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Catalytic resolution of (*RS*)-HMPC acetate by immobilized cells of *Acinetobacter* sp. CGMCC 0789 in a medium with organic cosolvent

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Abstract

Kinetic resolution of a chiral alcohol, 4-hydroxy-3-methyl-2-(2′-propenyl)-2-cyclopentenone (HMPC), a key intermediate for the production of prallethrin insecticides, was successfully carried out by enantioselective hydrolysis of (*RS*)-HMPC acetate using calcium alginate gel-entrapped cells of a newly isolated esterase-producing bacterium *Acinetobacter* sp. CGMCC 0789. When the effect of different cosolvents was investigated, it was found that isopropanol could markedly enhance the activity and enantioselectivity of the immobilized cells. The optimum concentration of isopropanol was 10% (v/v) where immobilized cells still showed good operational stability. After 10 cycles of reaction, no significant decrease in the enzyme activity was observed. The catalytic specificity constants (V_{max}/K_m) for both enantiomers of the substrate were determined with partially purified enzyme, giving 0.0184 and 0.671 h^{-1} for the (*S*)- and (*R*)-ester, respectively.

Keywords: *Acinetobacter* sp. esterase; Enantioselectivity; Enzymatic resolution; 4-Hydroxy-3-methyl-2-(2′-propenyl)-2-cyclopentenone; Immobilized cells; Isopropanol

1. Introduction

Chiral alcohol 4-hydroxy-3-methyl-2-(2′-propenyl)-2-cyclopentenone (HMPC) is the alcohol moiety of prallethrin, a widely employed synthetic pyrethroid insecticide for household use. It has been known that the insecticidal activity of prallethrin is mostly related to the (*S*)-HMPC. The insecticidal activity of (*S*)-isomer prallethrin is several times higher than the (*R*)-antipode [1–3]. Therefore, much effort has been made to produce optically pure (*S*)-HMPC. A highly enantioselective hydrolysis of *rac*-HMPC acetate has been successfully developed using a commercially available *Arthrobacter* lipase [3]. When the substrate concentration was as high as 80% (w/v), the lipase-catalyzed reaction could still produce almost optically pure (*R*)-HMPC at 50% conversion. In the same report, however, when the biocatalytic resolution of (*RS*)-HMPC acetate using various microbial cells was attempted, the enantioselectivity of those tested microorganisms seemed not so satisfactory. In the best case of *Chromobacterium chocolatum* IFO-3758, for instance, the enantiomeric excess of product (ee_p) was merely 90.2% even at a low conversion (11.7%) of substrate in a low concentration (0.5%, w/v) [3]. On the other hand, there were also some reports on the application of immobilized lipase to prepare (*S*)-HMPC by transesterification [4,5]. However, to the best of our knowledge, there is not yet any other report about utilizing immobilized cells for the hydrolytic resolution of (*RS*)-HMPC acetate.

To develop a simpler and cheaper process with a whole-cell biocatalyst for the optical resolution of (*RS*)-HMPC, our laboratory has succeeded in isolating a new strain of bacterium nominated as *Acinetobacter* sp. CGMCC 0789 [6]. In a previous report [7], we investigated the optical resolution of *rac*-HMPC acetate by using free cells of the bacterial strain, where the (*R*)-enantiomer of *rac*-HMPC acetate was preferentially hydrolyzed while the (*S*)-ester was kept unchanged (Scheme 1). However, the recovery and reusability of free cells as catalysts are not convenient, so immobilization of microbial cells was attempted for solving such a problem. Moreover, cell immobilization was

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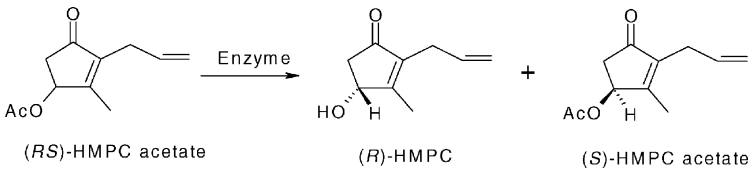

Scheme 1. Kinetic resolution of (*RS*)-HMPC acetate with a hydrolase.

considered helpful for stabilizing the whole-cell biocatalyst. In this paper, the classical method of gel entrapment with calcium alginate was chosen for the cell immobilization due to ease of operation and low cost of the gel.

The enantioselectivity of biocatalysis can be quantitatively described as the ratio of catalytic specificity constant (V_{max}/K_m) between a given pair of stereochemical isomers [8]. It may be varied in several ways [9]: (a) changing the enzyme, via either screening for novel catalysts or protein engineering of existing catalysts; (b) modification of the substrates; or (c) changing the reaction conditions, such as the type and composition of the reaction medium.

It has been shown that the modification of a reaction medium by adding a little amount of polar and low-molecular weight solvents can increase the catalytic activity [10] and, in some cases, even the enantioselectivity [11–15] of an enzyme. As a rule, when the cosolvent concentration was relatively low, the enzyme activity increases with the addition of the cosolvent, and reaches the maximum at a concentration between 10 and 20% (v/v) [16,17].

Here we describe the effect of cosolvent on the hydrolytic resolution of *rac*-HMPC acetate with immobilized cells of *Acinetobacter* sp. CGMCC 0789 expressing an intracellular esterase activity. Furthermore, the mechanism of solvent-induced enhancement of enantioselectivity for the bioreaction was also briefly discussed based on the kinetic constants measured.

2. Materials and methods

2.1. Chemicals

(*RS*)-HMPC acetate was provided by Yangzhou Agrochemicals Co. Ltd., Jiangsu Province, China. All other chemicals were obtained commercially and of reagent grade.

2.2. Microbial strain

Acinetobacter sp. CGMCC 0789 was used throughout this study. It was isolated from soil by Zhao et al. [6] and deposited presently at China General Microorganism Collection Center with an accession number CGMCC 0789 [18]. The working culture was maintained on agar slants at 4 °C and subcultured periodically.

2.3. Medium and culture conditions

The cell culture of *Acinetobacter* sp. CGMCC 0789 was performed in a 5 l jar fermenter with 3 l of sterile medium containing (per liter): sucrose, 15.0 g; Tween-80, 1.5 g; peptone, 7.5 g; yeast extract, 7.5 g; KH_2PO_4, 2.0 g; NaCl, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; pH 7.0. The cells were harvested after 24 h of culture at 30 °C by centrifugation at 6000 × g for 20 min. After washing with 0.85% NaCl, the cells were lyophilized and preserved at 4 °C. This preparation was very stable, without significant loss of activity after 6 months of storage.

2.4. Immobilization of cells in alginate gel beads

Unless otherwise stated, the immobilization procedure was as follows: 1.4 grams of lyophilized cells were suspended in 20 ml of physiological saline solution. This suspension was mixed with sodium alginate solution so that the final slurry was 2.4% (w/v) with respect to alginate. Then the mixture was added drop by drop with a peristaltic pump through a fine micropipette tip attached to a silicon tube into 500 ml of 100 mM $CaCl_2$ solution with continuous agitation. The resultant beads with average diameter of 1–3 mm were cured in the same solution for 2 h and stored in 10 mM $CaCl_2$ solution at 4 °C prior to use [19].

2.5. Enzyme assay

The activity of the intracellular esterase was measured using the (*RS*)-HMPC acetate as substrate. Immobilized cells (0.6 g, wet weight) were suspended in 10 ml Tris-HCl buffer (100 mM, pH 8.0) containing 50 mM $CaCl_2$. The reaction was started by the addition of (*RS*)-HMPC acetate to a final concentration of 100 mM. Cosolvents were added to the system when required. The mixture was incubated at 30 °C in a rotatory shaker at 160 rpm. During the course of reaction, samples (0.5 ml) were taken and both the alcohol produced and the ester unreacted were extracted with 0.5 ml of ethyl acetate and subjected to GC analysis.

2.6. Preparation of cell-free enzyme and optically pure HMPC acetate

The method for isolation and partial purification of the esterase from *Acinetobacter* sp. CGMCC 0789 cells has been described elsewhere [20]. Optically pure (*S*)-HMPC acetate

(>98% e.e.) and optically enriched (*R*)-HMPC were obtained by enantioselective hydrolysis of (*RS*)-HMPC acetate up to 54% conversion with 6 g immobilized cells suspended in 100 ml Tris-HCl buffer containing 10% isopropanol, followed by purification via silica gel column chromatography using petroleum ether/ethyl acetate (4:1, v/v) as elution solvent. The enantiopure (*R*)-HMPC acetate was obtained by enzymatic transesterification of the enantiomerically enriched (*R*)-HMPC (92% e.e.) with vinyl acetate using Lipase PS (Amano, Japan) as described recently in another paper [21].

2.7. GC analysis

The substrate and product concentrations were determined by GLC using BETA DEX^{TM} 120 column (oven temperature, 150 °C; injector and detector temperature, 280 °C) to determine the degree of hydrolysis and enantiomeric excesses of substrate (ee_s) and product (ee_p). The retention times were 15.4, 16.1, 20.6 and 21.2 min for (*R*)-HMPC acetate, (*S*)-HMPC acetate, (*S*)-HMPC and (*R*)-HMPC, respectively. Enantiomeric ratio (*E*-value) was calculated using the equation of literature [22]:

$$E = \ln\left[\frac{(1 - ee_s)/(1 + ee_s/ee_p)}{(1 + ee_s)/(1 + ee_s/ee_p)}\right]$$

3. Results and discussion

3.1. Effect of cosolvents added to the reaction system

Addition of some cosolvents to aqueous buffer can enhance activities of some enzymes. It has been reported that the activity of penicillin G acylase was improved in water-isopropanol mixture compared with that in neat aqueous buffer [10]. Similar results were also reported with respect to a commercial lipase [12] and yeast cells [23,24].

In this work, effects of some water-miscible cosolvents on both the activity and the enantioselectivity of the esterase inside the immobilized cells were first examined at a concentration of 20% (v/v). As shown in Fig. 1, significant increases in both the conversion and the *E*-value were observed upon the addition of alcohols, especially isopropanol, whereas the conversion decreased to some extent when other non-alcohol solvents were added. The potential reasons for this phenomenon could not simply explained by the increase of substrate solubility with the help of isopropanol because other cosolvents should have also similar effects. We speculate that some specific interactions probably exist between the enzyme and isopropanol or other alcohol-type cosolvents, since these alcohols may act as potential substrates or inhibitors of the esterase. The mechanism responsible for the significant improvement of enantioselectivity after addition of isopropanol seems more complicated, since the overall kinetics of enantiomer resolution involves not only the enzyme reaction itself but also the mass transfer limitation of substrate/product through both the alginate gel layer and the cell membrane of the immobilized cells.

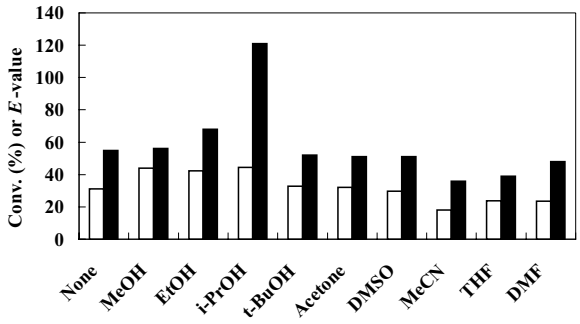

Fig. 1. Effects of various cosolvents (20%, v/v) on the reaction rate and enantioselectivity. The reaction was performed in Tris-HCl buffer (100 mM, pH 8.0) at 30 °C and 160 rpm for 12 h. Symbols: (□) Conversion, (■) *E*-value.

The concentration of a cosolvent also has profound influence on the enzyme activity. Usually, the enzyme activity increases with the addition of a cosolvent at low concentrations but cosolvents may also show inhibitory effects at high concentrations. The effects of isopropanol concentration on the initial rate and the conversion at 12 h were investigated (Fig. 2**)**. At a lower concentration (≤10%, v/v), the activity of immobilized cells increased obviously with the addition of isopropanol, up to a maximum activity (6.22 mM min^{-1} g DCW^{-1}) at 10% (v/v) isopropanol, which is proximately 150% higher than that in the neat aqueous buffer (4.09 mM min^{-1} g DCW^{-1}). The activity decreased when more (>10%, v/v) isopropanol was added. Even at the concentration of 20% (v/v), the activity (4.29 mM min^{-1} g DCW^{-1}) was still slightly higher than that in the pure aqueous buffer. At the same time, the influence of isopropanol concentration on the final conversion

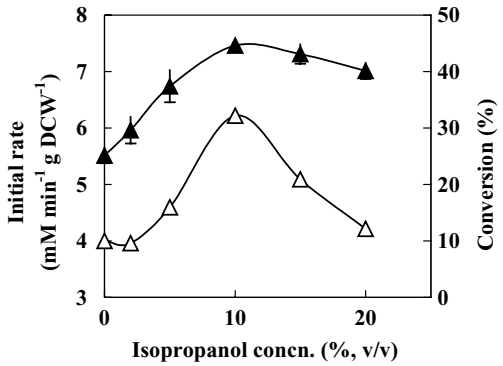

Fig. 2. Effect of isopropanol concentration on the biocatalytic hydrolysis of (*RS*)-HMPC acetate (100 mM) using immobilized cells. The activity was assayed under the standard conditions except that different amounts of isopropanol were added to the reaction mixture. Symbols: (△) Initial rate; (▲) Conversion of reaction at 12 h.

of HMPC acetate at 12 h was similar to that on the initial rate (Fig. 2). The conversion of reaction at 12 h increased from 27.9 to 43.5% when the isopropanol concentration was changed from 2 to 10% (v/v) and there was no obvious decrease in the final conversion when the concentration was raised up to 20% (v/v). The results clearly indicate that 10% (v/v) of isopropanol produces the best effect on the immobilized cell activity and the product yield. This result is in accordance with that reported by Arroyo and his co-workers [10]. They supposed a mechanism for Penicillin V acylase that the deacylation of the acyl-enzyme intermediate is affected not only by water but also by other competing nucleophilies, such as alcohol, to release the product in a faster manner, thus enhancing the catalytic activity.

Cosolvent affects not only the activity of enzymes, but also the membrane permeability of whole cell biocatalysts [23,24]. In order to gain insight into the effect of isopropanol on immobilized cells, they were pretreated before reaction in 10% isopropanol-containing Tris-HCl buffer for a certain time. Variation of the esterase activity with the pretreatment time was demonstrated in Fig. 3. As expected, the activity of the immobilized cells increased steadily with the incubation time and the maximal activity was observed after 24 h of pretreatment, which was 162% higher than that of the untreated cells. Moreover, we compared the progress curve of the reaction catalyzed by 24 h permeabilized cells with that by the untreated cells. As a result, the conversion and *E*-value of the permeabilized cells measured after 24 h of reaction were almost the same as that of the untreated cells (data not shown). This indicates that the cell permeability has little influence on the enantioselectivity, in contrast to its significant effect on the activity. The approaching of the two reaction curves at that moment (24 h) was reasonable because both the two reactions have arrived at nearly 50% conversion where the fast-reacting enantiomer has almost completely been exhausted, as similar to the reaction profiles in Fig. 4.

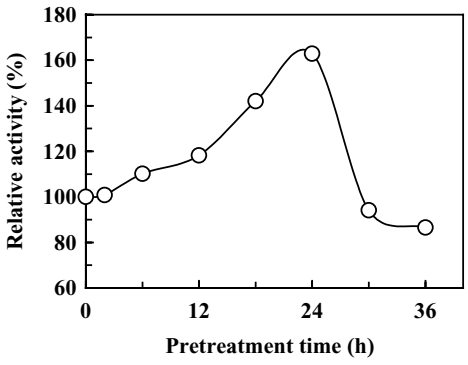

Fig. 3. Effect of isopropanol-pretreatment time on the activity of immobilized cells of *Acinetabacter* sp. CGMCC 0789 cells. Immobilized cells (0.6 g) were pretreated at 30 °C and 160 rpm in 100 mM Tris-HCl buffer (pH 8.0) containing 10% isopropanol for different times before activity assay. The activity of 100% corresponds to that of untreated cells (4.09 mM min^{-1} g DCW^{-1}).

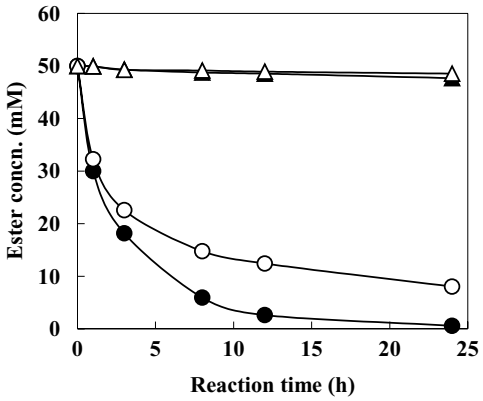

Fig. 4. Enzymatic hydrolysis of (*R*)- or (*S*)-HMPC acetate in a medium with or without isopropanol (10%, v/v). The concentration of each enantiomer of the substrate (100 mM) was plotted against the reaction time. Symbols: (●) (*R*)-Ester (with cosolvent); (▲) (*S*)-Ester (with cosolvent); (○) (*R*)-Ester (without cosolvent); (△) (*S*)-Ester (without cosolvent).

3.2. Enantioselective hydrolysis of (RS)-HMPC acetate

Isopropanol, as added into the reaction system, not only enhanced the activity of immobilized cells, but also improved the enantioselectivity (Fig. 1). Therefore, progress curves of the enantioselective hydrolysis of *rac*-HMPC acetate by immobilized cells were monitored and compared in the presence or absence of 10% (v/v) isopropanol (Fig. 4). It is obvious that the (*R*)-ester in the isopropanol-containing system decreased faster than that in control system and almost exhausted after 24 h of reaction. However the change in (*S*)-ester concentration was similar in both systems. In accordance to the non-linear regression of the experimental data, the *E*-value was 94 ± 6 in isopropanol-containing system, which is much higher than that in the absence of isopropanol ($E = 54 \pm 6$).

The catalytic specificity constant and the enantioselectivity for enzyme catalysis can be represented by the ratio of V_{max}/K_m and the ratio of $(V_{max}/K_m)_R/(V_{max}/K_m)_S$ respectively. To further elucidate the mechanism responsible for the enantioselectivity enhancement in the presence of isopropanol, we measured the kinetic constants by using a partially (3.1-fold) purified cell-free esterase of *Acinetobacter* sp. CGMCC 0789 and optically pure enantiomers of the substrate (i.e., (*R*)- and (*S*)-HMPC acetate) in the presence or absence of isopropanol. The results were summarized in Table 1. In the presence of 10% isopropanol, the V_{max} of (*R*)-isomer was 2.4-fold higher than that of control, while the V_{max} for the (*S*)-isomer was adjacent. On the other hand, when 10% (v/v) isopropanol was added, both the K_m values for (*R*)- and (*S*)-isomers were increased. The K_m of (*S*)-isomer was increased almost 2-fold while that of the (*R*)-isomer increased only 1.5-fold. Owing to the combined effect in these two aspects, the catalytic efficiency ($k_{cat} = V_{max}/K_m$) of (*R*)-isomer increased from 0.434 to 0.671 h^{-1}, while that (k_{cat}) for (*S*)-isomer decreased from 0.0269 to 0.0184 h^{-1}. Therefore, the enantioselectivity (*E*-value), ex-

Table 1
Kinetic parameters of HMPC acetate ester hydrolysis catalyzed by partially purified esterase (0.385 U mg^{-1} protein) from *Acinetobacter* sp. CGMCC 0789 with or without 10% (v/v) isopropanol as cosolvent[a]

Isopropanol (%, v/v)	$(V_{max})_R$ (mM h^{-1} mg^{-1} protein)	$(V_{max})_S$ (mM h^{-1} mg^{-1} protein)	$(K_m)_R$ (mM)	$(K_m)_S$ (mM)	$(V_{max}/K_m)_R$	$(V_{max}/K_m)_S$	E[b]
0	0.351	0.134	0.808	4.98	0.434	0.0269	16.1
10	0.852	0.174	1.27	9.47	0.671	0.0184	36.5

[a] The reactions were carried out by shaking the reaction mixture at 160 rpm and 30 °C in Tris-HCl buffer (100 mM, pH 8.0) with or without 10% isopropanol.
[b] The enantioselectivity (E) was defined as $E = (V_{max}/K_m)_R/(V_{max}/K_m)_S$.

pressed by $(V_{max}/K_m)_R/(V_{max}/K_m)_S$, increased from 16.1 to 36.5. This result supports the hypothesis that the solvent molecules might affect the substrate combination (K_m) and/or transformation (k_{cat}) of one enantiomer more than the other, as Secundo et al. [25] have suggested. In addition to the general effect of colsolvent on the medium properties, the geometry of isopropanol may also of importance if one consider its possibility of binding with the active center of the esterase as a potential acyl acceptor which will compete with HMPC and water molecules. The reason why solvent can affect the enantioslectivity of an enzyme might also be interpreted by computer-assisted model of enzyme and substrate [26].

3.3. Influence of substrate concentration on reaction kinetics

Higher substrate concentration is beneficial for industrial production because it can minimize the cost of product isolation. The effect of substrate concentration (from 50 to 300 mM) on the product formation was investigated with the same amount of catalyst. As shown in Fig. 5, the product formation rate was increased when substrate concentration increased from 50 to 125 mM. At higher substrate concentration, the production rate was kept nearly constant. When the ratio of substrate/catalyst was kept constant, the conversion at 24 h decreased a little with the increase in substrate concentration from 125 to 300 mM, but the enantioselectivity remained almost unchanged.

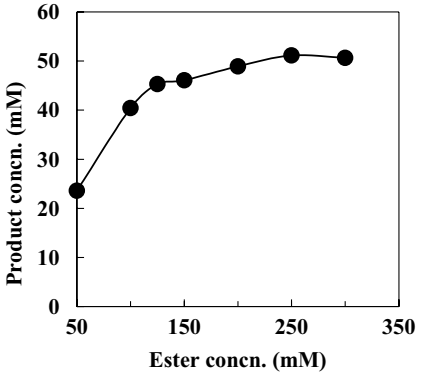

Fig. 5. Effect of substrate concentration. Reaction conditions: 0.6 g immobilized cells in 10 ml Tris-HCl buffer (100 mM, pH 8.0), 30 °C, 160 rpm and different amounts of substrate added to the reaction mixture. The concentrations of product (*S*- and *R*-) were monitored at 6 h.

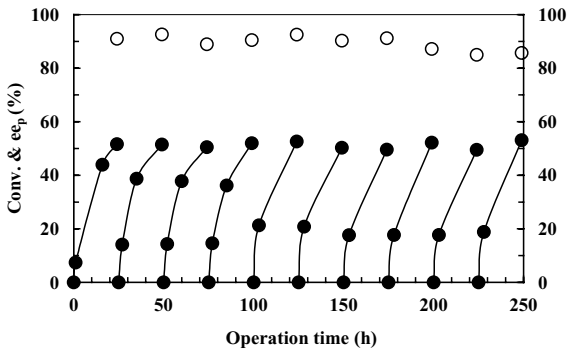

Fig. 6. Operational stability of the immobilized cells of *Acinetobacter* sp. CGMCC 0789 in repeated batch reactions. Reaction conditions: 0.6 g immobilized cells in 10 ml Tris-HCl buffer (100 mM, pH 8.0), 100 mM (*RS*)-HMPC acetate, 30 °C, 160 rpm. Symbols: (●) Conversion; (○) ee_p.

3.4. Repeated batch hydrolysis of (RS)-HMPC acetate

The operation stability of immobilized cells was investigated in the presence of 10% (v/v) isopropanol. As shown in Fig. 6, nearly 50% conversion with high enantioselectivity was reached at 24 h for each batch of reaction. The immobilized cells were very stable. After ten batches of reaction, the activity of immobilized cells was maintained very well, without any significant loss of enzyme activity. The long-term operation stability of the immobilized cells indicates that the calcium alginate gel-entrapped cells of *Acinetobacter* sp. CGMCC 0789 may serve as a good catalyst for industrial production of (*S*)-HMPC acetate.

Acknowledgements

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