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Journal of Molecular Catalysis B: Enzymatic 42 (2006) 55–63
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Immobilization of lipase by filtration into a specially designed microstructure in the CA/PTFE composite membrane

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Received 10 January 2006; received in revised form 31 May 2006; accepted 12 June 2006
Available online 1 August 2006

Abstract

A specially designed microstructure in the composite membrane with a porous hydrophobic layer and a relatively dense hydrophilic layer is proposed for lipase immobilization. Firstly, the composite membrane was prepared by coating the cellulose acetate layer with the average pore size of 1.40 nm on the hydrophobic PTFE layer with the average pore size of 76.3 nm. Then, enzymes were absorbed in pores of PTFE layer and deposited on the interface between the two layers by the filtration process. The relatively dense CA layer was used to reject the enzymes controlling the enzyme loading which prevented enzymes from being dissolved into the aqueous phase. The porous PTFE layer supplied a hydrophobic environment and a large specific surface area for the immobilization of lipases which were propitious to the activation of lipase. The activity of immobilized lipase membrane based on hydrolysis of olive oil was assayed in the biphasic membrane reactor and the maximum specific activity (1.20 ± 0.04 μmol-FFA min^{-1} cm^{-2}) was found to be higher than the value reported in some literature. The kinetic parameters of the immobilized lipases, K_m and k_{max} were fitted with the Michaelis–Menten equation. The Thiele modulus (φ) was used to evaluate the effect of the mass transfer through the membrane on the performance of reaction systems. The optimum enzyme loading (0.020 ± 0.002 mg-protein cm^{-2}) was obtained with the highest activity and without the diffusion-limited. Furthermore, the immobilized lipases retained 80% residual activity after ten hydrolysis cycles. The composite membrane was easily regenerated and lipases immobilized in the regenerated membrane remained a high activity.

Keywords: Composite membrane; Cellulose acetate; PTFE; Lipase immobilization; Filtration

1. Introduction

Lipase (EC 3.1.1.3) is an important enzyme with a broad variety of industrial applications due to the multiplicity of reactions it catalyzes. Due to their unique structural characteristics [1], lipases can catalyze reactions including organic substrates at the interface of organic and aqueous phases and can preserve their catalytic activity in organic solvents, biphasic systems [2] and in micellar solutions [3]. Application of the lipase can be achieved more economically and efficiently by immobilization to enhancing their activity, selectivity and operational stability. The immobilization of enzymes onto insoluble carriers is an important topic in enzyme technology and is fundamental for their application to industrial processes [4]. Numerous efforts have been focused on the preparation of lipases in immobilized forms involving a variety of both support materials and immobilization methods [5].

Membrane-immobilized enzymes may serve as model systems for enzymes, naturally bound to membranes, or may find practical application in enzyme reactors as less expensive, more stable and reusable alternatives to free enzymes [6,7]. Obviously, the materials of the membranes used in enzyme immobilization are important since their interaction with the enzyme may have an influence on the stability and kinetics [8,9]. There are various membrane materials available for enzyme immobilization, which can be classed into three general types: inorganic materials, synthetic polymers and natural macromolecules. The inorganic materials, such as porous glass [10], silica [11] and magnetic iron oxide [12,13], normally exhibit good mechanical stability, rigidity and regeneratability but are expensive and their biocompatibilities are poor. Consequently, so far inorganic membranes are rarely applied for enzyme immobilization. On the other hand, the synthetic polymers such as polypropylene [14,15], PTFE [16], polyacrylonitrile [17] and nylon [18], can

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doi:10.1016/j.molcatb.2006.06.007

Nomenclature

A	membrane area (cm^2)
C_1 and C_2	triolein concentrations in source and receiver cells, respectively (mol/L)
C_{10} and C_{20}	initially triolein concentrations in source and receiver cells, respectively (mol/L)
C_E	enzyme loading per unit membrane area (mg-protein/cm^2)
C_p	FFA concentration in iso-octane (mol/L)
C_s	olive oil concentration in isooctane based on ester-bond (mol/L)
D	diffusion coefficient of the substrate through the membrane (m^2/h)
K_m	apparent Michaelis constant (mol/L)
k_{max}	the highest possible velocity constant (μmol-FFA mg^{-1} protein min^{-1})
L	thickness of the membrane (μm)
r	initial hydrolysis rate (μmol-FFA min^{-1})
r_{max}	maximum velocity of the reaction (μmol-FFA L^{-1} min^{-1})
S	olive oil concentration (g/cm^3)
t	reaction time (h)
V	organic phase volume (mL)
V_1 and V_2	volumes of the source and the receiver cells (mL)

Greek symbol

φ	Thiele modulus

be used for enzyme immobilization because of good mechanical stability, availability of reactive functional groups for direct reactions with enzymes and ease of preparation in different geometrical configurations. Nevertheless, there are also some disadvantages for them, including imperfect biocompatibility and hydrophobicity. Thus, surface modification to enhance biocompatibility and hydrophobicity of the membrane surface is a potential and interesting subject [19,20]. The natural macromolecules, including chitosan [8,21], cellulose [22,23] and agarose [24], with excellent biocompatibility, are non-toxic, biodegradable and inexpensive. However, the weak mechanical stabilities of these natural materials have limited their applications greatly.

A variety of methods have been developed for immobilization of enzymes onto membranes. These may be divided into two main categories: physical methods based on molecular interactions between the enzyme and carrier, and chemical methods based on the formation of covalent bonds. Chemical methods including covalent attachment [25] and cross-linking [26] are highly stable, but have the disadvantage of denaturing the native enzyme during the binding process. Furthermore, it is difficult to replenish the denatured enzyme. Therefore, this technique is only useful if the initial denaturation step is negligible. Physical methods of immobilization include adsorption and entrapment. Adsorption may have a higher commercial potential than other methods because adsorption is relatively simple and less expensive, and a high catalytic activity may be retained [27]. The method also offers the reusability of expensive supports after inactivation of immobilized enzyme. However, adsorption is generally not very strong, and some of the adsorbed protein will desorb during washing and operation. Thus, immobilization via adsorption requires a strong hydrophobic or electrostatic interaction between the enzyme and support [28]. Entrapment is seldom used in membrane-immobilized enzymes due to difficult and complicated preparation technique.

In recent years, a new method of retaining the enzyme on the membrane dense surface or inclusion in sponge layer of asymmetric membranes by filtration has been used in a number of investigations [29–31]. Due to the simplicity of the immobilization procedure and high enzyme loading, this method was used commercially for lipase-catalyzed chiral resolution of a pharmaceutical intermediate [4,31]. Nevertheless, when lipases were retained on the membrane surface, they can be removed by the flow of organic or aqueous phase in the biphasic membrane reactor because of low binding force and cross-linking often required to improve their stability [29]. When lipases were embedded in the sponge layer of asymmetric membranes, the high mass-transfer resistance of water through hydrophobic membranes or organic substrates through hydrophilic membranes can induce the reaction system to work in a diffusion-limited regime.

In the present study, a composite membrane with the porous hydrophobic layer and the relatively dense hydrophilic layer was prepared for enzyme immobilization. Polytetrafluoroethylene (PTFE) membrane, an excellent hydrophobic support for immobilization, was employed for the porous hydrophobic layer. A biocompatible material of cellulose diacetate (CA) was used to prepare the hydrophilic layer of the composite membrane. Enzyme molecules were adsorbed and deposited in pores of the hydrophobic layer and on the interface between the hydrophilic layer and the hydrophobic layer by filtration. The structure of CA/PTFE composite membrane was investigated. The activity of the immobilized enzyme membranes, based on hydrolysis of olive oil was determined in a biphasic membrane reactor. The system parameters such as the kinetics parameter, the effect of enzyme loading, operation conditions, the reuse stability of immobilized enzyme, and membrane regeneration were also studied.

2. Materials and methods

2.1. Materials

Candida Rugosa lipase type VII was purchased from Sigma–Aldrich (Dorset, UK). PTFE flat membranes (with pore size, thickness and porosity of 0.1 μm, 85 μm and 55%, respectively) were purchased from Beijing Plastic Institute. Highly refined olive oil was obtained from Shanghai Agent Company with a saponification value of 192. Cellulose acetate (CA: 39.6% acetyl content, average M_w = 50,000) was obtained from Wuxi Chemical Plant. Other solvents and chemicals were of analytical grade and used without further purification.

2.2. Preparation and characterization of CA/PTFE composite membranes

PTFE flat sheet membranes were used as the support layer in the experiments. Before use, the membranes were rinsed with 0.1 M NaOH and 0.1 M HCl, and then washed with de-ionized water. Fifteen gram of cellulose acetate was dissolved in 85 g of DMF, and the casting solution for the hydrophilic layer was spread on PTEF flat membranes using a tailor-made stainless steel blade with the thickness of 65 ± 5 μm. After 20 min of evaporation at 25 ± 2 °C and relative humidity of 10 ± 5%, the cast film was immersed into a de-ionized water bath of 25 ± 2 °C for at least 1 h. The composite membranes were kept in the de-ionized water bath.

Scanning electron microscope (SEM, Hitachi S-450, Japan) was used to observe both surface and cross-section of membranes. The composite membrane was treated with liquid nitrogen to split the two layers. The structure of the CA layer and the PEFE layer was determined by the BET method (Quantachrome Autosorb-1C Chemisorption–Physisorption Analyzer) and the mercury intrusion method (Autopore IV 9510 mercury porosimeter, America), respectively.

2.3. Preparation of immobilized enzyme membranes

Lipase solutions were prepared by adding appropriate amounts of lipase powder to a phosphate buffer solution (0.05 M, pH7.5). The insoluble portion of the enzyme solution was removed by filtration with a filter membrane (pore size: 0.44 μm). The CA/PTFE composite flat membrane was placed into the dead-end filtration cell with a magnetic stirring apparatus. The PTFE layer was placed upward and the enzyme aqueous solution was filtrated under pressure by the compressed nitrogen of 0.6 MPa. After filtration, the membrane was taken out and rinsed with the phosphate buffer solution for three times. The original enzyme protein concentration and the concentration after filtration with the composite membrane were measured by the Lowry method [32] with UV–vis spectrophotometer (Agilent 8453 UV–vis spectrophotometer). Different volumes of the enzyme aqueous solution were filtrated to vary the amount of enzyme loading on membranes.

2.4. Activity assay of the free lipase

In order to investigate the effect of pH and temperature, the activity of the free lipase was measured in variety conditions and determined by the method of Yamada and Machida [33] with some modifications. The measurement was based on the action of enzyme on olive oil. The substrate emulsion was prepared by thoroughly mixing 50 mL olive oil with 150 mL of PVA (polyvinyl alcohol) solution (4 wt.%) and stored at 4 °C. A sample of 5 mL substrate emulsion was mixed with 5 mL of phosphate buffer (0.05 M). The emulsion was incubated in a water bath at a certain temperature for several minutes, and then pH was adjusted to the desired value with a NaOH solution. One milliliter of lipase solution was added into the emulsion. The pH was held constant for 10 min by continuously adding 0.01 M NaOH standard solution. The consumed volume of the NaOH standard solution was recorded. The blank value was measured using the same procedure. One unit corresponded to the release of 1 μmol of free fatty acid (FFA) per minute under the assay conditions. In order to compare with the immobilized lipase, the assay conditions of the free lipase including temperature and pH were the same as those of the immobilized lipase.

2.5. Biphasic enzyme membrane reactor

Fig. 1 shows the experimental set-up of the biphasic membrane reactor. It was made of stainless steel and consisted of two identical flat channels which were 0.5 cm deep, 2.0 cm wide and 6.0 cm long. The channels were separated by a biocatalytic membrane. A gasket made of 1.0 mm flat Teflon was used to seal the assembled membrane element. In order to perform an enzymatic reaction measurement, 75 mL of olive oil solution in isooctane was circulated through the PTFE side of the module, while 75 mL of a 0.05 M phosphate buffer solution at pH 7.0 was circulated through the CA side using a two channel peristaltic pump (NP-D-140, NS, Tokyo, Japan). Two water baths with thermostats were used to keep the temperature constant. Unless specified, all experiments were carried out at 37 °C and the flux of both phases was maintained at 15 mL/min. The transmembrane pressure from the organic phase to the aqueous phase was controlled by a valve to prevent the leakage of the buffer solution. The substrate concentration, based on the ester-bond concentration of olive oil and with the unit of mol/L, was calculated as follows:

$$C_s\left(\frac{\text{mol}}{L}\right) = \frac{192S(\text{g/cm}^3)}{56.1} \quad (1)$$

where S (g/cm^3) stands for the substrate concentration with the unit of g/cm^3. A series of oil olive concentration (0.05–1.0 M) in isooctane were used to examine the effect of substrate concen-

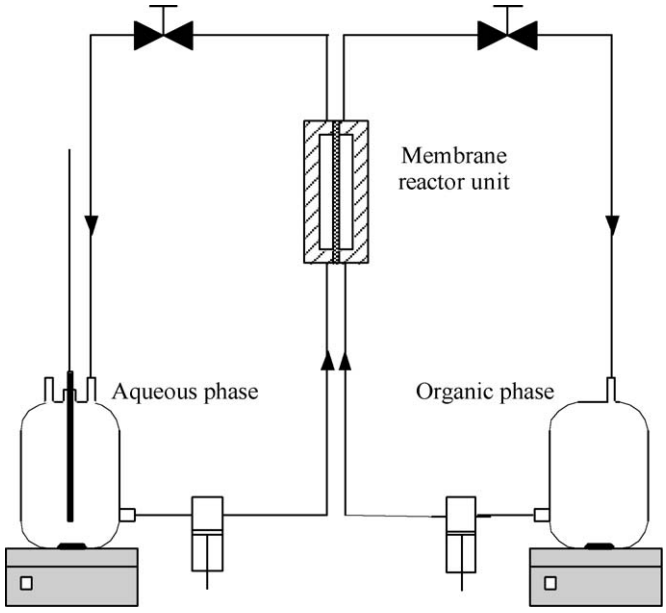

Fig. 1. Scheme of the experimental set-up of the biphasic membrane reactor.

tration. Samples of 0.5 mL were taken from the organic phase at different time intervals. The FFA concentration was determined by the method of Kwon and Rhee [34] with some modifications: 0.5 mL of the sample dissolved in 5 mL isooctane was heated at 50 °C and stirred. After 15 min, 1 mL of copper reagent was added (1% solution of copper acetate in water, the pH was adjusted with pyridine to 6.2). The sample was stirred vigorously for 120 s. The absorbance A_{715} of the supernatant was measured by UV–vis spectrophotometer. The blank experiment was carried out with non-hydrolyzed substrate solution.

2.6. Determination of diffusion coefficient of triolein through membranes with and without enzymes

In order to study the effect of the membrane enzyme loading to the diffusion of the substrates, a diffusion coefficient of triolein through the membranes, with and without enzymes, was determined. A stirred diffusion cell, composed of a source and a receiver cell, was used to determine the mass transfer coefficient of triolein through membranes. A PTFE membrane with an effective area of 7.0 cm^2 was inserted into two cells. A magnetic stirring bar at 300 rpm was used in each cell to reduce the concentration gradient on the membrane surface. The composite membrane with immobilized enzyme was cross-linked with 5% glutaraldehyde solution at 50 °C for 2 h. The cross-linking fixed the enzymes on surface and in pores of PTFE membranes. The composite membrane was then treated with liquid nitrogen to split the two layers of the composite membrane. Unless specified, these assays were carried out in a water bath maintained at 37 °C. All membranes were immersed in isooctane containing the required concentration of triolein for 12 h before they were used. The triolein concentration was determined by the spectrophotometric method.

The source cell was filled with 12.5 mL of isooctane containing the desired concentration of triolein. An equal volume of isooctane without triolein was placed in the receiver cell. The triolein concentration of each cell was determined 10 h later. By assuming a pseudo-steady-state of solute diffusion through the membrane, the mass balance equations for the two compartments can be combined and solved to give the following equation [9]:

$$\ln\left[\frac{C_{10}-C_{20}}{C_1-C_2}\right] = \frac{DAt}{L}\left[\frac{V_1+V_2}{V_1V_2}\right] \quad (2)$$

where A is the effective membrane area, t is the reaction time, C_{10}, C_{20}, C_1 and C_2 represent the solute concentrations in the source and receiver cells initially, those at the reaction time of t, D is the diffusion coefficient of triolein through the membrane, L is the thickness of the membrane and V_1 and V_2 are volumes of the source and the receiver cells.

3. Results and discussion

3.1. Characterization of the CA/PTFE composite membrane

The surface structure of the PTFE layer, as well as the surface and cross-sectional structure of CA layer are presented in Fig. 2(A–C), respectively. From these images, it was observed that the structure of PTFE layer was very porous, and the structure of CA layer was relatively dense.

The structure of PTFE layer and CA layer were determined by the mercury intrusion method and the BET method, respectively. The average pore size and pore size distribution of PTFE layer were calculated by a modified version of the Laplace equation in pressure range of 25–60,000 Pisa. Those of CA layer were calculated from the desorption branches using the Barrett–Joyner–Halenda (BJH) method. Results of the pore size distribution are shown in Fig. 3 (CA) and Fig. 4 (PTFE). It was found that the pore size distribution of CA layer (Fig. 3) was in

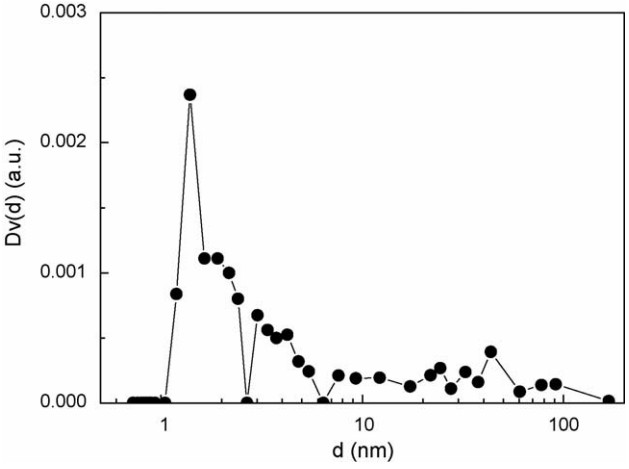

Fig. 3. Pore size distribution of the CA layer of composite membrane.

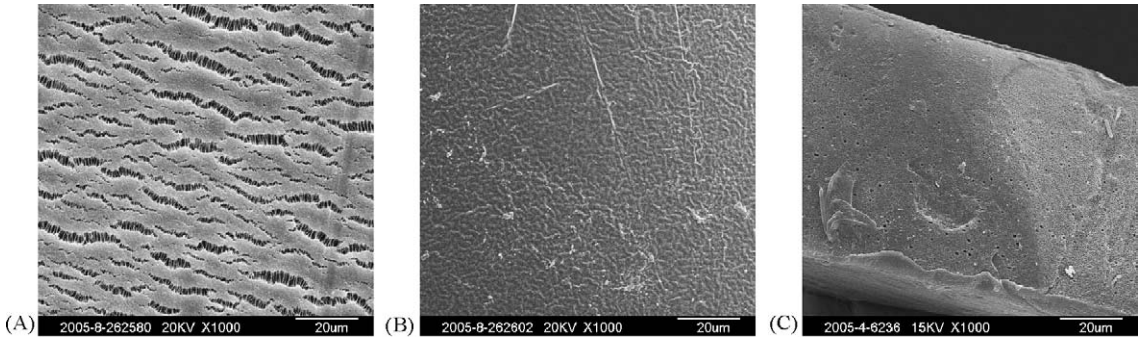

Fig. 2. SEM micrographs of the surface of PTFE (A) and CA (B), and the cross-section of CA (C).

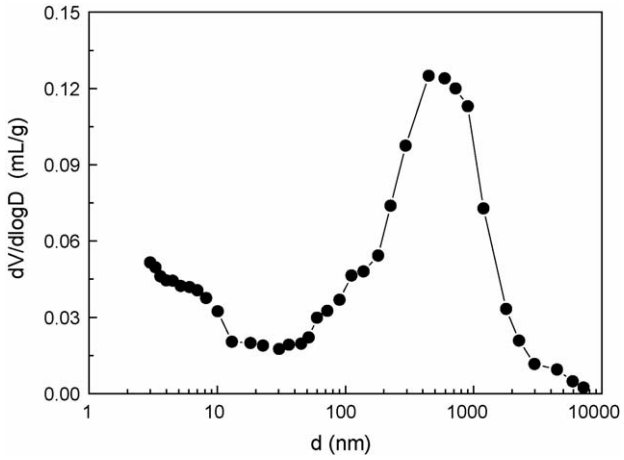

Fig. 4. Pore size distribution of the PTFE layer of composite membrane.

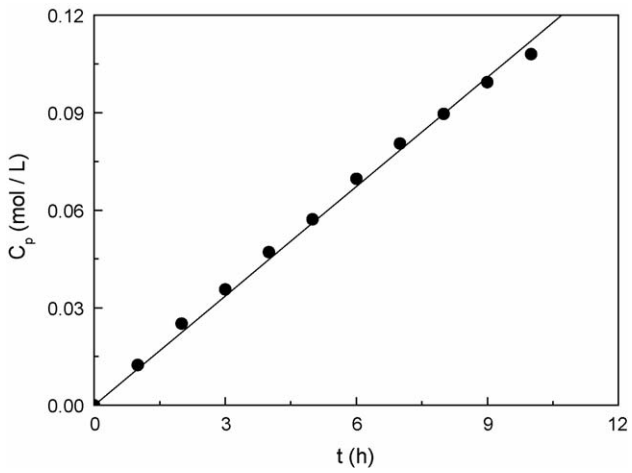

Fig. 6. Time-course variations of FFA concentration, $C_s = 1.0$ mol/L, $C_E = 0.020 \pm 0.002$ mg-protein cm^{-2}.

range of 1–100 nm. A majority of pore diameters were below 10 nm, and the average pore size was 1.40 nm. The pore size of CA layer was on the same order of magnitude as the size of lipase molecule, and therefore the lipase molecules were easily retained in pores. On the other hand, the pore size of PTEF layer showed a wide distribution from 3 nm to 10 μm (shown in Fig. 4), and the average pore size of 76.3 nm. The specific surface area of PTEF layer was calculated by the BET method and found to be 83 times its exterior surface area. This resulted in a large external surface area for the immobilization of lipases.

3.2. Immobilization of lipase by filtration into a special microstructure in the CA/PTFE composite membrane

Lipases were immobilized in this specially designed microstructure in the CA/PTFE composite membrane by filtration. The process of lipase immobilization is illustrated in Fig. 5. Pressure was applied using the compressed nitrogen, forcing the lipase molecules in the phosphate buffer solution into the pores of PTFE layer. Due to the action of CA ultrafiltration layer, the lipase solution was concentrated in pores of PTFE layer. This resulted from the concentration polarization, which intensified the lipase adsorption on the surface of pores. When the process of filtration was over, some lipase molecules were adsorbed and more were deposited on the interface between two layers. In this immobilization procedure, pressure used in filtration process forced the enzyme solution through the pores of the hydrophobic layer. As a result, the effective area available for immobilization of the enzymes increased remarkably. Furthermore, enzymes in the pores of membrane were more stable than those on surface. When this immobilized lipase membrane was used in biphasic membrane reactor, the relatively dense CA layer prevented enzymes from being dissolved into the aqueous phase. The diffusion resistance of water through a hydrophilic membrane to immobilized enzymes was lower than that through a hydrophobic membrane. This was favorable to reducing the effect of diffusion.

Fig. 6 illustrates the time-course variations of FFA concentration in the hydrolysis of olive oil catalyzed by the immobilized lipase membrane. The fatty acid production over time shows a linear trend and was regressed using linear function (correlation ratio > 0.99 for most cases) to determine the reaction rate. The linear trend confirms that the system behaved as though it there was no obvious product inhibition [2]. In this case, the immobilized enzyme activity per unit of membrane area was calculated to be 1.20 ± 0.04 μmol-FFA/cm^2 min. Table 1 presents a comparison of the immobilized enzyme activity per unit membrane area, where various membranes were used as the carrier for the lipase and various methods were used to immobilize the enzyme. Although experimental conditions of lipase loading, substrate source and concentration, reaction temperature, membrane type and operation mode were different in all cases, the initial enzyme activity obtained in this study is higher than those of other simi-

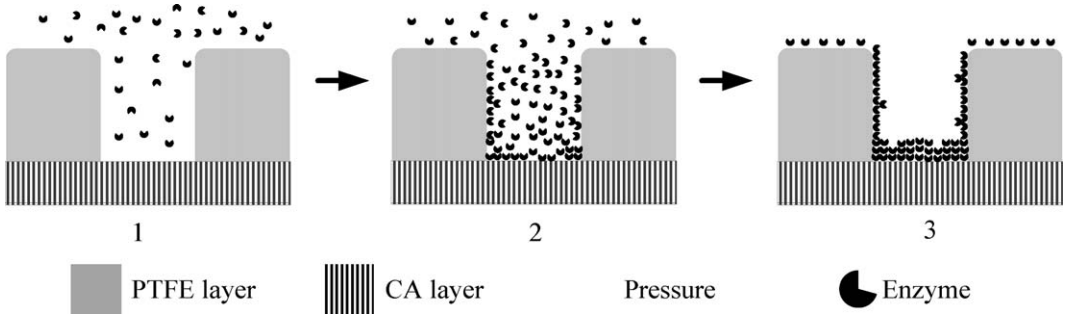

Fig. 5. Schematic representative for lipases immobilization process in CA/PTFE composite membrane by filtration.

Table 1
Hydrolysis of lipids by immobilized lipase membranes

Lipase source	Membrane material	Immobilization method	Activity (μmol-FFA min^{-1} cm^{-2})	Reference
C.R.	PP	Adsorption	0.118–0.189	[14,15]
R.S.	PTFE	Cross-linking	0.124	[16]
C.R.	PSF	Adsorption	0.171	[9]
C.R.	PVDF	Adsorption	0.543	
C.R.	Modified PP	Adsorption	0.657	[20]
C.R.	CA/PTFE	Filtration	1.24	This work

lar studies. In general, the order of magnitude of enzyme activity per unit membrane area is 0.1–1.0 μmol-FFA/cm^2 min at about 37 °C by applying various membranes as the carrier for lipases in lipids hydrolysis.

3.3. Effect of substrate concentrations

Fig. 7 shows the effect of substrate concentration on the initial hydrolysis rate per unit membrane area. Increasing the substrate concentration resulted in an enhancement of enzyme activity. Assuming that the system was in the reaction control, Goto et al. [35] and Ye et al. [36] analyzed their data by using the Michaelis–Menten equation to obtain the kinetic parameters of the immobilized enzymes. Therefore, the data in Fig. 7 were analyzed by the following equation [37]:

$$\frac{r}{A} = \frac{k_{max} C_E C_s}{C_s + K_m} \quad (3)$$

where the product inhibition was not considered. The parameters of r/A, K_m, k_{max}, C_E and C_s were the initial hydrolysis rate per unit membrane area, the apparent Michaelis constant, the highest possible velocity constant, the enzyme loading per unit membrane area and the concentration of olive oil. The parameters of K_m and k_{max} were fitted as 0.214 mol/L and 74.05 μmol-FFA mg^{-1}protein min^{-1} by using a two dimensional curve-fitting program from sigma-plot computer processor. The theoretical results presented in Fig. 7 were in good agreement with the experiments. The standard deviation of the experimental data from the theoretical result was less than 2%. Tsai and Shaw [9] have measured K_m and k_{max} of 0.31 mol/L and 13.2 mg-FFA cm h^{-1} mg^{-1} for the lipase adsorbed on the polyvinyllidene difluoride membrane in a stirred diffusion cell at 200 rpm, where the adsorption equilibrium of lipase between the membrane and the enzyme solution existed. Although the reaction operation modes in both systems are not the same, a comparison of the parameters implies that membrane materials and immobilization method does affect the interaction between the enzyme and substrate.

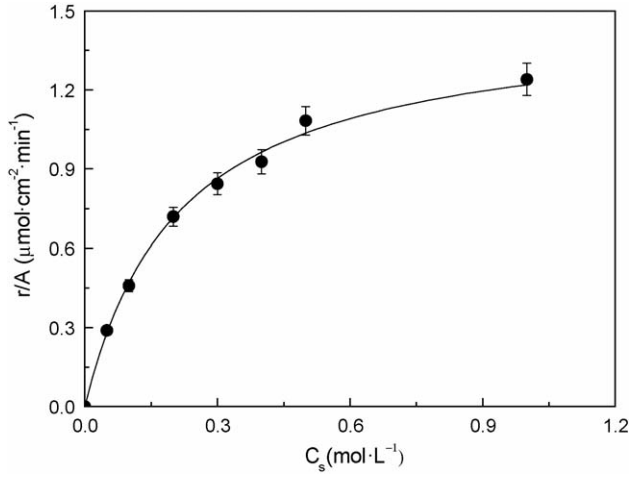

Fig. 7. Variation of initial hydrolysis rate of olive oil per unit membrane area with substrate concentration (C_E = 0.020 ± 0.002 mg-protein cm^{-2}). (—), theoretical results.

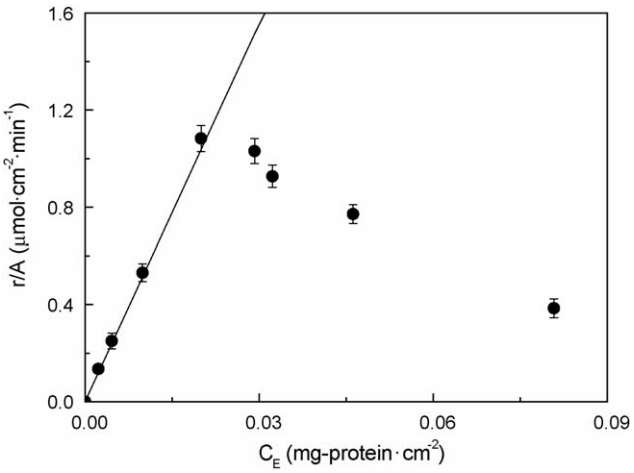

Fig. 8. Variation of initial hydrolysis rate of olive oil per unit membrane area with enzyme loading (C_s = 0.5 mol/L). (—), theoretical results.

3.4. Effect of enzyme loading

The enzyme loading on the membrane was easy to control by filtration with different volume of enzyme solution. Fig. 8 shows the relation between the initial hydrolysis rate per unit membrane area and the enzyme loading per unit membrane area. When the enzyme loading is less than 0.02 mg-protein cm^{-2}, the experiment data corresponds to the Michaelis–Menten Eq. (3). With further increasing of the enzyme loading, the initial hydrolysis rate per unit membrane area decreased. A possible explanation for this behavior is that too many enzymes fill in pores of the PTFE layer which remarkably reduces the pore size and porosity of the membrane. Low substrate concentration and high FFA concentration in the reaction microenvironment of the immobilized enzymes inhibited their activity. Therefore, the enzyme loading must be optimized to adjust the relationship between the activity per unit membrane area of immobilized enzyme and the diffusion of the substrate and product.

When enzymes are within the membrane, in order for the reaction to occur, the substrate has to be transported through the membrane to the catalyst and the product has to be transported from the reaction site to the other side of the membrane. In general, it is the mass-transport resistance that primarily influences the performance of these reaction systems. In order for a reactor to function at its optimal performance, it should work in a reaction-limited regime rather than a diffusion-limited

regime. The parameter that can give a measure of the condition is the Thiele modulus [38], which is given by the following equation [39]:

$$\phi = L\sqrt{\frac{V_{max}}{DK_m}} \quad (4)$$

where φ is Thiele modulus, L the thickness of the membrane, r_{max} the maximum velocity of the reaction and D is the diffusion coefficient of the substrate through the membrane. r_{max} can be calculated by following equation:

$$r_{max} = \frac{k_{max}C_E A}{V} \quad (5)$$

where V is the volume of the organic phase. In Eqs. (4) and (5), K_m and k_{max} had been calculated from Eq. (3). The overall mass transfer coefficients of the substrate in isooctane through the PTFE layer with different amount of enzyme loading were determined in reaction condition and shown in Table 2. By using Eq. (4), the Thiele modulus was calculated. φ has the physical meaning of a ratio between the reaction rate and the diffusion rate. When φ is sufficiently large ($\varphi \geq 3$), diffusion of substrate is slow relative to consumption. In such a situation with a diffusion-limited rate, it may be assumed that all substrate is utilized in a thin region within the membrane adjacent to immobilized enzymes. When $\varphi \leq 0.3$, the system is essentially controlled by kinetics and the mass-transfer limitation is negligible [38]. Thiele modulus of immobilized enzyme membranes was calculated and shown in Table 2. It is found that the effect of mass-transfer on overall kinetics of immobilized enzyme membranes was larger and larger with the increasing of enzyme loading. This could be attributed to the reducing of pore size and porosity of membranes because of the blocking action of enzymes and other macromolecule impurities as well as the compression action by liquid nitrogen during filtration process. Therefore, the enzyme loading should be optimized to ensure that the mass-transfer limitation is negligible. In this study, enzyme loading of 0.020 ± 0.002 mg-protein/cm^2 was the optimal value with the highest activity per unit membrane area and without any mass-transfer limitation.

3.5. Effect of operating variables on performance of the biphasic enzyme membrane reactor

Fig. 9 shows the effect of the aqueous phase pH on activity of the free and the immobilized lipases. It was found that the

Table 2
Effect of mass transfer on overall kinetics of immobilized enzyme membranes

C_E (mg-protein/cm^2)	$D \times 10^{-8}$ m^2/h	Φ	Limiting rate process	Extent of mass-transfer limitation
0.002	9.350	0.074	Reaction	Negligible
0.010	3.995	0.253	Reaction	Negligible
0.020	2.134	0.490	Both	Little
0.040	0.3995	1.601	Both	Medium
0.080	0.09350	4.680	Diffusion	Large

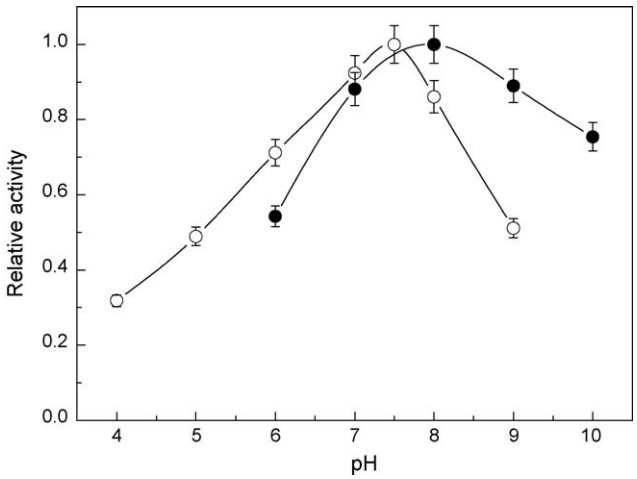

Fig. 9. Effect of pH on the activity of free and immobilized lipases: (◯) free lipase, (●) immobilized lipases.

optimum pH for the free lipase was about 7.5, whereas those for the immobilized lipases shifted to the alkaline region at about 8.0. It could be explained as that upon immobilization, the active site became more exposed to solvent than that in the globular, folder, dissolved lipase form. Therefore, proton transfer to the amino acid residues at the active site became less hindered [40]. But the shift of optimum pH was not great and it implied that interaction between enzymes and carries was not strong because of the physical immobilization method.

As shown in Fig. 10, the immobilization made the optimum temperature for lipase activity shift from about 35 °C of the free enzyme to 40 °. It could be attributed to a low restriction in the diffusion of the substrate and products at higher reaction temperature. In addition, the improved resistance of protein to thermal denaturation was also an important factor.

3.6. Reuse stability of the immobilized enzyme membrane and regeneration of membranes

Reuse stability for the immobilized enzyme is very important in economics, and an increased stability can make the immobi-

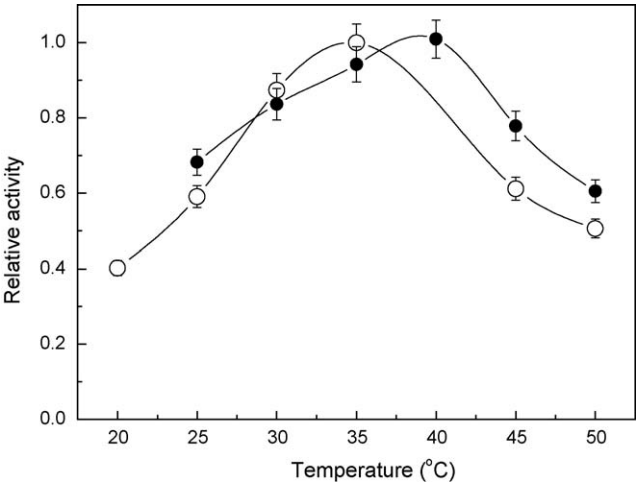

Fig. 10. Effect of temperature on the activity of free and immobilized lipases: (◯) free lipase, (●) immobilized lipases.

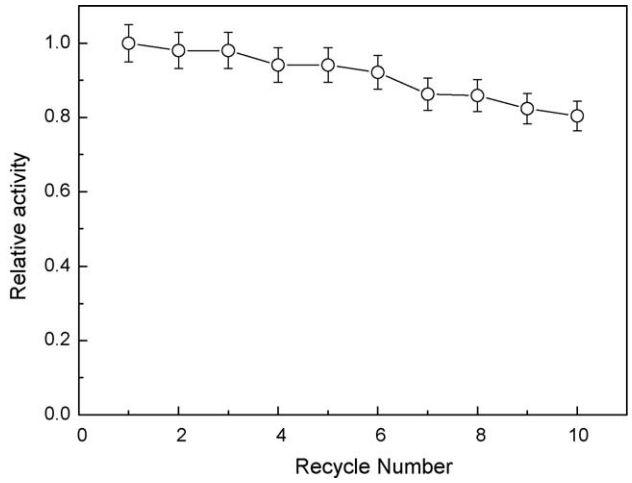

Fig. 11. Reuse stability of the immobilized lipases.

lized enzyme more advantageous than its free counterpart. To investigate the reuse stability, the enzyme-immobilized membranes were washed with phosphate buffer solution (0.05 M, pH 7.5) after one catalysis run and reintroduced into a fresh substrate solution for another hydrolysis at default operation conditions for 5 h. The results of the reuse stability of the immobilized lipase are shown in Fig. 11. After being used 10 times, 80% of activity remained. These results could be explained by the inactivation of the enzyme by the denaturation of the protein and the leakage of protein from the support upon use.

When immobilized enzymes were deactivated after a half-life time operation, cleaning the membrane to regenerate the membrane reactor was needed. A backwash process with ethanol and de-ionized water was carried out to take away inactive enzymes, and the regenerated membrane was used to immobilize lipases again. A comparison of immobilized lipases activity on a fresh membrane and a regenerated membrane is shown in Fig. 12. It could be found that the activity on the regenerated membrane was lower than on the fresh membrane but still remained at a high level. This indicated that a long-term use of this composite membrane for immobilization of the lipases, while maintaining high activity, was possible.

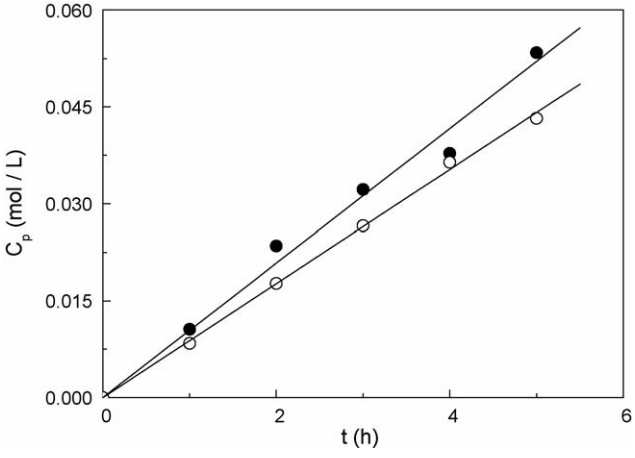

Fig. 12. Time-course variations of FFA concentration, $C_s = 0.5$ mol/L, $C_E = 0.020 \pm 0.002$ mg-protein cm^{-2}: (◯) for a regenerated membrane, (●) for a fresh membrane.

4. Conclusion

In the present study, a specially designed microstructure in the composite membrane composed of porous PTFE layer and relatively dense CA layer was prepared. Lipases were successfully immobilized on the composite membrane by the filtration process. The relatively dense CA layer rejected enzymes controlling the enzyme loading which prevented enzymes from being dissolved into the aqueous phase. The porous PTFE layer supplied a hydrophobic environment and a large specific surface area for the immobilization of lipases which were propitious to the activation of lipase. High activity (1.20 ± 0.04 μmol-FFA/cm^2 min) was obtained from lipase immobilized in the CA/PTFE composite membrane. The kinetic parameters of the immobilized lipases were calculated from the experiment data which was in good agreement with the Michaelis–Menten equation. Thiele modules were used to evaluate the effect of the mass transfer through the membrane with different enzyme loading. An optimum enzyme loading of 0.020 ± 0.002 mg-protein cm^{-2} obtained the highest activity without being diffusion-limited. Effects of temperature and pH on the immobilized lipase were investigated and compared with those of free lipase. The immobilized lipase retained 80% residual activity after 10 hydrolysis cycles. We conclude that the composite membrane with this structure was an effective carrier of the enzyme immobilization. However, the performance of the reactor with a flat membrane is not very good because of the small surface area. In subsequent work, this technology will be incorporated into a hollow fiber reactor to increase the surface area, which is more suited for industry applications.

Acknowledgements

This work was supported by National Science Foundation of China (20206012) and the National Basic Research Program (973 Plan) (No. 2003CB615701).

References

[1] P. Grochulski, Y.G. Li, J.D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin, M. Cygler, J. Mol. Catal. B: Enzym. 268 (1993) 12843.
[2] G. Pugazhenthi, A. Kumar, J. Membr. Sci. 228 (2004) 187.
[3] D. Han, J.S. Rhee, Biotechnol. Bioeng. 28 (1986) 1250.
[4] J.L. Lopez, S.L. Matson, J. Membr. Sci. 125 (1997) 189.
[5] V.M. Balcao, A.L. Paiva, F.X. Malcata, Enzyme Microb. Technol. 18 (1996) 392.
[6] A.E. Ivanow, M.P. Schneider, J. Mol. Catal. B: Enzym. 3 (1997) 303.
[7] Q.Z.K. Zhou, X.D. Chen, J. Food Eng. 48 (2001) 69.
[8] T.-C. Hung, R. Giridhar, S.-H. Chiou, W.-T. Wu, J. Mol. Catal. B: Enzym. 26 (2003) 69.
[9] S.W. Tsai, S.S. Shaw, J. Membr. Sci. 146 (1998) 1.
[10] J. Parrado, F. Millan, J. Bautista, Proc. Biochem. 30 (1995) 735.
[11] H. Ma, J. He, D.G. Evans, X. Duan, J. Mol. Catal. B: Enzym. 30 (2004) 209.
[12] A. Dyal, K. Loos, M. Noto, S.W. Chang, C. Spagnoli, K.C.P.M. Shafi, A. Ulman, M. Cownam, R.A. Gross, J. Am. Chem. Soc. 125 (2003) 1684.

[13] T. Itoh, N. Ouchi, Y. Nishimura, H.S. Hui, N. Katada, M. Niwa, M. Onaka, Green Chem. 5 (2003) 494.
[14] M.M. Hoq, T. Yamane, S. Shimidzu, J. Am. Oil Chem. Soc. 62 (1985) 1016.
[15] M.M. Hoq, T. Yamane, S. Shimidzu, Agric. Biol. Chem. 49 (1985) 3171.
[16] M. Rucka, B. Turkiewicz, Biotechnol. Lett. 11 (1989) 167.
[17] T. Godjevargova, K. Gabrovska, J. Biotechnol. 103 (2003) 107.
[18] A. De Maio, M.M. El-Masry, M. Portaccio, N. Diano, S. Di Martino, A. Mattei, U. Bencivenga, D.G. Mita, J. Mol. Catal. B: Enzym. 21 (2003) 239.
[19] H.T. Deng, Z.K. Xu, J. Wu, P. Ye, Z.M. Liu, P. Seta, J. Mol. Catal. B: Enzym. 28 (2004) 95.
[20] H.T. Deng, Z.K. Xu, Z.M. Liu, J. Wu, P. Ye, Enzyme Microb. Technol. 35 (2004) 437.
[21] I.A. Alsarra, S.S. Betigeri, H. Zhang, B.A. Evans, S.H. Neau, Biomaterials 23 (2002) 3637.
[22] A. Fishman, I. Levy, U. Cogan, O. Shoseyov, J. Mol. Catal. B: Enzym. 18 (2002) 121.
[23] U. Bora, K. Kannan, P. Nahar, J. Membr. Sci. 250 (2005) 215.
[24] S.S. Betigeri, S.H. Neau, 23 (2002) 3627.
[25] H.A. Sousa, C. Rodrigues, E. Klein, C.A.M. Afonso, J.G. Crespo, Enzyme Microb. Technol. 29 (2001) 625.
[26] N. Hilal, R. Nigmatullin, A. Alpatova, J. Membr. Sci. 238 (2004) 131.
[27] S.T. Bouwer, F.P. Cuperus, J.T.P. Derksen, Enzyme Microb. Technol. 21 (1997) 291.
[28] M.Y. Arica, H.N. Testereci, A. Denizli, J. Chromatogr. A 799 (1998) 83.
[29] Z. Sroka, J. Membr. Sci. 97 (1994) 209.
[30] T.H. Anna, A. Noworyta, Desalination 144 (2002) 427.
[31] K. Sakaki, L. Giorno, E. Drioli, J. Membr. Sci. 184 (2001) 27.
[32] R.L. Li, Biochemistry Experiment, Publishing Company of Wuhan University, WUhan, 1998, p. 93.
[33] B. Stellmach, Bestimmungsmethoden Enzyme, Chinese Light Industry Press, Beijing, 1992, p. 229.
[34] D.Y. Kwon, J.S. Rhee, J. Am. Oil Chem. Soc. 63 (1986) 1.
[35] M. Goto, M. GoTo, F. Nakashio, K. Yoshizuka, K. Inoue, J. Membr. Sci. 74 (1992) 207.
[36] P. Ye, Z.K. Xu, A.F. Che, J. Wu, P. Seta, Biomaterials 26 (2005) 6394.
[37] J.M. Lee, Biochemical Engineering, Prentice Hall, New Jersey, 1992, p. 24.
[38] J.E. Bailey, D.F. Ollis, Biochemical Engineering Fundamentals, McGraw-Hill, 1986, p. 157.
[39] L. Giorno, E. Drioli, Trends Biotechnol. 18 (2000) 339.
[40] S. Duinhoven, R. Poort, D. Van der Voet, W.G.M. Agerof, W. Rorde, J. Lyklema, J. Colloid Interface Sci. 170 (1995) 340–350.