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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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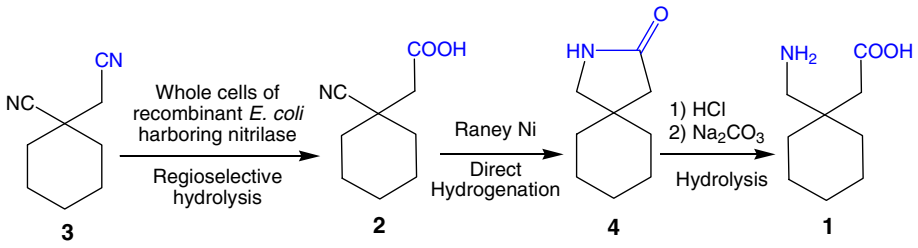

Scheme 1. Chemoenzymatic route to gabapentin.

(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

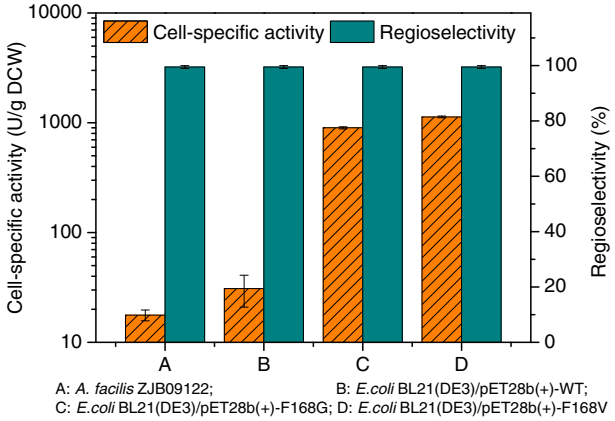

Fig. 1. Cell-specific activity and regioselectivity for the hydrolysis of dinitrile **3** by microbial catalysts.

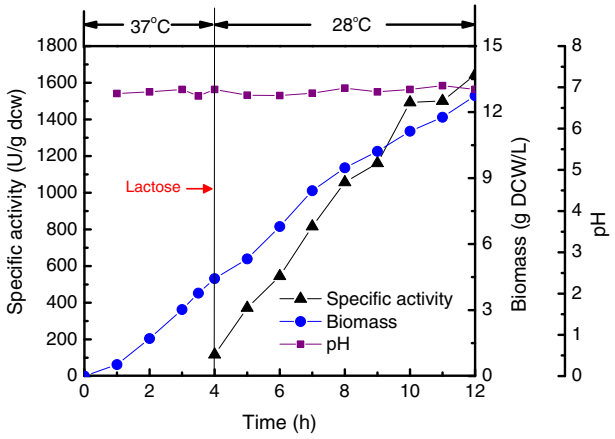

Fig. 2. Time course of nitrilase fermentation in 500-L fermentator with working volume of 300 L at 37 °C and 240 rpm with aeration (1.4 vvm) for 4 h. The temperature was decreased to 28 °C, and 12.5 g/L of lactose was added to the fermentation broth to induce nitrilase.

identified that the point plays an important role in enzyme catalysis and substrate specificity. It was selected and mutated by site-directed mutagenesis. Two mutants Mut-F168V (1131.0 U/g DCW) and Mut-F168G (900.4 U/g DCW) with higher cell-specific activity were obtained. Compared with native nitrilase, a 36.6-fold and a 29.1-fold increase in cell-specific activity were obtained for F168V and F168G mutant in *E. coli* BL21(DE3), respectively (Fig. 1). Overall increases in cell-specific activity of 63.9-fold and 50.9-fold were obtained compared to *A. facilis* ZJB09122 by improvement in both cell-specific activity and protein expression level in *E. coli* BL21(DE3), while the regioselectivity was kept at >99%. The mutant nitrilases showed 44.68% sequence identity to the nitrilase bll6402 from *B. japonicum*. The greatly improved nitrilase represents a promising biocatalyst for large-scale production of intermediate **2**.

Table 1
Regioselective hydrolysis of 1-cyanocyclohexylacetonitrile by recombinant *E. coli* BL21(DE3)-F168V at various concentrations of substrate [a].

Substrate (M)	Reaction time (h)	Product (M)	Yield (%)	Regioselectivity (%)
0.25	1	0.248	99.2	>99
0.50	4	0.495	99.0	>99
1.00	8	0.943	94.3	>99
1.50	24	1.000	66.8	>99
2.00	24	1.020	51.1	>99

[a] Reactions were performed at 40 °C and 150 rpm in sodium phosphate buffer (0.2 M, pH 7.0) with a concentration of 13.57 g DCW/L biocatalyst.

To capitalize further on this potential, we selected the recombinant *E. coli* BL21(DE3) harboring plasmid pET28b(+)-F168V to develop a feasible procedure for producing the regioselective nitrilase to demonstrate its cost-effectiveness and scalability. After optimizing the medium composition, a two-stage temperature control strategy was developed for nitrilase production, in which 37 °C was used for fermentation for the first 4 h and the temperature was then switched to 28 °C. Lactose (12.5 g/L) instead of IPTG was then added to induce the nitrilase expression. The higher temperature at the early stage favors biomass growth. Lower temperature is beneficial for the biosynthesis of active nitrilase, avoiding the formation of inclusion body. At the end of fermentation, the biomass and cell-specific activity were 12.74 g DCW/L and 1643.1 U/g DCW, respectively (Fig. 2). The nitrilase production was calculated to be as high as 20,933 U/L, which is the highest value for regioselective nitrilase fermentation so far. By centrifuging the fermentation broth, whole cells were collected for regioselective hydrolysis of **3**.

3.2. Regioselective hydrolysis of intermediate **3** by *E. coli* BL21(DE3)/pET28b(+)-F168V

The effects of pH and temperature on the activity and regioselectivity of nitrilase in *E. coli* BL21(DE3)/pET28b(+)-F168V are shown in Fig. 3. The optimum pH and temperature were 7.0 and 40 °C, respectively. Whole cells could tolerate against a pretty high concentration of up to 1.0 M of the highly toxic substrate (Table 1) without any effect on its regioselectivity (>99%). Intermediate **2** was produced in 0.94 M with a substrate loading of 1.0 M. Nitrile compounds are known to be detrimental to nitrilases, making the substrate concentrations used in their biotransformations usually very low [19–22]. However, *E. coli* BL21(DE3)/pET28b(+)-F168V was demonstrated to be a robust biocatalyst with

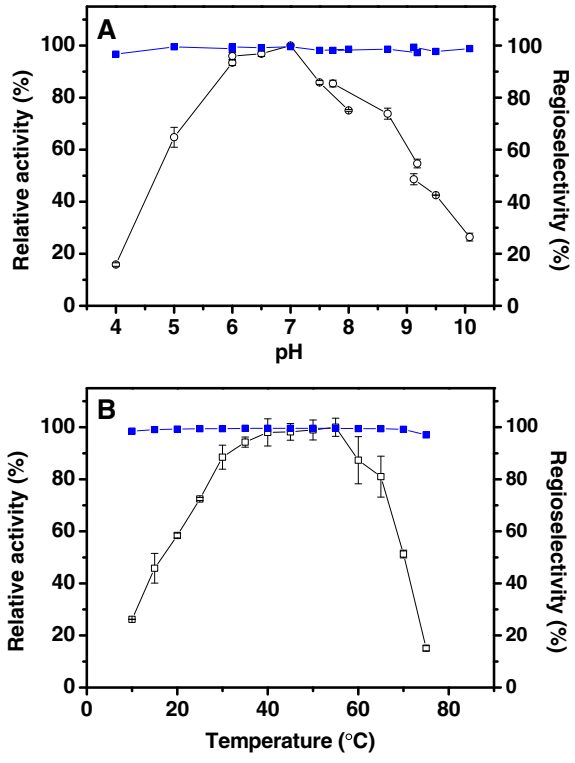

Fig. 3. Effect of pH (A) and temperature (B) on the activity (○) and regioselectivity (■) of the nitrilase in *E. coli* BL21(DE3)/pET28b(+)-F168V in 10 mL of buffer solution (0.2 M) with 0.2 M substrate **3**. pH 4.0–6.0: Na_2HPO_4-citric acid buffer; pH 6.0–8.0: Na_2HPO_4–NaH_2PO_4 buffer; pH 7.5–9.0: Na_2HPO_4–KH_2PO_4 buffer; pH 9.0–10.0: Na_2CO_3–NaH_2CO_3 buffer.

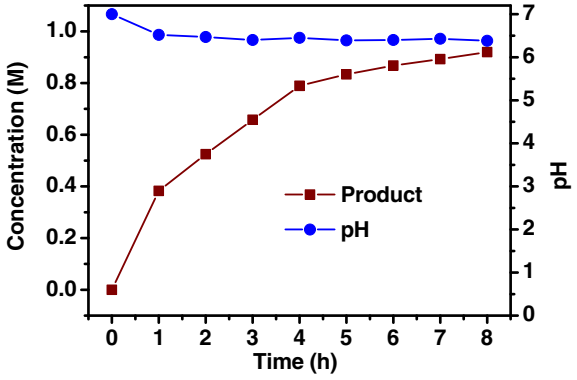

Fig. 4. Regioselective hydrolysis of **3** for synthesizing **2** in a 5-L reactor containing 1.0 L reaction mixture. The experiments were performed at 40 °C and 150 rpm with 1.0 M substrate and 13.57 g DCW/L biocatalyst.

Table 2
Hydrogenation of **2** to lactam **4** in aqueous solution without isolation of intermediate 2[a].

Entry	Catalyst (wt.%)[b]	Temperature (°C)	Reaction time (h)	Conversion of **2** (%)	Yield of **4** (%)
	5% Pd/C	70	12	17.4	0.9
2	10% FTH-Ni011	70	12	60.6	0.2
3	10% RTH-3110	70	12	99.3	51.8
4	10% RTH-6110	70	12	30.8	10.1
5	10% RTH-4110	70	12	99.4	65.2
6	5% RTH-4110	70	12	90.1	19.1
7	20% RTH-4110	70	12	99.5	64.4
8	10% RTH-4110	90	12	98.4	69.0
9	10% RTH-4110	110	9	99.5	95.3
10	10% RTH-4110	130	7	100	93.5
11	10% RTH-4110	150	5	100	91.3

[a] Reactions were performed at 1000 rpm, 290 psig hydrogen.
[b] wt.% based on weight of **2**.

excellent tolerance towards high substrate loading. We then compared the catalytic performance between phosphate buffer (0.2 M, pH 7.0) and purely aqueous solution (initial pH 7.0). No significant changes in the yield of product and regioselectivity were observed (Fig. S1). The pH of the reaction mixture changed only slightly over the course of the reaction due to the formation of ammonium salt of the carboxylic acid. Purely aqueous solution (initial pH 7.0) could be used for the preparative scale reaction to avoid the presence of too many salts in the subsequent steps.

To further evaluate the feasibility of the biocatalytic process for production of **2** for practical application, a 5.0 L scale reaction system for the regioselective hydrolysis of **3** (pH 7.0, Temp. 40 °C, rotation 200 rpm, 13.57 g DCW/L biocatalyst) was performed in a stirred-tank reactor. As a result, hydrolysis of substrate **3** (1.0 M) over 8 h provided the desired product with a conversion of 92% and a regioselectivity of >99% (Fig. 4), representing an outstanding space-time yield of 461 g/L/day, which markedly exceeded the average space-time yield of industrial bioprocess (372 g/L/day) [23].

3.3. Direct hydrogenation of *2* to *4* in the aqueous product mixture

Due to the high concentration of **2** accumulated in the reaction mixture, the aqueous product mixture could be used directly for the efficient production of lactam **4** in subsequent hydrogenation. All the catalysts showed the ability to produce **4** by hydrogenation of **2** in aqueous solution (Table 2). Clearly, the highest substrate conversion (99.4%) and product yield (65.2%) were obtained when RTH-4110 was used as catalyst. In entries 2–3 and 5–6 in Table 2, the major by-products were secondary amine, amide, dimine and diamine, which were formed because of the formation of C – N, C – O and C – C bond [24]. The concentration of gabapentin **1** was lower than 6.5%. The pool selectivity of catalysts (FTH-Ni011, RTH-3110, and RTH-4110) leads to a high conversion with a low yield.

The reaction temperature has a significant effect on hydrogenation. The yield of **4** increased with increase of temperature from 70 to 110 °C. The cyclization was promoted by increasing the reaction temperature. A further increase of the reaction temperature led to a decrease of the product yield. This result may be because the cyclization reaction was reversible in aqueous solution [25]. Maximum product yield (95.3%) was obtained at 110 °C, which was attributed to a shift of the equilibrium which favors the formation of **4**. Based on the above results, 10% catalyst loading and 110 °C were chosen as the optimal catalyst amount and reaction temperature. Under the optimal conditions, the highest substrate conversion (99.5%) and product yield (95.3%) were obtained at 9 h (Fig. S2).

3.4. Synthesis of gabapentin *1* from lactam *4*

The lactam **4** was collected and then convert to gabapentin **1** by hydrolysis and basification. After crystallization, the pharmaceutical grade gabapentin was obtained with above 99.9% HPLC purity. The yield of **1** from **4** was 88.2%. The overall yield of **1** form starting compound **3** was 77.3%, which is higher than the previously reported chemical methods (61.6%) [12].

4. Conclusions

An efficient industry-relevant chemoenzymatic route for preparing gabapentin **1** was developed. Protein engineering and overexpression of nitrilase, combined with optimized fermentation of *E. coli* BL21(DE3)/pET28b(+)-F168V resulted in a highly active and robust nitrilase biocatalyst. The enzymatic process used to prepare the key intermediate **2** features high substrate loading, high conversion, excellent regioselectivity and unprecedented space-time yield. Downstream chemical transformations have been developed to efficiently convert the enzymatic product to gabapentin. The complete process represents an efficient, practical and economical route to gabapentin.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.catcom.2015.03.035.

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