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Immobilization of Lipase with Alginate Hydrogel Beads and the Lipase-Catalyzed Kinetic Resolution of α-Phenyl Ethanol

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ABSTRACT: The immobilization of enzymes is one of the key issues in both the field of enzymatic research and industrialization. In this article, we report a facile method for immobilizing *Candida antarctica* lipase B in an alginate carrier. In the presence of calcium cations, an enzyme–alginate suspension was crosslinked to form beads with a porous structure at room temperature, and the enzymes were well dispersed in the beads. The chiral resolution of α-phenyl ethanol in the organic phase was tested by the enzyme–alginate beads. The effects of the reaction parameters, such as the enzyme concentration, temperature, and molar ratio of the substrate to the solvent, on the resolution behavior are discussed. Reuse cycle experiments for the chiral resolution of α-phenyl ethanol demonstrated that the activity of the enzyme–alginate beads was maintained without marked deactivation up to five repeated cycles. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40178.

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INTRODUCTION

The use of biocatalysts in organic synthesis has become an interesting alternative to conventional chemical methods. Biocatalysis has many attractive features in the context of green chemistry, including mild reaction conditions (physiological pH and temperature) and an environmentally compatible catalyst and solvent (often water) combined with high activities and chemoselectivity, regioselectivity, and stereoeslectivity in multifunctional molecules.[1–3] The use of enzymes generally circumvents the need for functional-group activation and allows one to avoid the protection and deprotection steps required in traditional organic syntheses.[4,5]

Enzymes show extremely high catalytic activity under normal temperature and pressure conditions and greatly promote the relative study of their varieties and aspects.[6–8] The free state of the enzyme is less stable to heat, acid, alkali, and high ionic strength.[9] The greatest disadvantage of the free enzyme is its difficulty in separation with reactants and products and recycling. Studies of free enzymes have mainly focused on the optimization of the selection and conditions of the enzyme sources.[10] To obtain an ideal biocatalyst to meet the requirements of industrialization, the immobilization of the enzyme is needed. The use of immobilized enzymes can reduce costs by enabling efficient separation, recycling, and the reuse of expensive enzymes. In addition, immobilized enzymes typically exhibit greater stability and enzyme activity over a broader range of pH and temperature.[11] The immobilization of enzymes also contributes to the development of continuous processes, and immobilized enzymes are adaptable to a variety of configurations and specific processes carried out in reactors. Enzyme immobilization has now attracted a wide range of interests, from fundamental academic research to many different industrial applications.[12–14] Bayramoglu's group[15–18] systematically carried out research on the immobilization of lipase onto different polymers and further studied the enzymatic reactions in these coated beads. In the immobilization of the enzyme, the enzyme is attached to a carrier; the properties of the carrier (chemical and mechanical) influence the catalytic activity of the enzyme as well and make it even more important to find a proper immobilization method. Generally, the choice for the best immobilization method depends on the enzyme, the type of reaction, and the reaction environment. Different immobilization methods can lead to different activities and stabilities under identical circumstances. Immobilization by entrapment involves the enzyme being retained in a

Additional Supporting Information may be found in the online version of this article.

membrane device, such as a hollow fiber, polymeric network, or microcapsule.[1,19] In this case, the enzyme was covalently attached to the polymer to prevent diffusion out of the carrier.

Alginate is by far the most widely used polymer for immobilization and microencapsulation technologies.[10,20] In our previous works, we developed a general and green approach to synthesize and stabilize uniform noble metal nanoparticles in alginate.[21] In the presence of calcium cations, the metal–alginate suspension was crosslinked to form beads with a porous structure at room temperature, and the metal nanoparticles were evenly dispersed in the beads. The as-synthesized metal–alginate could be expediently designed in the form of a homogeneous or heterogeneous carrier according to different real applications.

There are a large number of chiral molecules in nature and in organisms. In a chiral environment, the role and purpose of the pairs of enantiomers often have the opposite effect or a completely different role.[22] This difference exists not only in chiral pharmaceutical products[23] but also in pesticides,[24] spices,[25] food materials,[26] intermediates, and other areas of production.[27,28] Particularly in the pharmaceutical production, a single chiral molecule can often play a therapeutic role, and its enantiomer often has no effect or even negative effects. Therefore, chiral separation has become an increasingly important research topic.[29] The enzymatic kinetic resolution of racemate is an important method for the preparation of various chiral compounds.[30] Lipases with high enantioselectivity, high reactivity, and high stability can adapt to a wider range of substrate characteristics and usually do not require a coenzyme to be involved. Therefore, they have been widely used in the preparation of derivative of chiral alcohols,[31] carboxylic acids,[32] esters,[33] and amines.[34] However, biological catalytic processes for chiral separation often have a very long reaction time, and stability cannot be guaranteed; recycling and reuse also encounter many challenges. As an important chiral secondary alcohol, α-phenyl ethanol is widely used as a chiral intermediate in synthetic or natural products of modified chiral drugs.[35] The search for an effective and simple resolution method for the identification of optically active substances is essential.

In this study, *Candida antarctica* lipase B (CALB) was successfully immobilized in an alginate carrier. In the presence of calcium cations, the enzyme–alginate suspension was crosslinked to form beads with a porous structure at room temperature, and the enzymes were well dispersed in the beads. The chiral resolution of α-phenyl ethanol in the oil phase was systematically examined through enzyme–alginate composites. The resolution of α-phenyl ethanol on the lyophilized enzyme–alginate beads was investigated from different perspectives, including the temperature, molar ratio of vinyl acetate to (R, S)-1-phenylethanol, reaction time, and reaction solvents. High enantiomeric excess (e.e) and substrate conversion were achieved by catalysis in the lyophilized enzyme–alginate beads.

EXPERIMENTAL

Materials

Sodium alginate (low viscosity, 250 cps, molecular weight = 12,000–80,000) was obtained from Alfa Aesar Co. Anhydrous calcium chloride was purchased from Sigma (China). Potassium dihydrogen phosphate (KH_2PO_4; 0.995 mass fraction, Sinopharm Co., Ltd., Beijing, China), dipotassium hydrogen phosphate (K_2HPO_4; 0.995 mass fraction, Sinopharm Co.) and (±)-1-phenylethanol (Acros Organics, Fairlawn, NJ; >98% GC) were used. An aqueous solution of CALB (26.5 mg of enzyme/mL, activity = 5000 U/g) was purchased from Novozymes (China). CALB was used as received. Vinyl acetate, ethyl acetate, dichloromethane, hexane, acetonitrile, tetrahydrofuran, and toluene were supplied by Sinopharm Chemical Reagent Co. Doubly deionized water (electrical conductivity $\leq 1 \cdot \times 10^{-4}$ S/m) was used in this study. All other reagents were analytical grade and were used without further purification.

Fabrication of the Enzymatic Catalyst

In a typical experiment, the desired amount of sodium alginate was dissolved in pure water and stirred until the alginate was fully dissolved at room temperature. It was then left to stand for about 0.5 h. A volume of 50–100 μL of CALB (initial activity = 5000 U/g) was added to 2 mL of sodium alginate aqueous solution (0.02 g/mL), and the mixture was shaken to an even state in the vibration bed. The enzyme–alginate suspension was added dropwise to 30 mL of a $CaCl_2$ (10 wt %) aqueous solution without stirring, and it formed colorless transparent gel beads with a diameter of about 2–5 mm. After they were embedded for 60–100 min, these beads were repeatedly washed with deionized water in a Buchner funnel filtration process. Then, these beads were transferred to a clean glass dish, which was sealed with a sealing membrane. The beads were stored in a refrigerator (temperature = −11°C, relative humidity = 78%) and used within a week. The top of the sealing cap of the dish was punctured with a syringe needle before freeze-drying. The sample environment and the sample itself were held to be clean and to prevent any entry of moisture during the preparation processes.

Chiral Resolution of α-Phenyl Ethanol

The reaction of the chiral resolution catalyzed by the freeze-dried sodium alginate beads with immobilized enzyme and was conducted in a 12-mL glass-stoppered bottle. A mixture of 500 μL of racemic α-phenyl ethanol and 5 mL of vinyl acetate was reacted in a constant-temperature water bath with stirring at a rate of 1000 rpm. In the reaction, vinyl acetate was used as an acyl donor and also served as the reaction solvent. The samples were taken after 10 h of reaction times. The e.e and analytic conversion were determined on an Agilent 1100 HPLC instrument equipped with a chiral column (OD—H, 4.0 mmΦ × 10 mL, particle size = 5 μm) with a UV detector at 254 nm. The high performance liquid chromatography (HPLC) was performed with *n*-hexane/isopropyl alcohol (98/2, HPLC grade, Fisher Scientific Co.) as the mobile phase at a flow rate of 1.0 mL/min, and the column temperature was 28°C. NMR was used as qualitative judgments for the product. All of the experiments were performed three times to calculate the mean, stand deviation, and confidence interval of the mean at 95%.

RESULTS AND DISCUSSION

In this study, we took the chiral resolution of α-phenyl ethanol as a model reaction to evaluate the catalytic activity of CALB in different forms, including liquid free enzyme, calcium alginate

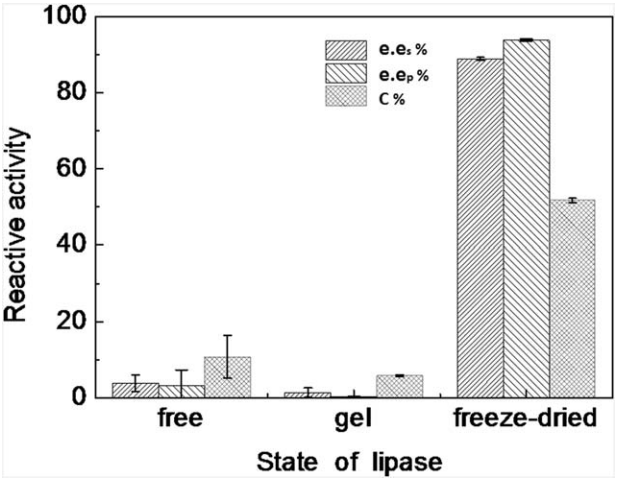

Figure 1. Reactive activity in free, gel, and freeze-dried enzymes. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 3 mL of vinyl acetate, 1 mL of CALB, 40°C, and 10 h of reaction time [e.e$_S$ = enantiomer excess value of (s)-α-phenethyl alcohol; e.e$_P$ = enantiomer excess value of (*R*)-phenylethyl acetate; *C*% = conversion rate of the substrate].

gel enzyme, and freeze-dried alginate gel beads. As shown in Figure 1, the free enzymes and calcium alginate gel enzymes had low catalytic activities. The freeze-dried enzyme showed a better asymmetric reaction selectivity. Generally, the use of microporous supports might improve the catalytic properties of immobilized lipase compared to those of free lipase because the substrate concentration of the liquid–solid interface is higher than that in solution. In a nonaqueous enzymatic catalytic resolution reaction, lipase needs a certain amount of bound water to maintain its active conformation; this makes the enzyme maintained in catalytic activity.[8] The optimum water content could be obtained by the regulation of the freeze-drying time. The water contents in the free enzyme and calcium alginate gel enzyme were far greater than the optimum value to maintain the enzyme activity and ultimately decreased the enzymatic activity. The effects of the reaction parameters on the chiral resolution of α-phenyl ethanol on the freeze-dried alginate gel beads were systematically investigated.

Effect of the Enzyme Concentration

The amount of enzyme catalyst in the biocatalytic reaction system directly affected the product yield of the bioconversion. As shown in Figure 2 and Table S1 (in the Supporting Information), the conversion of the product increased rapidly with increasing enzyme concentration when the enzyme concentration was smaller than 800 μL/mL and leveled out when the enzyme concentration was greater than 800 μL/mL. The conversion and the e.e of S-1-phenylethyl acetate decreased slightly when the enzyme concentration increased up to 1000 μL/mL. An excessive amount of enzyme made the enzyme protein unfolding insufficient and the spatial structure curly and resulted in a stack of multilevel structures of the enzyme, which led to a decrease in the enzymatic activity.

Effects of the Temperature and Optimization of the Reaction Time

The reaction temperature can affect the activity and stability of enzymes. An increase in the temperature makes the activation

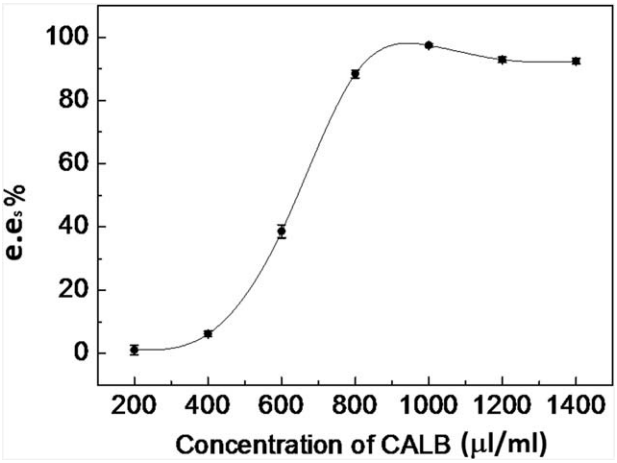

Figure 2. Effect of the enzyme concentration on e.e$_s$. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 3 mL of vinyl acetate, 40°C, and 10 h of reaction time.

energy for the objective reaction easier to reach, and the mass transfer of the substrate and product can be improved. On the other hand, as the temperature increases, the enzyme protein gradually denatures and is inactivated, and this results in a decrease in the reactivity. The combined effects of temperature are closely related to time because temperature-promoting enzyme protein denaturation is cumulative over time. As shown in Figure 3 and Table S2 (in the Supporting Information), the conversion and e.e of S-1-phenylethyl acetate increased with increasing reaction time. As expected, the conversion leveled out after 10 h. The reaction time was designated as 10 h in following experiments. The optimum temperature for an enzyme is not the value characterized by physical constants, which are often subject to the purity of the enzyme, substrate, activating agents, and inhibitors.[10] Therefore, the optimum temperature for a kind of enzyme is generally determined by experimentation. The effect of the reaction temperature on different amounts of enzyme was investigated, as shown Figure 4 and

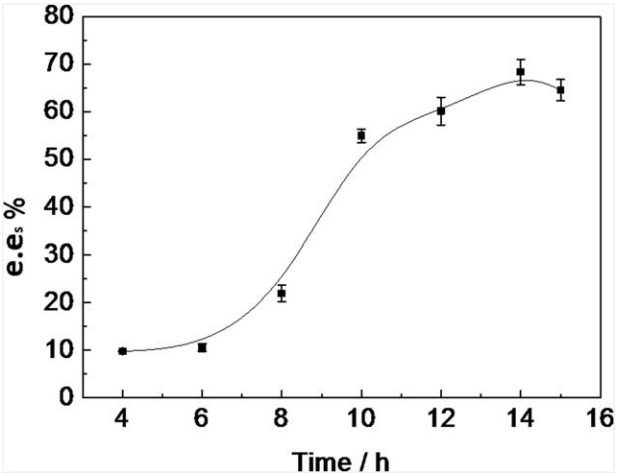

Figure 3. Profile of e.e$_s$ with the reaction time for the freeze-dried enzyme. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 3 mL of vinyl acetate, 600 μL of CALB, and 40°C.

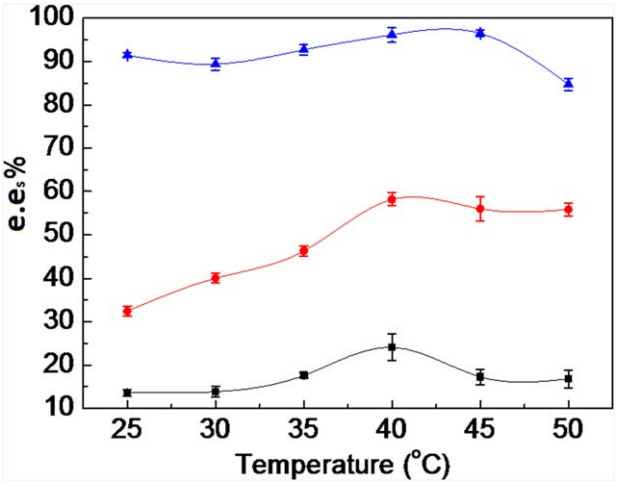

Figure 4. Profile of e.e$_s$ at different reaction temperatures for the freeze-dried enzyme. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 3 mL of vinyl acetate, 40°C, 10 h of reaction time. (■) 300, (●) 500, and (▲) 1000 μL of CALB. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

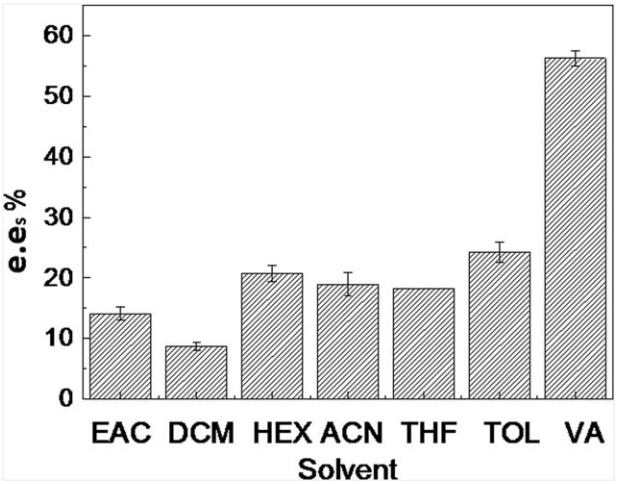

Figure 5. Reactive activity of the freeze-dried enzyme in different reaction solvents. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 500 μL of CALB, 3 mL of solvent, molar ratio of the substrate to the acyl donor = 1:2, 40°C, and 10 h of reaction time. EAC = ethyl acetate; DCM = dichloromethane; HEX = hexane; ACN = acetonitrile; THF = tetrahydrofuran; TOL = toluene; VA = vinyl acetate.

Table S3 (in the Supporting Information), and the results indicate that the conversion and e.e of S-1-phenylethyl acetate increased with increasing temperature. The values of the conversion and the entantiomeric excess of (s)-α-phenethyl alcohol (e.e$_s$) reached a maximum at 40°C and started to decease after the temperature exceeded 40°C. As shown in Figure 4, the reactive dependence on the temperature weakened with increasing enzyme concentration.

Selection of the Reactive Solvents

The structures and functions of an enzyme in the water phase and organic phase are very different. In many cases, the organic phase solution in the anhydrous system is often used as the enzyme-catalyzed reaction environment, so the solvent selection is of crucial importance for improving the activity and selectivity of the enzyme reaction. We investigated the influence of different solvents, such as ethyl acetate, dichloromethane, hexane, acetonitrile, tetrahydrofuran, toluene, and vinyl acetate, on the enzyme-catalyzed transesterification. As shown in Figure 5 and Table S4 (in the Supporting Information), the enzyme activity was the best in the vinyl acetate esters. In a kinetic resolution in a nonaqueous environment, solvents have greater impacts on the reaction rate and stereoselectivity. Stronger hydrophobic solvents favor the kinetic resolution. This is mainly due to the fact that hydrophobic solvents can be maintained around the enzyme molecule of water, and the combination of the enzyme molecule of water is the basis for maintaining its structure and physiological activity.[36] Log *P*, where *P* is the partition coefficient of a given solvent between *n*-octanol and water, is a widely used parameter for describing the hydrophobicity/hydrophilicity of organic solvents and their effects on enzyme activities.[37] Table S4 shows that hydrophobicity in solvent has little effect on the substrate conversion for the chiral resolution of α-phenyl ethanol in this study. Typically, vinyl acetate or ethyl acetate has been used as an acyl donor in the kinetic resolution of secondary alcohols. When vinyl acetate was used, because it is an unstable product, vinyl alcohol was formed in stoichiometric amounts and quickly reacted further to form acetaldehyde, so acylation was irreversible, and the reactive rate was higher. If ethyl acetate was used, ethanol was formed as a byproduct, and the reaction was reversible, and ethanol decreased the reaction rate by increasing the hydrophilicity of the reaction mixture.

Effects of the Molar Ratio of the Substrate and Acyl Donor on the Reaction

Typically, vinyl acetate has been used as an acyl donor in the kinetic resolution of secondary alcohols. The acyl donor has a major impact on the efficiency and selectivity of the reaction

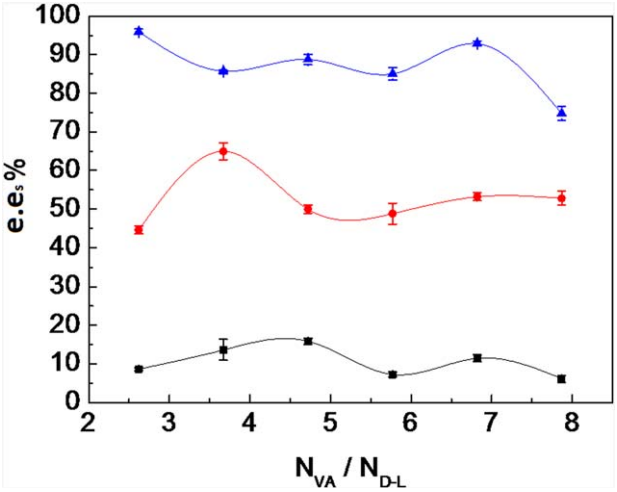

Figure 6. e.e$_s$ values at different molar ratios of the substrate to the acyl donor for the freeze-dried enzyme. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 3 mL of vinyl acetate, 40°C, and 10 h of reaction time. (■) 300, (●) 500, and (▲) 700 μL of CALB. N_{VA} = molar number of vinyl acetate; N_{D-L} = molar number of α-phenyl ethanol. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

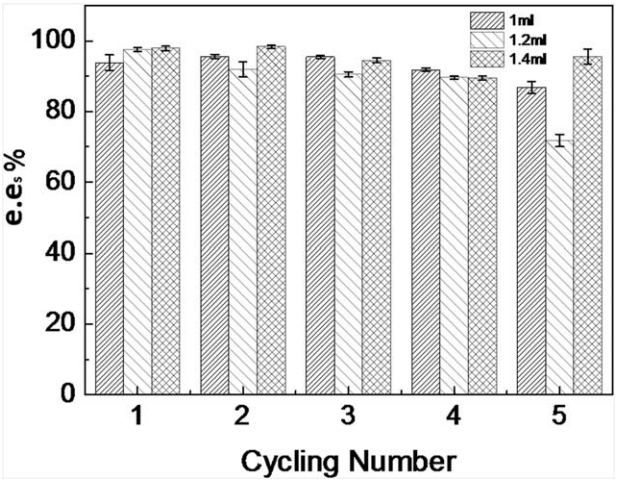

Figure 7. Recycling stability of the lyophilized enzyme. Reaction conditions: 4 mmol of (±)-1-phenylethanol, 3 mL of vinyl acetate, 40°C, 10 h of reaction time, and CALB contents of 1000, 1200, and 1400 μL.

system. To examine the dependence of the molar ratio of the substrate and acyl donor on the chemical equilibrium of the lipase-catalyzed reaction and enzymatic esterification, six different substrate to acyl donor molar ratios (1:2.62, 1:3.67, 1:4.72, 1:5.77, 1:6.82, and 1:7.87) were investigated. The molar ratios of the substrate to the acyl donor with different enzyme contents had dissimilar effects on the resolution reaction. The $e.e_s$ had its maximum value at the 1:4.72 molar ratio in 300 μL of enzyme. For an enzyme content of 500 μL, the yield and $e.e_s$ reached a maximum value at a 1:3.67 molar ratio. The $e.e_s$ had its maximum value at the 1:2.62 molar ratio in 700 μL of enzyme. The $e.e_s$ remained roughly unchanged over the scope from 1:4.72 to 1:6.82 for the three reactive systems. An increase in the vinyl acetate concentration could accelerate the product formation in chemical equilibrium. However, the concentration of vinyl acetate increased to a certain extent, and the promotion effect on the chiral selectivity on the immobilized enzyme gradually disappeared. Figure 6 and Table S5 (in the Supporting Information) demonstrates that the effect of the molar ratio on the chiral selection became weak with increasing content of enzyme. As shown in Table S5 (in the Supporting Information), the optimum molar ratio of vinyl acetate to α-phenyl ethanol was about 4:1. Generally, good enzyme activity could be maintained in reactive media with a molar ratio from 5:1 to 7:1, and the activity decreased when the molar ratio was greater than 7:1 at all enzyme concentrations.

Reusability of the Lyophilized Enzyme

Enzymatic reproducibility is also of crucial importance for chiral resolution reactions. In these experiments, the reactive solution was carefully taken out with a dropper, with the exception of the enzyme beads; the reaction vessel and enzyme beads were washed several times with *n*-hexane. The same amount of substrate was returned to the vessel and reacted in the same experimental conditions. The results show that enzyme beads still maintained a high activity after five cycles, as shown in Figure 7 and Table S6 (in the Supporting Information). This demonstrated that repeated use could be achieved for the resolution reaction also. In the kinetic resolution of secondary alcohols performed in the organic phase, the reactive conditions were harsh compared to the hydrolysis. In this case, the freeze-dried alginate beads still showed good catalytic activity and a high degree of reuse.

CONCLUSIONS

A simple and green pathway toward the immobilization of CALB in an alginate carrier was explored. In the presence of calcium cations, the enzyme–alginate suspension could be cross-linked to form beads with a porous structure at room temperature, and the enzymes were well dispersed in the beads. The activity of the enzyme–alginate beads was verified by the enzymatic hydrolysis reaction of *p*-nitrophenol butyrate in the aqueous phase. The chiral resolution of α-phenyl ethanol in the organic phase was tested by the same enzyme–alginate beads also. The effects of the reaction parameters, such as the enzyme concentration, temperature, and molar ratio of the substrate to the solvent, on the resolution behavior were discussed. To explore the advantage of the enzyme–alginate beads and their application, reuse cycles were tested for the chiral resolution of α-phenyl ethanol. The activity of the enzyme–alginate beads was maintained without marked deactivation for up to five repeated cycles. This study provided a new and simple way for designing and immobilizing enzymes in catalysis and other applications. The immobilization method provided by this study is expected to be expediently used in various organic reaction systems. Studies along these lines are now in progress.

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