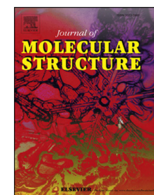
Journal of Molecular Structure 1219 (2020) 128600

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: http://www.elsevier.com/locate/molstruc



A novel strategy for efficient chemoenzymatic synthesis of D-glutamine using recombinant *Escherichia coli* cells

Qinglin Du [a, 1], Xiangyang Zhang [a, 1], Xinru Pan [a], Hongjuan Zhang [b], Yu-Shun Yang [a, *], Junzhong Liu [a, **], Qingcai Jiao [a, ***]

[a] *State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, 210093, PR China*
[b] *School of Pharmacy, Nanjing Medical University, Nanjing, 211166, PR China*

ARTICLE INFO

Article history:
Received 24 February 2020
Received in revised form
26 May 2020
Accepted 2 June 2020
Available online 6 June 2020

Keywords:
D-glutamine
DL-glutamic acid
Chemoenzymatic synthesis
L-aminopeptidase

ABSTRACT

D-glutamine is a D type stereoisomer of glutamine which is involved in many metabolic processes. Seeking lower-cost and industrially scalable approaches for the synthesis of D-glutamine is very valuable both in academic career and potential applications. Herein, we developed a novel efficient chemo-enzymatic strategy for producing D-glutamine. Initially, DL-glutamine was chemically prepared with cheap and accessible DL-glutamic acid as raw material. Subsequently, the L-glutamine among the racemic mixture was selectively hydrolyzed to L-glutamic acid by *Escherichia coli* whole-cell system which expressed L-aminopeptidase D-Ala-esterase/amidase (DmpA) from *Ochrobactrum anthropi*. The left D-glutamine was obtained by isoelectric point precipitation with 70% of the theoretical yield. Furthermore, we optimized enzymatic resolution conditions to determine the optimum parameters as pH 8, 30 °C, 0.1% (v/v) Triton X-100, and 1 mM Mn^{2+}. These results suggested that our strategy might be potentially usable for the synthesis of D-glutamine in industrial productions.

© 2020 Elsevier B.V. All rights reserved.

1. Introduction

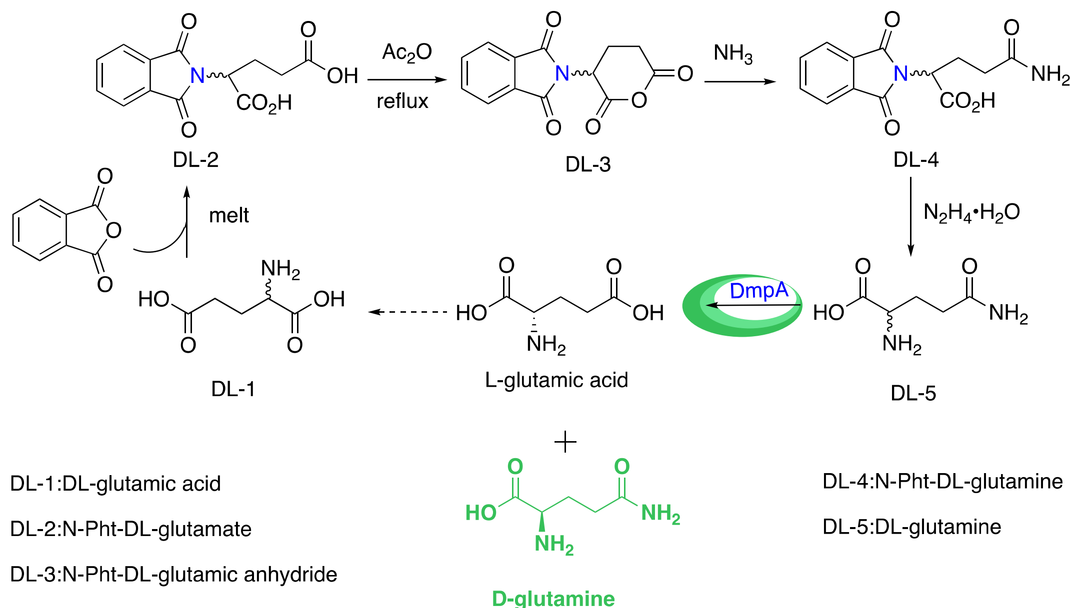
D-amino acids and their amide derivatives, the non-proteinogenic forms of amino acids, are naturally recognized as the relevant components in the biosynthesis of the peptide antibiotic and bacterial cell walls [1,2]. With significant biological activity, D-amino amides indicate great potential in nutrition and disease treatment [3–5]. Especially, the amide form of D-glutamic acid, D-glutamine, is essential for the oxidative stress, nitrogen metabolism and mitochondrial function [6,7], so as a key raw material which is widely used in the production of food additives and pharmaceutical products [8]. Therefore, many researches on lower-cost and scale-up synthesis of D-glutamine have been conducted [9].

The previous chemical synthesis strategy of DL-glutamine and the acquisition process of D-glutamine were both difficult and not industrial because of the low stereoselectivity and yield [10]. Nowadays, enzymatic or chemoenzymatic synthesis of pharmaceutical intermediates has exhibited a great potential, which benefits from the stereo-specificity and high efficiency brought by biological enzymes [11,12]. There are two common kinds of enzymes associated with the synthesis of D-glutamine. One of them directly catalyzes the formation of glutamine from glutamate and ammonium ion [13,14], for example, Glutamine synthetase (E.C. 6.3.1.2). This kind of enzyme is one of the key enzymes in nitrogen metabolism, but has low reactivity in external catalysis reaction and need expensive coenzyme ATP. The other kind first synthesizes D, L-amino amide, then selectively degrades L-form to obtain the undegraded D-amino amide [15], for example, L-glutamate decarboxylase (E.C. 4.1.1.15). Undoubtedly, it leads to a serious waste of raw material.

Herein, distinguishing from the above-mentioned two approaches, we introduced a novel strategy for the efficient chemoenzymatic synthesis of D-glutamine as shown in Scheme 1. This strategy included two procedures, the chemical preparation of DL-glutamine, and the biological resolution by an enzyme which has not yet been reported for such a utility. To avoid the unavailable and high cost L-glutamine, the cheaper raw material DL-glutamic acid was used to synthesize DL-glutamine through a four-step chemical

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: ys_yang@nju.edu.cn (Y.-S. Yang), junzhongliu2414@163.com (J. Liu), jiaoqc@nju.edu.cn (Q. Jiao).
[1] Both authors contributed equally to the work.

https://doi.org/10.1016/j.molstruc.2020.128600
0022-2860/© 2020 Elsevier B.V. All rights reserved.



Scheme 1. Chemoenzymatic synthesis of D-glutamine using DL-glutamic acid as raw material with whole-cell catalysis harboring recombinant DmpA.

reaction series. Afterwards, *L-aminopeptidase D-Ala-esterase/amidase* (DmpA, E.C. 3.4.13.20), as a key factor of this strategy, was utilized in the following resolution to achieve D-glutamine. DmpA exists widely in bacteria and naturally catalyzes the hydrolysis of β^3-or β^2-amino acid residues from amides and peptides. With the privileges of heterologous expression in *E. coli* and cofactor-free catalytic reaction [16], DmpA is an ideal enzyme for the resolution of DL-glutamine and has not been reported for this function yet. Combining the chemical preparation of DL-glutamine as substrate and the biological resolution by recombinant expressed DmpA in *Escherichia coli*, we optimized our strategy by refining the parameters and finally achieved the efficient chemoenzymatic synthesis of D-glutamine with industrial potential.

2. Materials and methods

2.1. Chemicals

D-glutamine standard was purchased from Shanghai Macklin Biochemical Co. Ltd., (1-Fluoro-2,4-dinitrophenyl)-5-L-alaninamide (FDAA) was purchased from Tianjin Heowns Biochemical Technology Co. Ltd., Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Nanjing Jitian Biotechnology Co., Ltd., Kits for genetic manipulation were purchased form Takara Bio (Dalian, China). All other chemicals were analytical grade.

2.2. Synthesis

2.2.1. Synthesis of N-Pht-DL-glutamate

DL-glutamic acid (29.4 g) and phthalic anhydride (29.6 g) were added into a 500 mL round-bottomed flask. *N*-Pht-DL-glutamate was prepared by the melting method [17]. Yield: 88%, 48.8 g white acicular crystal.

2.2.2. Synthesis of N-Pht-DL-glutamic anhydride

N-Pht-DL-glutamate (41.6 g) was refluxed in 45 mL acetic anhydride for 15 min, cooled naturally, crystallized, filtered and washed with ethyl acetate for crystallization. Yield: 95%, 36.9 g colorless acicular crystal. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.00–7.89 (m, 4H), 5.48 (dd, J = 13.0, 5.8 Hz, 1H), 3.17–3.07 (m, 1H), 3.03–2.92 (m, 1H), 2.68–2.55 (m, 1H), 2.19–2.07 (m, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 167.18, 166.77, 166.11, 135.52, 131.58, 124.09, 48.15, 29.92, 20.87.

2.2.3. Synthesis of N-Pht-DL-glutamine

N-Pht-DL-glutamic anhydride (20.7 g) was dissolved in 2 M ammonia (80 mL) at ambient temperature with stirring for 20 min. Subsequently, the solution was acidified to pH 3 with hydrochloric acid (6 M). The precipitate was filtered, washed with ultrapure water for three times. The filtered precipitate dried at 70 °C for 12 h to afford the *N*-Pht-DL-glutamine. Yield: 76%, 16.8 g white acicular crystal. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 13.21 (s, 1H), 7.20 (s, 1H), 6.72 (s, 1H), 4.75 (dd, J = 11.0, 4.5 Hz, 1H), 3.36 (s, 1H), 2.41–2.32 (m, 1H), 2.31–2.22 (m, 1H), 2.10 (t, J = 7.5 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 173.55, 170.91, 167.88, 135.26, 131.70, 123.82, 51.80, 31.84, 24.44.

2.2.4. Synthesis of DL-glutamine

N-Pht-DL-glutamine (15.2 g) was added to an aqueous solution containing 2.5 g hydrazine hydrate at room temperature for 48 h. The reaction liquid was acidified with 6 N hydrochloric acid to pH 3. Phthalic hydrazine precipitated was removed by filtration. DL-glutamine was precipitated by decompression. Yield: 90%, 8.0 g white needle crystal. ^{1}H NMR (600 MHz, D_2O) δ: 3.67 (t, J = 6.2 Hz, 1H), 2.43–2.29 (m, 2H), 2.09–1.95 (m, 2H). ^{13}C NMR (151 MHz, D_2O) δ: 177.55, 173.91, 54.06, 30.75, 26.12.

2.3. Bacterial strains, plasmids, and culture conditions

The *dmpA* gene from *Ochrobactrum anthropi* LMG7991 (GeneBank accession number CAA66259.1) was synthesized by TsingKe. *E. coli* BL21(DE3). The pETDuet-1 plasmids were used as the host cells as well as expression vector, respectively. The *E. coli* BL21(DE3) carrying the recombinant plasmid pET-Duet1-dmpA was constructed in our laboratory. The recombinant strain was inoculated in 3 mL of LB medium supplemented with 100 mg ampicillin/L and cultivated in a rotary shaker (200 rpm) at 37 °C for 12 h. After that, 5% seed culture was inoculated into 30 mL LB medium in 100 mL

flask and was cultured at 37 °C and 200 rpm in a rotary shaker. When the optical density of the culture at 600 nm reached 0.5, the expression of DmpA was induced by adding IPTG to a final concentration of 0.5 mM, and the culture was incubated at 28 °C and 200 rpm for 8 h. Cells were harvested via 5 min centrifugation at 10,000×*g* and 4 °C, and washed with 0.2 M phosphate buffer (pH 7.5). Then the obtained cell pellet was maintained at 4 °C for further studies.

2.4. DmpA activity assay

The whole-cell strains expressing DmpA were kept in store in the freezing condition. Before the activity assay, the whole-cell strains were weighted to control the steadiness, and then initially recovered with a 20-min incubation. This pre-incubation has been convinced to recover the high DmpA activity. Afterwards, the recovered whole-cell strains were used directly as an enzyme source to produce L-glutamic acid from DL-glutamine. Activities of DmpA was measured by detecting the formation of L-glutamic acid. The standard assay solution (final volume, 2 mL) containing 200 mM DL-glutamine and the whole-cell (0.02 g) were further incubated for 20 min at 30 °C, pH 8. One unit (U) of enzyme activity was defined as the amount of whole-cell strain catalyzing the conversion of substrate to the product at a rate of 1 μmol/min [18].

2.5. Analytical methods

The chemical structures of components synthesized in Scheme 1 were determined by Bruker DRX-600 MHz spectrometer. D-glutamine and L-glutamine were separated and detected by HPLC after the derivatization with the chiral derivative reagent (1-Fluoro-2, 4-dinitrophenyl)-5-L-alaninamide (FDAA) according to the literature [19]. The elution conditions were as follows: solvent A, 20 mM ammonium acetate in aqueous solution; solvent B, acetonitrile; flow rate, 0.6 mL/min; 0–8 min 95% A and 5% B; 8–30 min 87% A and 13% B. Ultimate XB-C18 (4.6 × 100 mm, 5 μm) was used for compound separation. The compounds were monitored at UV = 340 nm.

2.6. Molecular docking analysis

In order to hint the mechanism of DmpA preferentially hydrolysis L-glutamine than D-glutamine, we performed molecular docking analysis thus the possible patterns could be visualized before a further exploration. The three-dimensional structures of D-glutamine and L-glutamine were constructed using Chem. 3D ultra 19.0 software, then they were energetically minimized by using Minimize Small Moleculars function under CharMm forcefield by Discovery Stutio (version 3.5). The crystal structure of DmpA (PDB code: 1B65) complex was downloaded from the RCSB Protein Data Bank. All bound waters and ligands were eliminated from the protein and the polar hydrogen was added to the proteins after the Prepare Protein procedure in the same software [20]. Molecular docking of compounds L-glutamine and D-glutamine into the three-dimensional X-ray structure of DmpA was carried out using the CDOCKER protocol Discovery Stutio (version 3.5).

2.7. Enzymatic resolution of DL-glutamine by DmpA

Enzymatic resolution reaction was in 10 mL tube filled with 2 mL of reaction mixtures containing Na_2CO_3/$NaHCO_3$ buffer (50 mM), the whole-cell catalyzing system, and 200 mM DL-glutamine. Reaction condition optimization was performed under the following parameter variation: temperature variation from 20 to 45 °C; pH variation from 6 to 11; surfactants including Sodium dodecyl sulfate (SDS) , Tween-80 and Triton X-100; metal ions including Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+}.

3. Results and discussion

3.1. Docking simulation of the DmpA between L-glutamine and D-glutamine

D-glutamine and L-glutamine were docked into the enzyme, respectively. The docking results were shown in Fig. 1. The active center we selected from over 30 supposed receptor cavities for docking was consistent with the previous report [21], and we knew that Ser250 at the active site was the most important catalytic residue. The results of molecular docking showed that the CDOCKER Interaction Energy (interaction energy between the ligand and receptor) of the enzyme in combination with L glutamine (−27.9136 kcal/mol) was lower than that in combination with D glutamine (−25.4735 kcal/mol), indicating that L-glutamine was the preferred binding configuration of the enzyme. Moreover, seen in Fig. 1B, C, L-glutamine might form two possible hydrogen bonds with Ser250 (O···H–N: 2.66 Å, 119.566°; O···H–N: 1.87 Å, 141.012°), whereas in Fig. 1E, F, D-glutamine only form one hydrogen bond with Ser250 (O···H–N: 2.75 Å, 117.525°) [22]. The analysis of the above molecular docking results inferred that, it possibly gave priority to the hydrolysis of L-glutamine theoretically when DmpA catalyzed the hydrolysis of the substrate DL-glutamine.

3.2. Effect of temperature and pH on DmpA activity

The preferences of DmpA under different temperature and pH conditions exhibited consistency with that of the previous report [23]. The reaction was carried out in a 2 mL mixture containing 200 mM DL-glutamine and 0.02 g recovered whole-cell strain. The optimal temperature of the enzyme was evaluated using the Na_2CO_3/$NaHCO_3$ (pH 8). The temperature stability of DmpA was determined after pre-incubation of the recovered whole-cell strain at a broad temperature range of 20–45 °C. The enzymic activity was measured after 2 h. The highest relative activity and residual activity of 100% denoted 66.7% and 55.9% conversion of L-glutamine. The data were presented as mean ± standard deviation (SD) from three independent experiments. Subsequently, the effect of temperature on DmpA activity was investigated within a range from 20 to 45 °C. Along with the increase of temperature, the activity of DmpA also enhanced. The highest activity of DmpA was observed at 30 °C and then its activity decreased significantly with a further increase of temperature (Fig. 2A). The thermostability of DmpA was examined by determination of its residual activity after incubation at 20, 30 and 40 °C, respectively. Accordingly, 30 °C was chosen as a favorable temperature for glutamine synthesis (Fig. 2B).

In terms of pH, the reaction was carried out the same as Fig. 2. The optimal pH of the enzyme was evaluated using the following buffers (50 mM): sodium phosphate (pH 6–7), Na_2CO_3/$NaHCO_3$ (pH 8–10). The pH stability of DmpA was determined after pre-incubation of the recovered whole-cell strain at a broad pH range of 6–10. The enzyme activity was measured after 2 h. The highest relative activity and residual activity of 100% denoted 18.8% and 50.1% L-glutamine, respectively. The mixture was kept in a shaking plastic tube at 30 °C. The data were presented as mean ± standard deviation (SD) from three independent experiments. The maximum activity of DmpA for the hydrolysis of L-glutamine was observed at pH 8 in 50 mM Na_2CO_3/$NaHCO_3$ buffer, while it decreased significantly with pH lower than 7 or higher than 9, as shown in Fig. 3A. Therefore the optimum pH of this reaction was considered as pH 8. Similarly, the pH stability of the enzyme was also the best at pH 8 (Fig. 3B).

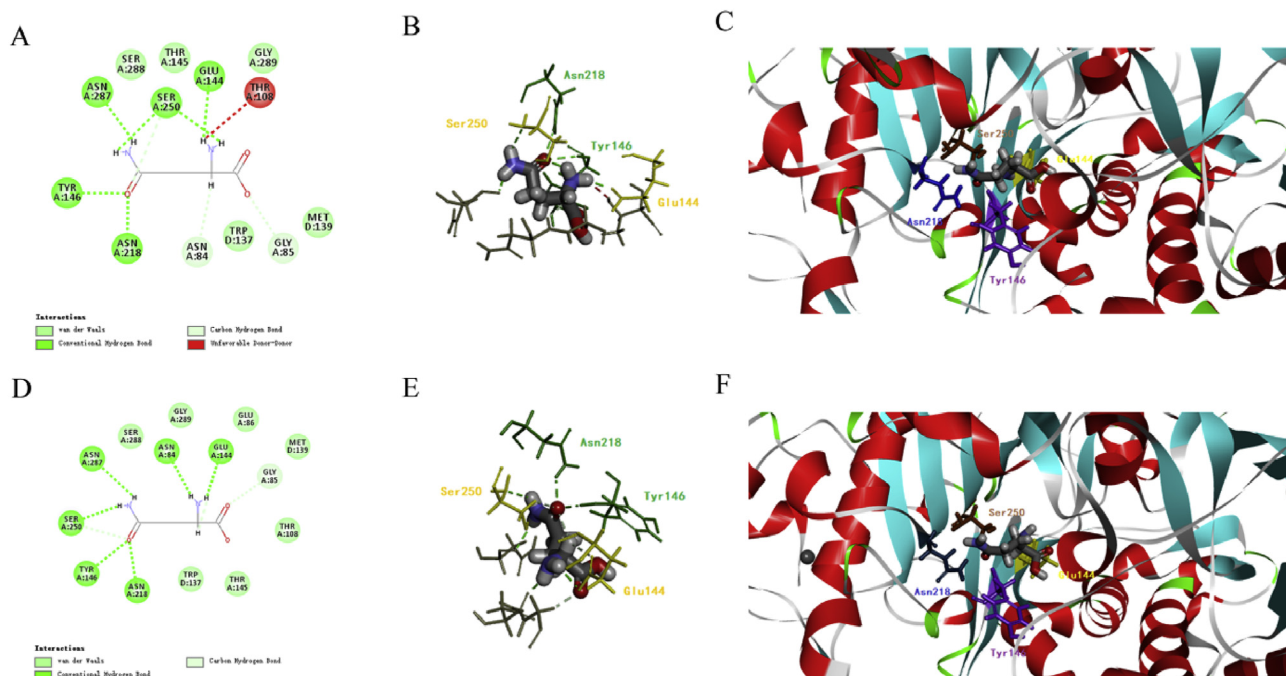


Fig. 1. Molecular docking analysis of L-glutamine and D-glutamine binding site on DmpA (PDB 1B65). (A) The 2D and (B and C) 3D docked images of the L-glutamine combined with protein. (D) The 2D and (E and F) 3D images of the D-glutamine into DmpA. Significant interactions and residues were exhibited. The H-bonds were displayed as green dotted lines. The catalytic residue Ser250, the stabilizing residues Tyr146 and Asn218, and other key residues are shown in stick representation.

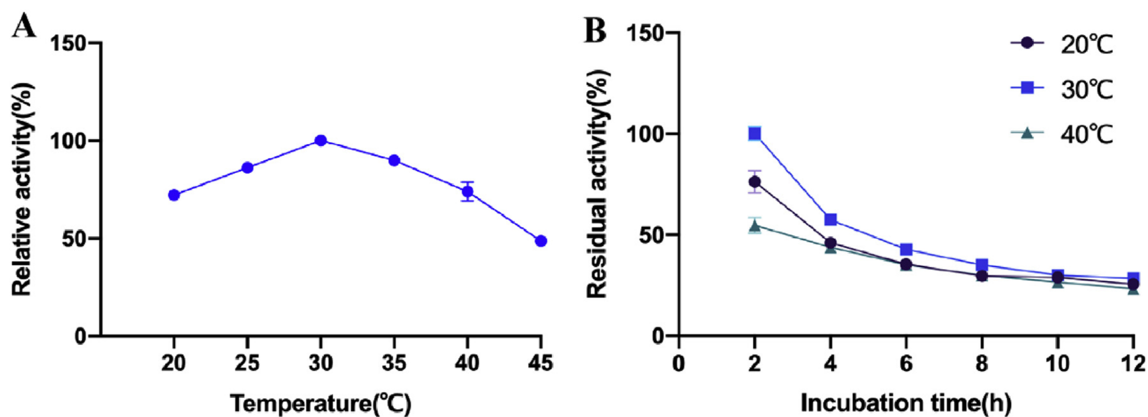


Fig. 2. Effect of temperature on the activities and stabilities of DmpA.

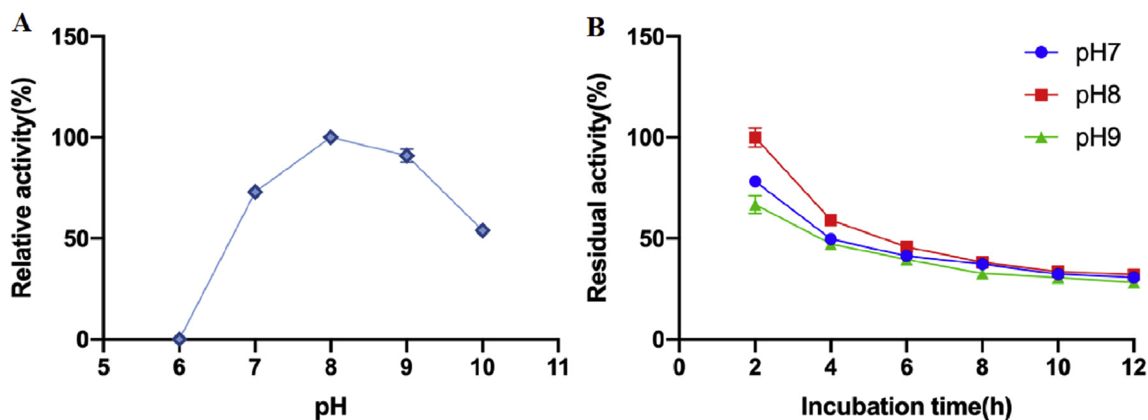


Fig. 3. Effect of pH on the biocatalytic activity of DmpA and on the stability of DmpA.

3.3. Effect of surfactants and divalent cations on DmpA activity

Generally, surfactants can obviously improve the cell membrane permeability [24]. In the present study, the effect of three surfactants on DmpA activity was researched by following the release of L-glutamic acid. The reaction was carried out in the conditions of pH 8 and 30 °C. Concentration of SDS, Tween-80 and Triton X-100 ranges from 0.01%, 0.02%, 0.03%, 0.04%, 0.05% and 0.06% (v/v), respectively. The mixture components were the same as those mentioned in Fig. 2. The enzymic activity was measured after 2 h. The highest relative activity of 100% denoted 40.2% L-glutamic acid on the condition of 0.04% Triton X-100. The data were shown as mean ± SD of three independent experiments. Fig. 4A showed the relative activity of DmpA in different surfactants. Accordingly, SDS had almost no effect on DmpA relative activity. On the contrary, Tween-80 caused a little broad, simultaneously weak enhancement on DmpA relative activity, and Triton X-100 led to the emergence of the highest relative activity. It was noted that the addition of Triton X-100 could distinctly improve the enzymatic activity of DmpA, but the addition of the other two kinds of surfactants could not.

The stimulatory or inhibitory effect of common divalent cations including Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, and Ca^{2+} on the DmpA activity was negligible as shown in Fig. 4B. The reaction was carried out in the conditions of pH 8 and 30 °C. Concentration of Mg^{2+}, Mn^{2+}, $Zn^{2+}$and Co^{2+} ranges from 100, 10, 1, 0.1 and 0.01 mM, respectively. The mixture components were the same as those mentioned in Fig. 2. The enzyme activity was also measured after 2 h. The highest relative activity of 100% denoted 16.7% L-glutamic acid on the condition of 1 mM Mn^{2+}. The data were presented as mean ± SD of three independent experiments. The highest relative activity of DmpA was observed at 1 mM of Mn^{2+}, and then decreased shapely with a further increase of concentration of Mn^{2+}. When the concentration of metal ions was 0.1 and 0.01 mM, the system hardly promoted enzymatic activity. Cu^{2+} and Ca^{2+} had no effect on the activity of DmpA.

3.4. Effect of substrate concentration

The substrate concentration effect on the conversion rate from DL-glutamine to L-glutamic acid was also studied [25,26]. The reaction was carried out in a 2 mL mixture containing 50 mM Na_2CO_3/ $NaHCO_3$ buffer (pH 8), 0.02 g recovered whole-cell strain and DL-glutamine with different concentrations. The mixture was kept shaking at 30 °C. The highest conversion rate of 100% denoted 25 mM L-glutamic acid. The data were presented as mean ± SD of three independent experiments. The conversion rate time course under different substrate concentrations was shown in Fig. 5. The results indicated that with substrate concentration increasing form 50 mM−200 mM, the time required to achieve the highest conversion rate decreased. But when the substrate concentration was 400 mM, it took 8 h to reach the highest conversion rate. Reaction inhibition was observed at high substrate concentration. Taking production efficiency into consideration, the optimal substrate concentration would be 200 mM.

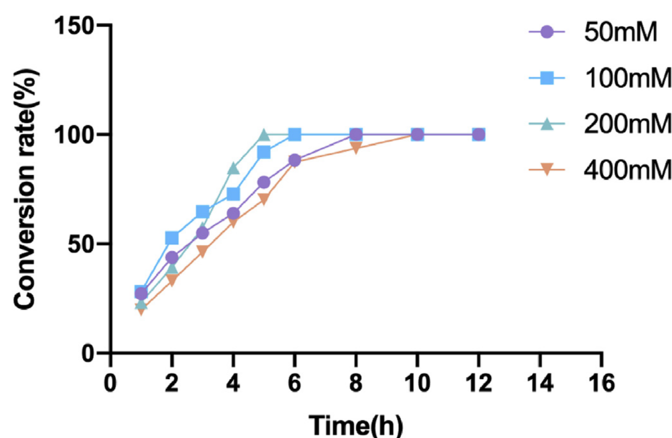


Fig. 5. Effect of concentration of DL-glutamine on DmpA activity.

3.5. Biotransformation process

We discovered the process of enzymatic resolution of DL-glutamine to D-glutamine. The reaction mixture (final volume, 1 L) contained 10 g/L recovered whole-cell strain with 200 mM DL-glutamine. Error bars show one standard deviation, as determined from triplicate experiments.

Obviously, with the increase of reaction time, the concentration of L-glutamic acid increased gradually. In Fig. 6, with the extension of the reaction time, the content of L-glutamine decreased. After 12 h of reaction, only 12.06% of L-glutamine was not hydrolyzed, whereas the content of D-glutamine remained unchanged. This result could indirectly indicate that the enantiomeric excess (ee) value at this time was greater than 99%. After 13 h of reaction, although only 6.5% of L-glutamine remained unhydrolyzed, the D-glutamine began to decrease (Fig. 6). Therefore, it seemed necessary to control the time of the biological resolution reaction within 12 h, which ensured the content and purity of the product D-glutamine.

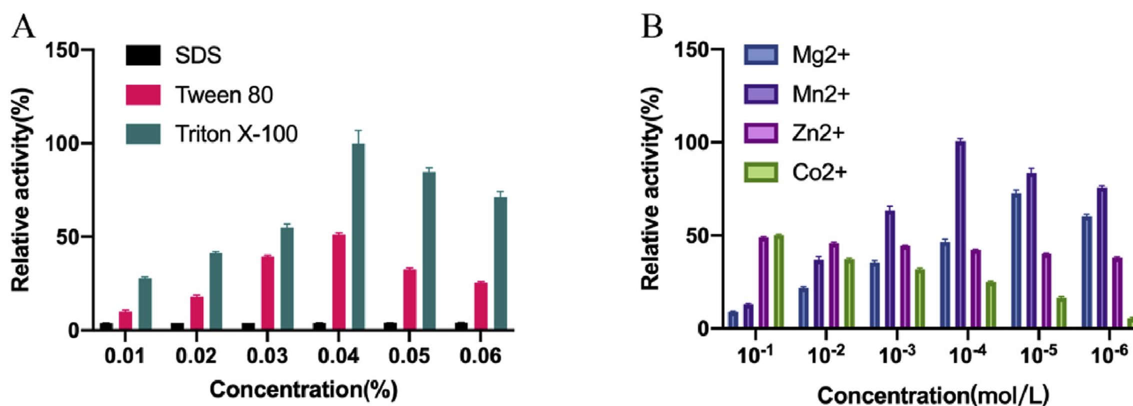


Fig. 4. Effect of surfactants (A) and divalent cations (B) on enzymatic activity.

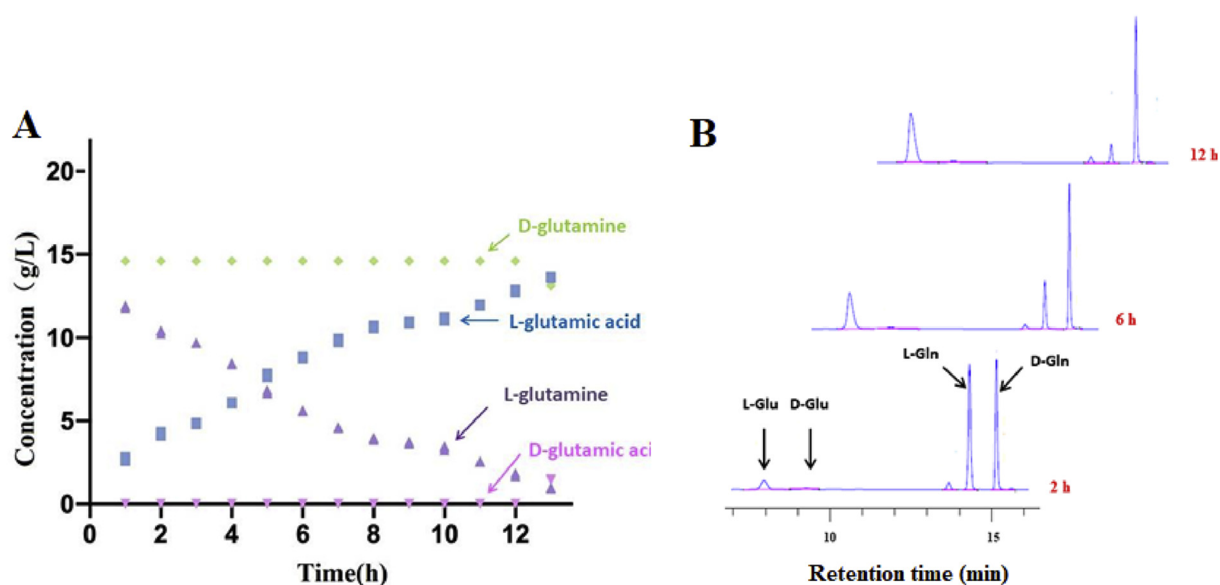


Fig. 6. (A) Biotransformation of L-glutamine to L-glutamic acid using dry whole cell expressing DmpA at optimum reaction conditions (pH 8, 30 °C). (B) HPLC detection of the DmpA-mediated hydrolysis of DL-glutamine (Retention time as follows: L-glutamic acid 8.2 min, D-glutamic acid 9.4 min, L-glutamine 14.2 min, D-glutamine 15.0 min).

3.6. Isolation and identification of D-Glutamine

The reaction mixture included 30 g/L DL-glutamine, 10 g recovered whole-cell strain, 1 L $Na_2CO_3/NaHCO_3$ buffer (pH 8), 0.04% (v/v) Triton X-100, 1 mM Mn^{2+}. After incubation at 30 °C for 12 h, the mixture was centrifuged at 12000×g for 10 min to remove the insoluble fraction. And the resulting supernatant was decolorized by active carbon at 60 °C for 30 min. After that, adjusting the pH of the conversion solution to 3.22, L-glutamic acid precipitated at its isoelectric point. Finally, 8.45 g L-glutamic acid was obtained by drying precipitated crystals [27]. Then the reaction mixture was adjusted to pH 5.65, and concentrated to 30 mL under vacuum, thus D-glutamine crystallized. At last, 10.6 g D-glutamine was obtained, with 70% of theoretical yield. Specific rotation of D-glutamine was $[\alpha]_D^{20} = -7.3$ ($c = 1$, H_2O).

3.7. Discussion

In this study, we have successfully developed an efficient chemoenzymatic synthesis method of D-glutamine. A simple chemical method was discovered to produce DL-glutamine using low price material with high efficiency. Moreover, products from each step of the chemical reaction were easy to crystallize and separate. The whole procedure was strong operability, and very suitable for industrial application. Besides, it seemed a better choice to take advantage of bio-resolution rather than chemical resolution [28]. Compared with chemical resolution, bio-resolution was environmentally friendly and more cost-effective due to using a starting material (DL-glutamic acid) which could be produced from biomass. More importantly, compared with the acylation-hydrolysis pathway, the direct degradation method could obtain a higher yield of D-amino acid [29,30].

In this work, enzymes were mainly used for biological resolution. The essence was to use enzymes to hydrolyze the γ-amide bond of L-glutamine efficiently and rapidly. As we knew, there were many enzymes that could hydrolyze amide bonds, such as the aminopeptidase used here, as well as proteolytic and amidase. DmpA is a member of serine aminopeptidase. According to the catalytic mechanism, the aminopeptidase superfamily can be divided into metal aminopeptidase, cysteine aminopeptidase and serine aminopeptidase [22,24]. D-aminopeptidase (DAP) and D-aminopeptidase B (DmpB), like DmpA, also belong to Serine aminopeptidase. Due to their strict substrate specificity, DAP and DmpB can only catalyze substrates in the D-configuration, thus they are not suitable for biological resolution of DL-glutamine. In contrast, DmpA has a broader substrate specificity and is more suitable for biological resolution of DL-glutamine. However, Amidases are an important class of biocatalysts that act on lactam bonds and catalyze the hydrolysis of amides to the corresponding carboxylic acids and ammonia [31,32]. In the past 20 years, although a large number of amidase-producing microorganisms have been screened from natural sources, they have faced real difficulties. Due to the low expression of amidases in wild bacteria and the stereoselectivity in the same microorganism, these are several reasons to limit their large-scale application. With the advent of genetic engineering technology, the use of *E. coli* as a host to express amylase from different sources (*B. stearothermophilus* BR388, *C. acidovorans* KPO-2771-4 and Pseudomonas sp. MCI3434) has gradually become a common method for industrial applications [33–35]. There have been a large number of reports using genetic engineering bacteria to produce a variety of substances, such as *S*-2,2-dimethylcyclopropane carboxylic acid, 1-CCHAA and (*R*)-TFHMA [36–38]. Of course, there have been reports in which the researchers used genetically engineered bacteria to heterologously express amidase with L-glutamine as a substrate, but the specific enzyme activity is only 17%, being far lower than the enzyme activity of DmpA [39].

In summary, the heterologous expression of DmpA for the biological resolution of DL-glutamine seemed the most suitable method to the best of our knowledge. Moreover, the combined use of chemical synthesis and enzymatic resolution to produce D-glutamine in this work provided a certain theoretical basis for potential industrial applications. There might be more suitable enzymes for biological resolution of DL-glutamine in the near future. At least, this work provided a good idea for the production of D-amino acids.

4. Conclusion

To sum up, we developed a novel efficient chemoenzymatic synthesis method of D-glutamine using DL-glutamic acid as the raw material. This was the first report that D-glutamine could be prepared by the resolution of DL-glutamine with recombinant DmpA which was expressed in *E. coli*. Meanwhile, it was also the first time for DmpA to be recruited in the synthesis of D-amino acids. Attractively, the D-glutamine was obtained by isoelectric point precipitation with the theoretical yield as high as 70%. Furthermore, the optimum parameters were determined as pH 8, 30 °C, 0.1% (v/v) Triton X-100, and 1 mM Mn^{2+} all through the control variable optimizing procedures. Stepping closer and closer to the productive requirements, our strategy seemed potential for the industrialized preparation of D-glutamine, thus might boost the corresponding applications such as chemical engineering and biological medicine.

Declaration of competing interest

All authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Qinglin Du: Methodology, Writing - original draft, Formal analysis. **Xiangyang Zhang:** Resources, Investigation. **Xinru Pan:** Validation, Visualization. **Hongjuan Zhang:** Validation. **Yu-Shun Yang:** Writing - review & editing. **Junzhong Liu:** Conceptualization, Supervision. **Qingcai Jiao:** Project administration, Funding acquisition.

Acknowledgments

This study was financially supported by the National Key R&D Program of China (2017YFC0506005).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molstruc.2020.128600.

References

[1] K. Hamamoto, Y. Kida, Y. Zhang, T. Shimizu, K. Kuwano, Antimicrobial activity and stability to proteolysis of Small linear cationic peptides with D-amino acid substitutions, Microbiol. Immunol. 11 (2002) 741–749.
[2] F. Cava, M.A. De Pedro, H. Lam, B. M Davis, M.K. Waldor, Distinct pathways for modification of the bacterial cell wall by non-canonical D-amino acids, EMBO J. 30 (2011) 3442–3453.
[3] A. Wessolowski, M. Bienert, M. Dathe, Antimicrobial activity of arginine- and tryptophan-rich hexapeptides: the effects of aromatic clusters, D-amino acid substitution and cyclization, Chem. Biol. Drug Des. 64 (2010) 159–169.
[4] A.A. Cluntun, M.J. Lukey, R.A. Cerione, J.W. Locasale, Glutamine metabolism in cancer: understanding the heterogeneity, Trends in Cancer 3 (2017) 169–180.
[5] G. Dranoff, G.B. Elion, H.S. Friedman, G.L. Campbell, D.D. Bigner, Influence of glutamine on the growth of human glioma and medulloblastoma in culture, J. Canc. Res. 45 (1985) 4077–4081.
[6] J.M. Matés, J.A. Segura, A. José, Campos-Sandoval, C. Lobo, L. Alonso, F.J. Alonso, J. Márquez, Glutamine homeostasis and mitochondrial dynamics, Int. J. Biochem. Cell Biol. 41 (2009) 2051–2061.
[7] J. Coloff, J. Murphy, C. Braun, I. Harris, L. Shelton, K. Kami, S.P. Gygi, L.M. Selfors, J.S. Brugge, Differential glutamate metabolism in proliferating and quiescent mammary epithelial cells, Cell Metabol. 23 (2016) 867–880.
[8] C. Bin, J.S. Panek, S. Amar, Convergent synthesis of novel muramyl dipeptide analogs: inhibition of porphyromonas gingivalis-induced pro-inflammatory effects by high doses of muramyl dipeptide, J. Med. Chem. 59 (2016) 6878–6890.
[9] Y.N. Belokon, N.B. Bespalova, T.D. Churkina, I. Císařová, M.G. Ezernitskaya, S.R. Harutyunyan, R. Hrdina, H.B. Kagan, P. Kočovský, K.A. Kochetkov, O.V. Larionov, K.A. Lyssenko, M. North, M. Polášek, A.S. Peregudov, V.V. Prisyazhnyuk, S. Vyskočil, Synthesis of α-amino acids via asymmetric phase transfer-catalyzed alkylation of achiral nickel(II) complexes of glycine-derived schiff bases, J. Am. Chem. Soc. 125 (2003) 12860–12871.
[10] S.H. Wu, F.Y. Chu, C.H. Chang, K.T. Wang, The synthesis of D-isoglutamine by A chemoenzymatic method, Tetrahedron Lett. 32 (1991) 3529–3530.
[11] J.M. Ding, Y. Zhou, H.J. Zhua, M. Deng, Y.X. Gao, Y.J. Yang, Z.X. Huang, Characterization of EstZY: a new acetylesterase with 7-aminocephalosporanic acid deacetylase activity from Alicyclobacillus tengchongensis, Int. J. Biol. Macromol. 148 (2020) 333–341.
[12] K. Shi, J. Jing, L. Song, T.T. Su, Z.Y. Wang, Enzymatic hydrolysis of polyester: degradation of poly(ε-caprolactone) by Candida Antarctica lipase and Fusarium solani cutinase, Int. J. Biol. Macromol. 144 (2020) 183–189.
[13] G. Harth, S. Maslesa-Galić, M.V. Tullius, M.A. Horwitz, All four Mycobacterium tuberculosis glnA genes encode glutamine synthetase activities but only GlnA1 is abundantly expressed and essential for bacterial homeostasis, Mol. Microbiol. 58 (2005) 1157–1172.
[14] D.S. Murray, N. Chinnam, N.K. Tonthat, T. Whitfill, M.A. Schumacher, Structures of the Bacillus subtilis glutamine synthetase dodecamer reveal large intersubunit catalytic conformational changes linked to a unique feedback inhibition mechanism, Biol. Chem. 288 (2013) 35801–35811.
[15] G. Richardson, H. Ding, T. Rocheleau, G. Mayhew, E. Reddy, Q. Han, B.M. Christensen, J. Li, An examination of aspartate decarboxylase and glutamate decarboxylase activity in mosquitoes, Mol. Biol. Rep. 37 (2010) 3199–3205.
[16] T. Heck, B. Geueke, Bacterial β-Aminopeptidases: structural insights and applications for biocatalysis, Chem. Biodivers. 9 (2012) 2388–2409.
[17] J.H. Billiman, W.F. Harting, Amino acids. V.1Phthalyl derivatives, J. Am. Chem. Soc. 70 (4) (1948) 1473–1474.
[18] J. Yu, J. Li, X. Gao, S. Zheng, H. Zhang, J. Liu, Q. Jiao, Dynamic kinetic resolution for asymmetric synthesis of Lnoncanonical amino acids from D-serine via tryptophan synthase and alanine racemase, Eur. J. Org Chem. 39 (2019) 6618–6625.
[19] K. Fujii, Y. Ikai, H. Oka, M. Suzuki, K. Harada, A nonempirical method using LC/MS for determination of the absolute configuration of constituent amino acids in a peptide: combination of Marfey's method with mass spectrometry and its practical application, Anal. Chem. 69 (1997) 5146–5151.
[20] J. Xu, T. Wang, Q. Yuan, Y. Yang, H. Zhu, Discovery and development of novel rhodanine derivatives targeting enoyl-acyl carrier protein reductase, Bioorg. Med. Chem. 27 (2019) 1509–1516.
[21] C. Bompard-Gilles, V. Villeret, G.J. Davies, L. Fanuel, B. Joris, A new variant of the Ntn hydrolase fold revealed by the crystal structure of L-aminopeptidase D-Ala-esterase/amidase from Ochrobactrum anthropic, Structure 8 (2000) 153–162.
[22] H. Zhang, Z.Y. Wu, Y.Z. Wang, D.D. Zhou, D.Q. Li, On-line immobilized trypsin microreactor for evaluating inhibitory activity of phenolic acids by capillary electrophoresis and molecular docking, Food Chem. 310 (2020) 125823.
[23] L. Fanuel, C. Goffin, A. Cheggour, The DmpA aminopeptidase from Ochrobactrum anthropi LMG7991 is the prototype of a new terminal nucleophile hydrolase family, Biochemical 341 (1999) 147–155.
[24] G. Zhao, J. Liu, K. Dong, F. Zhang, H. Zhang, Q. Liu, Q. Jiao, Enzymatic synthesis of L-tryptophan from hair acid hydrolysis industries wastewater with tryptophan synthase, Bioresour. Technol. 102 (2011) 3554–3557.
[25] W. Li, P. Zheng, J.Q. Zhang, X.Y. Shan, Z.Y. Wang, M. Zhang, The effect of substrate concentration fluctuation on the performance of high-rate denitrifying reactor, Bioresour. Technol. 167 (2014) 53–60.
[26] S.Y. Chen, J.G. Lin, Effect of substrate concentration on bioleaching of metal-contaminated sediment, J. Hazard Mater. 82 (2001) 77–89.
[27] C. Wu, J. Li, Q. Zeng, J. Yuan, J. Hu, C. N. Patent 107760735-A, 2018.
[28] Z.F. Zhang, Y.M. Sheng, K.Y. Jiang, Z. Wang, Q. Zhu, Bio-resolution of glycidyl (o, m, p)-methylphenyl ethers by Bacillus megaterium, Biotechnol. Lett. 32 (2010) 513–516.
[29] R.V. Ulijn, N. Bisek, S.L. Flitsch, Enzymatic optical resolution via acylation–hydrolysis on a solid support, Org. Biomol. Chem. 4 (2003) 609–761.
[30] B.K. Gogoi, R.L. Bezbaruah, S. Singh, Racemic resolution of some DL-amino acids using Aspergillus fumigatus L-amino acid oxidase, Curr. Microbiol. (2011) 63–94.
[31] Z.M. Wu, R.C. Zheng, Y.G. Zheng, Exploitation and characterization of three versatile amidase superfamily members from Delftia tsuruhatensis ZJB-05174, Enzym. Microb. Technol. 86 (2016) 93–102.
[32] F.M. Guo, J.P. Wu, L.R. Yang, G. Xu, Soluble and functional expression of a recombinant enantioselectiveamidase from Klebsiella oxytoca KCTC 1686 in Escherichia coli and itsbiochemical characterization, Process Biochem. 50 (2015) 1264–1271.
[33] T.K. Cheong, P.J. Oriel, Cloning of a wide-spectrum amidase from Bacillus stearothermophilus BR388 in Escherichia coli and marked enhancement of amidase expression using directed evolution, Enzym. Microb. Technol. 26 (2000) 152–158.
[34] K. Yamamoto, K. Otsubo, A. Matsuo, Production of R-(2) Ketoprofen from an amide compound by Comamonas acidovorans KPO-2771-4, Appl. Environ. Microbiol. 62 (1996) 152–155.
[35] H. Komeda, H. Harada, S. Washika, A novel Rstereoselective amidase from Pseudomonas sp . MCI3434 acting on piperazine-2-tert-butylcarboxamida, Eur. J. Biochem. 271 (2004) 1580–1590.
[36] R.C. Zheng, Y.S. Wang, Y.G. Zheng, Kinetic resolution of (R, S)-2,2-dimethylcyclopropanecarboxamide by Delftia tsuruhatensis ZJB-05174: role

of organic cosolvent in reaction medium, Catal. Commun. 18 (2012) 68–71.
[37] Z.M. Wu, R.C. Zheng, X. Ding, Enzymatic production of key intermediate of gabapentin by recombinant amidase from Pantoea sp. with high ratio of substrate to biocatalyst, Process Biochem. 51 (2016) 607–613.
[38] N.M. Shaw, A. Naughton, K. Robins, Selection, purification, characterisation, and cloningof a novel heat-stable stereo-specific amidase from Klebsiella oxytoca, and its application in the synthesis of enantiomerically pure (R) - and (S) -3, 3, 3-trifluoro-2-hydroxy-2-methylpropionic acids and (S) -3, 3, 3-trifluoro-2-hydroxy-2-methylpropionamide, Org. Process Res. Dev. 6 (2002) 497–504.
[39] W.L. Shen, H.H. Chen, K.Z. Jia, X. Yan, S.P. Li, Cloning and characterization of a novel amidase from Paracoccus sp. M-1, showing aryl acylamidase and acyl transferase activities, Appl. Microbiol. Biotechnol. 94 (2012) 1007–1018.