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Journal of Molecular Catalysis B: Enzymatic 38 (2006) 119–125
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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# Resolution of *N*-(2-ethyl-6-methylphenyl)alanine via free and immobilized lipase from *Pseudomonas cepacia*

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Received 13 October 2005; received in revised form 23 November 2005; accepted 10 December 2005
Available online 18 January 2006

**Abstract**

A biocatalytic approach for the production of (*S*)-*N*-(2-ethyl-6-methylphenyl)alanine, a key intermediate for (*S*)-Metolachlor, has been developed by the use of lipase-catalyzed hydrolytic kinetic resolution. Compared with other selected lipases, the lipase from *Pseudomonas cepacia* (PSL) gives good conversion (48.2%) and excellent enantioselectivity (*E*-value > 100) to obtain enantiomerically pure (*R*)-acid (99% $e.e._p$). Then, a simple second resolution procedure is used to prepare (*S*)-acid product from the remaining (*S*)-ester without any reduction in enantiomeric excess (98% $e.e._p$) using the lipase B from *Candida antarctica* (CAL-B). Subsequent PSL immobilized on mesoporous SBA-15 molecular sieve is studied. The resulting supported enzyme catalyst exhibits higher activity, stability as well as reusability, compared with free enzyme.
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*Keywords:* *Pseudomonas cepacia* lipase; Resolution; (*S*)-*N*-(2-ethyl-6-methylphenyl)alanine; SBA-15; Immobilization

## 1. Introduction

The use of lipases for the kinetic resolution of racemic substances has been a major research area in the last few years [1,2], because these enzymes are fairly stable in organic solvents [3] and they can catalyze hydrolysis [4] as well as synthesis reactions of water-insoluble oily substances encountered in many processes [5,6]. (*S*)-(−)-*N*-(2-ethyl-6-methylphenyl) alanine ((*S*)-NEMPA) is an important precursor in the synthesis of most widely used herbicides such as (*S*)-Metolachlor [7]. (*S*)-NEMPA is currently produced by chemical synthesis in large quantities [8]. Nevertheless, the chemical method requires drastic reaction conditions that may cause racemization, decomposition or side reactions. In contrast, enzyme-catalyzed reactions are less hazardous, polluting, and energy-intensive than conventional chemistry-based.

The aim of the present study is to develop a stereoselective hydrolytic process for the production of the optical enantiomer of NEMPA via lipase. For commercial applications, the lipase must be sufficiently enantioselective to resolve the racemic mixture into enantiomerically pure forms and, moreover, the enzyme should be highly active and stable during the reaction. In this paper, the catalytic properties of different lipases are compared, and the effects of various racemic alkyl esters, operation temperature, enzymatic concentration and surfactants on the activity and enantioselectivity of free PSL for the kinetic resolution are initially investigated in order to obtain the optimum reaction conditions, and then the immobilization of PSL is investigated.

Immobilization of lipases on solid supports [9,10] offers several advantages, including simple recovery allowing repeated use of the catalyst, easy separation of enzyme from product, possibility of continuous operation, and improvement of enzyme stability. The various matrices [11,12], including inorganic and organic materials, have been explored to use as the supports for an immobilization of the enzyme catalyst to improve its catalytic efficiency and reduce the cost of using enzyme catalyst. Compared with the organic support, the inorganic support [13] can be regenerated easily and reused in case of the deactivation of enzyme immobilized, which can reduce the cost of support. In addition, the surface properties and structures of the inor-

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doi:10.1016/j.molcatb.2005.12.003

ganic support can be controlled more easily and more stable. As inorganic support materials, molecular sieves [14,15] offer interesting properties, such as high surface areas, hydrophobic or hydrophilic behavior and electrostatic interactions, as well as mechanical and chemical resistance, which make them attractive for enzyme immobilization.

Herein, the hexagonal mesoporous SBA-15-type molecular sieve [16,17] is used for the immobilization of PSL. The suitability of SBA-15 for enzyme immobilization is examined, with focus on the effect of the chemistry of SBA-15 on the activity and enantioselectivity of enzyme, the leaching problem following enzyme immobilization, and the activity, stability as well as reusability of immobilized enzyme.

## 2. Experimental

### 2.1. Materials

*Pseudomonas* sp. lipase (PSL) and *Candida cylindracea* A.Y. lipase (AYL) were purchased from Amano Pharmaceutical Co. Ltd. (Japan). *Candida antarctica* lipase B (CAL-B) was kindly donated by Novo Nordisk Industries (China). *Porcine pancreatic* lipase (PPL) was purchased from Shanghai Dongfeng Biochemical Reagent Co. Ltd. (China). *Candida lipolytic* lipase (CLL) was provided by Wuxi enzyme preparation plant (China). *Penicillium expansum* lipase (PEL) was provided by Nantong Pharmaceutical Co. Ltd. (China). Molecular sieve MCM-41, MCM-48 and SBA-15 were kindly donated by College of Chemistry, Jilin University (Changchun, China). Tween-80 was bought from Tianjin Chemical Reagent, China. Bis(2-ethylhexyl)sodium sulfosuccinate (AOT) was bought from Shanghai Reagent, China. Hexadecyltrimethyl ammonium bromide (CTAB) was bought from Shanghai Huishi Biochemical Reagent, China. The authenticity of racemic esters prepared during the study was confirmed by spectroscopic analysis including 300 MHz NMR (Mercury-300B, VARIAN, USA) and GC–MS (Saturn 220, VARIAN, USA). Reactions were routinely monitored on silica gel plates (Qingdao Haiyang Chemical Co. Ltd., China) using UV light for detection of the spots. All the organic solvents were reagent grade and used without further purification. Other reagents were all analytical grade or better.

### 2.2. Determination of conversion and enantiomeric excess ($e.e._p$)

The analysis of the reaction mixtures and the determination of enantiomeric excesses of (*S*)-NEMPA were performed by capillary zone electrophoresis (P/ACE MDQ, Beckman, USA) with a 59 cm (49 cm to detector) × 50 μm i.d. eCAP$^{TM}$ neutral capillary (Beckman, USA). The conversion of reaction was determined by using 100 mmol/L triethylamine/acetic acid buffer (TEAA, pH 5.5) as background electrolyte. The enantiomeric excess of (*S*)-NEMPA was successfully analyzed in 100 mmol/L TEAA, pH 5.5 by using 40 mmol/L 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD, Beckman, USA) as a buffer additive. The analysis was performed with applied voltage at −20 kV, and the absorbance was recorded at 200 nm.

Enantiomeric ratio (*E*) of hydrolysis of racemic *N*-(2-ethyl-6-methylphenyl) alanine methyl ester was calculated from the conversion ($c$) and enantiomeric excess ($e.e._p$) of (*S*)-NEMPA, using the equation [18]: $E = \ln[1 - c(1 + e.e._p)]/\ln[1 - c(1 - e.e._p)]$, where $e.e._p = (c_S - c_R)/(c_S + c_R)$, while $c_S$ and $c_R$ are concentrations of the (*S*)- and (*R*)-enantiomers, respectively. The absolute configuration of the enantiomers was established by comparison of the measured optical rotation with the literature data [8].

### 2.3. Preparation of racemic esters of NEMPA

The reaction mixture of 2-ethyl-6-methylaniline (8.4 mL, 60 mmol), $NaHCO_3$ (5.5 g, 65 mmol) and alkyl 2-bromopropionate (180 mmol), was stirred under nitrogen atmosphere and slowly heated up to 120–125 °C in 1 h. Then the dark reaction mixture was continually kept at the temperature for 18 h with stirring. After it was cooled, the reaction mixture was transferred into 30 mL of ice water and extracted with ethyl acetate. The ethyl acetate fractions were dried over sodium sulfate and concentrated in a rotary evaporator at 40 °C. The resulting ester after normal work up was purified by column chromatography on silica gel using ethyl acetate and petroleum ether (1:5) as the eluant to furnish the corresponding ester. *N*-(2-ethyl-6-methylphenyl) alanine methyl ester (NEMPA-ME, 8.9 g): GC–MS (*m/z*): 221 (25, $M^+$), 162 (100, $M^+$-(C=O)$OCH_3$), 133 (30, $M^+$-(C=O)$OCH_3$–$C_2H_5$), 77 (11, Ph); GC: 98% area. $^1H$ NMR ($CDCl_3$), δ: 7.02–6.96 (m, 2H, Ph), 6.88–6.83 (t, 1H, Ph, J = 7.2 Hz), 3.96–3.94 (q, 1H, CH, $J$ = 6.9 Hz), 3.81 (S, 1H, NH), 3.66 (S, 3H, $CH_3$), 2.69–2.66 (m, 2H, $CH_2$), 2.30 (S, 3H, $CH_3$), 1.38–1.35 (d, 3H, $CH_3$, $J$ = 6.9 Hz), 1.26–1.21 (t, 3H, $CH_3$, $J$ = 7.5 Hz); $^{13}C$ NMR ($CDCl_3$), δ: 176.2, 143.6, 135.3, 129.6, 129.1, 126.9, 122.4, 55.8, 52.2, 24.6, 19.9, 19.2, 14.7.

### 2.4. Preparation of SBA-15 immobilized PSL

Crude enzyme powder (PSL, 20 mg) was added in phosphate buffer (25 mM, pH 8.0, 5.0 mL). The mixture was stirred for 1 h at 4 °C, then the supernatant was separated by centrifugation and the volume was measured. Solid support SBA-15 (0.3 g) was suspended in supernatant solution (pH 8.0) for 2 h at 4 °C under stirring. The immobilization of PSL had previously been found to be optimum at pH 8.0 (unpublished results). The supernatant was first separated from the solid materials by centrifugation. The solid was washed with phosphate buffer (pH 8.0) and air-dried. The amount of enzyme immobilized on the solid support was measured by the Lowry method with bovine serium albumin (BSA) as a standard for protein concentration [19].

### 2.5. Determination of immobilized PSL activity

The method is based on the hydrolysis of tributyrin by the enzyme and titrating the butyric acid produced [20]. The 6.0 mL phosphate buffer (25 mM, pH 8.0) was incubated in vessel at 37 °C and stirred sufficiently. Then 50 μL tributyrin was added and the pH-stat was started to keep the pH at 8.0. When the

pH stabilized, 10 mg immobilized PSL was added and the consumption of 0.05 mol/L NaOH were determined. In addition, a blank experiment without added enzyme was carried by the above assay procedure. From the amount of alkali consumed, the equivalent amount of butyric acid in the samples was calculated and the enzyme activity was determined. One lipase unit (IU) is defined as the amount of enzyme required to produce 1 μmol of free fatty acid per min at 37 °C.

### 2.6. Preparation of (S)-NEMPA via two-step resolution

To the aqueous phosphate buffer (100 mmol/L, pH 8.0) containing surfactant CTAB (40 mg/mL) 50 mL, free PSL (300 mg) and (*R*,*S*)-NEMPA-ME (5.0 mmol, 1.1 g) were added. The resulting mixture was shaken at 50 °C and the pH was maintained using 0.1 mol/L NaOH solution. Aliquots were periodically drawn and analyzed on capillary electrophoresis. When the hydrolysis reached 50% conversion, a saturated solution of $NaHCO_3$ (100 mL) was added to the reaction mixture. The mixture was first extracted with ether (3× 100 mL) to remove (*S*)-NEMPA-ME. The aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3× 100 mL) to remove the acid product. Acid extracts were dried over anhydrous $Na_2SO_4$ and evaporated to give (*R*)-(+)-NEMPA (0.50 g, e.e.$_p$ = 99.0%). The hydrolysis catalyzed by immobilized PSL (9.6 g) was performed by the same procedure.

To the aqueous phosphate buffer (100 mmol/L, pH 8.0) containing diethyl ether (15%, v/v) 50 mL, CAL-B (100 mg) and (*S*)-NEMPA-ME (0.5 g) were added. The mixture was stirred at 25 °C and the pH was maintained using 0.1 mol/L NaOH solution. When the hydrolysis reached 49% conversion, a saturated solution of $NaHCO_3$ (100 mL) was added to the reaction mixture. The mixture was first extracted with ether (3 × 100 mL), the aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3 × 100 mL) to remove the acid product. Acid extracts were dried over anhydrous $Na_2SO_4$ and evaporated to give (*S*)-(−)-NEMPA (0.48 g, e.e.$_p$ = 98.0%).

Table 1
Lipase-catalyzed hydrolysis of (*R*,*S*)-NEMPA-ME[a]

| Lipase | Time (h) | Conversion (%) | e.e.$_p$ (%) | *E*-value | Major enantiomer |
|---|---|---|---|---|---|
| CAL-B | 2 | 48.2 | 69.4 | 10.6 | *S*-(−) |
| PSL | 60 | 48.2 | 99.0 | >100 | *R*-(+) |
| PPL | 72 | 1.7 | 99.0 | >100 | *R*-(+) |
| CLL | 72 | 0.24 | 98.0 | 99.2 | *S*-(−) |
| AYL | 72 | 2.0 | 78.8 | 8.6 | *S*-(−) |
| PEL | 72 | 0.12 | 58.5 | 3.8 | *S*-(−) |

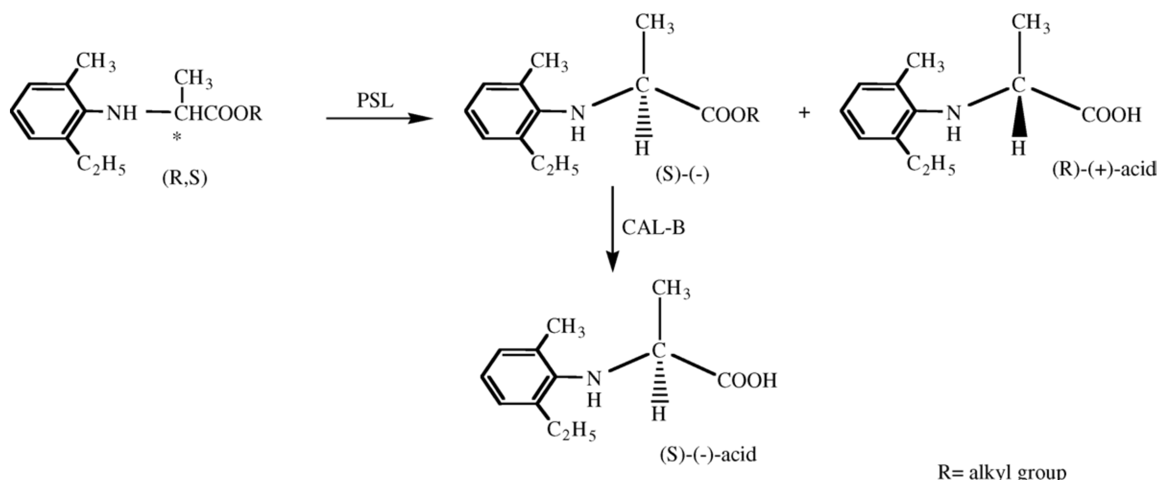
[a] *Reaction conditions*: buffer, 100 mmol/L phosphate buffer (pH 8.0, 5.0 mL); substrate, 0.5 mmol; enzyme, 105 U; *T*, 37 °C.

## 3. Results and discussion

### 3.1. Selection of enzyme

This study focused on the identification of suitable lipase for the enantiomeric resolution of (*R*,*S*)-NEMPA (Scheme 1). Six lipases were screened for the enantioselective hydrolysis of racemic *N*-(2-ethyl-6-methylphenyl) alanine methyl ester (NEMPA-ME) to yield chiral NEMPA, and the screening results were listed in Table 1. The lipase CAL-B was very active and 48.2% of NEMPA-ME was hydrolysis after 2 h, but displayed poor enantioselectivity since it catalyzed the hydrolysis of both (*R*)- and (*S*)-enantiomers of NEMPA-ME, thus leading to the low enantioselectivity of enzyme (*E* = 10.6). With the aim to improve the enantioselectivity of CAL-B, the reaction conditions of CAL-B-catalyzed resolution of (*R*,*S*)-NEMPA-ME to produce (*S*)-NEMPA had been optimized by us, and reported in the previous paper [21]. The optimized results show that the highest enantioselectivity of CAL-B (*E* > 100) is achieved in diethyl ether/water (15%, v/v).

The *E*-value of >100 and enantiomeric excess (e.e.$_p$) of >98% were lipase PSL and PPL, but the conversion of PPL-catalyzed reaction was only 1.7. From the results, as far as the higher enantioselectivity is concerned, it is also significant that the lipase PSL is selected for further investigation, although it preferably




Scheme 1. Preparation of (*S*)-(−)-NEMPA by two-step resolution.

Table 2
Hydrolysis of racemic esters of NEMPA using PSL[a]

| Entry (*R*) | Reaction time (h) | Conversion (%) | e.e.$_p$ (%) | *E*-value |
|---|---|---|---|---|
| $CH_3$ | 60 | 48.2 | 99.0 | >100 |
| $C_2H_5$ | 60 | 45.8 | 99.0 | >100 |
| *n*-$C_3H_7$ | 96 | 42.5 | 99.0 | >100 |
| *i*-$C_3H_7$ | 96 | 10.7 | 99.0 | >100 |
| *n*-$C_4H_9$ | 96 | 28.6 | 99.0 | >100 |
| *i*-$C_4H_9$ | 96 | 29.2 | 99.0 | >100 |
| *t*-$C_4H_9$ | 96 | 32.6 | 99.0 | >100 |
| *n*-$C_5H_{11}$ | 120 | 24.1 | 90.5 | 26.5 |
| *i*-$C_5H_{11}$ | 120 | 30.2 | 84.9 | 17.5 |
| *t*-$C_5H_{11}$ | 120 | 36.5 | 99.0 | >100 |
| *n*-$C_6H_{13}$ | 120 | 9.8 | 84.6 | 13.1 |
| *n*-$C_8H_{17}$ | 120 | 1.0 | 80.0 | 9.1 |
| $CH_2C_6H_5$ | 60 | 38.5 | 99.0 | >100 |

[a] *Reaction conditions*: buffer, 100 mmol/L phosphate buffer (pH 8.0, 5.0 mL); substrate, 0.5 mmol; enzyme, 105 U; *T*, 37 °C.

produces the (*R*)-(+)-acid. Then, utilizing the higher activity of CAL-B and stereospecificity towards the (*S*)-enantiomer, simple second resolution procedure is used to prepare (*S*)-acid from the remaining (*S*)-ester without any reduction in enantiomeric excess (98% e.e.$_p$) (Scheme 1). Thus, by two-step resolution, we may obtain higher optically pure (*R*)- and (*S*)-NEMPA, respectively.

Other enzymes investigated in this paper gave both low conversion and enantioselectivity, practically making them useless.

### 3.2. Effect of various racemic alkyl esters on the activity and enantioselectivity of PSL

To date, little has been reported on the effectiveness of PSL with respect to various racemic alkyl esters of NEMPA, so, several esters of NEMPA are prepared to investigate its behavior. Table 2 summarizes the results of the hydrolysis of various racemic alkyl esters using PSL. In all the experiments, the biocatalytic system shows the same stereopreference for racemic esters giving the (+)-(*R*) acid and the unreacted (−)-(*S*) ester as hydrolysis products. The enantioselectivity of PSL was almost independent from the short-chain alkyl esters (*E*-value > 100 for all esters from methyl ester to butyl ester). The lowest enantioselective ratio ($E = 9.1$) was observed when *n*-octyl ester was used as a substrate. It was found that the reaction rate was dependent on the molecular size of esters, the larger the size of the alkyl groups in the substrate molecular, the lower the efficiency. It may be ascribed the bulkiness and steric effects of the substituents, which make them inaccessible to the active site of enzyme or cause obstruction in the interactions with enzyme. For the methyl ester of racemic NEMPA (NEMPA-ME), the enzyme displayed a fairly good conversion (48.2%) and yielded e.e.-values of 99% for the acid at a relatively short time, and then was selected as substrate for further optimization studies.

### 3.3. Enhancement of the reaction rate

From the above results, it may be observed that the reaction rate of PSL-catalyzed hydrolysis is not fast enough to obtain the target product (it needs 60 h to be up to 48.2% conversion at 37 °C), implying that further optimization studies such as the influence of reaction temperature, enzymatic concentration as well as surfactants to fasten the reaction rate are needed.

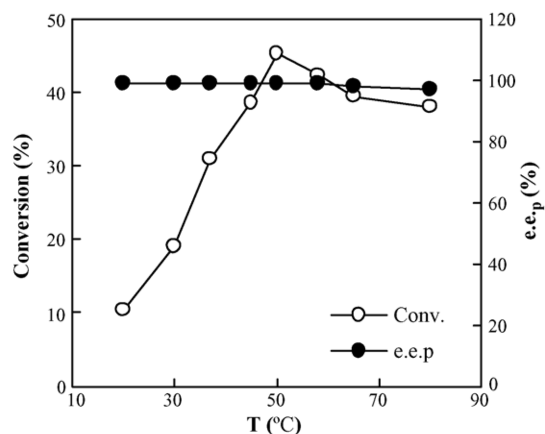


Fig. 1. Effect of temperature on the conversion and enantiomeric excess in the kinetic resolution of NEMPA.

The PSL-catalyzed hydrolysis of (*R*,*S*)-NEMPA-ME at pH 8.0 in a temperature range from 20 to 80 °C was investigated. The results plotted in Fig. 1 show that the increase of temperature generally led to increase the reaction rates, and the highest activity of PSL was obtained at 50 °C (the conversion is 45.3% only after 35 h). The little enantiomeric excess changes occurred in this range (e.e.$_p \sim 99\%$), which suggest the selectivity of PSL-catalyzed resolution of NEMPA is not sensitive to the temperature.

The enzymatic concentration used in the reaction is another parameter worthy of careful optimization. The effect of enzyme concentration on the reaction rate was studied at pH 8.0 and 50 °C. As can be seen from Fig. 2, increasing the enzyme concentration increased the reaction rates, and the maximum yield (49.3% after 17 h) was obtained at enzyme concentration of 6.0 mg/mL, which may be attributed to the enhanced hydrolysis of (*R*,*S*)-NEMPAME with increasing enzymatic concentrations.

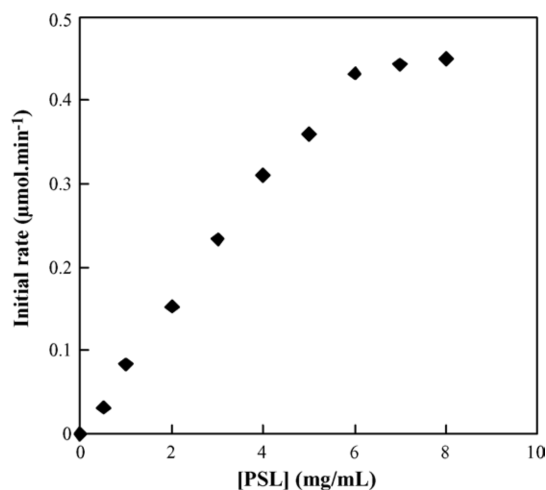


Fig. 2. Effect of enzymatic concentration on the initial rate in the resolution of NEMPA.

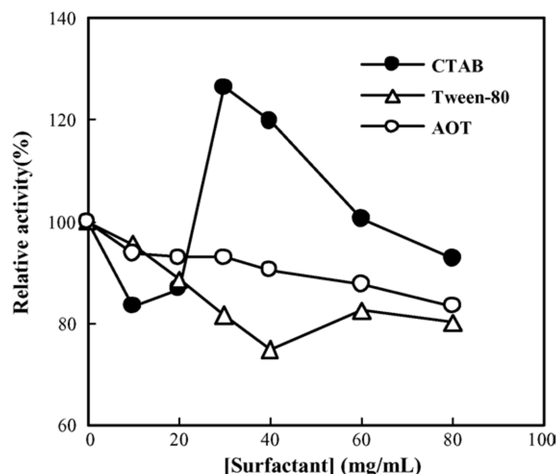



Fig. 3. Effect of suractants concentration on the activity of PSL in the resolution of NEMPA.

However, continue to increase the amount of enzyme, in practice, the increase in the reaction rate will become less, which may be ascribed to the increasing viscosity of enzyme or a finite amount of substrate present once that has been converted to product the enzyme becomes ineffective.

It is well known that surfactants can be applied in lipase assay to increase the lipid–water interfacial area, which, in turn, enhances the observed rates of lipase-catalyzed reactions [22,23]. For ensuring the effects of surfactants on the catalytic activity of PSL, three kinds of surfactants (non-ionic, cationic and anionic) are selected as additives to be systematically investigated. The results (Fig. 3) show that the activity of PSL is drastically improved in the presence of the cationic surfactant (CTAB), whereas the anionic surfactant (AOT) and non-ionic surfactant (Tween-80) make the enzyme activity decrease. At the optimum additive condition (40 mg/mL CTAB), the activity of PSL was about 1.3-fold as that in pure buffer medium.

All in all, the approaches used in this paper to fasten the reaction rate are effective. Under the optimized conditions (reaction temperature: 50 °C; enzymatic concentration: 6.0 mg/mL; CTAB additive concentration: 40 mg/mL), the reaction time is evidently shortened (from 60 to 8 h), and the enantiomeric excess of (*R*)-acid is 99.0% $e.e._p$ at 49.5% conversion.

### 3.4. PSL Immobilization

From an application point of view, it is necessary for the study of enzyme immobilization, because it may combine the physical properties of support material with the basic biochemical activity of enzyme, and offer an operational advantage over free enzyme, and consequently decrease the use cost of enzyme [24]. Adsorption is one of the simplest methods of physical immobilization of enzyme with the added advantage of being inexpensive and “mild” towards the enzyme [9]. This method profits from the interactions between the support surface and the outer shell of the enzyme.

In this paper, three different types of mesoporous molecular sieves (MCM-41, MCM-48 and SBA-15) were selected because of the differences in their pore dimensions and structures (Table 3). The enzyme chosen in this study was PSL (MW = 31 000, average molecular diameter about 50 Å). From the immobilization results (Table 3), it was found that the adsorbed amount of protein, immobilization efficiency and activity retention were dependent on the pore diameter of molecular sieves. On increasing the channel size of molecule sieve, the immobilized PSL showed a significant improvement in terms of activity. So, the hexagonal structure of SBA-15 could be more suitable for enzyme immobilization. This may be explained as that the small pore diameter of MCM-41 and MCM-48 appears not to be large enough for PSL molecules to enter the structure, and the enzyme would only be adsorbed to the outer surface of particles, resulting in rapid inactivation, the lower immobilization efficiency and activity; whereas the pore diameter of SBA-15 was measured to be 62 Å, which is slightly larger than the PSL molecule (50 Å), indicating that the PSL molecules were able to diffuse into the channels of SBA-15, and the silanols located on the pore walls of SBA-15 could also promote adsorption through hydrogen bonding interactions with the hydrophilic residues of enzyme. However, the immobilization environment would easily induce the changes of immobilization results, it is necessary to determine the optimum immobilization conditions at which the enzyme-support interactions were savored. Herein, optimum immobilization was achieved using the phosphate buffer (25 mmol/L, pH 8.0), the ratio between enzyme and molecular sieve (1:15), the temperature (4 °C) and the immobilization time (2 h). The maximum amount of protein bound is 4.0 mg/g, the maximum immobilization efficiency is 69.2% and the activity retention is 59.9% under the optimum conditions.

### 3.5. Comparing the thermal stability of immobilized lipase with that of free enzyme

After the optimization of immobilization conditions, thermal stability in the presence of surfactant CTAB was examined, as it

Table 3
Physicochemical properties of mesoporous silica and the results of immobilization[a]

| Sample | Pore diameter (Å) | BET surface Area ($m^2\,g^{-1}$) | Pore volume ($cm^3\,g^{-1}$) | Adsorbed amount ($mg\,g^{-1}$) | Immobilization efficiency (%) | Activity retention (%) |
|---|---|---|---|---|---|---|
| MCM-41 | 16 | 1321 | 0.63 | 0.4 | 9.6 | 3.5 |
| MCM-48 | 24 | 1206 | 1.16 | 0.9 | 18.5 | 10.6 |
| SBA-15 | 62 | 832 | 1.29 | 4.0 | 69.2 | 59.9 |

[a] *Immobilization conditions*: phosphate buffer, 25 mmol/L (pH 8.0); enzyme/molecular sieve, 1:15; temperature, 4 °C; 2 h.

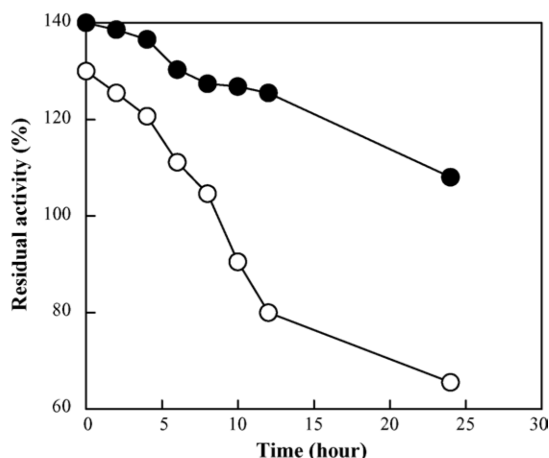



Fig. 4. Effect of thermal (50 °C) incubation of free (○) and immobilized (●) PSL on enzymatic activity.

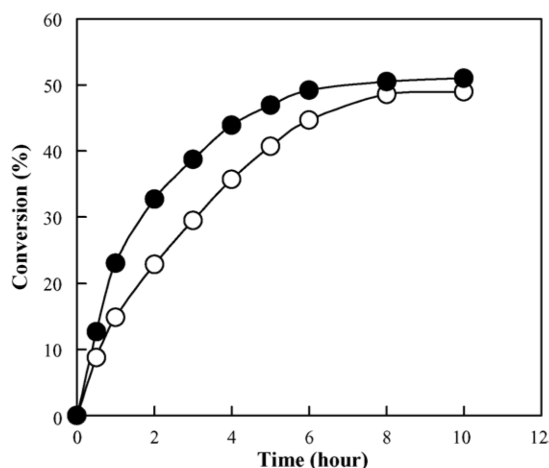



Fig. 5. The time-course for enantioselective hydrolysis of (*R*,*S*)-NEMPA-ME catalyzed by the free (○) and immobilized (●) PSL.

is important for industrial applications. It should be pointed out that the thermal stability was studied in the presence of CTAB, exactly because it was confirmed as a useful additive that can improve the activity of both immobilized PSL and free PSL up to 1.4- and 1.3-fold, respectively, compared to that in pure buffer media. In addition, we believe it is more significant to study the thermal stability under the most optimum conditions of hydrolyzing (*R*,*S*)-NEMPA-ME. Immobilized PSL and free PSL were incubated at 50 °C and pH 8.0 in the presence of CTAB (40 mg/mL) for 25 h, and the activity was measured periodically during the time. Herein, relative activity was calculated by assuming the initial activity of free PSL in the pure buffer medium (no CTAB) was 100%. As shown in Fig. 4, the thermal stability of both the enzymes were diminished to a certain content, and the free lipase lost 64.5% activities after 24 h, while only 32.0% activity was lost for the immobilized lipase. It may be concluded that the stability of enzyme could attain a satisfactory level through immobilization.

### 3.6. Comparing the catalytic activity of immobilized lipase with that of free enzyme

The correspondent protein amounts of free and immobilized lipases were tested in the optimized conditions of hydrolysis of (*R*,*S*)-NEMPA-ME. Kinetic studies were performed to determine the Michaelis constant, $K_m$, and the maximum velocity, $V_{max}$, of the free and immobilized lipase. The values of these parameters were determined from the Lineweaver–Burk plots. The value of $K_m$ of immobilized enzyme is almost identical to that of the free enzyme ($K_m$ is 0.1 mol/L), thus indicating that the affinity toward the substrate is not significantly modified. However, the values of $V_{max}$ are quite different ($V_{max}$ of the immobilized and free enzyme is 6.5 and 5.0 μmol min$^{-1}$, respectively), suggesting that the activity of immobilized lipase increases in the course of immobilization. From the time-course of the hydrolysis of (*R*,*S*)-NEMPA-ME catalyzed by the free and immobilized PSL (Fig. 5), we had also observed that the immobilized PSL exhibited a little higher activity, and the time of reaching the balance point in the immobilized PSL-catalyzed reaction was obviously shortened (about 6.2 h), compared with that of free lipase-catalyzed reaction (about 8 h). This can be accounted for that the free PSL may be dispersed equally on the support during immobilization, which decreases the chance of free enzyme convergence in the pure buffer, thus improving accessibility for the substrate to the active site of enzyme molecule and enhancing the activity of immobilized enzyme.

### 3.7. Recovery and reusability of immobilized lipase

In general, free enzymes are difficult to recover and re-use. Recovery and reusability of immobilized enzymes are, therefore, important aspects of the study. So, in this section, we will study the durability of the immobilized PSL in the repeated batch hydrolysis of (*R*,*S*)-NEMPA-ME. After 6 h for each run, the immobilized lipase was reused with fresh substrates. The relative activity of lipase immobilized on SBA-15 after recycling several times was given in Fig. 6. The initial activity of

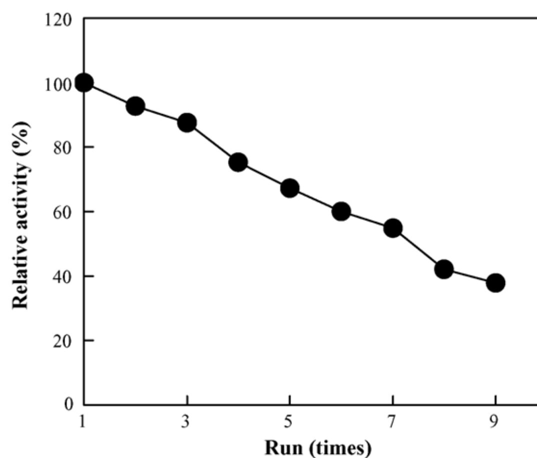



Fig. 6. Reusability of the immobilized PSL.

freshly prepared immobilized lipase in the first run was defined as 100% activity. Immobilized lipase could be used with little loss of activity for the first cycle. After six cycles, immobilized lipase still shows 54.8% of its initial activity in (*R*,*S*)-NEMPA-ME hydrolysis. It should also be stressed that the enantiomeric excesses of (*R*)-NEMPA were maintained over 99% throughout the eight repeated reactions.

The results indicated the possibility of re-using enzyme immobilized on SBA-15, but it was still not ideal because the activity of immobilized enzyme was decreased during the successive reuse, which may have a close relationship with the leaching of PSL from the support to give free enzyme. The leaching of PSL is possibly due to the weak interactions between PSL molecules and the support surface. Leakage of adsorbed enzyme into solution is a common disadvantage of the adsorption immobilization technique. A second possibility is the mechanical loss during the reaction and recovery of immobilized enzyme.

## 4. Conclusion

In conclusion, we had successfully developed a convenient and scalable enzymatic procedure for the preparation of both (*R*)- and (*S*)-NEMPA, taking advantage of the excellent enantioselectivity of lipase PSL and higher activity of lipase CAL-B. The method was easy to perform with standard equipment and could be widely applied. The kinds of esters strongly influenced the reaction rate, and the methyl ester was found to be the best substrate in terms of the reaction rate and enantiomeric excess. Improving the reaction temperature, enzymatic concentration and adding surfactant CTAB in the reaction medium can drastically enhance the reaction rate of PSL-catalyzed hydrolysis, and shorten reaction time (from 60 to 8 h). In addition, the immobilization of PSL had also been studied, and found that the activity, stability and reusability of immobilized enzyme were superior to those obtained from free PSL. It shows mesoporous molecular sieve SBA-15 had a higher potential in the immobilization of PSL, but leaching of enzyme during applications was still a major problem. This might be minimized by surface modification or covalent attachment of the enzyme to the solid. Further work in the area including decreasing the leakage of immobilized enzyme and studying the mechanism of immobilization is being done in our laboratory, and will be reported in due course.

## Acknowledgements

The authors are grateful for the financial support from National Natural Science Foundation of China (No. 20432010) and the Foundation of Research Program of Jilin University, China.

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