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Resolution of (±)-menthol by immobilized *Candida rugosa* lipase on superparamagnetic nanoparticles

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Abstract

Lauric acid-stabilized magnetic particles were prepared by coprecipitation in the presence of lauric acid and used for the covalent immobilization of *Candida rugosa* lipase via carbodiimide activation. Size analysis by transmission electron microscopy (TEM) and measurement of magnetization curves revealed that the immobilized lipase was superparamagnetic. Resolution of (±)-menthol was performed by the immobilized lipase-catalyzed enantioselective esterification with propionic anhydride. Effects of various reaction parameters, such as enzyme load, solvents, water activity, substrate concentration, reaction time and temperature, on the conversion as well as enantioselectivity were investigated. As a result, (−)-menthyl propionate with a yield higher than 96% and over 88% enantiomeric excess of products was obtained. Better conversion and enantioselectivity could be expected for the immobilized lipase-catalyzed reaction performed at 30 °C for 2.5 h with 0.2 mol/l of (±)-menthol. Hexane was found to be the most suitable solvent, and the activity as well as enantioselectivity of the immobilized lipase decreased gradually with increasing water activity. Good durability of the immobilized lipase to catalyze the resolution of (±)-menthol was also observed.

Keywords: Immobilized lipase; Superparamagnetic nanoparticle; Resolution; Menthol; Enantioselectivity

1. Introduction

Biocatalytic revolution of racemic mixtures in organic media has become a potentially important method to acquire optically active enantiomers (Berglund, 2001; Persson, Costes, Wehtje, & Adlercreutz, 2002; Sakurai, Margolin, Russel, & Klibanov, 1988). Practical interests in this subject primarily arose from the fact that biocatalysis in organic media has many advantages such as higher solubility of hydrophobic compounds in the reaction system, good enantioselectivity and durability of biocatalyst, shifting many enzymatic reactions to the production of desired products and avoidance of bacterial contamination of bioreactors (Carrea, Ottolina, & Riva, 1995; Klibanov, 2001; Wescott, Noritomi, & Klibanov, 1996). Lipases (EC 3.1.1.3) are ubiquitous and highly stereoselective biocatalysts, which are of great value for the modern chemical and pharmaceutical industries, especially in enzymatic resolution of racemic mixtures of organic compounds (Ducret, Trani, & Lortie, 1998; Margolin, 1993; Wu, Xu, & Tsang, 2004). Therefore, there has been much work involving screening of lipases for racemate resolution, development of novel carriers for efficient application in enzymatic resolution, as well as optimization of reaction systems (Furukawa, Ono, Ijima, & Kawakami, 2001; Margolin, 1993; Wang, Nag, Lee, & Shaw, 2002).

Currently, the development of new immobilization methods and carriers remains one of the main subjects of research in enzyme engineering, because immobilization of lipase helps improve its stability, separation and reusability (Dyal et al., 2003; Guo, Bai, & Sun, 2003;

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Huang, Liao, & Chen, 2003). Of the various supports, magnetic nanoparticles have received considerable attention not only due to their higher specific surface area for the binding of larger amount of enzymes (Dyal et al., 2003), lower mass transfer resistance and less fouling (Curtis & Wilkinson, 2001; Huang et al., 2003), but also the ease in the separation of immobilized enzymes from a reaction mixture by the application of a magnetic field (Halling & Dunnill, 1980).

(*l*)-(−)-Menthol is widely used in industry (Gandhi, 1997) because of its refreshing flavor, whereas *d*-(+)-menthol has an undesirable taste. Previous work has demonstrated that *Candida rugosa* lipase can catalyze the enantioselective esterification of *l*-(−)-menthol from (*dl*)-menthol to obtain optically pure l-(−)-menthol (Wang et al., 2002; Wu, Akoh, & Phillips, 1996). However, the practical application of the methods depends on the yield and purity of product, reaction efficiency and enzyme stability. In this work, we developed lauric acid-stabilized Fe_3O_4 nanoparticles and coupled *C. rugosa* lipase for the enantioselective esterification of *l*-(−)-menthol. The effects of various reaction parameters on the enzymatic conversion and the enantioselectivity of the bioreaction as well as the stability of the immobilized lipase were investigated.

2. Materials and methods

2.1. Materials

(±)-Menthol, (−)-menthol, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), bovine serum albumin (BSA), coomassie brilliant blue G-250 and *C. rugosa* lipase (CRL, Type VII) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propionic anhydride was obtained from Fluka (Buchs, Switzerland). $FeSO_4$, $FeCl_3$ and lauric acid were of analytic grade from local sources. All other reagents and solvents were obtained from local sources and purified before use. A permanent magnet (maximum field strength $3. \times 105$ A/m) provided by the Research Institute of Rare Earth Elements (Baotou, China) was used for magnetic separations.

2.2. Immobilization of lipase on magnetic nanoparticles and activity assay

$FeCl_3$ (100 ml of 0.2 mol/l), 100 ml of 0.32 mol/l $FeSO_4$ and 2.0 g lauric acid were mixed at 50 °C in a 1000 ml steeless reactor equipped with a jacketed heater by circulated water and mechanic agitator. Forty ml of 25% $NH_3 \cdot H_2O$ was slowly added to the reaction mixture under vigorous agitation and the reaction was allowed to proceed for 30 min. The magnetic particles were collected by magnetic separation and washed with 0.5% (v/v) ammonia. The resultant magnetic particles and 1.0 g lauric acid were dispersed in 100 ml distilled water at 80 °C and held at this temperature for another 30 min. The particles were finally harvested by magnetic separation and routinely washed to pH 5–6 with dilute HCl and distilled water. The magnetic particles were lyophilized and stored at room temperature for future use.

Enzyme coupling onto the magnetic nanoparticles was performed by the EDC activation method (Huang et al., 2003). The immobilized lipase collected in magnetic field was lyophilized and stored at −20 °C before use. Magnetic particles with or without lipase were subjected to magnetism characterization. The size and morphology of particles were observed by transmission electron microscopy (TEM) using a JEM-100CX II system (JEOL, Japan) as described previously (Guo et al., 2003). The magnetization curves of the particles were recorded with an LDJ 9600-1 vibrating sample magnetometer (LDJ Electronics, MI, USA). The magnetism of particles was measured with ST-I Tesla meter (Baotou Steel Plant, Inner Mongolia, China).

The amount of lipase protein in supernatant was determined by the Bradford method (Bradford, 1976) using BSA as a standard. The amount of protein bound onto the particles was calculated by mass balance. Activities of the native and the immobilized lipase were determined with the olive oil method (Cho & Rhee, 1993). One unit (U) of the activity was defined as the amount of lipase which liberates 1 μmol fatty acids per minute under the assay conditions.

2.3. Esterification of menthol and its assays

Esterification of (±)-menthol was performed in 10 ml screwed vials. In a typical experiment, 0.5 mmol menthol and 0.5 mmol propionic anhydride were dissolved in 3 ml solvent followed by the addition of 0.5 mmol $NaHCO_3$ and lipase. The reaction mixture was shaken at 200 rpm at a desired temperature (20–40 °C). The progress of the reaction was monitored by analyzing the aliquots of reaction mixture by gas chromatography on an Agilent 6590N system (Agilent Technologies, DE, USA) equipped with a splitless/split injector and a flame-ionization detector. A Cyclosil-B chiral column (30 m length, 0.5 mm I.D.) was used to analyze (±)-enantiomers of menthol and their respective esters. The injector was set at 200 °C and detector at 225 °C. The flow rate of the carrier gas N_2 was 2 ml/min. The initial column temperature of 110 °C was held for 12 min and then raised to 150 °C at a rate of 4 °C/min and finally held at 150 °C for 3 min.

Enantiomeric excess of menthyl propionate [*ee*(*P*)%] based on the GC analyses were calculated as described by Wang et al. (2002). Enantioselectivity (*E*) was then calculated by the following equation (Chen, Fujimoto, Girdaukas, & Sih, 1982):

$$E = \frac{\ln[1 - c(1 + ee(P))]}{\ln[1 - c(1 - ee(P))]}, \quad (1)$$

where c and $ee(P)$ denote (±)-menthol conversion and enantiomeric excess of menthol propionate ester, respectively.

2.4. Effects of water activity and solvents

The water activity of the reaction system was controlled via direct addition of salt hydrates of $LiCl \cdot H_2O$ ($a_w = 0.023$), $CuSO_4 \cdot 5H_2O$ ($a_w = 0.35$), $Na_4P_2O_4 \cdot 10H_2O$ ($a_w = 0.52$), $ZnSO_4 \cdot 7H_2O$ ($a_w = 0.62$) and $NaH_2PO_4 \cdot 12H_2O$ ($a_w = 0.85$) at 30 °C (Alston & Freeman, 2002). In a typical procedure, 120 mg of a salt hydrate, 3 ml hexane and 0.5 mmol menthol and 0.5 mmol $NaHCO_3$ were mixed in a 10-ml sealed vial and allowed to reach equilibrium for a week with continuous shaking at 30 °C. The reaction was initiated by the addition of 0.5 mmol propionic anhydride and 200 U of the immobilized CRL at 30 °C, which was performed on a shaking bath at 200 rpm for 2.5 h.

To investigate the effects of solvents on the reaction, the solvents with log P values, a kind of measure to indicate the polarity or hydrophobicity of a solvent, of 2.0–4.5 (chloroform log $P = 2.0$, toluene 2.5, methane tetrachloride 3.0, *n*-hexane 3.5, *n*-heptane 4.0 and *n*-octane 4.5) were chosen to examine their effects on the activity and enantioselectivity of the immobilized lipase on the magnetic nanoparticles. The reaction was also performed as described above except employing different solvents.

2.5. Recycled use of the immobilized lipase

To test the stability of the immobilized lipase in repeated use, batch esterification of 0.5 mmol (±)-menthol and 0.5 mmol propionic anhydride was conducted by the addition of 0.5 mmol $NaHCO_3$ and 200 U of native or immobilized lipase to 3 ml hexane. The reaction was allowed to proceed at 30 °C for 2.5 h in each cycle. The enzyme recovered by centrifugation for the native and by magnetic separation for the immobilized CRL was reused for the next batch reaction under the same conditions. The relative activity of the native and the immobilized lipase based on the conversion of (±)-menthol obtained in their first run was defined as 100%, respectively.

3. Results and discussion

3.1. Characteristics of the immobilized lipase on magnetic particles

Image analyses of the size and morphology of the magnetic particles without or with loaded lipase obtained by transmission electron microscopy (TEM) revealed that both of them have a mean diameter of less than 20 nm. No significant size variation was found after lipase was bound to the particles. It has been well known that ultrafine magnetic particles less than a critical size of 25 nm exhibit superparamagnetism (Bean & Livingston, 1959; Huang et al., 2003), which can be readily captured in magnetic field and redispersed after the removal of the external field. The size of the magnetic particles prepared in this work is obviously smaller than the critical size (25 nm) of the granules that acquire single magnetic domain and exhibit superparamagnetism (Bean & Livingston, 1959; Guo et al., 2003).

Measurement of the magnetic particles without or with bound lipase gave the similar saturation magnetization of 56.0 emu/g, and the magnetic particles showed the remanent magnetization of less than 0.85 emu/g and coercivity below 8.1 Oe. Such weak hysteresis indicated that the lipase-bound magnetic particles could be considered as superparamagnetism. Measured with a Tesla meter, no magnetic force around the particles was detected. Little agglomeration of the immobilized lipase was observed in either aqueous solution or solvents when employed as biocatalyst. All the observations showed that the nanoparticles with coupled lipase were superparamagnetically discrete and monodisperse.

The activities of the native and the immobilized lipase have been determined and compared in our previous work (Liu, Bai, & Sun, 2004). It revealed that the immobilized lipase showed a specific activity of 1.8-fold higher than the native one. The result indicates that the magnetic nanoparticles were a good carrier for lipase immobilization. So, in this work, we investigated its application for the resolution of racemic menthol.

3.2. Effect of lipase load

Solid powder of $NaHCO_3$ was added to the reaction system in all experiments for esterification of menthol with propionic anhydride in this work. It is to neutralize the generated propionic acid for instant removal of the byproduct and to shift the equilibrium to the formation of menthyl propionate.

Enzyme load is a crucial factor for its potential in industrial application. An optimum enzyme load is preferable to increase its application efficiency. Fig. 1(a) shows the effect of the immobilized lipase load on the conversion of racemic menthol, enantiometric excess of products and enantioselectivity (E). The total conversion of (±)-menthol and enantiometric excess of products ($ee(P)$%) increased rapidly with lipase load till a value of 63.3 U/ml. When the enzyme load exceeded 63.3 U/ml, however, the percentage conversion of (±)-menthol and the $ee(P)$% increased less. Continuous increase of the E value against enzyme load are primarily due to the sensitivity of E to the $ee(P)$ and c value, especially when these values are close to the theoretical ones

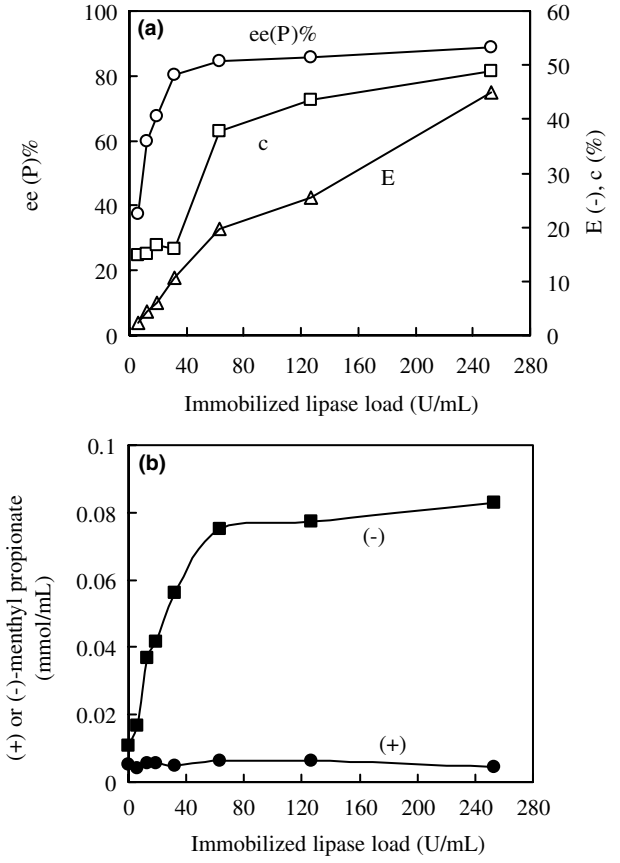

Fig. 1. (a) Effects of the immobilized CRL load on (±)-menthol conversion (*c*) (□), enantiomeric excess of menthyl propionate [*ee*(*P*)%] (○) and enantioselectivity (*E*) (△). (b) Changes of the concentrations of (−)-menthyl propionate (■) and (+)-menthyl propionate (●) as a function of the immobilized lipase load. Reactions were performed at 30 °C in 3 ml hexane with 1:1:1 mole ratio of (±)-menthol (0.158 mmol/ml), propionic anhydride and $NaHCO_3$ for 2.5 h.

(Wang et al., 2002). From the standpoint of practical application, an enzyme load of 63.3 U/ml has given an acceptable reaction efficiency and relatively high *E* value (20). Moreover, Fig. 1(b) shows that at the immobilized lipase load higher than 63.3 U/ml, the concentration of (−)-menthyl propionate increased little. Therefore, from commercial consideration, 63.3 U/ml is a better preference. With the enzyme loading, 70% (−)-menthol conversion and 84.5% *ee*(*P*) were obtained in 2.5 h.

3.3. Effects of reaction time and temperature

Fig. 2 indicates the time course of the conversion of racemic menthol, enantiomeric excess of menthyl propionate and enantioselectivity at 30 °C. The conversion showed a linear increase till 2.5 h, and thereafter the increase slowed down. In contrast, the enantiometric excess kept approximately unchanged at about 80% before 2.5 h, and then decreased with the reaction time. The maximum *E* value of 20 was obtained at 2.5 h. It is

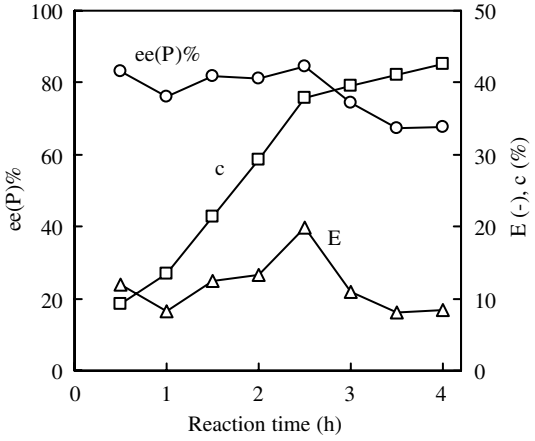

Fig. 2. Time course of (±)-menthol conversion (*c*) (□), *ee*(*P*)% (○) and enantioselectivity (*E*) (△) catalyzed by the immobilized CRL. Reactions were performed at 30 °C in 3 ml hexane with 63.3 U/ml immobilized CRL. Other conditions were the same as those in Fig. 1.

obvious that the decrease of optical selectivity was related to the changes of the relative content of menthol enantiomers with the evolution of the reaction. That is, with the progress of the reaction, the (+)-menthol concentration became higher, and became more competitive to react with propionic anhydride, leading to the decrease of the enantiometric excess. Therefore, we chose 2.5 h as a favorable reaction time to achieve higher yield of (−)-menthyl propionate and higher enantioselectivity.

Previous experiments have proved that the enantioselectivity of enzyme is dependent on reaction temperature (Athawale, Manjrekar, & Athawale, 2001). It is considered that enantioselectivity depends on the difference of the activation free energy ($\Delta\Delta G$) between *l*- and *d*-enantiomers and there exists a racemic temperature (T_r) (Pham, Phillips, & Ljungdahl, 1989). When reaction temperature is close to or higher than T_r, the discrimination ability of enzyme between *l*- and *d*-enantiomers will be lost or give an adverse preference. The results presented in Fig. 3 clearly show the decreasing tendency of the enantiomeric excess with increasing temperature from 20 to 40 °C. Higher enantioselectivity of 21 at 25 °C and 20 at 30 °C were obtained, respectively. However, a maximum conversion was achieved at 30 °C, which is in agreement with the previous report using native CRL (Athawale et al., 2001). It indicates that the optimal temperature of the CRL for the reaction did not change after immobilization.

3.4. Effect of substrate concentration

Previous experiments were performed at a (±)-menthol concentration of 0.158 mmol/ml. To examine the reaction capacity, we carried out experiments at extensive substrate concentrations at the mole ratio of 1:1:1.

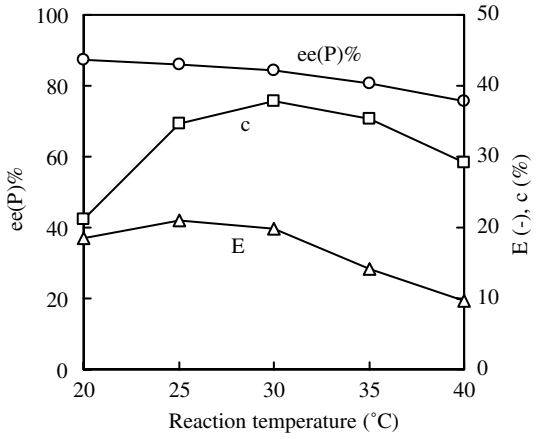

Fig. 3. Temperature dependency of (±)-menthol conversion (*c*) (□), *ee*(*P*)% (○) and enantioselectivity (*E*) (△). Reactions were performed in 3 ml hexane with 63.3 U/ml immobilized CRL. Other conditions were the same as those in Fig. 1.

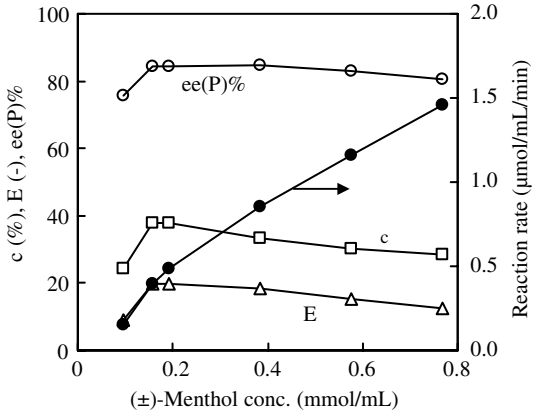

Fig. 4. Effects of substrate concentrations on (±)-menthol conversion (*c*) (□), *ee*(*P*)% (○), *E* (△) and reaction rate (●). (±)-Menthol, propionic anhydride and $NaHCO_3$ were in the mole ratio of 1:1:1 for each reaction using 63.3 U/ml immobilized CRL. Other conditions were the same as those in Fig. 1.

Fig. 4 shows the effect of substrate concentration. The esterification rate continuously increases with the increasing (±)-menthol concentration, and does not show any saturation effect in the test range. This suggested that the immobilized lipase has the potential to catalyze the conversion of substrate at higher concentration, which is important for industrial application. However, the conversion of the total (±)-menthol began to decline when more than 0.192 mmol/ml (±)-menthol was employed, because the increasing rate of substrate exceeds its consuming rate within the test time. The decline of the conversion of (±)-menthol also resulted in the decrease of enantioselectivity. On the other hand, it is notable that *ee%* and *E* even hold at a relatively high level at the substrate concentration up to 0.385 mmol/ml, and the decrease of conversion could be compensated by prolonging the reaction time. So the (±)-menthol concentrations ranged from 0.158 to 0.385 mmol/ml are better preference by the integrated consideration of the conversion and enantioselectivity. The results also indicate higher reaction capacity of the immobilized CRL developed in this work.

3.5. Effect of solvent (log *P*)

The nature of organic media not only influences the activity of enzyme but also the enantioselectivity, which has been verified by the work of several groups (Carrea et al., 1995; Klibanov, 2001; Persson et al., 2002). Log *P*, logarithm of the partition coefficient of a given solvent between *n*-octanol and water, is now widely used to denote the polarity or hydrophobicity of a solvent. In this study, effects of the solvents on the conversions and enantioselectivity of the immobilized CRL were indicated in Fig. 5. As expected, higher conversions were obtained for the reactions using the solvent with higher log *P* than three as reaction media. Although, highest yield of (−)-menthyl propionate was achieved in methane tetrachloride, the enantiomeric excess is significantly lower than in *n*-hexane and *n*-heptane. *n*-Hexane gave the maximum enantioselectivity and only a slightly lower yield than the maximal value in tetrachloride. The impairment of polar solvents to enzyme by depriving the essential water binding to the active site seems to be incontrovertible. In another way, the effects of reaction media on the activity of the immobilized lipase might be also related to the activity of the substrate molecules dissolved in these media as well as the water activity. Striking effects of solvents on lipase enantioselectivity remain unclear; some explanation suggested solvent molecules coordinated to the active site interfere differently with the substrate enantiomers (Hirose et al., 1992). While our results seem to suggest that the nature

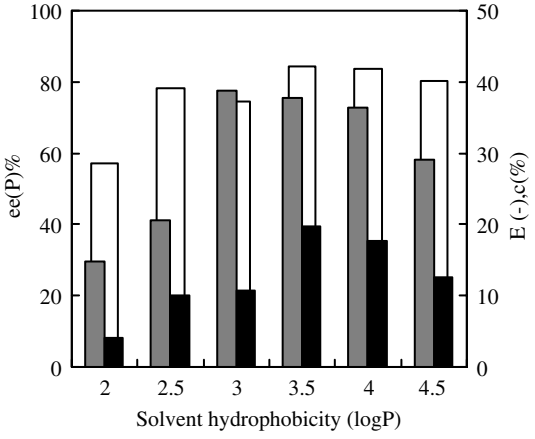

Fig. 5. Conversion of (±)-menthol (*c*) (▩), *ee*(*P*)% (□) and enantioselectivity (*E*) (■) in different solvents (log *P*). Reactions were performed in 3 ml different solvent employing 63.3 U/ml immobilized CRL, respectively. Other conditions were the same as those in Fig. 1.

of the carrier might affect the lipase enantioselectivity by creating different microenvironment to influence the solvation and diffusion of substrate and product in organic media (Wescott & Klibanov, 1994). In conclusion, the present results suggested that *n*-hexane appears to be the most suitable solvent for the immobilized lipase on the palmatic acid-modified magnetic nanoparticles to catalyze enantioselective synthesis of (−)-menthyl propionate.

3.6. Effect of water activity

Fig. 6 shows the water activity dependence of the esterification activity and enantioselectivity of immobilized lipase. At the lowest water activity, the conversion of (±)-menthol, enantioselectivity and enantiomeric excess of menthyl propionate all achieve the maximal values. The conversion of (±)-menthol undergoes a significant decline with the increasing water activity, in contrary to the indistinctive decreases of enantiomeric excess and enantioselectivity. The observation is similar to the results reported by Furukawa et al. (2001), who conducted the esterification of (−)-menthol with butyric acid employing the *C. rugosa* lipase deposited on Celite. The authors suggested that the decrease of the activity of immobilized lipase is related to the higher water content-induced deformation of the activated structure pertaining to the immobilized lipase at lower water activity. We proposed another possible reason for the decline of the conversion of racemic menthol may be related to the decrease of the activity of menthol molecules at higher water activity, that is, the hydration of menthol molecule might decrease its affinity to the lipase immobilized on the hydrophobic carrier. However, the deformation of the activated structure at higher water activity may account for the decrease of enantioselectivity.

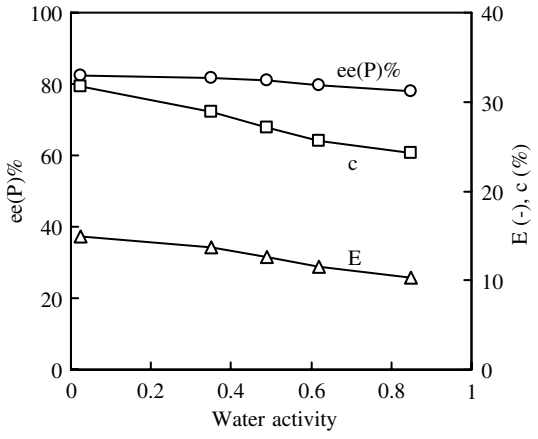

Fig. 6. Water activity dependence of (±)-menthol conversion (*c*) (□), *ee*(*P*)% (○) and enantioselectivity (*E*) (△).

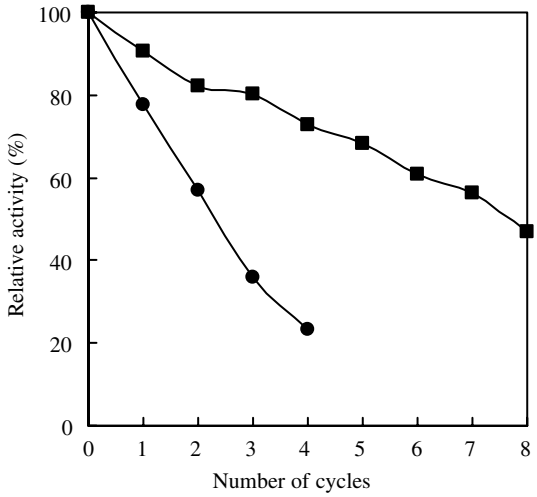

Fig. 7. Operation stability of the native (●) and immobilized (■) lipase in catalyzing (±)-menthol esterification with propionic anhydride in *n*-hexane.

3.7. Reusability of the immobilized lipase

The duration of a biocatalyst is an important feature for its potential application in industry. As shown in Fig. 7, the activity of immobilized lipase in repeated use does not significantly decrease as the native lipase; the remaining activity was about 56.4% of the original use after seven cycles, similar to the activity of the native lipase used at the second cycle. That is to say, about 94% activity of the immobilized lipase was recovered in each cycle. The results indicated that the immobilized *C. rugosa* lipase on the superparamagnetic nanoparticles has a good durability and magnetic recovery.

4. Conclusions

Size analyses and magnetic measurement showed that the magnetic nanoparticles coupling the CRL was superparamagnetic, so good dispersancy and high magnetic response of the immobilized lipase in the hydrophilic and hydrophobic solvents was observed. For the immobilized catalyzed resolution of (±)-menthol, enzyme load, substrate dosage, reaction time, temperature, nature of solvents and water activity were found to have profound effects on the conversion and enantioselectivity. Racemic menthol conversion and enantiomeric excess of menthyl propionate as high as 48.8% and 88.8%, respectively, were obtained under the optimized conditions. All the results indicate that the immobilized CRL can be readily recovered by magnetic separation and reused for enantioselective esterification of menthol. It is expected to find use in a preparative production of optically active enantiomers.

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