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Immobilization of *Bacillus subtilis* esterase by simple cross-linking for enzymatic resolution of DL-menthyl acetate

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ABSTRACT

A recombinant esterase (EC 3.1.1.1) cloned from *Bacillus subtilis* 0554 (BSE) was carrier-freely immobilized with the method of cross-linked enzyme aggregates. The conditions for preparing the cross-linked aggregates of BSE (CLA-BSE) were optimized, including the type and concentration of precipitants, and the concentration of cross-linker, and a simple and efficient procedure for preparing CLA-BSE was developed, consisting of a precipitation step with 0.5 g mL^{-1} $(NH_4)_2SO_4$ and a cross-linking step with 60 mM glutaraldehyde for a period of 3 h as the cross-linking time. As a result, about 70% of the initial free BSE activity was incorporated into the CLA-BSE. The thermal stabilities of the immobilized enzyme at 30 °C and 50 °C were >360 and 14 times those of free BSE, respectively. More importantly, the operational stability of CLA-BSE was also considerably improved. In the kinetic resolution of DL-menthyl acetate to produce L-menthol with CLA-BSE gave ee_p > 94% at conversion of >40% and the CLA-BSE could be reused for 10 times with only about 8% reduction in activity. Therefore, the new biocatalyst immobilized through the methodology of CLEAs could significantly decrease the manufacturing cost of L-menthol and would be more beneficial for its practical applications.

1. Introduction

In the last decades, the use of lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) to produce chiral fine chemicals and drugs has become an alternative to chemical synthesis, as they are capable of enantioselective hydrolysis or esterification in a cheap and environmentally friendly process. *Bacillus subtilis* esterases have also drawn much attention because of their excellent catalytic properties and been used in the production of valuable chiral intermediates, such as tertiary alcohols, naproxen, and (*S*)-1,2-*O*-isopropylidene glycerol [1–3].

L-Menthol as an important flavoring chemical is extensively used in oral products, pharmaceuticals, tobacco products, confectionaries and shaving products. Enantioselective hydrolysis of racemic menthyl esters in aqueous medium mediated by a specific hydrolase is an attractive approach for the production of L-menthol. A few examples of preparing L-menthol in this manner have been reported [4,5]. We have also reported an esterase from *B. subtilis* ECU0554 that could produce L-menthol through enantioselective resolution of DL-menthyl acetate at a very high concentration [6]. In addition, the gene encoding the *B. subtilis* esterase (BSE) has also been successfully cloned and overexpressed in *Escherichia coli* BL21, and the recombinant esterase exhibited high activity, extraordinary substrate/product tolerance and excellent enantioselectivity in the production of L-menthol but poor stability under operational conditions (30 °C) and the half-life was only 2.8 h [7]. So its industrial application was hampered by poor operational stability, and difficult recovery and reuse of its free form.

These drawbacks can generally be overcome by applying a suitable method of immobilization [8]. Immobilized enzymes have many advantages in practical application, such as easy separation of biocatalyst from the product, reduced costs of downstream processing, reuse of biocatalyst, better stability, especially that against organic solvents and heat, and easy realization of continuous production. Therefore, many conventional immobilization methods associated with carriers, such as synthetic organic polymers, biopolymers or inorganic polymers, have been successfully applied to a variety of enzymes for industrial application [9–11]. Nonetheless, the use of an extra carrier usually leads to 'dilution of activity', owing to the introduction of a large portion of non-catalytic ballast from 90% to >99%, which results in lower space-time yields and productivities [12]. Moreover, immobilization of an enzyme on a carrier often leads to the loss of more than 50% native activity [13], especially at high enzyme loadings [14]. More recently, a novel family of carrier-free immobilized enzymes, cross-linked enzyme aggregates (CLEAs), have emerged and gained much attention in recent years [15]. The method comprises the aggregation of free enzyme from an aqueous solution using a precipitation

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agent, followed by cross-linking of the formed aggregates using a bifunctional cross-linker. Compared with other kinds of immobilized methods, this immobilization technology exhibits many advantages, for instance, simple preparation procedure, highly concentrated enzyme activity in the catalyst, high stability and low production costs due to the exclusion of an additional (expensive) carrier. Another important feature is that one does not have to start with a purified enzyme preparation [16]. Due to these advantages, many enzymes, such as penicillin G acylase, lipase, hydroxynitrile lyase, laccase, nitrilase, tyrosinase, etc., have been successfully immobilized through this immobilization method [17–20].

Herein, the cross-linked aggregates of *B. subtilis* esterase (CLA-BSE) expressed by recombinant *E. coli* BL21 were prepared. The effect of some key factors, including the type and concentration of precipitants and the concentration of cross-linking reagent on the activity of CLA-BSE was examined. In particular, the thermal stability and kinetic parameters of CLA-BSE were compared with those of the free enzyme and its reusability in the production of L-menthol was also investigated.

2. Experimental

2.1. Chemicals

Racemic menthol was purchased from Alfa Aesar (Tianjin, China). Racemic menthyl acetate was prepared from racemic menthol and acetyl chloride [6]. *p*-Nitrophenyl butyrate (*p*NPB) was synthesized by ourselves [21]. Tryptone and yeast extract were obtained from Oxoid (Shanghai, China). A 25 wt.% solution of glutaraldehyde in water was obtained from Shanghai Ling-Feng Chemical Reagents Co., Ltd. (Shanghai, China). All other regents or chemicals used were of analytical grade and obtained from local suppliers.

2.2. Enzyme preparation

BSE was prepared with the recombinant *E. coli* BL21/pBSE [7]. Cells of *E. coli* BL21/pBSE were grown for 8 h in LB medium containing kanamycin (50 μg mL^{-1}) at 37 °C. The cells were subsequently diluted (1:14, v/v) in 2.8 L fresh 2× LB medium and grown in a 5-L fermenter at 30 °C. IPTG (0.3 mM) was added to induce the expression of esterase gene when the optical density at 600 nm ($OD_{600\,nm}$) of bacterial cultures reached about 10. The cells were harvested by centrifugation (13,500 × *g*, 4 °C, 5 min) when the esterase activity did not increase, washed twice with physiological saline and resuspended in 300 mL sodium phosphate buffer (50 mM, pH 7.0). The cells were disrupted 3 times using a high-pressure homogenizer (AH110B, ATS Engineering Inc.) and the homogenate was centrifuged (13,500 × *g*, 4 °C, 30 min) to remove the cells debris. Ammonium sulfate was added slowly into the supernatant (soluble protein) with stirring at 0 °C, and the protein precipitated between 30% and 60% saturation of ammonium sulfate was collected by centrifugation (13,500 × *g*, 4 °C, 30 min) and dialyzed in 2 L sodium phosphate buffer (10 mM, pH 7.0). Then the dialysate was dried using a vacuum freeze drier, and the dry powder of crude BSE was stored at 4 °C and used for further experiments.

2.3. Activity assay

The activities of free esterase and CLEAs were measured using *p*NPB as a substrate. Esterase or blank solution (50 μL) was added to sodium phosphate buffer (2.920 mL, 100 mM, pH 7.0). After preincubation at 30 °C for 3 min, the reaction was initiated by a quick mixing of the reaction mixture with *p*NPB (30 μL, 100 mM) solution in dimethyl sulfoxide (DMSO) and the variation in absorbance at 405 nm was recorded [22]. One unit of activity was defined as the amount of enzyme releasing 1.0 μmol of *p*-nitrophenol per minute under such conditions.

2.4. Protein assay

Protein concentration was determined according to the method described by Bradford using bovine serum albumin (BSA) as the standard protein [23].

2.5. Precipitation study

The crude BSE (10 mg enzyme powder) was dissolved in sodium phosphate buffer (500 μL, 100 mM, pH 7.0). Various precipitants were slowly added to the esterase solution under gentle stirring at 0 °C until no more activity and protein were detected in the supernatant. A 25% aqueous glutaraldehyde solution (10 μL) was then added to the aggregates under gentle stirring at 0 °C and the mixture was continuously stirred at 0 °C for an additional 3 h. The insoluble CLA-BSE was collected by centrifugation (6000 × *g*, 4 °C, 5 min) and washed 3 times with sodium phosphate buffer (100 mM, pH 7.0) to remove all remaining glutaraldehyde and non-cross-linked protein. The CLA-BSE was resuspended in 500 μL of sodium phosphate buffer (100 mM, pH 7.0) and the activity was measured.

Optimization of ammonium sulfate concentration (0.2–0.7 g mL^{-1}) was carried out using the method described above.

2.6. Cross-linking study

The crude BSE (10 mg enzyme powder) was dissolved in sodium phosphate buffer (500 μL, 100 mM, pH 7.0). Ammonium sulfate (0.25 g) was slowly added to the esterase solution under gentle stirring at 0 °C, and continuously stirred at 0 °C for 10 min. A 25% aqueous glutaraldehyde solution (6, 8, 10, 12, 14 and 16 μL) was then added to the mixture. The suspension was stirred at 0 °C for 3 h and the CLA-BSE were separated by centrifugation (6000 × *g*, 4 °C, 5 min). The CLA-BSE was resuspended in 500 μL of sodium phosphate buffer (100 mM, pH 7.0) and the activity was measured.

2.7. Enzyme stability

Thermal stability experiments of both the free BSE and CLA-BSE were carried out at 30 and 50 °C under shaking condition (1000 rpm). Thermal stability results were presented as percentage of residual activities, taking their initial activities of the biocatalysts as 100%.

2.8. Kinetic analysis

Kinetic parameters of free BSE and CLA-BSE were determined by using different concentrations of L-menthyl acetate in the range of 1–32 mM in sodium phosphate buffer (200 mM, pH 8.0) at 30 °C. K_m and V_{max} of both the free and immobilized enzymes were calculated by the Lineweaver–Burk double-reciprocal method.

2.9. Enantioselective hydrolysis of racemic menthyl acetate with the immobilized BSE

Batch reactions were performed in a 250-mL three-necked, round-bottomed flask with a mixed solution of sodium phosphate buffer (90 mL, 200 mM, pH 8.0) and EtOH (10 mL). The resulting CLA-BSE prepared from free BSE (0.3 g) was added to the mixed solution. The reactions were initiated by addition of racemic menthyl acetate (20 g) at a constant stirring speed of 200 rpm. A temperature-controlled water bath was used to control the reaction temperature at 30 °C, and the pH of reaction was adjusted to 8.0

constantly by automatic titration with 2 M NaOH. Samples (250 μL each) were withdrawn at different time intervals and immediately extracted with ethyl acetate (500 μL). After centrifugation (6000 × *g*, 5 min), the samples were analyzed by GC.

2.10. Scanning electron micrograph (SEM) analysis

The CLA-BSE preparations were dehydrated on a vacuum freeze drier and mounted on aluminum SEM stubs using a double sticky tape. The mounted samples were sputter coated with a thin layer of gold and recorded by scanning electron microscopy (Jeol Electron Microscope, JSM 6360LV, Japan).

2.11. Analytical methods

Samples from the hydrolysis reaction mixture were analyzed on a GC-14 gas chromatography (Shimadzu, Tokyo, Japan) equipped with an FID detector. The ee_s was determined with a Beta Dex™ 120 chiral column (30 m × 0.25 mm, 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA) using N_2 as carrier gas. The column, injector and detector were held at 130, 280 and 350 °C, respectively. The ee_p was determined with a Gamma Dex™120 chiral column (30 m × 0.25 mm, 0.25 μm film thickness) also from Supelco (Bellefonte, PA, USA) using N_2 as carrier gas. The injector and detector were held at 280 and 350 °C, respectively. The oven temperature was programmed from 110 °C, held for 15 min, then raised to 150 °C at a rate of 10 °C min^{-1} and finally held at 150 °C for 1 min. The ee_s, ee_p and substrate conversion were calculated according to Chen et al. [24].

3. Results and discussion

3.1. Optimization of CLA-BSE preparation

The selection of precipitation parameters is a critical step in the preparation of CLEAs with a high recovery of enzyme activity [25,26]. Therefore, several types of precipitants on the activity recovery of CLA-BSE were first tried, including inorganic salt ($(NH_4)_2SO_4$), non-ionic polymer (polyethylene glycol (PEG)) and organic solvents (alcohols and acetone). These precipitants were slowly added into the enzyme solution until no more activity and protein were detected in the supernatant. The results were shown in Fig. 1. The activity recoveries of CLA-BSE were all relatively low when organic solvents were used as precipitants. This might be due to the fact that a high concentration of organic solvent causes partial denaturation of all enzymes including BSE. The better results were obtained with inorganic salt ($(NH_4)_2SO_4$) and non-ionic polymer (PEG) as precipitants. Especially, CLA-BSE gave the maximum activity recovery (51%) when $(NH_4)_2SO_4$ was utilized. So the very cheap $(NH_4)_2SO_4$ was selected as the optimized precipitant for preparation of CLA-BSE.

The concentration of precipitant also affects the activity recovery of CLEAs. Therefore, the adding amount of $(NH_4)_2SO_4$ was also investigated (Fig. 2). The activity recovery of CLA-BSE increased gradually with the increase of $(NH_4)_2SO_4$ added. However, an excessive addition of $(NH_4)_2SO_4$ resulted in the partial deactivation of the BSE and therefore the reduced activity recovery of CLA-BSE was observed. The maximum activity recovery (63%) of BSE-CLEAs was achieved at 500 g $(NH_4)_2SO_4$ per liter. Therefore, the optimum concentration of $(NH_4)_2SO_4$ was 0.5 g mL^{-1} for 20 mg mL^{-1} of the free BSE solution.

Subsequently, an investigation on the cross-linking process is also necessary. This is because that cross-linking using a bifunctional cross-linker, such as glutaraldehyde, periodate oxidized dextran [27], poly(ethyleneimine) (PEI) and their mixtures [28], can "lock" the enzyme into its active state and prevent redissolution

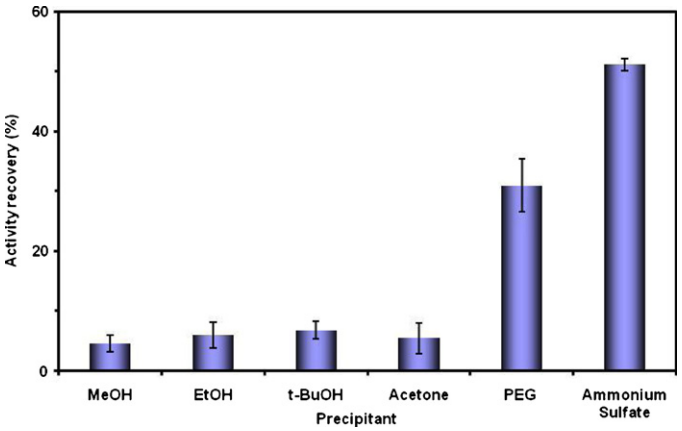

Fig. 1. Activity recovery of CLA-BSE with various types of precipitants. Various precipitants were slowly added to the esterase solution (500 μL, 100 mM, pH 7.0) containing 10 mg BSE powder under gentle stirring at 0 °C until no more activity and protein were detected in the supernatant. A 25% aqueous glutaraldehyde solution (10 μL) was then added to the aggregates under gentle stirring at 0 °C and the mixture was continuously stirred at 0 °C for an additional 3 h. The CLA-BSE centrifugated was resuspended in 100 mM phosphate buffer (pH 7.0) for activity assay.

(leaching) during reaction [16]. Moreover, cross-linking determines the particle size and makes the biocatalyst more robust against deleterious effects such as organic solvent or vigorous stirring [29]. Glutaraldehyde is generally employed as the cross-linking agent, as it is inexpensive and readily available in commercial quantities [16]. Therefore, glutaraldehyde was selected as cross-linking agent in this study.

Effect of glutaraldehyde concentration on the activity recovery of CLA-BSE was investigated (Fig. 3). It was observed that the activity recovery increased with an increase of glutaraldehyde concentration, and reached a maximum value (70%) when 60 mM glutaraldehyde solution and 3 h cross-linked time were adopted, and then reduced. The excessive cross-linking might influence the

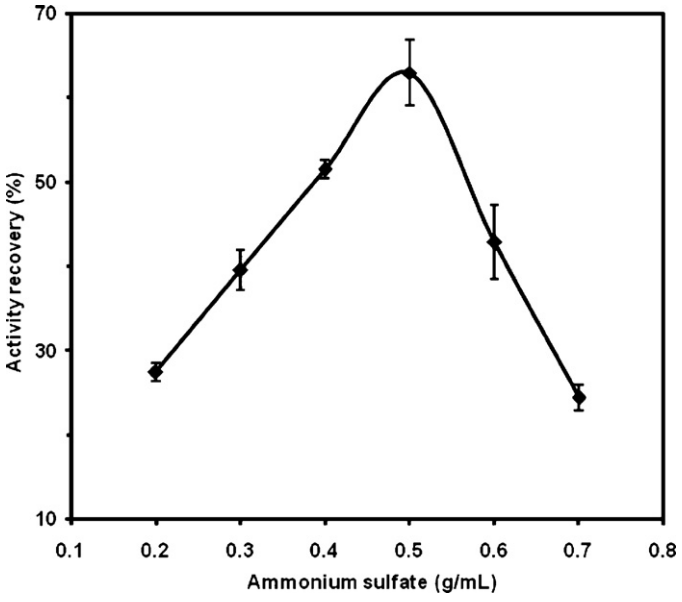

Fig. 2. Effect of precipitant concentration on the activity recovery of CLA-BSE. Various concentration of ammonium sulfate were slowly added to the esterase solution (500 μL, 100 mM, pH 7.0) containing 10 mg BSE powder under gentle stirring at 0 °C and the mixture was stirred for 10 min. Then, A 25% aqueous glutaraldehyde solution (10 μL) was then added to the aggregates under gentle stirring at 0 °C and the mixture was continuously stirred at 0 °C for an additional 3 h. The CLA-BSE centrifugated was resuspended in 100 mM phosphate buffer (pH 7.0) for activity assay.

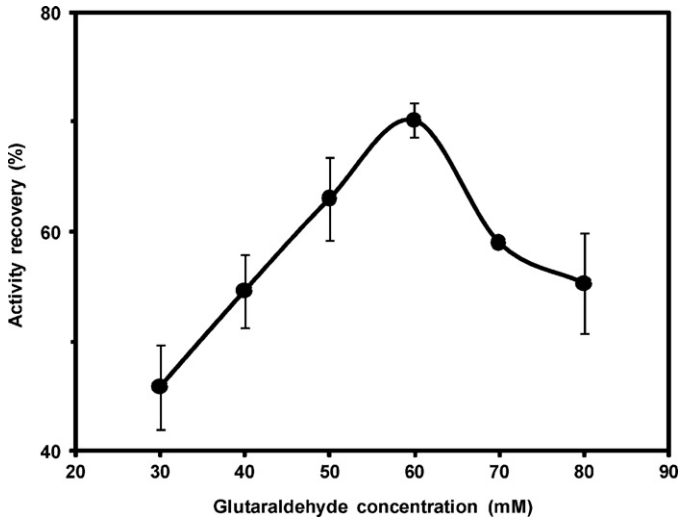

Fig. 3. Effect of glutaraldehyde concentration on the activity recovery of CLA-BSE. 0.25 g ammonium sulfate was slowly added to the esterase solution (500 μL, 100 mM, pH 7.0) containing 10 mg BSE powder under gentle stirring at 0 °C for 10 min. A 25% aqueous glutaraldehyde solution (6, 8, 10, 12, 14 and 16 μL) was then added to the mixture and the suspension was stirred at 0 °C for 3 h. The CLA-BSE centrifugated was resuspended in100 mM phosphate buffer (pH 7.0) for activity assay.

active site availability and therefore decrease the activity recovery of CLA-BSE [30].

Herein, CLA-BSE was prepared under the above-mentioned optimal conditions. Finally, 70% of free BSE was effectively recovered in the CLA-BSE. The scanning electron microscopy images of CLA-BSE were given in Fig. 4. The surface structure of CLA-BSE was observed, and it looks similar to the structures of other CLEAs given in the literature [31]. The particle size dispersion, as measured by laser diffraction, was homogeneous and narrow, from 2 to 5 μm (*Z*-average: 3.910 μm; PdI: 0.369, see Fig. 5).

3.2. Thermal stability of free and immobilized BSE

For any industrial application, the stability of immobilized preparation is of great importance. Thermal stabilities of both the free BSE and CLA-BSE were examined at different temperatures (30 °C and 50 °C) and time intervals (Fig. 6). The results showed that the stability of the enzyme in CLEAs was increased significantly at both temperatures, especially at the typical operation temperature (30 °C). At 50 °C, after 10 h of incubation, the free BSE almost inactivated completely, but the CLA-BSE still retained more than 60% of its initial activity. Notably, an obvious hyperactivation phenomenon was observed for CLA-BSE when incubated at 30 °C and nearly 60% enhancement of its initial activity was obtained after 250 h of incubation. This increase in the activity might be that a long period of shaking during incubation helped to disperse the CLA-BSE to smaller particles and minimized size-imposed kinetic barriers to efficient catalysis. The hyperactivation phenomenon has also been observed by others [20]. The CLA-BSE retained nearly 130% of its initial activity after being incubation for as long as 1000 h. However, the same amount of activity loss of the free BSE occurred within about 1 h at the same temperature, and the activity almost was lost completely after 72 h. The initial first-order thermal inactivation rate constant (k_d) was calculated from the initial slope of the plot of the logarithmic form of the residual activity versus the incubation time. The half-life of CLA-BSE increased >360-fold and 14-fold respectively at 30 °C and 50 °C as compared with those of the free enzyme (Table 1). This enhancement in stability can be due to inter- and intramolecular covalent cross-linking that prevent conformational changes. Thermal inactivation rate constant (k_d) and observed half-life ($t_{1/2}$) observed also suggest that, thermal stability of BSE was increased significantly in the CLA-BSE form under both conditions.

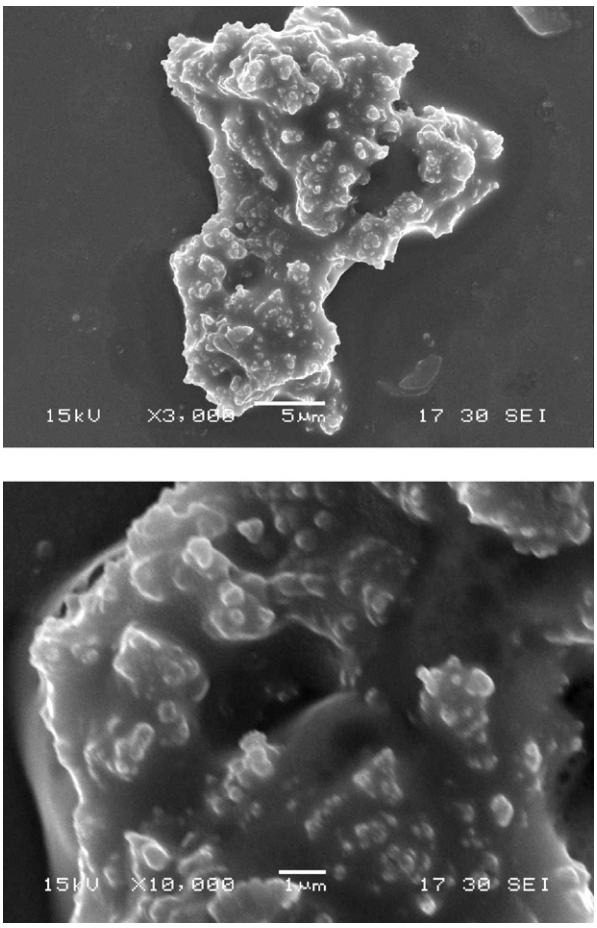

Fig. 4. SEM photographs of CLA-BSE at 3000× (top) and 10,000× (bottom) magnification.

Table 1
First order inactivation rate constant (k_d) and half life ($t_{1/2}$) of free BSE and CLA-BSE.

Temperature [°C]	Biocatalyst form	k_d [h^{-1}]	$t_{1/2}$ [h]
30	Free BSE	0.248	2.8
30	CLA-BSE	<0.001	>1000
50	Free BSE	0.770	0.90
50	CLA-BSE	0.055	12.5

Inactivation was performed in 100 mM KPB (pH 7.0) at 30 °C and 50 °C, respectively.

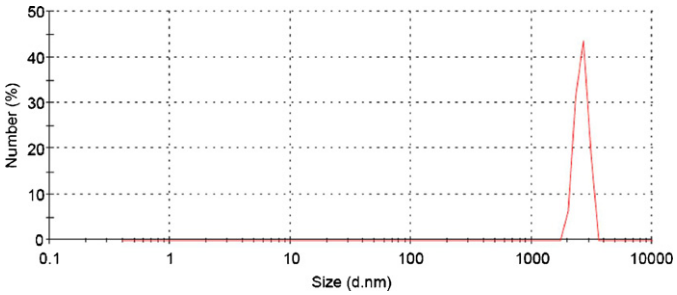

Fig. 5. Particle size distribution of CLA-BSE prepared under optimal conditions.

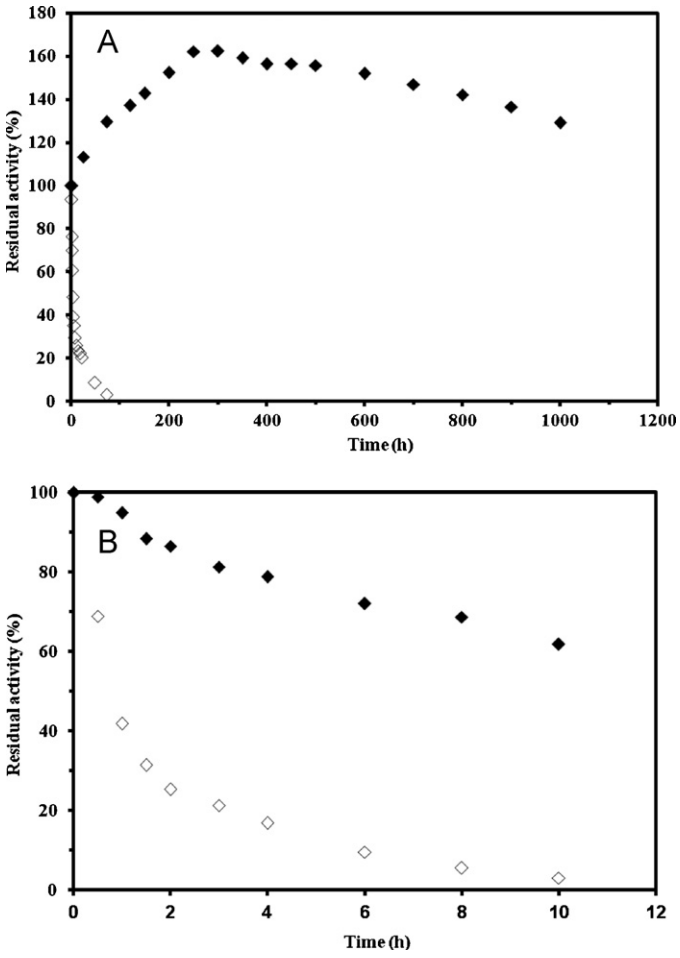

Fig. 6. Thermal inactivation profiles of free BSE and CLA-BSE at 30 °C (A) and 50 °C (B). Inactivation was performed in 100 mM KPB (pH 7.0) at 30 °C and 50 °C, respectively. (♦): CLA-BSE; (□): free BSE.

3.3. Kinetic studies of free and immobilized BSE

Kinetic parameters of the free BSE and CLA-BSE were examined by measuring initial rates of reaction at various L-menthyl acetate concentrations. For both the free and immobilized enzymes, Michaelis–Menten type kinetic behavior was observed. As Table 2 shows, V_{max} values of the free and CLA-BSE were nearly equal, which indicates the rate of L-menthyl acetate hydrolysis by BSE did not changed after immobilization. The slightly higher K_m value in CLA-BSE suggests a reduction of affinity for its substrate. This might be explained that conformational changes due to immobilization result in an unfavorable position of active sites.

3.4. Enantioselective hydrolysis of racemic menthyl acetate with the immobilized BSE

From an industrial point of view, the quality of a given kinetic resolution not only depends upon the degree of enantioselectivity,

Table 2
Comparison of kinetic parameters between free BSE and CLA-BSE.

	V_{max} [mM h^{-1}]	K_m [mM]
Free BSE	0.526	1.5
CLA-BSE	0.485	2.2

Kinetic parameters of the free BSE and CLA-BSE were examined by measuring initial rates of reaction at various L-menthyl acetate concentrations at 30 °C and pH 8.0.

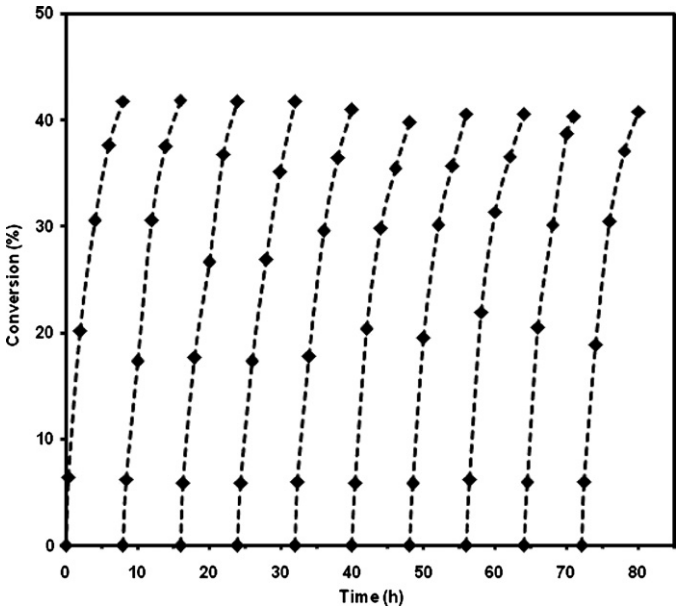

Fig. 7. Reusability of CLA-BSE in kinetic bioresolution of DL-menthyl acetate to produce L-menthol. Reaction was performed at 30 °C in aqueous solution containing 10% EtOH and 1 M DL-menthyl acetate. The pH is kept at 8.0. After each batch of reaction, the CLA-BSE was centrifuged and washed after each use, and then resuspended again in a fresh reaction mixture.

but also on the activity and the possibility of recycling and reusing the lipase. We therefore studied all of these factors in a test reaction involving the hydrolysis reaction-kinetic resolution of high concentration DL-Menthyl acetate (200 g L^{-1}, ~1.0 M) to produce L-menthol. The CLA-BSE in each case were centrifuged and washed after each use and then resuspended again in a fresh reaction mixture to measure the conversion and residual activity. The residual activity was calculated by taking the enzyme-catalyzed activity of the first cycle as 100%. The substrate conversion and residual activity of CLA-BSE during 10 rounds of recycling were shown in Fig. 7. The resolution reactions with CLA-BSE gave L-menthol (ee_p) > 94% at conversion of >40%. After 10 cycles, the CLA-BSE still showed as much as 92% of its initial activity. Immobilized CLA-BSE exhibited high enantioselectivity, high conversion, high operational stability and fast recovery of product.

4. Conclusions

A significantly stabilized biocatalyst of *B. subtilis* esterase was developed by using the method of cross-linked enzyme aggregates. The optimal conditions for preparing CLA-BSE were obtained with $(NH_4)_2SO_4$ as the precipitant and glutaraldehyde as the cross-linker, *i.e.*, $(NH_4)_2SO_4$, 0.5 g mL^{-1}; enzyme solution, 20 mg mL^{-1}; glutaraldehyde, 60 mM; and cross-linking period, 3 h. It was shown that the thermal stability of CLA-BSE was far superior to that of free BSE. The CLA-BSE also exhibited excellent operational stability, retaining 92% of its initial activity after 10 cycles of reuse. This indicates that CLA-BSE is very promising as an immobilized biocatalyst for the kinetic bioresolution of DL-menthyl acetate to produce L-menthol in industrial application.

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