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Short communication

Chemoenzymatic synthesis of gabapentin by combining nitrilase-mediated hydrolysis with hydrogenation over Raney-nickel

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ABSTRACT

An efficient chemoenzymatic process is devised for synthesizing high-purity gabapentin. 1-Cyanocyclohexaneacetic acid was first produced in 0.94 M from 1.0 M 1-cyanocycloalkaneacetonitrile by a greatly improved nitrilase from *Acidovorax facilis* ZJB09122, resulting in a commercially attractive bioprocess with an outstanding space-time yield of 461 g/L/day. The resulting aqueous 1-cyanocycloalkaneacetic acid was then directly converted to 2-azaspiro [4.5] decan-3-one without further purification in subsequent hydrogenation by Raney-nickel, followed by simple chemical steps to afford gabapentin in high purity and 77.3% overall yield from 1-cyanocyclohexylacetonitrile. The simplicity of the process makes this new pathway suitable for large-scale preparation.

1. Introduction

Gabapentin (**1**) is a structural analog of the inhibitory neurotransmitter gamma-aminobutyric acid [1,2]. Currently, it is recommended as a first line agent for the treatment of neuropathic pain arising from diabetic neuropathy, post-herpetic neuralgia, and central neuropathic pain [2,3].

Due to its importance, a number of chemical routes have been developed for synthesizing gabapentin [4–10]. The developed chemical process via the formation of 1-cyanocyclohexaneacetic acid (**2**) from 1-cyanocyclohexylacetonitrile (**3**) followed by hydrogenation seems to be a promising approach to afford gabapentin [11,12]. Unfortunately, the chemical procedure to convert **3** to **2** affords product in low yield and purity due to the low regioselectivity of catalysts. Moreover, the process requires strong acids and bases and large amounts of organic solvent, thus producing unwanted byproducts and considerable amount of inorganic wastes. Alternatively, nitrilase-catalyzed hydrolysis of nitriles offers a "greener" protocol with eco-efficiency [13,14]. This biotransformation of dinitrile to cyanocarboxylic acid can be occurred with high regioselectivity, where only one of the two nitrile groups is hydrolyzed to the corresponding carboxylic acid in one single step. Wong and Burns [15] reported that whole microbial cell enzyme catalysts *Acidovorax facilis* 72W and its mutants harboring aliphatic nitrilase activity catalyzed the selective conversion of **3** to **2**. Recently, Zhu et al. [16] reported that nitrilase bll6402 from *Bradyrhizobium japonicum* strain USDA110 efficiently catalyzed the selective hydrolysis of α,ω-dinitriles to exclusively afford ω-cyanocarboxylic acids. Despite the reported nitrilases or microorganisms possessing nitrilase activity have the ability to hydrolyze **3** to **2**, the drawbacks including low space-time yield, low substrate loading or poor regioselectivity restricted the industrial applications.

In our recent work, *A. facilis* ZJB09122 harboring a nitrilase with excellent regioselectivity was isolated. The gene encoding *A. facilis* ZJB09122 nitrilase was cloned and expressed in *Escherichia coli* BL21(DE3) [17]. This nitrilase converts a wide variety of dinitriles to the cyanocarboxylic acids, including **3** to **2**. The cell-specific activity is still insufficient for industrial application. Therefore, protein engineering was used to increase the nitrilase specific activity [17]. As a result, the bioconversion of **3** to **2** demonstrated to meet the targets for yield and productivity. Herein, we seek to take advantage of the promising features of regioselective nitrilase mutant to develop an efficient chemoenzymatic route to gabapentin. The nitrilase catalyzed regioselective hydrolysis of **3** to **2**, followed by direct hydrogenation to 2-azaspiro [4.5] decan-3-one (**4**) over Raney-nickel. The resulting **4** was then converted to **1** with simple chemical steps (Scheme 1).

2. Experimental

2.1. Chemicals and catalysts

Compounds **3** and **2** were provided by Zhejiang Chiral Medicine Chemicals Co., Ltd. (Hangzhou, China). Catalysts including Pd/carbon

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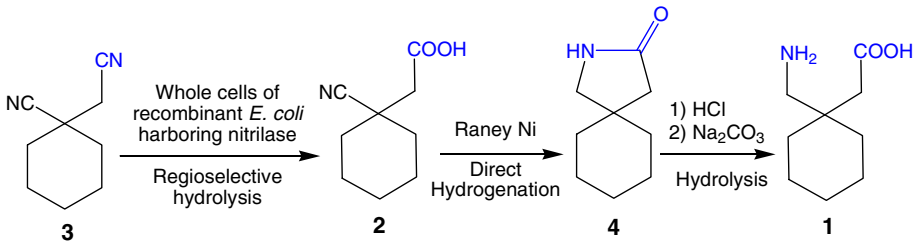

Scheme 1. Chemoenzymatic route to gabapentin.

(5% Palladium on carbon), FTH-Ni 011 (50% nickel on alumina), RTH-3110 (Molybdenum-promoted Raney-nickel), RTH-4110 (Chromium-promoted Raney-nickel) and RTH-6110 (Raney-cobalt) were purchased from Dalian Tongyong Chemical Co. Ltd. (Dalian, China).

2.2. Strain, gene cloning and mutagenesis of nitrilase

E. coli JM109 and *E. coli* BL21(DE3) were used as hosts for cloning and expression, respectively. The gene (GenBank No. KJ001820) encoding *A. facilis* ZJB09122 nitrilase was cloned, mutated and expressed in *E. coli* BL21(DE3) as described previously [17].

2.3. Fermentation of nitrilase in a 500-L fermentor

The optimized medium composition was as follows: peptone, 15 g/L; yeast extract, 12 g/L; NaCl, 10 g/L; glycerol, 15 g/L; $(NH_4)_2SO_4$, 5 g/L; $K_2HPO_4 \cdot 3H_2O$ 4.1 g/L, KH_2PO_4, 6.8 g/L; $MgSO_4 \cdot 7H_2O$, 1.125 g/L (pH 7.0). Cells were firstly transferred to 750-mL flasks containing 150 mL of LB medium from the colony and incubated at 37 °C and 150 rpm. Kanamycin (50 mg/L) was added to the medium at the beginning of inoculation. When cells were grown to the end of exponential growth phase, 900 mL of the culture broth was transferred to a 50-L fermentator containing 30 L of LB medium. Cells were cultivated at 37 °C for 3 h with aeration at 1.1 vvm and agitation at 500 rpm. 15 L of the culture broth was then transferred to 500-L fermentator containing 300 L of optimized fermentation medium. Fermentation was carried out at 37 °C with aeration at 1.4 vvm and agitation at 240 rpm for 4 h. The fermentation temperature was then decreased to 28 °C, and lactose (12.5 g/L) was added to induce the nitrilase activity. After an 8 h fermentation, whole cells were harvested by centrifugation.

2.4. Preparation of **4** by hydrogenation

Into a 500-mL stirred autoclave was added 150 mL of aqueous solution containing **2** and 10 wt.% catalyst (based on the weight of **2**). After flushing the reactor with nitrogen, the mixture was stirred at 1000 rpm, 110 °C and 290 psig of hydrogen for 9 h. After hydrogenation, the pH of the mixture was increased due to the released ammonia. The mixture was cooled to 60 °C, and filtered to remove the catalyst. The filtrate was adjusted to pH 7.0 with 6 M HCl and sodium chloride was added to saturate the solution. The resulting solution was extracted with equivoluminal dichloromethane for three times. The organic phases were combined and then evaporated to obtain yellowish liquid. After cooling to −20 °C for a few hours, a white crystalline solid was collected and dried at 40 °C, to obtain the compound **4**.

2.5. Preparation of **1**

Lactam **4** (15.3 g), water (50 mL) and hydrochloric acid (50 mL) were added to a 250-mL round bottomed flask with a mechanical stirrer, and refluxed for 4 h at 150 rpm. The mixture was then cooled to room temperature, and washed twice with dichloromethane (50 mL each). The organic phases were combined, dried by $CaCl_2$ and filtered. The resulting filtrate was evaporated to remove the organic, and the starting material **4** was recovered. The aqueous phase was cooled to 0–4 °C. After 1 h, a white crystalline solid was collected by filtration. The solid was dried at 40 °C to obtain gabapentin hydrochloride. The mother liquors were recovered and reused in next reaction.

36.4 g of gabapentin hydrochloride and 50 mL water were added to a 250-mL round bottomed flask. The mixture was stirred at 40 °C to dissolve the gabapentin hydrochloride, 12.5 mL of methylbenzene was added and the pH was adjusted to 7.5 with 200 g/L sodium carbonate aqueous solution. After 30 min, the mixture was cooled to 4 °C for a few hours. The solid was separated, and flushed with methylbenzene. The crude gabapentin was obtained. After crystallization by methanol/isopropanol, the pure **1** was obtained. The mother liquors were reused in next reaction.

2.6. Analytical methods

Biomass was measured by dry cell weight (DCW) [18]. The concentrations of gabapentin **1**, cyanocarboxylic acid **2**, and lactam **4** were determined by HPLC as described previously [17]. Cell-specific activity was measured at 40 °C using 6.79 g DCW/L in sodium phosphate buffer (0.2 M, pH 7.0) containing 0.2 M substrate. The regioselectivity was the molar ratio of the desired product to the total amount of carboxylic acid products formed.

3. Results and discussion

3.1. Improvement of biocatalyst specific activity and nitrilase fermentation

In the initial experiment, we evaluated the activity of the regioselective nitrilase in *E. coli* transformant that expresses wild type nitrilase [*E. coli* BL21(DE3)/pET28b(+)-WT]. The cell-specific activity was determined to be 30.9 U/g DCW. Although it was superior to the native strain (17.7 U/g DCW), the biocatalyst specific activity was still too low to achieve the yield and productivity targets. Based on homology modeling and "hot spot" mutation analysis [17], a key amino acid Phe168 was

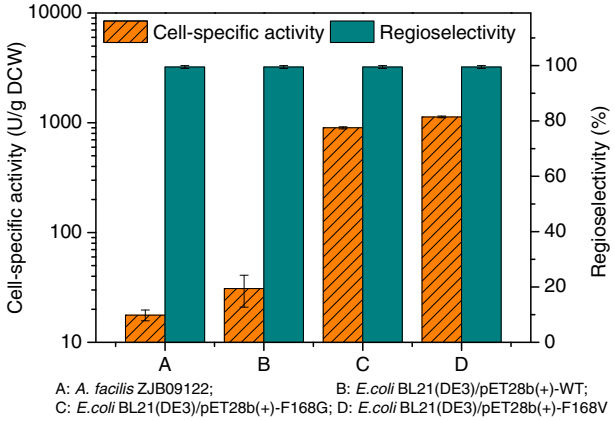

Fig. 1. Cell-specific activity and regioselectivity for the hydrolysis of dinitrile **3** by microbial catalysts.