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Short Communication

Kinetic resolution of (*R*,*S*)-2,2-dimethylcyclopropanecarboxamide by *Delftia tsuruhatensis* ZJB-05174: Role of organic cosolvent in reaction medium

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ABSTRACT

Both enantioselectivity and catalytic activity were greatly enhanced in *Delftia tsuruhatensis* ZJB-05174 catalyzed kinetic resolution of 2,2-dimethylcyclopropanecarboxamide in the presence of ethanol and acetonitrile. The enantiomeric ratio (E) rose from 27 to 140 and 90 by addition of 5% acetonitrile and ethanol, respectively. In the scaled-up biotransformation, the reaction time was shortened to 1.33 h with an ee_s of 99% and 12.4 g of optically pure product (43.5% total yield) was afforded. These results indicated that addition of cosolvent was a simple and practical tool to improve amidase properties and would be extensively applied in amidase-catalyzed bioprocess.

1. Introduction

Amidases (EC 3.5.1.4) are widely distributed in both prokaryotic and eukaryotic organisms. They catalyze the hydrolysis of various endogenous and foreign aliphatic or aromatic amides by transferring an acyl group to water with the production of free carboxylate and ammonia [1]. Except for nitrogen metabolism, their biological role in nature is still not clearly defined [2]. However, with the boom in research on enzymatic nitrile hydrolysis in the 1980s, amidases found great potential in organic synthesis, especially in the production of homochiral molecules [3]. Compared with the other two classes of nitrile-converting enzymes, namely nitrilases and nitrile hydratases, amidases have the advantages of wider substrate specificities and stricter enantioselectivity [4], thereby attracting much attention as catalysts for stereospecific production of important chiral acids and amides [5–7].

In order to enhance volumetric productivity or reduce cost of a particular enzymatic process, the use of technologies such as immobilization, protein engineering or substrate modulation becomes feasible and at times indispensable approaches [8–10]. Nevertheless, the application of these technologies in amidase-mediated processes still remained scarce, which to a certain extent hampered the adoption of amidase-catalyzed hydrolysis into synthetic or manufacturing routes. Among the above mentioned strategies, medium engineering is a widely acknowledged practical tool for improving enzyme properties [11,12]. It has been shown that modification of a reaction medium by adding small amount of polar organic cosolvent can effectively increase catalytic activity and, sometimes even the enantioselectivity of lipase [13–16] and a range of other enzymes, such as arylacetonitrilase [17], esterase [18,19] and penicillin acylase [20,21]. In other examples, even inhibition of protease by high concentration of substrate was overcome by the addition of cosolvent [22]. However, to the best of our knowledge, there has been no report regarding influence of variations in reaction conditions, such as the type and composition of the reaction medium on the performance of amidase.

(*S*)-2,2-dimethylcyclopropanecarboxamide (**1**) is the key segment of cilastatin, a renal dehydropeptidase inhibitor administered with imipenem to prevent its degradation in the kidney [23]. Our laboratory has succeeded in isolating a new *R*-stereospecific amidase-producing strain *Delftia tsuruhatensis* ZJB-05174 [24], which exhibited great potential for the industrial production of (*S*)-**1** by asymmetric hydrolysis of the racemic mixture (Scheme 1). However, the amidase exhibited only moderate enantioselectivity ($E = 27$) for compound **1**, resulting in low productivity of the bioprocess. To further improve throughput of the biocatalytic process, herein we describe the effect of cosolvent on the hydrolytic resolution of (*R*,*S*)-**1** using free cells of *D. tsuruhatensis* ZJB-05174. Enhancement of both enantioselectivity and catalytic activity of the amidase was reported for the first time by addition of water-miscible organic solvents.

2. Materials and methods

2.1. Chemicals

(*R*,*S*)-**1** and (*R*,*S*)-**2** were kindly supplied by Huakang Chemicals Ltd., Zhejiang Province, China. Media components were purchased

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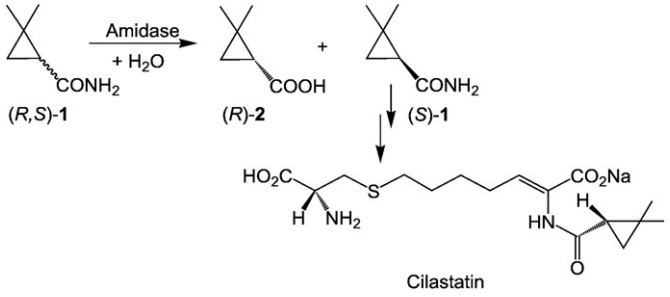

Scheme 1. Amidase-mediated stereospecific hydrolysis of (*R*,*S*)-**1** to (*S*)-**1**, a key segment of cilastatin.

from Huadong Medicine Group (Hangzhou, China). The other chemicals used in this work were of analytic grade from local suppliers.

2.2. Microorganisms and cultivation conditions

D. tsuruhatensis ZJB-05174 was isolated through a colorimetric high-throughput screening system [25]. The cell culture of *D. tsuruhatensis* ZJB-05174 was performed in 250 ml flasks with 40 ml of sterile medium containing (g/l): glucose 8.4, acetamide 3.56, yeast extraction 6.3, peptone 0.7, NaCl 1.0, KH_2PO_4 1.0, and K_2HPO_4 1.0 (pH 7.5). The cells were harvested after 20 h of incubation (150 rpm) at 30 °C by centrifugation at 9000×*g* for 10 min and stored at 4 °C for further use.

2.3. Biotransformation

Amidase-catalyzed hydrolysis of **1** was carried out at 30 °C in shaking flasks on an orbital shaker at 120 rpm. The reaction mixture consist of 4.5 ml Tris–HCl buffer (50 mM, pH 8.2), 30 mM (*R*,*S*)-**1**, 0.5 ml different organic solvent and 0.3 g wet resting cells (approximately 27 mg cell dry weight (cdw)). Samples (0.5 ml each) were withdrawn after 10 min and the reaction was quenched by addition of 30 μl HCl (5 M). After centrifugation, 200 μl supernatant was extracted with 800 μl ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 and subjected to GC analysis. All experiments were conducted in triplicate if not specified.

One unit of amidase activity was defined as the amount of enzyme required to produce 1 μmol of (*R*,*S*)-**2** per minute under the above conditions.

2.4. Scale-up resolution of (R,S)-2,2-dimethylcyclopropanecarboxamide

A 5-l scale reaction was performed in an 8-l stirring-tank reactor containing 100 g of wet free cells,, 28.5 g of (*R*,*S*)-**1**, 250 ml of acetonitrile and 4750 ml of Tris–HCl buffer (50 mM, pH 8.2). The resulting mixture was stirred at 120 rpm with the temperature maintained at 30 °C. After reaction for 140 min, the reaction broth was centrifuged to remove the cells. The supernatant was treated by activated carbon absorption (0.3%, w/v) for 30 min at 45 °C and filtration. Thereafter, the solvent was basified to pH 12 and (*S*)-**1** was purified via macroporous resin HP-1 using 80% ethanol as elution solvent. The eluent was evaporated under reduced pressure to afford white solid. The product was characterized by FT-IR spectroscopy, GC–MS, ^{1}H NMR and ^{13}C NMR. Optical purity of the product was determined by chiral gas chromatography and polarimetry.

(*S*)-2,2-dimethylcyclopropanecarboxamide was isolated as white powder; yield: 12.4 g (0.109 mol, 43.5%); $[\alpha]_D^{20}+82.2°$ (*c* 1.0, CH_3OH) {lit.: $[\alpha]_D^{20}+82°$ (*c* 1.0, CH_3OH) [26]}, >99% *ee* by GC; ν_{max}-(KBr) (cm^{-1}) 3384, 3194, 1650, 1621; *m/z* (EI) 113 (M^+, 63%), 98 (44), 96 (47), 81 (18), 72 (55), 70 (92), 55 (100), 41 (81); ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.76–0.79 (*q*, 1H, *J* = 4.4 Hz), 1.09–1.11(*t*, 1H, *J* = 4.8 Hz), 1.16 (*s*, 3H), 1.20 (*s*, 3H), 1.30–1.34 (*q*, 1H, *J* = 5.6 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 174.3, 28.3, 27.0, 21.8, 20.5, 18.6.

2.5. Gas chromatography analysis

Enantiomeric compositions of residual amide and the corresponding acid were determined by gas chromatography on a γ-cyclodextrin based chiral column BGB-175 [27]. Retention times for (*S*)-**1**, (*R*)-**1**, (*R*)-**2**, and (*S*)-**2** were 9.10 min, 9.30 min, 7.82 min and 8.65 min, respectively.

The enantiomeric ratio (*E*) and concentration of the four enantiomers in the reaction mixture were calculated based on ee_S and ee_P as the method developed by Rakels and us [28,29].

3. Results and discussion

3.1. Selection of cosolvents added to the reaction mixture

It has been reported in many cases that addition of water-miscible organic solvent can enhance the enantioselectivity and activity of biocatalysts [30], especially in hydrolase (lipase, esterase and protease) catalyzed reactions [18,19]. In this work, effects of 8 cosolvents on both catalytic activity and enantioselectivity of the amidase were first studied at a concentration of 10% (v/v). As shown in Fig. 1, addition of acetonitrile, ethanol, methanol and acetone significantly accelerated initial rate of the hydrolytic reaction. In particular, amidase activity was enhanced to 52.9 and 46.2 U/g cdw in the presence of acetonitrile and ethanol, which was 3.7 and 3.2 times higher than that in the neat aqueous buffer (14.3 U/g cdw). With regard to enantioselectivity, cells of *D. tsuruhatensis* only exhibited moderate enantiomeric ratio (*E* = 27) to (*R*,*S*)-**1** while a notable increase (*E* > 80) was observed upon the addition of acetonitrile, ethanol, and DMSO. Because catalytic activity was much lower in the presence of DMSO, acetonitrile and ethanol were selected as optimal additives for further investigation.

Solubility of **1** in aqueous solution was low (0.54 g/100 ml H_2O, 30 °C), but it was almost doubled with the addition of cosolvents, e.g. 12.7 g/l in the presence of 10% EtOH. The increase of substrate concentration and reduced mass-transfer resistance from membrane and wall of whole cells contributed to enhancement of amidase activity. The mechanism responsible for significant improvement of enantioselectivity after addition of cosolvents was more complicated and might lie in two aspects. Firstly, specific interactions probably exist between the amidase and the polar cosolvent, causing change of the substrate combination of *R*-enantiomer more than the other, and hence, higher enantioselectivity was observed. Secondly, the improved penetrability of cell membrane and wall led to an altered

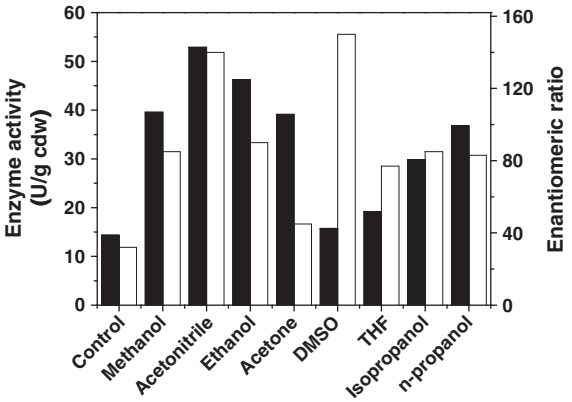

Fig. 1. Effect of various cosolvents (10%, v/v) on catalytic activity and enantioselectivity of the amidase catalyzed hydrolysis. Symbols: enzyme activity (■); enantiomeric ratio (□). Reaction conditions: 4.5 ml Tris–HCl buffer (pH 8.2), 0.5 ml of different organic solvent, 30 mM (*R*,*S*)-**1**, and 0.3 g wet cell, 30 °C, 10 min.

state of reaction kinetics due to the diffusion restriction [31]. This resulted in a change in kinetics of presentation of the enantiomers to the active site, and the *R*-enantiomer experienced less competition from the *S*-enantiomer, leading to an increase in enantioselectivity.

3.2. Optimization of cosolvent concentration

The concentration of a cosolvent has profound influence on the enzyme activity. Usually, cosolvents at low concentration reduce mass-transfer resistance of resting cells, while high concentration cosolvents show inhibitory effects [18]. In order to find an optimal cosolvent concentration, the effects of acetonitrile and ethanol concentration on amidase activity, conversion and enantioselectivity were investigated. As depicted in Fig. 2A, both amidase activity and the conversion of substrate were high even EtOH concentration was raised up to 24% (v/v). The amidase showed its maximal activity (47.1 U/g cdw) with an EtOH concentration of 15% (v/v). Nevertheless, the two parameters decreased significantly when more than 30% (v/v) of ethanol was added. With respect to acetonitrile, the conversion increased from 11.4% to 29.3% when its concentration was increased from 0% to 9% (v/v) (Fig. 2B). However, the two parameters rapidly dropped with further increase of acetonitrile concentration up to 12% (v/v). These results clearly indicated that ethanol and acetonitrile had different effects on the biocatalytic process and more ethanol was demanded to achieve high conversion of substrate. After addition of ethanol and acetonitrile, the *E* value was always higher than the control and was almost invariant with the rise of ethanol and acetonitrile concentration.

3.3. Effects of cosolvent on amidase stability

Cosolvents affect not only enzyme activity, but also the permeability of whole cell biocatalysts and stability of enzyme [32]. In order to evaluate effect of ethanol and acetonitrile on the amidase stability, resting cells of *D. tsuruhatensis* ZJB-05174 were pretreated before biotransformation. Variation of amidase activity with pretreatment time at different cosolvent concentrations (0%, 5%, 8%, v/v) was demonstrated in Fig. 3. In contrast to improvement of esterase activity by cosolvent treatment [33], the amidase was almost inactivated after preincubation in 5% and 8% EtOH for 21 h (Fig. 3A). As shown in Fig. 3B, the amidase retained 44.9% of its activity in the presence of 5% acetonitrile, indicating that it was more stable than in the same concentration of EtOH. Similarly, the amidase activity decreased significantly when pretreated in 8% acetonitrile. These results suggested that long time exposure of the whole-cell biocatalysts to cosolvent would lower stability of the amidase, which indicated a very complex effect of cosolvent and importance of choice of reaction time. Since the amidase experienced deactivation very slowly, it was not surprise that its initial activity and conversion was much higher than that in the absence of cosolvent. Therefore, 5% acetonitrile was chosen as the optimal cosolvent added to the hydrolytic reaction mixture.

3.4. Scale-up biocatalytic hydrolysis of (*R,S*)-2,2-dimethylcyclopropanecarboxamide

To further evaluate the feasibility of enhancing productivity of the bioprocess by cosolvent addition, a 5-l scale biotransformation was performed in an 8-l stirred-tank reactor. Fig. 4 plots the kinetic resolution progress of (*R,S*)-**1** in the presence of 5% acetonitrile. The hydrolytic reaction was ceased after 1.33 h when ee_s reached 99% (48.8% yield), giving an average *E*-value of 126. However, to achieve an ee_s of 99%, the reaction time should be prolonged to 8.5 h and the resulting yield was merely 42.1% when the bioconversion was performed in the absence of acetonitrile (Table 1). Finally, 12.4 g of (*S*)-**1** with 99% *ee* were obtained after resin adsorption and evaporated under reduced pressure. These results demonstrated that space-time

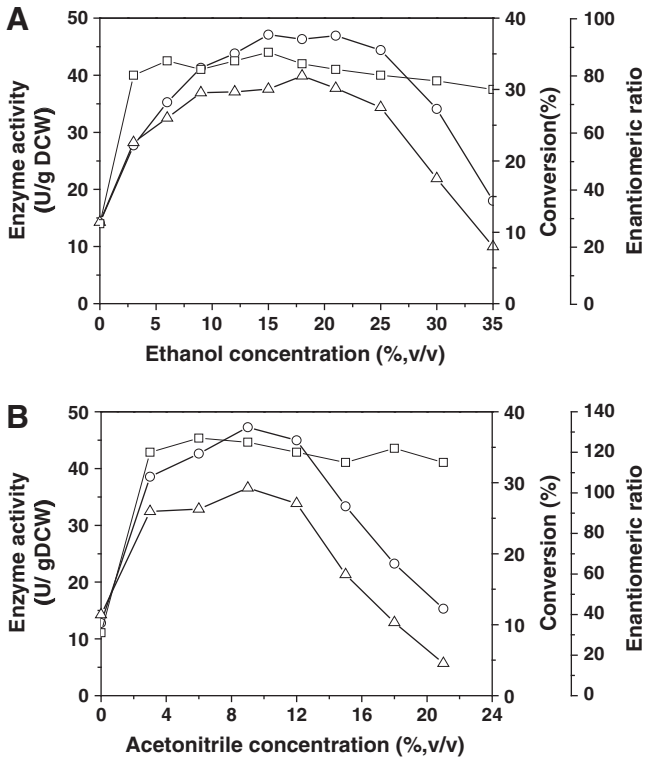

Fig. 2. Effect of ethanol and acetonitrile concentrations on amidase activity and conversion. (A) Ethanol and (B) acetonitrile. Symbols: amidase activity (○); conversion (△); enantiomeric ratio (□). Reaction conditions: 4.5 ml Tris–HCl buffer (pH 8.2), 0.5 ml of different organic solvent, 30 mM (*R,S*)-**1**, and 0.3 g wet cell, 30 °C.

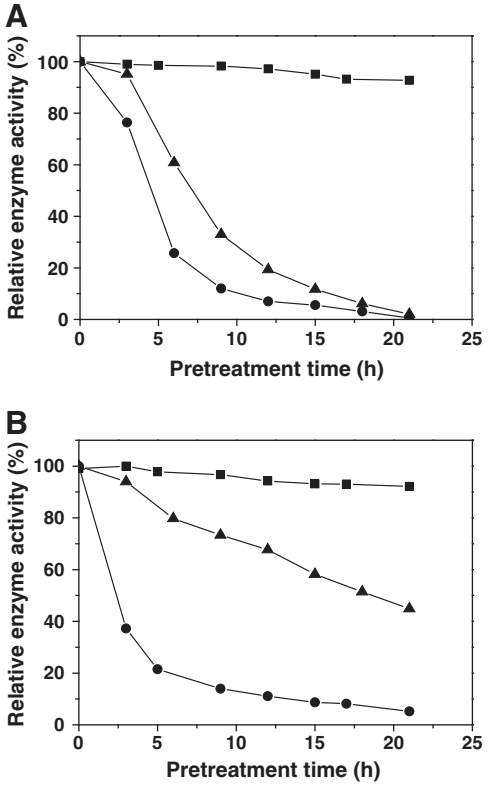

Fig. 3. Effect of cosolvent concentration and pretreatment time on amidase activity. (A) Ethanol and (B) acetonitrile. Symbols: no addition of cosolvent (■); 5% of cosolvent (▲); and 8% of cosolvent (●).

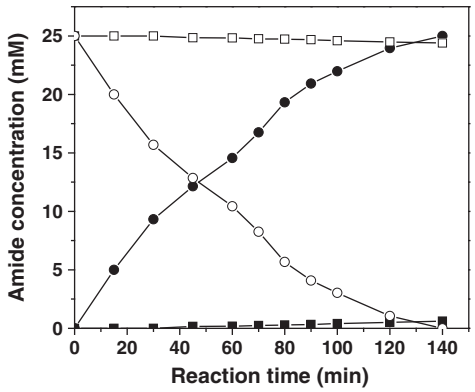

Fig. 4. Kinetic resolution progress of (*R*,*S*)-**1** by *D. tsuruhatensis* ZJB-05174 in the presence of 5% acetonitrile. Symbols: *S*-amide (□); *R*-amide (○); *S*-acid (■); and *R*-acid; (●).

Table 1
Comparison of process parameters in kinetic resolution of (*R*,*S*)-**1** with and without acetonitrile (5%,v/v) by *D. tsuruhatensis* ZJB-05174.

Acetonitrile (%, v/v)	Reaction time (h)	Yield (%)	Enantioselectivty	Space-time yield ($g_{product} l^{-1}$ day^{-1})
0	8.50	42.1	28	6.77
5	1.33	48.8	126	50.19

yield of the bioprocess was notably improved by adding low concentration of acetonitrile to the reaction medium. Moreover, the feasibility of the bioprocess for practical production of optical pure (*S*)-**1** was further confirmed.

4. Conclusions

In this work, the performance of *D. tsuruhatensis* ZJB-05174 amidase-catalyzed enantioselective hydrolysis of racemic 2,2-dimethylcycolpropaneamide was investigated in the presence of cosolvents. Several cosolvents examined proved to be effective for enhancing either enantioselectivity or catalytic activity. After optimization of type and composition of the reaction mixture, 5% acetonitrile was finally chosen as optimal cosolvent. The scaled-up biotransformation results definitely demonstrated that modification of reaction medium by adding small amount of cosolvent was a simple and effective approach to improve space-time productivity of the bioprocess and showed great potential in industrial production of (*S*)-2,2-dimethylcyclopropanecarboxamide.

Acknowledgements

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