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ORIGINAL ARTICLE

Improvement of the enantioselectivity and activity of lipase from *Pseudomonas* sp. via adsorption on a hydrophobic support: kinetic resolution of 2-octanol

CHUANG DU[1,2], BO ZHAO[1], CHUNYUAN LI[1], PING WANG[1], ZHI WANG[1], JUN TANG[3], & LEI WANG[1]

[1]*Key Laboratory of Molecular Enzymology and Engineering, Ministry of Education, Jilin University, Changchun, People's Republic of China,* [2]*Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences, Changchun, People's Republic of China and* [3]*College of Chemistry, Alan G. MacDiarmid Institute, Jilin University, Changchun, People's Republic of China*

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Abstract

Pseudomonas sp. lipase (PSL) was successfully immobilized on a novel hydrophobic polymer support through physical adsorption and the immobilized PSL was used for resolution of (*R*,*S*)-2-octanol with vinyl acetate as acyl donor. Enhanced activity and enantioselectivity were observed from the immobilized PSL compared with free PSL. The effects of reaction conditions such as temperature, water activity, substrate molar ratio and the amount of immobilized lipase were investigated. Under optimum conditions, the residual (*S*)-2-octanol was recovered with 99.5% enantiomeric excess at 52.9% conversion. The results also indicated that the immobilized PSL could maintain 94% of its initial activity even after reusing it five times.

Keywords: Pseudomonas *sp. lipase, hydrophobic support, immobilization, resolution, 2-octanol*

Introduction

Enzymatic catalysis in non-aqueous media for synthetic applications has gained considerable importance in recent years (Krishna 2002; Hudson et al. 2005). However, the industrial use of enzymes presents a number of difficulties. Enzyme agglomeration is a common problem that may be encountered when powdered enzyme is employed in organic solvents (Dixon et al. 1994; Young et al. 1999). Agglomeration can reduce the catalytic rate by blocking the active site through protein–protein contacts. Another problem is how to recover and reuse the enzyme with high residual activity. It is well known that these problems can be overcome by immobilization of the enzyme on a suitable support material by either covalent or non-covalent attachment.

The methods for immobilization are varied in complexity and efficiency, such as physical adsorption, covalent attachment and physical entrapment. Among the immobilization techniques, physical adsorption is one of the simplest methods with higher commercial potential than other methods. In the past, physical adsorption has been studied with different types of support materials including different polymers (Koops et al. 1999; Panzavolta et al. 2005), molecular sieves (Serralha et al. 2002; Yu et al. 2007), silica composites (Goradia et al. 2005; Montiel et al. 2007) and carbonaceous materials (Darowski 2001; Dalla-Vecchia et al. 2005). It is evident from these reports that the nature and morphology of the support material can greatly influence enzyme accessibility and the resultant enzyme activity.

Correspondence: Lei Wang, Key Laboratory of Molecular Enzymology and Engineering, Ministry of Education, Jilin University, Changchun 130023, People's Republic of China. Tel: +86-431-88498972. Fax: +86-431-88980440. E-mail: w_lei@jlu.edu.cn
Co-corresponding authors.
E-mail addresses: chemjtang@jlu.edu.cn (Jun Tang); wangzhi@jlu.edu.cn (Zhi Wang)

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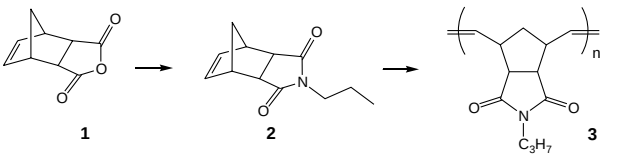
Recently, a number of papers have reported that the immobilization of lipases on hydrophobic supports can stabilize, activate and even enhance their properties (Reetz 1997; Fernández-Lorente et al. 2001, 2007; Palomo et al. 2002a,b,c, 2003; Nieto et al. 2005; Torres et al. 2006; Wilson et al. 2006; Mateo et al. 2007; Cunha et al. 2009; Hanefeld et al. 2009; Palomo 2009). Nieto et al. (2005) found that hydrophobic binding on Sepharose derivatives might result in hyperactivation of lipase through selective adsorption. Reetz (1997) reported that immobilization of lipases on hydrophobic sol–gels yields a higher activity recovery. Wilson et al. (2006) immobilized lipase QL from *Alcaligenes* sp. on a hydrophobic support (octadecyl-Sepabeads) by adsorption and found that the enzyme was hyperactivated after immobilization, the optimal temperature increasing from 50°C to 70°C. Furthermore, the enantioselectivity of the enzyme for the hydrolysis of glycidyl butyrate and its dependence on experimental conditions were significantly altered. The same group also found (Fernández-Lorente et al. 2007) that the immobilization of lipases on hydrophobic supports could greatly increase the specificity of lipases towards hydrophobic substrates. In general, a part of the lipase molecule covers the active site with a short α-helix called the 'lid' or 'flap'. The side of the α-helical lid facing the catalytic site, as well as the protein chains surrounding the catalytic site, are mainly composed of hydrophobic side chains (Miled et al. 2001). When lipases are immobilized on hydrophobic supports, the presence of hydrophobic interfaces on the hydrophobic supports may induce conformational rearrangement of the lipase, altering the corresponding functionality of the lipase immobilized on it.

In the present study, we synthesized a hydrophobic polymer **3** by the ring-opening metathesis polymerization method (ROMP, Scheme 1) and immobilized lipase from *Pseudomonas* sp. (PSL) on it through physical adsorption. The immobilized PSL was used in the resolution of 2-octanol through transesterification with vinyl acetate as acyl donor (Scheme 2) and the reaction conditions for the transesterification were optimized.



Scheme 1. Synthesis of hydrophobic polymer **3** by ROMP method.

Materials and methods

Materials

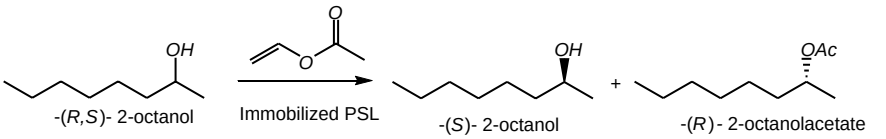
PSL was purchased from Sigma (Beijing, China). Folin substrate, 2-octanol, vinyl acetate and other organic solvents (analytical grade) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). *R*-(+)-1-phenylethyl isocyanate (*R*-(+)-PELC) was purchased from Fluka (Beijing, China). THF was distilled from sodium/benzophenone before use. *Cis*-5-norbornene-exo-2,3-dicarboxylic anhydride, propylamine, Grubbs Catalyst 1st and ethyl vinyl ether were purchased from Aldrich (Beijing, China) and used as received.

Preparation and characterization of hydrophobic polymer 3

*Synthesis of monomer **2** (N-propyl-exo-norbornene-5, 6-dicarboximide).* *Cis*-5-norbornene-exo-2, 3-dicarboxylic anhydride **1** (328 mg, 2 mmol) of in 10 mL of anhydrous benzene, 124 mg (2.1 mmol, 1.05 equiv.) of propylamine and 202 mg (2 mmol, 1 equiv.) of triethylamine were placed into a 50-mL round-bottomed flask equipped with a condenser and a Dean–Stark trap. The resulting mixture was allowed to reflux for 12 h. Rotary evaporation of the solvent left a yellow crude product which was purified by flash chromatography on silica gel with acetone–petroleum ether (1:4, v/v) to yield 365 mg (89%) of imine **2** as a white crystalline solid.

*Synthesis of hydrophobic polymer **3**.* Grubbs catalyst 1st (4.3 mg, 0.018 mmol, 1 equiv.) was dissolved in 1.5 mL of dry THF and stirred for 15 min. It was then added dropwise to monomer **2** (200 equiv.) which was dissolved in dry THF with vigorous stirring. The solution was stirred for 12 h at room temperature, and the reaction terminated by adding ethyl vinyl ether (600 equiv.) dropwise. The resulting mixture was stirred for 1 h and precipitated by dropwise addition into methanol. The precipitated polymer was purified by re-precipitation from THF–methanol (1:20, v/v) and dried *in vacuo* (yield 85%). The anhydrous reactions in synthesis of polymer **3** were performed under a positive pressure of N_2.

*Characterization of monomer **2** and polymer **3**.* The structure of monomer **2** and polymer **3** were characterized by ^{1}H NMR, using a Varian Unity-500 spectrometer (CA, USA), $CDCl_3$ as solvent and TMS as the internal reference. Monomer **2**. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 6.286 (2H, s, olefinic); 3.436 (2H, t, $J = 7.5$ Hz); 3.277 (2H, s); 2.676 (2H, d, $J = 1.0$ Hz); 1.588 (2H, dd, $J = 7.5$



Scheme 2. Resolution of 2-octanol by the immobilized PSL.

and 15.0 Hz); 1.514 (1H, dd, $J = 1.0$ and 9.5 Hz); 1.242 (1H, d, $J = 9.5$ Hz); 0.912 (3H, t, $J = 7.5$ Hz). Polymer **3**. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 5.761 (1H, m, olefinic); 5.520 (1H, m, olefinic); 3.340 (2H, br s); 3.019 (2H, m); 2.733 (1H, m); 2.117 (1H, m); 1.562 (4H, br s); 0.887 (3H, br s). The ^{1}H NMR spectrum for polymer **3** did not show resonances (6.286 ppm) corresponding to the olefinic proton of the norbornene skeleton, indicating that the polymerization took place in the ring-opening fashion.

The number-average (M_n) and weight-average (M_w) molecular weight of the polymer **3** were determined by GPC (Figure 1) using a Waters 410 GPC apparatus (MA, USA) equipped with a 10-μm Styragel HT6E column (300 mm × 7.8 mm) using linear polystyrene standards. THF was used as the eluent at a flow rate of 1 mL min^{-1}. The homogeneous system resulted in polymers with low polydispersities.

Preparation of the enzyme solution

PSL powder (5 g) was dispersed in phosphate buffer (250 mL, pH 8.0, 0.1 M) with stirring at 4°C for 2 h, and the insoluble impurity was removed by centrifugation (8000 rpm, 5 min). Finally the supernatant was lyophilized. Enzyme solution (10 mg mL^{-1}) was prepared by dissolving the lyophilized PSL (1 g) in phosphate buffer (100 mL, pH 8.0, 0.1 M).

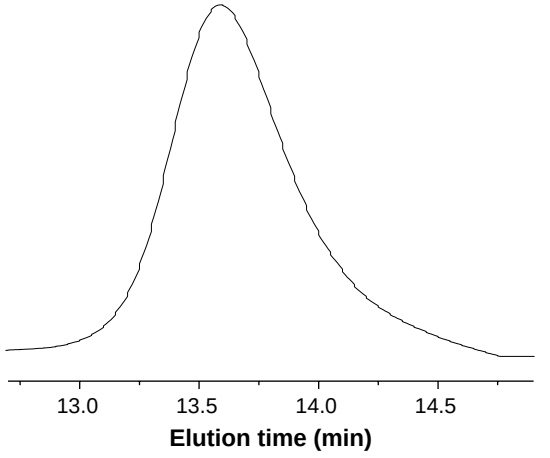


Figure 1. GPC trace of polymer **3** ($M_n = 38\,968$, PDI $= M_w/M_n = 1.14$).

Immobilization of PSL

Polymer **3** (50 mg) was dissolved in THF (5 mL) and then the enzyme solution (4 mL) was dispersed into the THF solution with vigorous stirring, which caused coacervation to occur forming a precipitate of lipase immobilized on the surface of polymer **3**. The immobilized PSL was dried overnight under vacuum and the amount of PSL immobilized on the support (260 mg g^{-1}) was measured by the Lowry method with bovine serum albumin as the standard for protein concentration (Lowry et al. 1951).

Resolution of (R,S)-2-octanol catalyzed by lipase

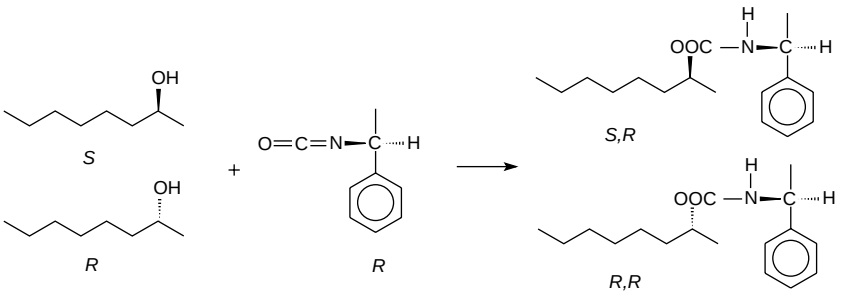
The reaction was performed in a round-bottomed flask containing (*R*,*S*)-2-octanol, vinyl acetate, n-hexane (10 mL) and immobilized PSL at controlled temperature and water activity. The mixture was incubated on a rocking bed at 200 rpm for 12 h. One unit of enzymatic activity (U) is defined as the amount of enzyme required to produce 1 μmol of 2-octanol acetate per minute in the first 0.5 h.

Reusability

To test the stability of the immobilized enzyme in repeated use, batch transesterification of 2-octanol (1 mmol) and vinyl acetate (4 mmol) was conducted by the addition of immobilized PSL (50 mg) in n-hexane (10 mL) at 55°C for 12 h with a water activity of 0.40. The immobilized PSL was recovered by centrifugation (3000 rpm, 15 min) after each batch, washed with water and hexane, and then dried. The dried immobilized PSL was reused for the next batch reaction under the same conditions.

Determination of enantiomeric excess values and enantioselectivity

The samples were withdrawn from the vials and analyzed directly by GC on a Shimadzu gas chromatograph (GC-14B, Japan) equipped with a FID detector and a column (EC-1000, 30 m × 0.25 mm × 0.25 μm; Alltech, MD, USA). The temperatures of the injector and the detector were 200°C and 290°C, respectively. N_2 was used as the carrier gas at a flow rate of 60 mL min^{-1}. Temperature programming between 110 and 210°C with an increment of 15°C min^{-1} was used to determine the



Scheme 3. Formation of isomer for pre-column derivation.

concentration of 2-octanol. (*S*)-2-Octanol or (*R*)-2-octanol was derivatized with *R*-(+)-PELC (Scheme 3) with a reaction system comprising the above mixture (8 μL), *R*-(+)-PELC (2 μL) and toluene (40 μL) and incubated in a shake flask for 3 h (200 rpm, 55°C). The discrimination of *S*- from *R*-enantiomers was achieved with temperature programming between 110 and 220°C with an increment of 10°C min^{-1}, giving retention times for the corresponding diastereoisomers of 12.36 and 12.84 min, respectively.

The degree of conversion (*C*) was calculated from the decrease in 2-octanol. The enantiomeric excess (ee_S) was determined by calculating the peak areas of the two derivatives and the enantiomeric ratio (*E*) was determined from *C* and ee_S by use of the following equations (Ma et al. 2002):

$$ee_S\ (\%) = \frac{[S] - [R]}{[S] + [R]} \times 100$$

and

$$E = \frac{\ln[(1 - C)(1 - ee_S)]}{\ln[(1 - C)(1 + ee_S)]}$$

where [S] and [R] represent the concentrations of the (*S*,*R*)-diastereoisomer and the (*R*,*R*)-diastereomer, respectively.

Results and discussion

Immobilization of PSL

In order to confirm that PSL was immobilized successfully on the hydrophobic polymer **3**, FTIR spectra of the free polymer (trace a), PSL (trace b) and immobilized PSL (trace c) were determined. As shown in Figure 2, the presence of an intense absorption band at 3350 cm^{-1} indicated that the hydrophobic polymer **3** had an abundance of N–H groups. However, the band was not seen in the PSL sample. This band could be found in the immobilized PSL. In addition, a band attributable to C–H groups (2900 cm^{-1}) was observed in the hydrophobic polymer **3** and PSL, and the intensity of this band increased dramatically in immobilized PSL compared with free PSL. These results confirmed that PSL had been successfully immobilized on the hydrophobic polymer **3**.

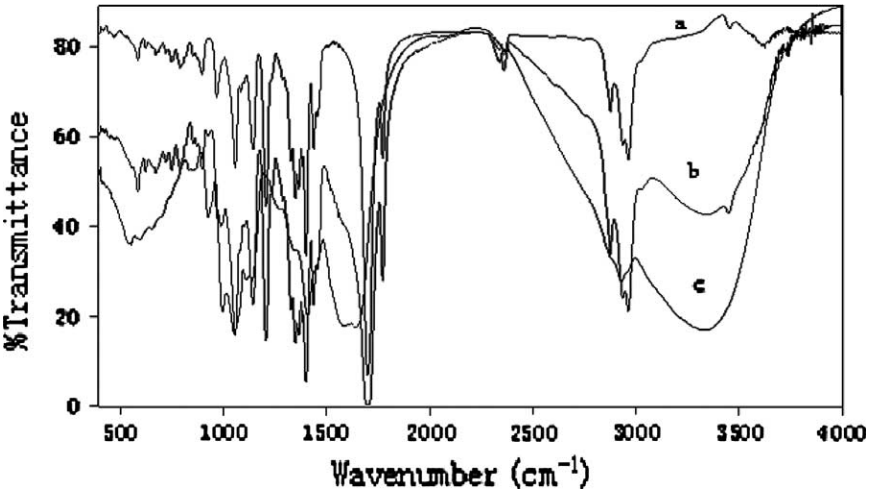


Figure 2. FTIR spectra of free PSL (trace a), immobilized PSL (trace b) and free polymer (trace c).

Table I. Comparison of the catalytic properties of free and immobilized PSL under optimum reaction conditions.

Sample	Bound protein (mg g^{-1})	Activity (μmol g^{-1} min^{-1})	Enantiose lectivity, *E*
Free PSL		124.6	49
Immobilized PSL	260	480	97
SBA-15-PSL (Yu et al. 2007)	250	184	114

Reactions were carried out in n-hexane (10 mL) with (*R*,*S*) -2-octanol (1 mmol), vinyl acetate (4 mmol), free (10.3 mg) or immobilized PSL (50 mg) that had the same protein content and water activity of 0.40 at 55°C for 12 h.

Comparison of the catalytic properties of immobilized PSL and free PSL

Table I shows the enzymatic activity and enantioselectivity of the free and immobilized PSL under the optimum reaction conditions. Compared with the free enzyme, the activity and enantioselectivity of PSL were dramatically improved after immobilization on hydrophobic polymer **3** and SBA-15, while the activity of immolibilized PSL on hydrophobic polymer **3** was clearly higher than that of immobilized PSL on SBA-15. An interesting observation was the loading capacity: that of polymer **3** was higher (260 mg g^{-1}) than for other hydrophobic supports, such as Duolite A-568, which is about 40 mg g^{-1} (Nanba et al. 1998). A probable reason may be that the small size of the immobilized carrier in our study provides a high surface area for PSL immobilization and some functional groups of the hydrophobic carrier may also improve the adsorption.

A promising property of lipase is its interfacial activation in the presence of a hydrophobic interface, which was first reported by Sarda & Desnuelle (1958). It is obvious that both hydrophobic substrate and hydrophobic support could provide the hydrophobic interface. In the presence of a hydrophobic support, lipases seem to become strongly adsorbed through a large hydrophobic surface that surrounds the catalytic site. The hydrophobic surface of the lipase involves residues from the internal face of the lid as well as from other protein chains (Chen et al. 1982). When the hydrophobic lid is 'adsorbed' onto the support interface, the 'open' form of the lipase may be fixed and then the activity of lipase can be improved. However, if the substrate is very large or hydrophilic, the proximity of the hydrophobic support surface may generate steric hindrance, reducing the activity of the lipase (Mateo et al. 2007). In the current study the substrates were 2-octanol and vinyl acetate, which are small and hydrophobic, so diffusion might have little effect on activity. When other hydrophobic surfaces of the protein chains are strongly adsorbed onto the hydrophobic support, a dramatic change in the enzyme conformation may occur, which results in alteration of enantioselectivity of the lipase.

Optimization of the reaction conditions

In order to optimize the reaction conditions, the effect on the reaction of several factors, including temperature, water activity, substrate molar ratio and amount of immobilized PSL, were studied.

Temperature. The activity and enantioselectivity of immobilized PSL in the resolution of 2-octanol were examined at various temperatures. The activity increased as the reaction temperature increased from 20°C to 55°C (Figure 3). The maximal enzyme activity of 480 μmol g^{-1} min^{-1} was observed at 55°C and was not further improved by increasing the temperature (55–65°C). With an increase in reaction temperature, the chance of collisions between enzyme and substrate molecules increases, which might help to form enzyme–substrate complexes and then improve the reaction rate. In addition, the enhanced fluctuation of protein caused by the higher temperature can relieve steric repulsion, which may also contribute to rate acceleration (Yu et al. 2007; Fernández-Lafuente et al. 1998). Further increasing the temperature may destroy the enzyme conformation by heat-induced destruction of non-covalent interactions (Shakeri & Kawakami 2008) and reduce the enzyme activity. An increased enantioselectivity (*E* value) was observed with a decrease in temperature in accordance with Bayramolu et al. (2004), who found that enzymes

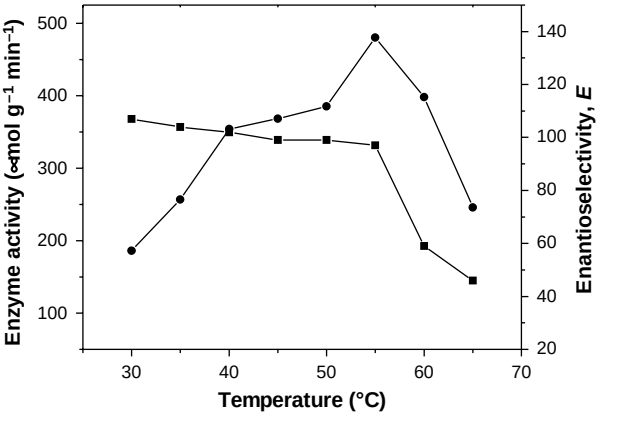


Figure 3. Effect of temperature on the activity (●) and enantioselectivity (■) of immobilized PSL in transesterification. Reactions were carried out in n-hexane (10 mL) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (4 mmol), immobilized PSL (50 mg) and water activity of 0.40 at different temperatures (30–65°C) for 12 h.

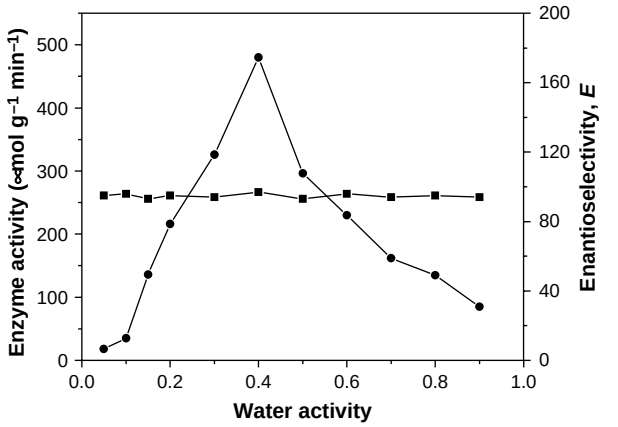


Figure 4. Effect of water activity on the activity (●) and enantioselectivity (■) of immobilized PSL in transesterification. Reactions were carried out in n-hexane (10 mL) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (4 mmol), immobilized PSL (50 mg) and differing water activity (0.05–0.95) at 55°C for 12 h.

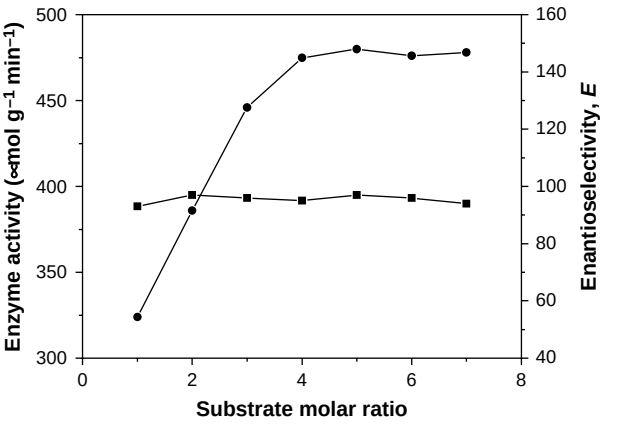


Figure 5. Effect of substrate molar ratio (vinyl acetate to 2-octanol) on the activity (●) and enantioselectivity (■) of immobilized PSL in transesterification. Reactions were carried out in n-hexane (10 mL) with immobilized PSL (50 mg) and water activity of 0.40 at 55 °C for 12 h. The concentration of 2-octanol was kept constant (1 mmol), whereas the concentration of vinyl acetate was varied from 1 mmol to 7 mmol.

exhibited their highest enantioselectivity at low temperatures. Since enzyme activity was found to be highest at 55°C while maintaining high enantioselectivity ($E = 96$), 55°C was selected as the optimal temperature for this reaction.

Water activity. When enzymes are used in non-aqueous media, water may play an important role in maintaining proper enzyme conformation so as to keep its catalytic activity. The amount of water available for enzymes in non-aqueous reaction systems is often quantified by the thermodynamic water activity (a_w) (Nirprit & Jagdeep 2002), which can be controlled by addition of salts or salt hydrates in the organic solvent or substrate as described by Andrade et al. (1991). In the present study, the reaction catalyzed by immobilized PSL was conducted at a wide range of initial a_w values (0.05–0.95), and the results are shown in Figure 4. The enzyme activity exhibited a bell-shaped curve with changing water activity (Figure 4). The immobilized PSL exhibited the highest activity when $a_w = 0.40$. At low water activity, the conformation of enzymes can be excessively rigid, which may disturb the 'induced-fit' process of PSL and decrease the enzyme activity (Halling 2002). The decrease in enzyme activity at higher initial a_w values can be attributed to the observed enzyme particle aggregation that may, in turn, limit access of the substrate to the enzyme active site. Additionally, substrate hydrolysis and acid release may also have a significant influence on enzymatic transesterification at higher initial a_w (Veum et al. 2005; Hara et al. 2007). Overall, these results suggest that water activity strongly influenced the hydration level of the enzyme which in turn affected its transesterification activity. Interestingly, E remained almost the same with the variation in a_w. A possible explanation could be that water was not acting as a competitive nucleophile for the acyl enzyme intermediate in this reaction, and so the enantioselectivity was not affected by water activity (Janssen et al. 1993).

Substrate molar ratio. The effect of molar ratio of vinyl acetate to 2-octanol from 1:1 to 7:1 on transesterification was investigated (Figure 5). It was shown experimentally that if the amount of the

Table II. Transesterification of 2-octanol in repeated batch process by PSL immobilized on polymer **3**.

Batch	Enzyme activity ($\mu molg^{-1}\,min^{-1}$)	Enantiomeric excess, ee_S (%)	Conversion (%)	Enantioselectivity, E
1	480	99.5	52.9	97
2	476	98.4	51.3	93
3	471	97.9	50.8	89
4	462	97.2	50.2	85
5	453	95.6	49.5	79
6	296	51.5	35.2	63
7	180	43.7	31.6	55

Reactions were carried out in n-hexane (10 mL) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (4 mmol), immobilized PSL (50 mg) and water activity of 0.40 at 55°C for 12 h.

enzyme was kept constant and the substrate ratio was increased, the enzyme activity increased gradually until it reached the maximum. Beyond the ratio of 4:1, the enzyme activity could not be improved with increasing substrate ratio. On the other hand, the enantioselectivity was not affected by the increase of substrate ratio.

Reusability of enzyme

In general, free enzymes are difficult to recover and reuse. The use of immobilized enzymes should help to drive down the product cost and make the enzymatic process economically viable (Ye et al. 2006). Studies on lipase reusability were carried out by using the recovered lipases over several cycles. The results (Table II) showed that 94% activity of the immobilized PSL remained after five cycles while enantioselectivity did not change significantly. However, the enzymatic activity started to decrease gradually from cycle 6. This indicates the possibility of reusing the immobilized lipase, but it is still not ideal because the activity and enantioselectivity of the immobilized enzyme eventually decrease with successive reuse, probably due to conformational changes. In addition, leaching of enzyme due to the weak interactions between PSL molecules and the hydrophobic support surface may be another reason for decrease in activity of the immobilized enzyme.

Conclusions

The lipase from *Pseudomonas* sp. was successfully immobilized on a novel hydrophobic polymer support through physical adsorption. The immobilized PSL exhibited a higher enzymatic activity and enantioselectivity in kinetic resolution of 2-octanol than the free enzyme. Under the optimum conditions, the residual 2-octanol was recovered with 99.5% enantiomeric excess at 52.9% conversion. The immobilized PSL also proved to be stable and lost little activity when it was subjected to repeated use.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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