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ORIGINAL PAPER

Enantioselective hydrolysis of (*R*)-2, 2-dimethylcyclopropane carboxamide by immobilized cells of an *R*-amidase-producing bacterium, *Delftia tsuruhatensis* CCTCC M 205114, on an alginate capsule carrier

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Abstract Immobilized cells of *Delftia tsuruhatensis* CCTCC M 205114 harboring *R*-amidase were applied in asymmetric hydrolysis of (*R*)-2, 2-dimethylcyclopropane carboxamide (*R* − 1) from racemic (*R*, *S*)-2, 2-dimethylcyclopropane carboxamide to accumulate (*S*)-2, 2-dimethylcyclopropane carboxamide (*S* − 1). Maximum *R*-amidase activity of 13.1 U/g wet cells (0.982 U/g beads) was obtained under conditions of 3% sodium alginate, 2.5% $CaCl_2$, 15 h crosslinking and 2 mm bead size, which was 53.9% of that of free cells (24.3 U/g wet cells). In addition, characterization of the immobilized cells was examined. The optimum *R* − 1 hydrolysis conditions were identified as follows: substrate concentration 10 mM, pH 8.5, temperature 35°C and time course 40 min. Under optimum conditions, the maximum yield and enantiomeric excess of (*R*)-2, 2-dimethylcyclopropanecarboxylic acid were 49.5% and >99%, respectively. This afforded *S* − 1 with a yield >49% and an *e.e.* of 97.7%. With good operational stability and excellent enantioselectivity, the immobilized cells could be potentially utilized in industrial production of *S* − 1.

Keywords Nitrile-converting enzyme · *R*-amidase · Immobilized cells · *Delftia tsuruhatensis* · (*S*)-2, 2-dimethylcyclopropane carboxamide

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Introduction

Production of economically important chiral carboxyl acid and its amide derivatives by biotransformation of nitrile using nitrile-converting enzymes (nitrile hydratase, amidase and nitrilase) has become a promising chiral synthesis method [3, 5, 14, 15, 18–20, 33]. With the ability to hydrolyze amides to corresponding optical pure compounds with high enantioselectivity, mild reaction conditions and environmental compatibility, amidases (EC 3.5.1.4) have been intensively studied [2, 4, 7, 8, 10, 12, 13, 16, 17, 21, 22, 26, 30]. However, current research on amidase, such as screening, characterization and its heterogeneous expression, is concentrated on the laboratory level, and applications of amidase on a large scale are quite few. This has often been partly caused by the difficulty in recycling the amidase, which might result in high costs for amidase preparation. Thus, it is important to improve the reuse of amidase for the scale-up of amidase-catalyzed bioprocesses.

Biotransformations of amides could be carried out with both purified or partly purified amidase and amidase-harboring microbial cells. The cells could be used in free or immobilized states. Compared to the free cells, the immobilized cells can be readily recovered from the reaction mixture and recycled [9]. To date, immobilization is one of the most successful technologies to extend the operational lifetime of biocatalysts and allow for easy recovery of biocatalysts from the reaction mixture. Therefore, their large-scale application is possible. Cells may be immobilized by various physical and chemical methods. Encapsulation is an important physical method for cell immobilization. Among the capsule-forming materials, alginate, a low-cost linear seaweed extract composed of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid, is by far the most widely applied because of its nontoxic nature and easy

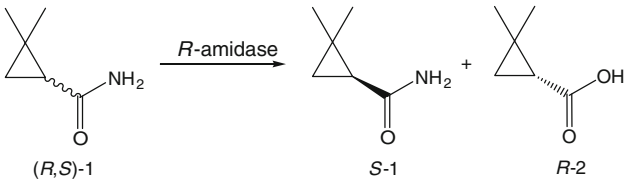

Fig. 1 Kinetic resolution of racemic (*R*, *S*) − 1 by *R*-enantioselective amidase

manipulation. Alginate can rapidly form gels by crosslinking the carboxyl group of α-L-guluronic acid with Ca^{2+} or Ba^{2+} ions under mild conditions.

Although amidases, a promising synthesis tool for chiral carboxyl acids and their derivatives, have been intensively investigated, reports on immobilization of amidases are rare. The *Delftia tsuruhatensis* CCTCC M 205114, an *R*-amidase-producing bacterial strain, has been isolated and used for *R*-enantioselective hydrolysis of *R*-2,2-dimethylcyclopropanecarboxamide (*R* − 1) from (*R*,*S*)-2,2-dimethylcyclopropanecarboxamide ((*R*,*S*) − 1) with the release of (*R*)-2,2-dimethylcyclopropanecarboxylic acid (*R* − 2) and accumulated (*S*)-2,2-dimethylcyclopropanecarboxamide (*S* − 1) (a key intermediate of cilastatin, a renal dehydropeptidase inhibitor administered with penem and carbapenem antibiotics to prevent their degradation in the kidney) (Fig. 1). Thus, the strain might be used as an *S* − 1 producer [30]. Presently, *S* − 1 is mainly chemically prepared via asymmetric synthesis or resolution of racemic substrates such as (*R*, *S* − 1) or (*R*, *S* − 2) [1, 25, 27, 28]. However, these methods suffered from certain flaws such as complex reactions, expensive chiral catalysts, harsh reaction conditions, low recovery and high cost. Biological methods based on asymmetric hydrolysis of *R* −1 from (*R*, *S*) − 1 using *R*-amidase producing microorganisms were also attempted [11, 24, 34]. Compared to the chemical methods, the biological methods involved simpler reactions and had higher product quality and fewer unit operations required. However, the reusability of the biocatalysts was poor.

The aim of this work was to improve the reusability of microbial cells harboring *R*-amidase by immobilizing *D. tsuruhatensis* CCTCC M 205114 cells with alginate entrapment. We report herein the immobilization of *D. tsuruhatensis* CCTCC M 205114 cells and the application of the immobilized cells in enantioselective hydrolysis of *R* − 2, 2-dimethylcyclopropane carboxamide.

Materials and methods

Materials

Compounds (*R*, *S*) − **1**, (*R*, *S*) − **2** and *S* − **1** were provided by Huakang Chemicals Ltd. (Shangyu, Zhejiang, China). Sodium alginate and yeast extract were purchased from Huadong Medicine Group (Hangzhou, Zhejiang, China). All other chemicals were of analytical grade.

Microorganism and growth conditions

The *D. tsuruhatensis* CCTCC M 205114 harboring *R*-amidase was isolated from a soil sample using a colorimetric method [30, 32] and preserved at the China Center for Type Culture Collection (CCTCC, Wuhan, China). For cell growth and amidase production, the strain was cultivated in 250-ml flasks containing 40 ml of optimized medium [in g/l: glucose 8.23, yeast extract 11.59, (*R*, *S*) − **1** 1.76, NaCl 1, KH_2PO_4 1, and K_2HPO_4, 1] aerobically on a rotary shaker at 30°C, 150 r/min [29]. After 20 h incubation, the cells were collected by centrifugation for 8 min at 9000×*g*, 4°C. Then the resulting cells were washed twice with 0.85% (w/v) NaCl solution and centrifuged at 9000×*g* for 8 min, 4°C. The harvested cells were stored at 4°C and used in the following experiments.

Entrapment of *D. tsuruhatensis* CCTCC M 205114

The sodium alginate solution (3%, w/v) was prepared by dissolving 1.8 g sodium alginate in 40 ml deionized water, heating and votexing. Then the cell suspension (3 g wet weight cells in 20 ml deionized water) was introduced to the sodium alginate solution and mixed thoroughly to obtain homogeneous cell/sodium alginate suspension. The immobilization was carried out using a peristaltic pump. The mixture was extruded through silicone tubing and dripped into a 2.5% (w/v) $CaCl_2$ solution to give spherical entrapment beads. The entrapped beads were maintained immersed in the same solution for 15 h at 4°C. The bead radius (2.0, 2.5 and 3.0 mm) was controlled by flow rate of the mixture. Then the beads were washed three times with distilled water and stored at 4°C.

Hydrolyzing of *R* − 1 by immobilized *D. tsuruhatensis* CCTCC M 205114 cells

Hydrolyzing of *R* − 1 was performed in a 50-ml Erlenmeyer flask with a screw cap. One gram of entrapped cells and 4 ml of 0.85% NaCl solution were added to the flask. Then the entrapped cells were preincubated at 35°C for 10 min in a reciprocal shaker at 150 r/min. One milliliter of 50 mM (*R*, *S*) − 1 solution was added to the flask, and the mixture was incubated at 35°C for 10 min with a reciprocal shaker at 150 r/min. The bead-free reaction broth (600 μl) was withdrawn after a specified period of time and used for determination of *R* − 1, *S* − 1, *R* − 2, *S* − 2 concentration

and *e.e.* of $R-2$. When the time course of (*R*, S) − 1 hydrolysis was examined, the bead-free samples were periodically withdrawn from separate reaction mixtures with identical conditions.

All samples were analyzed in triplicates. Then the entrapped cells were recovered by filtration and washed three times with distilled water. The resulting entrapped cells were maintained or reused.

Enzyme assay

Concentration of $R-1$, $S-1$, $R-2$ and $S-2$ was determined using an enantioselective capillary column BGB-175 (50% 2,3-diacetyl-6-tert-butyldimethylsilyl-gamma-cyclodextrin dissolved in 14% cyanopropyl-phenyl and 86% methylpolysiloxane, BGB Analytik, Boeckten, Switzerland). The assay was performed with a GC-14C instrument (Shimadzu, Kyoto, Japan), equipped with a flame ionization detector (FID). The column flow rate was 1.6 ml/min (helium). The GC was performed in the split mode with a split ratio of 40. The column temperature was maintained at 130°C for analysis of (*R*, *S*) − 2 and 170°C for analysis of (*R*, *S*) − 1. The inlet and detector temperatures were both kept at 220°C. Chromatographic data were collected and processed using a digital computer with a GC solution workstation [31].

One unit of the enzyme activity was defined as the amount of enzyme required to produce 1 μmol of (*R*, *S*) − 2 per minute at 35°C.

The conversion rate (*c*) was detected from the ratio of hydrolyzed substrate concentration ($[S_0]-[S]$) to the original substrate concentration ($[S_0]$).

$$c=\frac{[S_0]-[S]}{[S_0]}\times 100\%$$

in which $[S_0]$ is the initial (*R*, *S*) − 1 concentration, $[S]$ is the residual (*R*, *S*) − 1 concentration and $[S_0]-[S]$ is the hydrolyzed (*R*, *S*) − 1 concentration.

The enantiomeric excess (*e.e.*) of (*R*) − 2 and (*S*) − 1 was calculated as follows:

$$e.e._P=\frac{[R-2]-[S-2]}{[R-2]+[S-2]}\times 100\%$$

$$e.e._S=\frac{[S-1]-[R-1]}{[S-1]+[R-1]}\times 100\%$$

in which $[R-2]$, $[S-2]$, $[S-1]$ and $[R-1]$ are the concentration of $R-2$, $S-2$, $S-1$ and $R-1$, respectively.

Results and discussion

Entrapment of *D. tsuruhatensis* CCTCC M 205114

Effects of sodium alginate concentrations and bead sizes

To examine the effect of sodium alginate concentration on amidase activity of the immobilized cells, 20 to 35 g/l of sodium alginate was used for immobilizing the cells (Fig. 2a). As shown in Fig. 2a, there was a significant difference in amidase activity of the immobilized cells obtained under different sodium alginate concentrations. The amidase activity of the immobilized cells first increased when the sodium alginate concentration increased from 20 to 30 g/l. The maximal enzyme activity was obtained at 30 g/l of sodium alginate. Then the amidase activity of the immobilized cells decreased when the sodium alginate concentration further increased. We also found that when sodium alginate concentrations higher than 35 g/l were tested, it was difficult to form spherical beads. This was consistent with the earlier report on immobilization of cells from a nitrilase-producing bacterium, *Bacillus subtilis* ZJB-063, with sodium alginate [6]. Therefore, 30 g/l of sodium alginate was optimal for immobilization of *D. tsuruhatensis* CCTCC M 205114.

The bead size of the alginate capsule has a significant influence on the enzyme activity of the immobilized biocatalyst. To examine the size effect of beads on amidase activity of the immobilized cells, the beads with a radius of about 2.0, 2.5 and 3.0 mm were investigated (Fig. 2b). As shown in Fig. 2b, the bead size demonstrated a significant influence on amidase activity of the immobilized cells. The amidase activity of the immobilized cells decreased significantly when bead size increased from 2 mm to 3 mm. This could be due to the diffusion limitations of the substrate and products by the matrix that entrapped the cells. Therefore, bead size of approximately 2 mm was optimal for immobilization of *D. tsuruhatensis* CCTCC M 205114.

Based on the above results, 3.0 g wet cells (0.45 g dry cell weight) of *D. tsuruhatensis* CCTCC M 205114 was immobilized as follows: sodium alginate 3%, $CaCl_2$ 2.5%, crosslinking time 15 h and bead size of 2 mm. Under these conditions, 40.01 g of beads was obtained (approximately 0.075 g wet cells/g beads). The biocatalyst loading was 1.667 g wet cells/g alginate. The amidase activity of resulting immobilized cells was around 13.1 U/g wet cells (0.982 U/g beads), which was about 53.9% of the amidase activity of free cells (24.3 U/g wet cells).

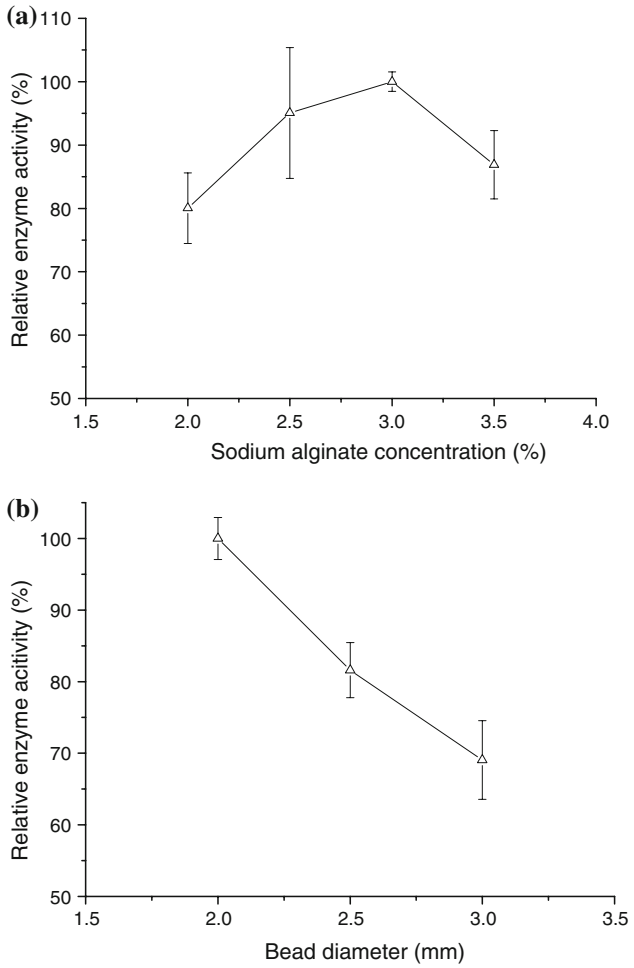

Fig. 2 Effect of **a** sodium alginate concentration and **b** bead size on amidase activity of immobilized cells of *D. tsuruhatensis* CCTCC M 205114. Reaction conditions: 1 g immobilized cells, 35°C, 10 min, 10 mM (*R*, *S*) − 1, 150 r/min, reaction mixture volume: 5 ml

Characterization of immobilized cells of *D. tsuruhatensis* CCTCC M 205114

Effect of pH on hydrolyzing of (R, S) − 1 by immobilized cells and free cells

In all enzymatic reactions, buffer pH plays an important role by affecting the activity and selectivity of enzymes. To examine the effect of pH on hydrolyzing of (*R*, *S*) − 1 by immobilized cells and free cells, the amidase activity of immobilized cells and free cells was determined between pH 6.0 and 10.0. The hydrolysis reaction was carried out in buffers as follows: 10 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0, 6.5, 7.0, 7.5, 8.0), 10 mM Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5, 9.0) and 10 mM sodium borate buffer (pH 9.2, 9.5, 10.0) (Fig. 3), which showed that buffer pH exhibited a significant influence on the reaction of both immobilized cells and free cells. The amidase activity of immobilized

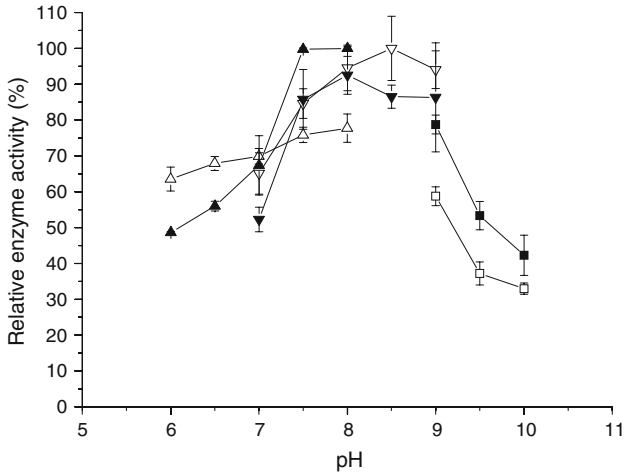

Fig. 3 Effect of pH on amidase activity of immobilized cells and free cells of *D. tsuruhatensis* CCTCC M 205114. Reaction conditions: 1 g immobilized cells or 0.076 g wet cells, 35°C, 10 min, 10 mM (*R*, *S*) − 1, 150 r/min; reaction mixture volume: 5 ml. Symbols: *filled square* NaH_2PO_4-Na_2HPO_4 buffer, *open square* Tris-HCl buffer and *open square* sodium borate buffer for immobilized cells. *Filled triangle* NaH_2PO_4-Na_2HPO_4 buffer, *filled inverted triangle* Tris-HCl buffer and *filled square* sodium borate buffer for free cells

cells increased when buffer pH increased from 6.0 to 8.5. However, a further increase in buffer pH above 8.5 resulted in a notable decline in amidase activity. As to free cells, amidase activity first increased when buffer pH increased from 6.0 to 8.0, and then dropped when buffer pH was above 8.0.

The results showed that Tris-HCl buffer was the optimal buffer for (*R*, *S*) − 1 hydrolysis by immobilized cells. The maximal amidase activity of immobilized cells was obtained at pH 8.5, whereas free cells exhibited maximal amidase activity at pH 8.0.

Effect of temperature on hydrolyzing of (R, S) − 1 by immobilized cells and free cells

It is reported that temperature plays an important role in the activity and selectivity of a biocatalyst [23]. To examine and compare the effect of temperature on hydrolyzing of (*R*, *S*) − 1 by the immobilized cells and free cells, the amidase activity of the immobilized cells and free cells was determined at 25, 30, 35, 40, 45, 50 and 55°C, respectively (Fig. 4a). It is indicated that temperature plays an important role in (*R*, *S*) − 1 hydrolyzing by the immobilized cells and free cells. The immobilized cells showed maximal amidase activity at 45°C, which was nearly 2 times higher than that of 25°C. Although free cells also exhibited maximal amidase activity at 45°C, their activity dropped faster at temperature above 45°C compared with immobilized cells. Therefore, immobilized cells were less sensitive to high temperatures than free cells. However, a significant

difference in enanotioselectivity of the immobilized cells against (*R*, *S*) − 1 was observed at the various temperatures. The *e.e.* of *R* − 2 remained above 99% at reaction temperatures from 25 to 35°C. When the reactions were carried out at temperatures from 40 to 45°C, the amidase activity of the immobilized cells increased significantly, but the *e.e.* of the product dropped remarkably. When the temperature was above 45°C, a further increase in reaction temperature led to a remarkable drop in amidase activity and the *e.e.* of the products. Furthermore, a thermal reverse in enanotioselectivity against *R* − 1 and *S* − 1 was observed at 45°C. The enanotioselectivity against (*R*, *S*) − 1 of free cells also displayed the same changes (Fig. 4a). The results indicated the enanotioselectivity of *D. tsuruhatensis* CCTCC M 205114 was highly temperature-dependent. This might be caused by changes in configuration of the amidase active site following heat treatment [32]. Taking into account the amidase activity and product *e.e.*, 35°C was considered as the optimal temperature for hydrolysis of (*R*, *S*) − 1.

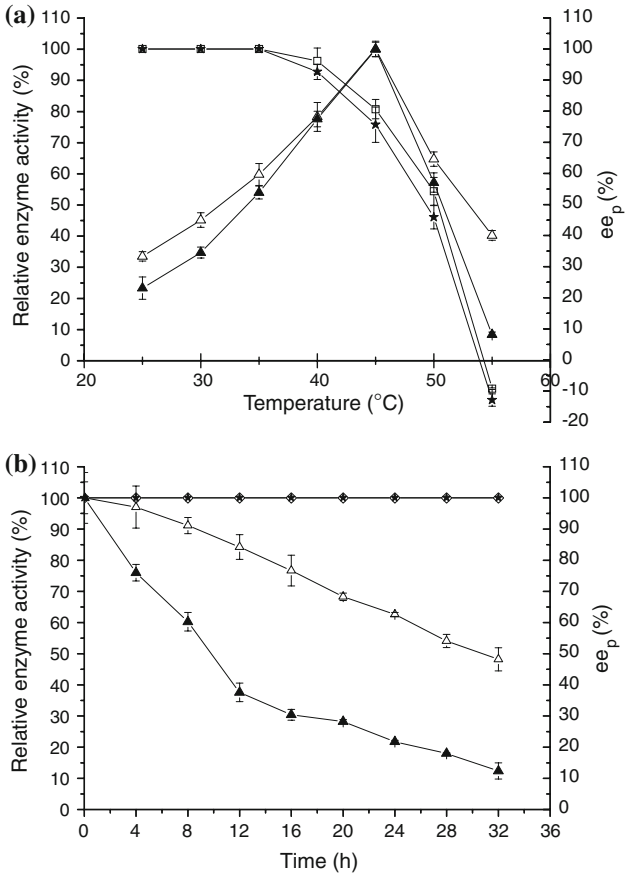

Fig. 4 Effect of temperature on **a** amidase activity and **b** stability at 35°C of immobilized cells and free cells of *D. tsuruhatensis* CCTCC M 205114. Reaction conditions: 1 g immobilized cells or 0.076 g wet cells, pH 8.5, 10 min, 10 mM (*R*, *S*) − 1, 150 r/min; reaction mixture volume: 5 ml. Symbols: *open square* relative amidase activity of immobilized cells, *filled triangle* relative amidase activity of free cells, *open square e.e.* of the products of immobilized cells, *open square e.e.* of the products of free cells

The stability of biocatalyst is important for all bioprocesses. The stabilities of the immobilized cells and free cells at 35°C were further determined (Fig. 4b). About 54% amidase activity of the immobilized cells remained after 28 h, whereas only 37.6% amidase activity of the free cells remained after 12 h. Therefore, immobilization can significantly improve the stability of the cells. The results also indicated that the enanotioselectivity against *R* − 1 of immobilized cells and free cells was quite stable (*e.e.* >99%) at 35°C. This suggests that the immobilized cells in this study would be beneficial if used on an industrial scale.

Effect of substrate concentration on hydrolyzing of (R, S) − 1 by immobilized cells and free cells

To find the optimal (*R*, *S*) − 1 concentration, 5, 10, 15, 20, 25 and 30 mM of (*R*, *S*) − 1 were examined (Fig. 5). The optimal (*R*, *S*) − 1 concentrations for (*R*, *S*) − 1 hydrolyzing by immobilized cells and free cells were all 10 mM. When (*R*, *S*) − 1 concentrations above 15 mM were tested, the substrate showed an apparent negative influence on the reaction for both immobilized cells and free cells. The details were shown in Fig. 5.

Time course of (*R*, *S*) − 1 hydrolyzing by immobilized and free cells

To examine the effect of reaction time on hydrolysis of (*R*, *S*) − 1 by immobilized and free cells, reaction times ranging from 20 to 140 min were used (Fig. 6). In case of free cells, 0.076 g weight cells were used instead of 1 g beads under identical conditions. Figure 6 shows the time course of asymmetric hydrolysis of (*R*, *S*) − 1 by immobilized and free cells. As shown in Fig. 6, 49.5% of the substrate was hydrolyzed by immobilized cells with *R* − 2 *e.e.* of 99.8% after 40 min. The yield and *e.e.* of accumulated *S* − 1 were 49.9% and 97.7%, respectively. In case of free cells, conversion of (*R*, *S*) − 1 and *e.e.* of *R* − 2 reached 54.0% and 85.3% after 20 min, respectively. The accumulated *S* − 1 had a yield of 46.0% and an *e.e.* >99%. Thus, the catalyst productivity for immobilized cells at 40 min was 492.8 μM/g wet cells/h (37.4 μM/g beads/h), which was much higher than that obtained with free cells of *Brevibacterium epidermidis* ZJB-07201 (58.7 μM/g wet cells/h) and immobilized cells of *Comamonas acidovorans* A: 18 (15.0 μM/g beads/h) [11, 24].

The conversion of the substrate increased with the extension of the reaction time. However, the *e.e.* of the products dropped significantly because of the formation of *S* − 2 resulting from the hydrolysis of *S* − 1 by immobilized cells when *R* − 1 in the substrate was used up. This

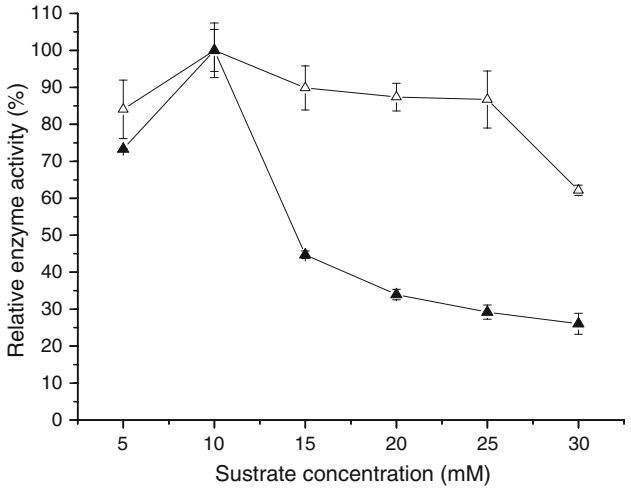

Fig. 5 Effect of substrate concentration on amidase activity of immobilized cells and free cells of *D. tsuruhatensis* CCTCC M 205114. Reaction conditions: 1 g immobilized cells or 0.076 g wet cells, 35°C, pH 8.5, 10 min, 150 r/min; reaction mixture volume: 5 ml. Symbols: *Filled triangle* relative amidase activity of immobilized cells, *filled inverted triangle* relative amidase activity of free cells

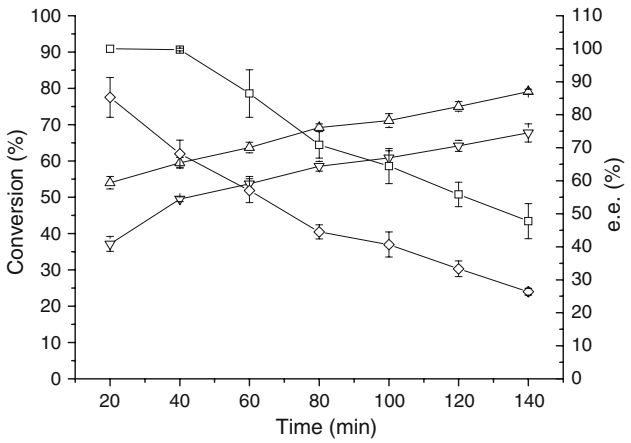

Fig. 6 Time course of hydrolysis of $R-1$ by immobilized and free cells of *D. tsuruhatensis* CCTCC M 205114. Reaction conditions: 1 g immobilized cells or 0.076 g wet cells, 35°C, pH 8.5, 10 mM $(R, S)-1$, 150 r/min; reaction mixture volume: 5 ml. Symbols: *open square* conversion of substrate by immobilized cells, *open square* conversion of substrate by free cells, *open square e.e.* of the products by immobilized cells, *open square e.e.* of the products by free cells

resulted in a higher *e.e* (>99%) and increasing low yield of $S-1$. The free cells also displayed the same trend. Finally, conversion of $(R, S)-1$ and *e.e.* of $R-1$ with immobilized cells were 47.8% and 67.7%, respectively. In case of free cells, conversion of $(R, S)-1$ and *e.e.* of $R-1$ at 140 min were 79.1% and 26.4%, respectively.

Operational stability of immobilized cells

To examine and compare the operation stability of immobilized and free cells, reuse of immobilized cells and free cells to hydrolyze $(R, S)-1$ was carried out. The results are shown in Fig. 7. After each hydrolysis cycle, the beads were recovered by filtration and washed three times with distilled water. Then the immobilized cells were collected and suspended in a fresh reaction mixture with 10 mM $(R, S)-1$ and reacted at 35°C for 40 min. In case of free cells, the cells were recovered by centrifugation ($9000\times g$ for 8 min, 4°C) and washed three times with distilled water. Then the cells were resuspended in 4 ml 10 mM Tris-HCl buffer followed by adding 50 mM $(R, S)-1$ and reacted at 35°C for 20 min. The same process was repeated up to 8 batches. The immobilized cells retained 83.1% of the recycling efficiency. However, in case of free cells, the recycling efficiency dropped remarkably to less than 50% after 3 batches. Therefore, the immobilized cells demonstrated good operational stability as compared to free cells.

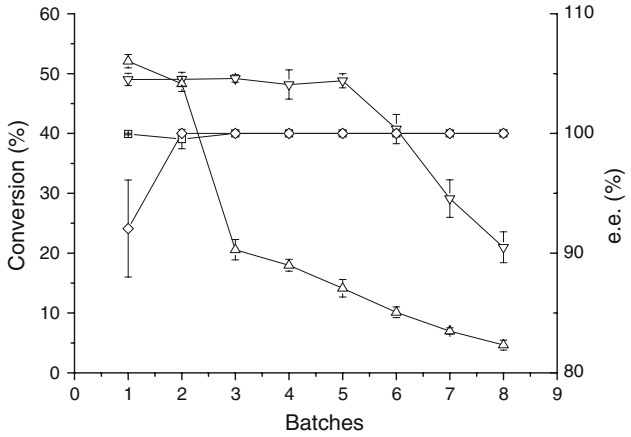

Fig. 7 Reuse of immobilized and free cells of *D. tsuruhatensis* CCTCC M 205114. Reaction conditions: 1 g immobilized cells or 0.076 g wet cells, 35°C, pH 8.5, 40 min, 10 mM $(R, S)-1$, 150 r/min; reaction mixture volume: 5 ml. Symbols: *open square* conversion of substrate by immobilized cells, *open square* conversion of substrate by free cells, *open square e.e.* of the products by immobilized cells, *open square e.e.* of the products by free cells

No significant difference in *e.e.* of the resulting acids was observed between the immobilized and free cells except that of batch 1 of free cells. This demonstrated that both immobilized and free cells possessed an excellent enanotioselectivity.

Conclusions

In summary, the feasibility of enantioselective hydrolyzing $(R, S)-1$ using alginate immobilized cells of *D. tsuruhatensis* CCTCC M 205114, an *R*-amidase producing bacterium, was investigated in this paper. The data demonstrated that the immobilized *D. tsuruhatensis* CCTCC M 205114 cells possess good operational stability and

excellent enanotioselectivity against *R*-1 together afforded high yield and high *e.e.* of $S-1$. Therefore, the immobilized *D. tsuruhatensis* CCTCC M 205114 cells might be used in large-scale enantioselective hydrolysis of $R-1$ in $(R, S)-1$ to produce $S-1$. The results also proved that it is possible to apply immobilized cells of amidase-producing microorganisms in biocatalysis. This study laid the foundation for the production of $S-1$ on the industrial scale.

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