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Industrial production of chiral intermediate of cilastatin by nitrile hydratase and amidase catalyzed one-pot, two-step biotransformation

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ABSTRACT

An industrial one-pot, two-step bioprocess catalyzed by nitrile hydratase (NHase) and amidase was developed for (*S*)-2,2-dimethylcyclopropanecarboxamide (**1**), the key chiral intermediate of cilastatin. The key unit operations of the whole process including fermentative production of enzymes, biotransformation, isolation of product, and recycling of by-product were reported for the first time. The volumetric enzyme activities of NHase and amidase in 1000-L fermentor were enhanced to 351,000 and 5880 U/L, respectively. The two-step, one-pot biotransformation of *rac*-2,2-dimethylcyclopropanecarbonitrile (**2**) took full advantage of both enzymes, leading to accumulation of (*S*)-**1** in 47% yield and 99.6% *ee*. (*S*)-**1** and the by-product (*R*)-2,2-dimethylcyclopropanecarboxylic acid (**3**) were obtained with yields of 38% and 45%, respectively, by a novel macroporous resin adsorption chromatography. Moreover, the total yield of (*S*)-**1** was further increased to 53% after one recycling of (*R*)-3. The new bioprocess dramatically improved process efficiency compared with the chemical route by elimination of six synthetic steps and proved to be a superior and more cost-effective approach towards (*S*)-**1**.

1. Introduction

Imipenem was the first carbapenem antibiotic selected for development because of its highly potent, broad-spectrum antimicrobial activity and good safety profile [1]. As imipenem is rapidly degraded by kidney dehydropeptidase I, it is administered in a 1:1 ratio with cilastatin, an inhibitor of dehydropeptidase I. Cilastatin not only prevents the renal metabolism of imipenem but also protects the kidneys against potential toxic effects exerted by higher doses of imipenem [2]. During the past two decades, imipenem/cilastatin has played an important role in both empirical and targeted treatments of severe and difficult to treat infections [3].

(*S*)-2,2-Dimethylcyclopropanecarboxamide (**1**) is the chiral intermediate of cilastatin. During the past years, considerable efforts have been devoted to the synthesis of this important building block. The chemical resolution route usually began with basic hydrolysis of *rac*-2,2-dimethylcyclopropanecarbonitrile (**2**), followed by chloroformylation to give the corresponding acid chloride. Resolution of the racemate was achieved by several steps of fractional crystallization using a chiral auxiliary, such as *L*-carnitine oxalate, (*S*)-mandelic acid methyl ester, and *L*-menthol. The resulting (*S*)-2,2-dimethylcyclopropanecarboxylic acid (**3**) was finally converted into optically pure (*S*)-**1** via acylation and ammonolysis (Scheme 1) [4–6]. The processes suffered from low overall yields (~20%) and the generation of large amounts of waste. Moreover, as the resolution depended on the solubility difference of the diastereomers, the optical purity of the product was not high enough to satisfy the pharmaceutical quality standards.

Biocatalysis is rapidly evolving into a key technology for the pharmaceutical industry owing to its high enantioselectivity, mild reaction conditions, and environmental friendliness [7]. As a result, several enzymatic routes have been exploited for the preparation

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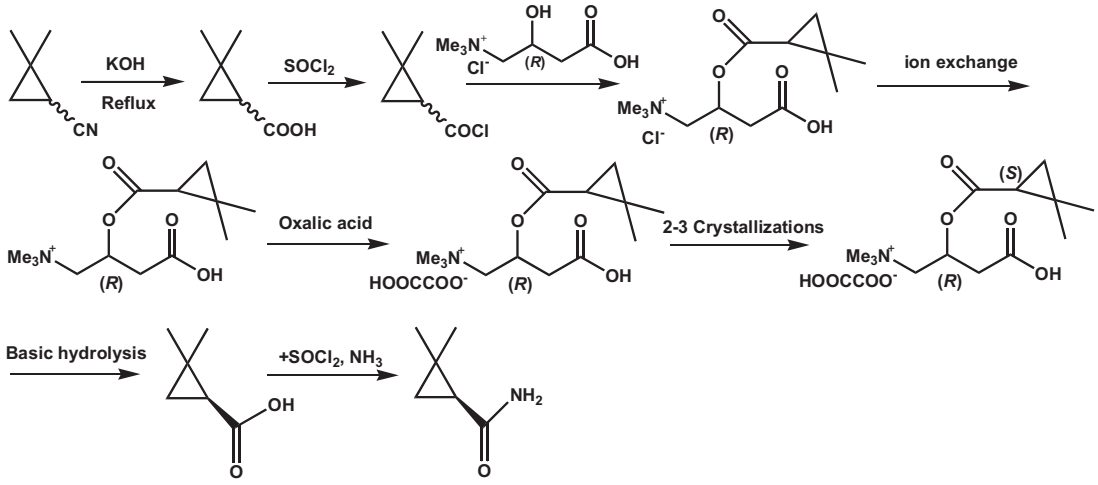

Scheme 1. Industrial chemical resolution of *rac*-**1**.

of enantiopure (*S*)-**1** or (*S*)-**3** [8–12]. Among these, the one-pot, two-step biocatalytic route developed by Lonza was most competitive [13]. This latter procedure involved a nitrile hydratase (NHase) and *R*-amidase catalyzed sequential biotransformation, and started from the existing, inexpensive racemic precursor **2**.

We started industrial production of (*S*)-**1** via an enzymatic system of NHase and amidase using strains discovered in our lab in 2005 [14] (Scheme 2). The manufacturing process was continuously improved over the past years, which made the bioprocess a superior and more cost-effective approach toward (*S*)-**1**. Herein, we report the key unit operations of the bioprocess, including fermentative production of the enzymes, biotransformation, purification of the product, and recycling of the by-product.

2. Materials and methods

2.1. Chemicals

The medium components were usual commercial products and used without further purification. *Rac*-**2** was synthesized according to Nelson et al. [15] with modifications. Macroporous resins were purchased from Shanghai Huazhen Sci. &Tech. Co. Ltd (Shanghai, China). All the other chemicals were of reagent grade purity and purchased from commercial sources.

2.2. Bacterial strains, media, and culture conditions

The NHase producing strain *Rhodococcus boritolerans* FW815 was screened using the enrichment culture technique [16]. It was deposited at the China Center for Type Culture Collection (CCTCC) under the accession number of CCTCC M208108. The *R*-enantioselective amidase producing strain *Delftia tsuruhatensis* ZJB-05174 was isolated employing a colorimetric method [17] and deposited at CCTCC under the accession number of CCTCC M205114. The heterologous expression of *R*-amidase gene (*dam*) from *D. tsuruhatensis* ZJB-05174 in *Escherichia coli* BL21 (DE3) has been previously described in detail [18].

2.3. Enzyme activity assay

The NHase activity to *rac*-**2** was determined according to the production of **1**. One milliliter of *R. boritolerans* FW815 fermentation broth was centrifuged and the resultant cell paste was resuspended in 50 mL phosphate buffer (50 mM, pH 7.0). *Rac*-**2** was added at a concentration of 12.2 g/L to initiate the reaction. After 5 min, 800 μL reaction samples were withdrawn and the reaction was quenched by 100 μL HCl (1.0 M), and then neutralized with 100 μL NaOH (1.0 M). After centrifugation at 12,000 × *g* for 3 min, the supernatant was analyzed by gas chromatography. One unit of NHase activity was defined as the amount of biocatalyst required for the production of 1 μmol **1** per min under the above reaction conditions.

Amidase activity was assayed using *rac*-**1** as substrate. A 1.0-mL aliquot of fermentation broth was centrifuged at 12,000 × *g* for 10 min. The cell pellet was suspended in 1.0 mL phosphate buffer (50 mM, pH 7.0) and mixed with 3.0 mL of the same buffer containing 8.0 g/L of **1**. The bioconversion was carried out at 35 °C for 10 min with reciprocal shaking (200 rpm). Samples (800 μL each) of the reaction mixture were withdrawn and the enzymatic reaction was quenched by removing biomass through centrifugation. The resulting supernatant was analyzed by gas chromatography. One unit of amidase activity is represented by the release of 1.0 μmol of **3** per minute under such conditions.

2.4. Fermentation of R. boritolerans FW815 in 1000-L fermentor

To prepare liquid inocula, a loop of cells from 24-hour-old slant cultures was initially inoculated into each 500 mL Erlenmeyer flask containing 80 mL of seed medium: 10 g of glucose, 2 g of peptone, 5 g of yeast extract, 1 g of NaCl, 0.2 g of $MgSO_4$, 0.1 g of K_2HPO_4, 0.1 g of KH_2PO_4, 0.01 g of $FeSO_4{\cdot}7H_2O$ per liter of tap water, and cultivated at 30 °C for 24 h with shaking at 200 rpm. Thereafter, 500 mL of the culture was transferred to a 15-L fermentor containing 10 L of the fresh seed medium. Cells in the seed reactor were cultivated at 30 °C for 22 h with aeration at 0.5 vvm (air volume/culture volume/minute) and agitation at 200 rpm. The culture broth was subsequently inoculated to a 1000-L fermentor containing 600 L of the same medium as that of seed culture with the exception that 1.0 g/L of ε-caprolactam was supplemented as inducer. The fermentations were carried out at 30 °C, agitated at 100 rpm and aerated at 0.4 vvm.

2.5. Fermentation of E. coli BL21 (DE3)/pET-dam in 1000-L fermentor

Escherichia coli BL21 (DE3)/pET-dam was first cultured in 5 mL LB medium and incubated for 10 h. It was then diluted into 500 mL flask cultures with 30 μg/mL kanamycin and grown at 35 °C overnight. As the OD_{600} reached 0.8–1.0, 100 mL of the culture was used to inoculate 10 L of 2 × YT medium composed of 10 g yeast