achieved in this set. It can be seen that the maximum activity was obtained with the immobilized enzyme prepared at a pH 6.0. The result of protein assay (i.e., Bradford's method) indicated that 2.4 mg of protein could be immobilized per gram of PANCMA membrane at this pH value and the immobilization yield was 11%. Lipase immobilization in the pH range of 5.5–6.5 provided relatively high activity values, because the enzyme conformation, which is vital for the enzymatic activity, changed with different pH values. Therefore, there

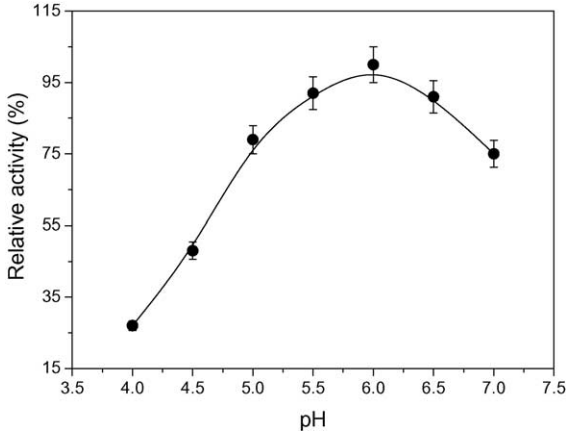

Fig. 2. Effect of immobilization pH on the relative activity of the immobilized lipase.

was an optimum pH value for enzyme activity in the enzyme immobilization process.

3.2. Effect of temperature on the lipase activity

Effect of temperature on the activity of the free and immobilized lipase preparations was studied in the temperature range of 20–55 °C. Typical results are shown in Fig. 3. For the free lipase preparation in the aqueous medium, an optimum temperature for enzyme activity was observed at 37 °C. However, the enzyme activity in heptane increased with the temperature increasing from 20 to 37 °C and then kept almost constant from 37 to 55 °C. For the immobilized lipase preparation in the aqueous medium, on the other hand, the optimum temperature for enzyme activity was nearly at 45 °C, while the activity in heptane increased with the temperature increase from 20 to 45 °C and then kept constant at higher temperature. These results demonstrated approximately that the immobilized enzyme preparations showed their catalytic activities at higher reaction temperature in both aqueous and organic media compared to the free ones. It could be attributed to either the creation of conformation limitations on the enzyme movements as a result of covalent bond formation between the enzyme and the matrix or a low restriction for the diffusion of substrate at higher temperature. The activity of both the free and immobilized lipase preparations kept almost constant at higher temperature in the organic medium could be due to the low water content which might affect the profile of activity against temperature [34].

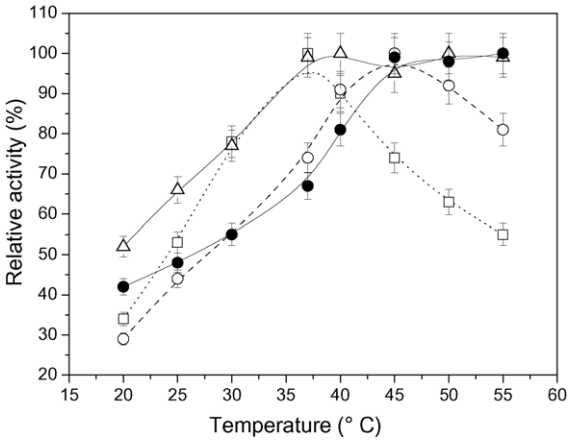

Fig. 3. Effect of temperature on the activity of the free and immobilized lipases. (□) Free lipase in the aqueous medium, (◯) immobilized lipase in the aqueous medium, (△) free lipase in heptane, (●) immobilized lipase in heptane.

3.3. Effect of added water on the lipase activity in the organic medium

Water plays a major role of "molecular lubricant" in enzyme resulting in conformational flexibility of enzyme, which leads to enhanced activity in non-aqueous media. A small amount of water molecules are absolutely essential to obtain a sufficient enzyme conformational flexibility for enzyme activity by forming multiple hydrogen bonds with enzyme molecule in organic media [35]. Water activity (a_w) has been shown to be the relevant parameter in such cases [36]. Pre-equilibration of all the components of the reaction mixture at a given a_w using salt hydrates is an efficient method to control water effects in organic media. In our work, the amount of water added was used as a key parameter instead of water activity for the reason of simplification.

Effect of the amount of water added on the lipase activity is shown in Fig. 4. Commercial lipase from *Candida rugosa* was used without treatment. Water content in this lipase sample was determined as 11 mg/g (1.1%, w/w) by heating the sample at 120 °C until constant weight. With no addition of water, the rate of *p*-NPP hydrolysis was 0.204 μmol/min mg for the free lipase preparation, 0.231 μmol/min mg for the immobilized lipase preparation. The increment of the amount of water from 5 to 60 μL added led to an increase in the activity

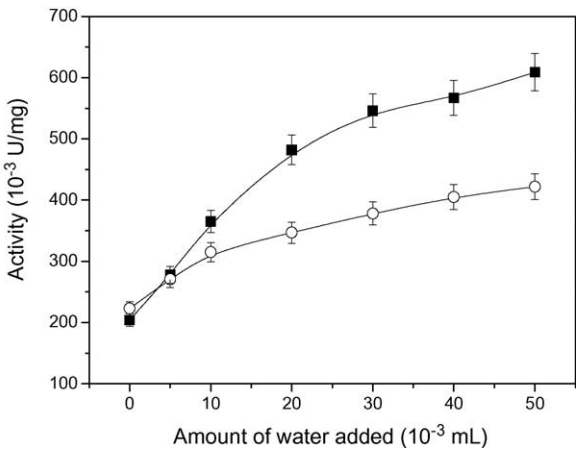

Fig. 4. Effect of amount of water added in heptane on the activity of the free and immobilized lipases. (◯) Immobilized lipase, (■) free lipase.

of both the free and immobilized lipase preparations. This activity increase could be attributed to the change of lipase conformation, which was more flexible and easy to access substrate for reaction. In addition to that, water might affect the reaction rate by its direct participation as a reactant in the hydrolysis reaction.

Obviously, it can be seen from Fig. 4 that the increase of activity was not at the same rate for the free and immobilized lipase preparations. The increase of activity was faster for the free lipase preparation than that for the immobilized lipase preparation with the increment of the amount of water added. This could be explained by the creation of conformation limitation on the enzyme moments as a result of covalent bond formation between the enzyme and the matrix. On the other hand, the carboxyl groups not reacted with EDC/NHS for steric hindrance reason on the membrane surface might attract some water molecule on the membrane surface. Therefore, the immobilized lipase preparation showed less sensitivity to the amount of water added in heptane in comparison with the free lipase preparation.

3.4. Kinetic constants and activity of the free and immobilized lipase preparations

Kinetic constants for the hydrolytic activity of the free and immobilized lipase preparations were assayed at substrate concentration from 1 to 14.4 mM in the aqueous medium, and from 1 to 50 mM in heptane. Higher concentrations should not be used because the substrate was not soluble upon this limit. The kinetic constants K_m and V_{max} from double reciprocal plot are shown in Table 1.

In aqueous medium, the K_m value for the free lipase preparation (0.45 mM) was found to be lower than that for the immobilized lipase preparation (1.35 mM), while the V_{max} value for the former preparation (46.4 U/mg) was found to be higher than that for the latter preparation (16.1 U/mg). So, the activity was 14.3 U/mg for the immobilized lipase preparation and 42.1 U/mg for the free one and the activity yield was 33.9% in aqueous medium. These results might be ascribed either to the conformation changes of the enzyme induced by the applied immobilization procedure, or to the limitation of the substrate dispersion to the active sites of the immobilized enzyme.

Kinetic constants K_m and V_{max} could be obtained in different media. The K_m value of free lipase preparation was 0.45 mM in the aqueous medium and 8.61 mM in heptane, respectively. The increase in K_m value was nearly 19-folds. The V_{max} value of free lipase preparation was 46.4 U/mg in the aqueous medium and 0.269 U/mg in heptane. The decrease in V_{max} value was about 172 folds. Therefore, it was found that the activity of the free lipase preparation in heptane (0.204 U/mg) was 0.48% of that in the aqueous medium (42.1 U/mg). There were several hypotheses to explain the relative low activity in the organic medium. Firstly, most of the enzyme might be inactivated by the organic medium, only partial enzyme was active. Secondly, the diffusion limitation of substrate might considerably increase and lead to the decrease of hydrolysis rate.

Interestingly, it was found that the activity for the free lipase preparation in heptane increased from 0.204 to 0.233 for the immobilized lipase preparation, and the activity yield was 114%. The K_m and V_{max} values were 8.61 mM and 0.269 U/mg for the free lipase preparation, but 3.86 mM and 0.277 U/mg for the immobilized lipase preparation, respectively. The increase of enzyme activity could be explained by several hypotheses. Firstly, free lipase aggregated because it was insoluble in the organic medium, while immobilized lipase scattered on a large membrane surface area could easily contact with substrates. Secondly, the formation of covalent bonds between the enzyme and the membrane surface increased the stability of the enzyme conformation against the organic medium. Thirdly, some properties of the membrane surface could benefit the activity of the immobilized lipase, which might include the hydrophobic interaction between the PANCMA main chains on the membrane surface and the hydrophobic domain around the lipases' active site. This hydrophobic interaction can stabilize the "open state" conformation of lipase and favor the active site accessibility to substrates. The carboxyl groups on the membrane surface, which are rare, but close to immobilized lipase due to the special structure of maleic acid, could also provide water molecular for immobilized lipase.

3.5. Operational stability of the immobilized lipase preparation

The operational stability of immobilized enzyme is very important economically, and an increased stability could make the immobilized enzyme more advantageous than its free counterparts. To investigate the operational stability, the enzyme-immobilized membranes were washed with PBS (0.05 M pH 7.5) or *n*-heptane after any run and reintroduced into a fresh aqueous or organic media, this being repeated up to 10 cycles. Fig. 5 shows the effect of repeated use on the activities of the immobilized lipase in both media. It was found that the immobilized enzyme retained an activity of

Table 1
Activity and kinetic parameters for the free and immobilized lipases

Sample	Medium	V_{max} (U/mg)	K_m (mM)	Activity (U/mg)	Activity yield (%)
Free lipase	Aqueous	46.4	0.45	42.1	-
Immobilized lipase	Aqueous	16.1	1.36	14.3	33.9
Free lipase	Organic	0.269	8.61	0.204	-
Immobilized lipase	Organic	0.277	3.86	0.233	114

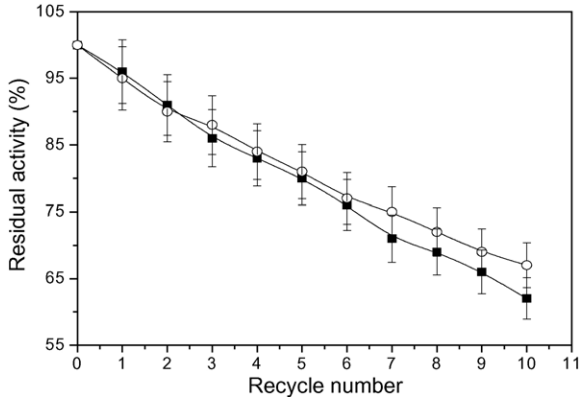

Fig. 5. Operational stability of the immobilized lipase. (◯) In heptane, (■) in the aqueous medium.

80% in aqueous media, 81% in organic media, respectively, after 5 reuses. After 10 reuses the residual activities of the immobilized enzyme were 62% in aqueous media and 67% in organic media. These results could be explained by the inactivation of the enzyme and the leakage of protein from the support upon use.

4. Conclusion

A new support, PANCMA ultrafiltration hollow fiber membrane, was successfully used to immobilize lipase from *Candida rugosa* by covalent binding. The hydrolysis reactions of *p*-NPP in aqueous and organic media by the immobilized enzyme were assayed and compared with those of the free enzyme. In the aqueous medium, maximum enzyme activity was observed at relative high temperature for the immobilized lipase than that for the free ones. In heptane, it seems due to the adsorbed water on the PANCMA membrane surface, the reaction rate for the immobilized lipase was less sensitive to the amount of added water than that for the free ones. The activity of the enzyme in this organic medium was improved with immobilization and the specific activity was 0.233 U/mg for the immobilized lipase, 0.204 U/mg for the free lipase, respectively. And the operational stability of enzyme was also increased upon immobilization in both media.

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