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Efficient biosynthesis of β-alanine with a tandem reaction strategy to eliminate amide by-product in the nitrilase-catalyzed hydrolysis

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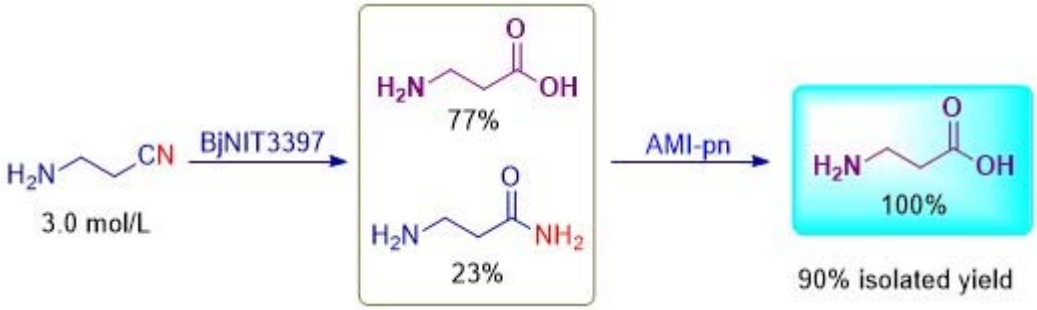
Graphical abstract



Research Highlights

- ✧ An amidase from *Pseudomonas nitroreducens* (AMI-pn) was characterized.
- ✧ It catalyzed the hydrolysis of 3-aminopropanamide (2.0 mol/L, 176 g/L) to β-alanine.
- ✧ An effective bienzymatic synthesis of β-alanine from 3-aminopropionitrile (3.0 mol/L) was achieved.
- ✧ Amidase effectively eliminated the by-product in the nitrilase-catalyzed hydrolysis of nitriles.

Abstract An efficient biosynthesis of β-alanine from 3-aminopropionitrile at high concentration has been developed using a one-pot bienzymatic cascade of a nitrilase and an amidase. The nitrilase BjNIT3397 from *Bradyrhizobium japonicum* strain USDA110 catalyzes the hydrolysis of 3-aminopropionitrile to β-alanine at the concentration up to 3.0 mol/L with the formation 23% of 3-aminopropanamide. In order to eliminate the by-product 3-aminopropanamide, we cloned and characterized a new amidase from *Pseudomonas nitroreducens* through gene mining. Under the optimal conditions (50 mmol/L Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0, 40°C), 2.0 mol/L (176 g/L) of 3-aminopropanamide was completely hydrolyzed within 12 h. A tandem reaction system was then established to eliminate the by-product 3-aminopropanamide and increase the production of β-alanine to 90% isolated yield

with 15.02 g/(L.h) space-time-yield. These results demonstrated that the tandem reaction strategy was an effective method of eliminating the amide by-products in the nitrilase-catalyzed hydrolysis at high substrate concentration.

Keywords: Amidase; Nitrilase; β-Alanine; Hydrolysis; Tandem reaction

1. Introduction

Nitrilases are becoming important and powerful biocatalysts with potential applications in the synthesis of high value-added carboxylic acids under mild conditions [1-8]. However, the undesirable amide by-products, which are generated from nitriles due to the nitrile hydratase activity contributed by nitrilase, have adverse effect on the yield and purity of the products [9-11]. For example, a recombinant nitrilase from *Arabidopsis thaliana* (AtNIT4) catalyzed the hydrolysis of 3-cyano-L-alanine and >60% amide was formed [12]. Similarly, a recombinant nitrilase originating from *Pseudomonas fluorescens* EBC191 is also prone to amide formation [13]. The nitrilase from *Gibberella intermedia* CA3-1 displays prominent catalytic efficiency towards 3- and 4-cyanopyridine to produce the corresponding nicotinic and isonicotinic acids, but with about 3% of amide in the products [14]. Therefore, development of practical methods to eliminate the amide by-products in nitrilase-catalyzed hydrolysis of nitriles is highly in demand.

Recently, Gong *et al* employed sequence analysis and saturation mutagenesis to obtain new mutant enzymes with the aim of reducing the by-product formation in the hydrolysis of 3-and 4-cyanopyridine catalyzed by a fungal nitrilase from *Gibberella*

intermedia. Their best mutant I128L-N161Q reduced the amount of amide to one third of that in the hydrolysis with wild-type enzyme [11]. As such, modification of nitrilases offers an approach to reduce the formation of amide by-product. However, only two protein crystal structures of nitrilases from the archaeon *Pyrococcus abyssi* [15] and *Synechocystis* sp. PCC6803 [16] have been reported up to now. The lack of the structural information hindered the rational modification of nitrilases to obtain the effective mutants without the formation of amide by-product.

On the other hand, the control of hydrolysis process offers an alternative strategy. Vejvoda *et al* reported that immobilization of fungal nitrilase and bacterial amidase resulted in the decrease of isonicotinamide in the products from 25% to 5% [17]. Chmura *et al* reported that the cascade reaction by a triple cross-linked enzyme aggregate of a (*S*)-selective oxynitrilase, a non-selective nitrilase and an amidase produced (*S*)-mandelic acid as the sole product at 10 mM substrate concentration [18]. In order to prevent the formation of 3-aminopropanamide, we constructed a tandem reaction strategy by using aspartate ammonia-lyase and fumaric acid to consume the by-product ammonia, resulting in the decrease of 3-aminopropanamide from 33% to 3% [19]. However, the mixture of L-aspartic acid, 3-aminopropanamide and β-alanine increases the downstream process cost because of additional purification steps.

In this study, we cloned and screened a series of amidases, and a new amidase from *Pseudomonas nitroreducens* was found to catalyze the hydrolysis of 3-aminopropanamide to β-alanine at high concentration. Furthermore, a tandem

reaction strategy was established to eliminate the 3-aminopropanamide by-product in the nitrilase-catalyzed hydrolysis by using a nitrilase and an amidase as the biocatalysts.

2 Materials and methods

2.1 Materials

3-Aminopropionitrile, 3-aminopropanamide hydrochloride, β-alanine and 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA) were purchased from TCI (Tokyo Chemical Industry Co. Ltd.), Bide Pharmatech Ltd, Sinopharm Chemical Reagent Co., Ltd. and Innochem (Beijing) Technology Co., Ltd., respectively. All chemical reagents were of analytical grade. The genes AMI-pa from *Pseudomonas aeruginosa* (GenBank: CEI05647.1), AMI-pp from *Pseudomonas putida* KT2440 (NCBI Reference Sequence: NP_745976.1), AMI-pc1 from *Pseudomonas chlororaphis* (NCBI Reference Sequence: WP_025806143.1) and AMI-pc2 from *Pseudomonas chlororaphis* (NCBI Reference Sequence: WP_023969146.1) were synthesized by Shanghai Xuguan Biotechnological Development Co., Ltd (Shanghai, China). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-Ⅲ 400 MHz NMR spectrometer. High-resolution MS was recorded on a Bruker microTOF-QII.

2.2 ***Gene cloning, functional expression and preparation of whole cells***

The full-length amidase gene (NCBI Reference Sequence: WP_024765375.1) was amplified by PCR from *Pseudomonas nitroreducens* using the following primer pair: pn-F (5'- GGGAATTCCATATGATGAAAGTAGAACTCGTCCAACT-3') and

pn-R (5'- CCGCTCGAGTCAGGCGGGAATGATCAGCTCGCGC-3'). *Nde*I and *Xho*I restriction sites (underlined) were used to clone the amidase gene into the same sites on pET32a (+), and the resulting plasmid was transformed into *E. coli* BL21 (DE3) strain. The constructed plasmid was confirmed by restriction analysis and DNA sequencing.

The *E. coli* BL21 (DE3) strain harboring the amidase gene was grown in LB medium containing 100 μg/mL ampicillin at 37°C and 200 rpm. When the optical density at 600 nm (OD600) reached 0.6 – 0.8, 0.1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce gene expression at 25°C for 12 h. The cells were harvested by centrifugation, washed once with deionized water and stored at -20°C for further use.

2.3 Purification of amidase and determination of the molecular mass

All purifications steps were performed at 4℃ using 50 mmol/L pH7.0 Na_2HPO_4/NaH_2PO_4 buffer (A). Cells were suspended in buffer A and disrupted by a high pressure slurry disruptor (APV 2000). Cell debris were removed by centrifugation at 6000 g, 4°C for 30 min. Poly(ethylene imine) (PEI, the final concentration of 5%) was added to the resulting cell free extract and stirred for 1 h. After centrifugation, the cell-free supernatant was subjected to precipitation with 60% ammonium sulfate overnight. The resulting precipitate was collected and dissolved in buffer A and dialyzed against the same buffer overnight. The dialysate was then applied to a DEAE-Sephadex column equilibrated with buffer A. The column was

washed with the same buffer, the absorbed protein was eluted by the buffer A supplementing with 0 – 1.0 mol/L NaCl, and the eluent was collected in 2.0 mL fractions. The resulting target protein solution was dialyzed in buffer A overnight. The purity of the protein was determined by SDS-PAGE analysis.

The enzyme sample was subjected to a Sephadex ™ 200 Increase 10/300GL column to determine the native molecular mass of the purified enzyme. The column was equilibrated with buffer A and eluted at a flow rate of 0.5 ml/min and the absorbance at 280 nm was monitored. The relative molecular mass of the enzyme was calculated by retention volume compared with those of the standard proteins, ovalbumin (44kDa), conalbumin (75kDa), aldolase (158kDa), ferritin (440kDa), and thyroglobulin (669kDa).

2.4 Amidase activity assay

Amidase activity was determined by measuring the release of ammonia using the phenol-hypochlorite ammonia assay method [20]. A standard reaction mixture (100 μL) contained Na_2HPO_4-NaH_2PO_4 (50 mmol/L, pH 7.0), 10 mmol/L substrate, and 2 μL of the purified enzyme or cell-free extract of *E. coli* BL21 (DE3) strain harboring the amidase gene. The reaction mixture was shaken at 40°C in an orbital shaker at 200 rpm for 10 min, then 350 μL of reagent A (0.59 mol/L phenol and 1 mmol/L sodium nitroprusside) was added. Color was developed by the addition of 100 μL reagent B (2.0 mol/L sodium hydroxide and 0.11 mol/L sodium hypochlorite) after 5 min of incubation at 60°C and the absorbance at 600 nm was measured. Control reactions

were performed without enzyme. Standard curve was prepared using NH_4Cl. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of NH_3 per min under standard assay conditions. All assays were performed in triplicate.

2.5 Effects of pH and temperature on the hydrolysis of 3-aminopropanamide

Similar to the procedure in Section 2.4, citrate buffer (50 mmol/L, pH 4.0 – 6.0) and sodium phosphate buffer (50 mmol/L, pH 6.0 – 8.0) were used to determine the optimum pH. The optimum temperature was established at pH 6.0 within the temperature range of 20 – 60°C. Stability of the enzyme at different pHs was studied by incubating the enzyme at pH 6.0 – 8.0 for an appropriate period of time (6 h) followed by measurement of the residual enzyme activity. The thermal stability of the enzyme was studied by incubation of the enzyme at 30 – 50°C for an appropriate period of time (12 h) followed by the measurement of the residual enzyme activity.

2.6 ***Substrate specificity***

All reactions were carried out at 40°C under standard conditions. Fifty μL of purified amidase (2.75 U) was added to 40 μL of sodium phosphate buffer (50 mmol/L, pH 6.0), and the mixture was pre-incubated for 5 min. Ten μL of amide (100 mmol/L in water) was added to the reaction mixture and incubated for additional 20 min. The reaction mixtures were treated and analyzed as described in Section 2.4. The activity of AMI-pn for 3-aminopropanamide was regarded as 100%.

2.7 Determination of kinetic constants

Apparent K_m value for 3-aminopropanamide was measured with various substrate concentrations (10 mmol/L – 500 mmol/L) in Na_2HPO_4-NaH_2PO_4 buffer (50 mmol/L, pH6.0). The reaction was carried out at 40°C for 3 min, quenched by equal volume of 10% sodium carbonate and derivatized with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA). The reaction rate was determined by HPLC analysis, which was performed on an Agilent 1200 series HPLC system with an Eclipse XDB-C18 column and a UV detector at wavelength of 340 nm as previously reported [19]. Initial reaction velocities measured at different substrate concentrations were analyzed by non-linear regression, which was performed using SigmaPlot program.

2.8 *Substrate and product inhibition*

The wet *E. coli* whole cells producing AMI-pn enzyme (50 mg, 41 U) were added to the phosphate buffer (1.0 mL, 50 mmol/L, pH 6.0) with appropriate amounts of 3-aminopropanamide, and the reaction mixture was shaken for 12 h at 200 rpm and 40°C on an orbital shaker. For substrate inhibition, the concentrations of 3-aminopropanamide were 0.5 – 3.0 mol/L. For product inhibition, the reaction was performed with 0.5 mol/L 3-aminopropanamide by adding 0.5 – 3.0 mol/L β-alanine. The effect of 3-aminopropionitrile was investigated at 0.5 mol/L 3-aminopropanamide by adding 0.5 – 3.0 mol/L 3-aminopropionitrile. The reactions in the presence of 3-aminopropionitrile and β-alanine were carried out for 30 min. Aliquots (50 μL) were taken and analyzed by HPLC analysis as described in Section 2.7.

2.9 *Biosynthesis of β-alanine through bienzymatic one-pot two-step cascade*

To a solution of 3-aminopropionitrile (4.20 g, 60 mmol) in water, which was adjusted to pH 7.0 with concentrated hydrochloric acid (12 mol/L), 1.6 g of whole cells of *E. coli* Rosetta2(DE3) strain expressing nitrilase BjNIT3397 (800 U) was added [19]. The initial volume of the reaction mixture was 20 mL. The suspension was stirred at 40°C for 8 hours. At the indicated time intervals, aliquots (50 μL) were taken and analyzed by HPLC analysis. After 3-aminopropionitrile was completely hydrolyzed to 3-aminopropanamide and β-alanine, the whole cells were removed by centrifugation at 6000 g, 4°C for 15 min. The supernatant was adjusted to pH 6.0 with concentrated hydrochloric acid (12 mol/L). The wet *E. coli* whole cells producing AMI-pn enzyme (1.6 g, 1312 U) was added to the mixture and the suspension was stirred at 40°C for additional 8 hours and monitored by HPLC analysis. Upon completion, the reaction was acidified to pH 1 with concentrated hydrochloric acid (12 mol/L). The supernatant was obtained by centrifugation at 6000 g, 4°C for 15 min, and run through a column with strong acid cation exchange resin. The product was eluted with an ammonia solution (about 7%, w/w) and detected by thin-layer chromatography (TLC), which was developed with a mixture of 1-butanol, water and glacial acetic acid (4/1/1, v/v), and visualized by 2, 2-dihdroxyindane-1, 3-dione. The desired product was obtained as pale yellow solid (4.81 g, 90% yield) by removal of the solvent. ^{1}H NMR (400 MHz, D_2O): δ 2.90 (t, *J*=6.8 Hz, 2 H), 2.34 (t, *J*=6.8 Hz, 2 H). ^{13}C NMR (100 MHz, D_2O): δ 179.88, 37.15, 36.69. High-resolution MS (ESI): m/z: calcd for $C_3H_6NO_2^-$: 88.0399 $[M-1]^-$, found: 88.0369.

3 Results and discussion

3.1 *Cloning and functional expression of AMI-pn*

NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) was carried out to identify a series of candidate amidase genes using an amidase from *Pseudomonas* sp. MCI3434 (AMI-ps, GenBank: BAE02667.1) as a template, because it has been reported to hydrolyze 3-aminopropanamide [21]. Five putative carbon-nitrogen hydrolases with the homology between 30% and 80% were selected, cloned into pET32a (+) vector and transformed into *E. coli* BL21 (DE3) strain for further detailed studies. The amidases AMI-ta from *Thermoplasma acidophilum* DSM 1728 (NCBI Reference Sequence: WP_010901615.1), AMI-gp from *Geobacillus pallidus* RAPc8 (GenBank: AAO23013.1) [22], AMI-rs from *Rhodococcus* sp. N-771 (GenBank: BAA36596.1) [23] and AMI-ms from *Microbacterium* sp. AJ115 (GenBank: CAG29798.1) [24] were available at our laboratory. After screening 10 amidases with 3-aminopropanamide at a substrate concentration of 500 mmol/L at pH 7.0 for 12h, we chose a carbon-nitrogen hydrolase (AMI-pn) from *Pseudomonas nitroreducens* (61.2% identity to AMI-ps) for further research (Table 1).

3.2 *Expression, Purification of amidase and determination of the molecular mass*

The AMI-pn gene was expressed in *E. coli* BL21 (DE3) strain as a soluble protein, and the SDS-PAGE analysis indicates that the subunit molecular weight of the enzyme is about 30 kDa (Figure 1). The recombinant enzyme was purified by PEI precipitation, ammonium sulfate fractionation and DEAE-Sephadex chromatography

(Table 2, Figure 2). Size exclusion chromatography reveals that the molecular weight of the native enzyme is 62 kDa, suggesting that the native enzyme is composed of two subunits (dimer). According to sequence alignment, we may conclude that AMI-pn belongs to nitrilase superfamily with the characteristic catalytic triad residues Glu-Lys-Cys(E-K-C) [25-29] (Figure 3). Some amidases belonging to the nitrilase superfamily also form dimers, such as the amidases from *Pyrococcus yayanosii* CH1 (Gene ID: 10837614) [30] and *Nesterenkonia* strain AN1 (GenBank: ACS35546) [31].

3.3 ***Effects of pH and temperature on AMI-pn activity and stability***

Effects of pH and temperature on the enzyme activity of AMI-pn were investigated at 10 mmol/L of substrate concentration. As shown in Figure 4, the optimal pH for AMI-pn was 6.0. Different buffering systems of pH6 gave quite different AMI-pn activities with deviation approaching 20%. We tested the assay method with ammonia standard solution in these two buffer systems, and the results showed no significant difference in the measured amount of ammonia. This suggests that the observed difference in AMI-pn activity should not be resulted from the effect of the buffers on the assay method, but these buffers do affect the AMI-pn activity, although it is not clear why these buffering systems affect the enzyme activity. The purified AMI-pn exhibited the maximum relative activities at 40°C (Figure 5).

The pH and thermal stability was investigated by measuring the remaining activity of the enzyme after incubation at different pH and temperatures, and the

initial activity without incubation was defined as 100%. As shown in Figure 6, the purified AMI-pn retained more than 85% of activity in the pH range of 6.0 − 8.0 for 6 h, suggesting that the enzyme is stable in this pH range. Although AMI-pn was not stable at 50°C, it retained 94% of activity after incubation at 40°C for 12 h (Figure 7). As such, AMI-pn shows relatively high thermal stability.

3.4 *Substrate spectrum and kinetic constants*

A number of amides were tested to investigate the substrate spectrum of AMI-pn (Table 3). AMI-pn showed activity toward the majority of aliphatic amides, such as butyrylamide, 3-hydroxypropionamide, 3-pyrrolidine formamide hydrochloride, piperidine-3-carboxamide and piperidine-4-carboxamide, but no or little activity towards aromatic amides and 2-amino amides.

The purified amidase showed apparent K_m for 3-aminopropanamide of 42.5±5.9 mmol/L and k_{cat} was 17.3±0.9 s^{-1}.

3.5 *Substrate and product inhibition*

The hydrolysis of 3-aminopropanamide of different initial concentrations was performed with 50 mg/mL of wet *E. coli* whole cells producing AMI-pn enzyme in phosphate buffer (50 mmol/L, pH 6.0) at 40°C. Up to 2.0 mol/L (176 g/L) of 3-aminopropanamide was completely hydrolyzed within 12 hours. When further increasing the initial concentration of the substrate to 2.5 mol/L and 3.0 mol/L, the conversions of the reaction were about 98% and 91%, respectively.

The results showed that low concentration of 3-aminopropionitrile could facilitate the hydrolysis of 3-aminopropanamide, while high concentration of 3-aminopropionitrile inhibited the activity of AMI-pn and the activity was 79% at 3.0 mol/L concentration relative to the control experiment (Figure 8). β-Alanine showed a little inhibitory effect on the activity of AMI-pn, and the activity at 3.0 mol/L of β-alanine was 75% relative to the control experiment (Figure 9).

3.6 *Biosynthesis of β-alanine through bienzymatic one-pot two-step cascade*

In the coupled reaction shown in Figure 10, the wet *E. coli* whole cells producing nitrilase BjNIT3397 (80 mg/ml) completely hydrolyzed 3-aminopropionitrile (3.0 mol/L) in 8 hours, producing 77% β-alanine and 23% 3-aminopropanamide. In the following 8 hours, the wet *E. coli* whole cells producing AMI-pn enzyme (80 mg/ml) completely hydrolyzed the by-product 3-aminopropanamide to β-alanine. After purification by strong acid cation exchange resin, the isolated yield of β-alanine was 90% and the space-time-yield was 15.02 g/(L.h). Thus, the one pot two-step cascade reaction strategy provided an efficient method to eliminate the by-product amide in the nitrilase-catalyzed hydrolysis.

4. Conclusion

A new amidase from *Pseudomonas nitroreducens* (AMI-pn) was found to catalyze the hydrolysis of 3-aminopropanamide (2.0 mol/L, 176 g/L) to β-alanine at pH 6.0 and 40°C within 12 h. Furthermore, the amidase was applied to eliminate the amide by-product of nitrilase, and an effective bienzymatic synthesis of β-alanine

from 3-aminopropionitrile at 3.0 mol/L of substrate concentration was achieved by using the wet *E. coli* whole cells producing AMI-pn and a nitrilase BjNIT3397 from *Bradyrhizobium japonicum* strain USDA110. This one-pot bienzymatic transformation provides a practical method to eliminate the by-product in the nitrilase-catalyzed hydrolysis of nitriles.

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Figure 1 The protein expression level of AMI-pn. Lane M: protein marker, Lane 1: the cell-free lysate, Lane 2: the supernatant of lysate, Lane 3: the precipitation of lysate.

Figure 2 The purification of AMI-pn. Lane M: protein marker, Lane 1: the column flow-through of the protein solution obtained from ammonium sulfate fractionation, Lane 2: 60% ammonium sulfate fractionation protein, Lane 3: impurity protein after 60% ammonium sulfate fractionation, Lane 4-5: purified AMI-pn after column.

Figure 3 Amino acid sequence alignment of amidases from different origins. The alignment was generated with DNAMAN. Identical amino acid residues are marked in black, similar residues are marked in pink. The blue arrows above the sequence indicate the residues of the characteristic Glu-Lys-Cys (E-K-C) catalytic triad residues.

Figure 4 Effects of pH on the activity of 3-aminopropanamide to β-alanine using purified enzyme. The buffers were citrate buffer (50 mmol/L, pH 4.0-6.0), sodium phosphate buffer (50 mmol/L, pH 6.0-8.0). The reaction activity in sodium phosphate buffer (50 mmol/L, pH 6.0) at 37 °C for 10 min was defined as 100%.

Figure 5 Effects of temperature on the activity of 3-aminopropanamide to β-alanine using purified enzyme in the temperature range of 20 - 60 °C. The reaction activity at 40 °C for 1 h in sodium phosphate buffer (50 mmol/L, pH 6.0) was defined as 100%.

Figure 6 The stability of purified enzyme at different pHs in the sodium phosphate buffer (50 mmol/L, pH 6.0-8.0) for 6h. The reaction activity without incubation in sodium phosphate buffer (50 mmol/L, pH 6.0-8.0) at 40 °C was defined as 100%.

Figure 7 The thermal stability of purified enzyme in the temperature range of 30 - 50 °C for 12 h. The reaction activity without incubation at 30 - 50 °C was defined as 100%.

Figure 8 Effects of 3-aminopropionitrile on the activity of AMI-pn. The transformation was carried out with 0.5 mol/L of 3-aminopropanamide and 50 mg/ml of *E. coli* whole cells producing AMI-pn enzyme in the presence of different concentrations of 3-aminopropionitrile. The activity without 3-aminopropionitrile was defined as 100%.

Figure 9 Effects of β-alanine on the activity of AMI-pn. The transformation was carried out with 0.5 mol/L of 3-aminopropanamide and 50 mg/ml of *E. coli* whole cells producing AMI-pn enzyme in the presence of different concentrations of β-alanine. The activity without β-alanine was defined as 100%.

Figure 10 Time course for the biotransformation of 3-aminopropionitrile to β-alanine by using the *E. coli* whole cells producing BjNIT3397 and AMI-pn. Reaction conditions: A: 3.0 mol/L 3-aminopropionitrile and 80 mg/ml *E. coli* whole cells producing BjNIT3397 at pH 7.0; B: then 80 mg/ml *E. coli* whole cells producing AMI-pn at pH 6.0.

Figure 1

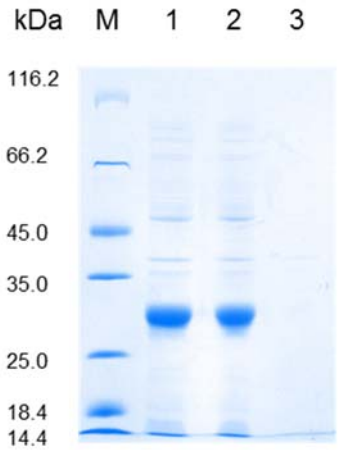


Figure 2

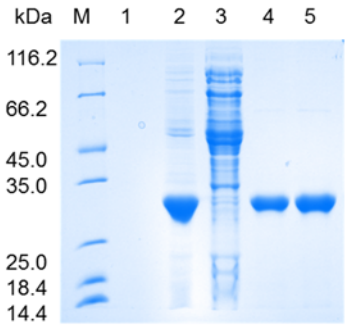
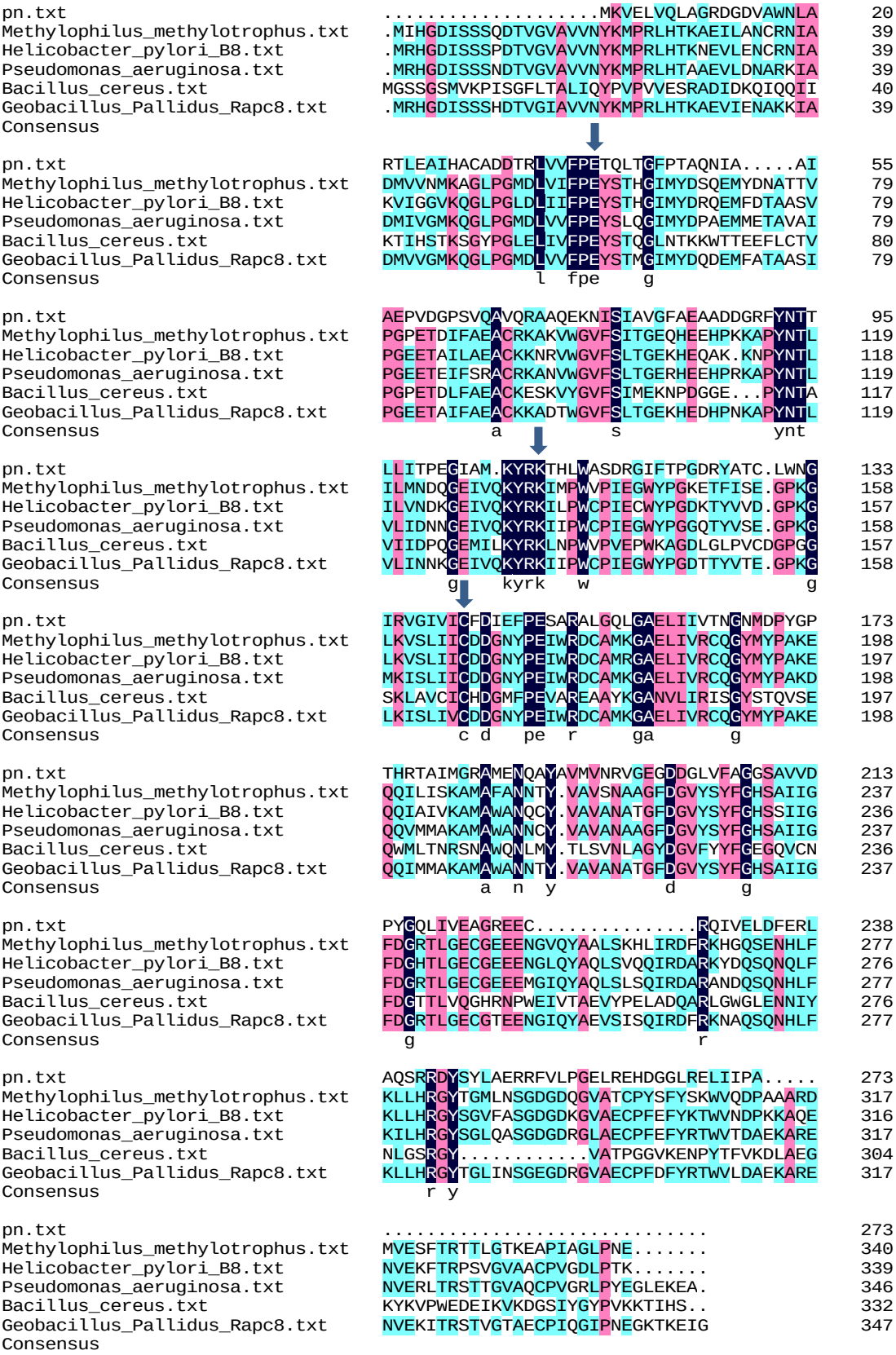


Figure 3



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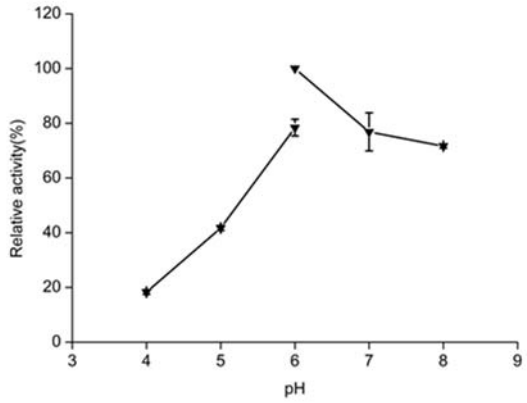

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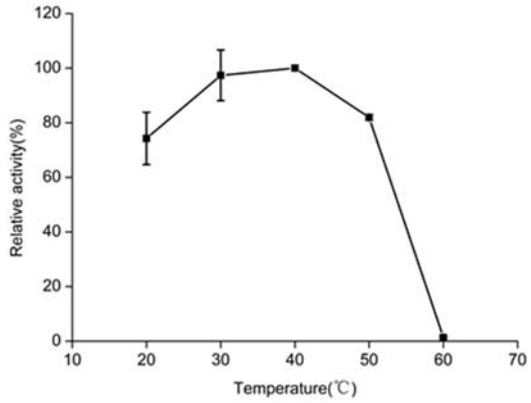

Figure 6

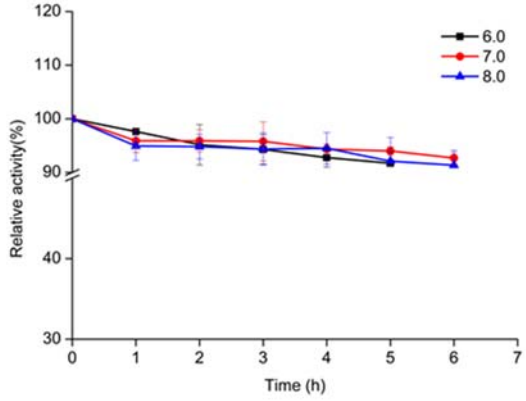

Figure 7

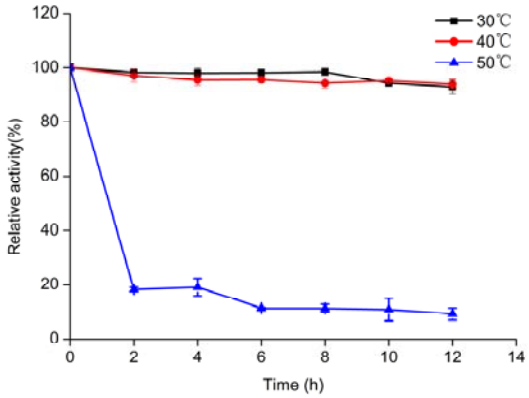

Figure 8

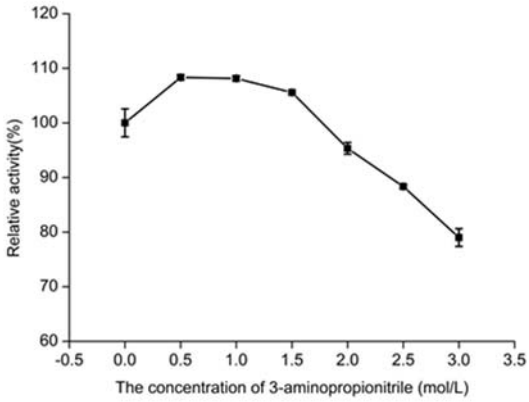

Figure 9

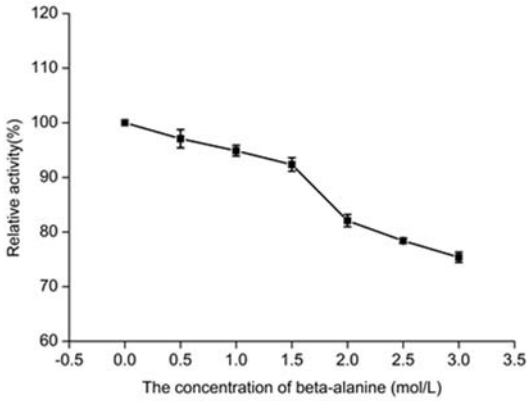

Figure 10

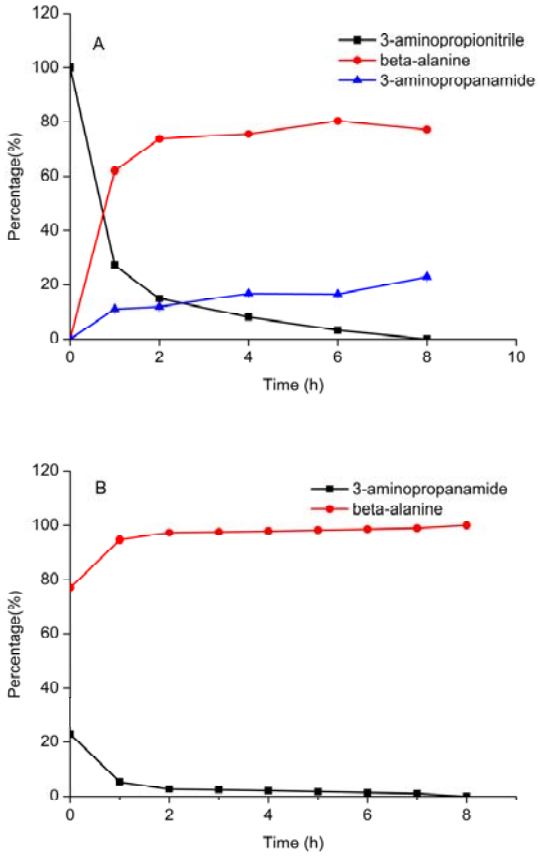

Table 1 Information of amidases from gene mining and the results of screening

Amidase	Source	GenBank	Identity (%)	Activity[b)]
AMI-ps	*Pseudomonas* sp. MCI3434	BAE02667.1	100	+
AMI-pn[a)]	*Pseudomonas nitroreducens*	WP_024765375.1	61.2	+++
AMI-pa[a)]	*Pseudomonas aeruginosa*	CEI05647.1	63.5	-
AMI-pp[a)]	*Pseudomonas putida* KT2440	NP_745976.1	71.5	++
AMI-pc1[a)]	*Pseudomonas chlororaphis*	WP_025806143.1	75.2	++
AMI-pc2[a)]	*Pseudomonas chlororaphis*	WP_023969146.1	34.8	+
AMI-ta	*Thermoplasma acidophilum* DSM 1728	WP_010901615.1	27.7	+
AMI-gp	*Geobacillus pallidus* RAPc8	AAO23013.1	17.2	+
AMI-rs	*Rhodococcus* sp. N-771	BAA36596.1	8.6	+
AMI-ms	*Microbacterium* sp. AJ115	CAG29798.1	9.8	+

a) Previously unreported amidases. b) The activity of amidase towards 3-aminopropanamide (500 mmol/L) at pH 7.0 for 12 h. "-" no activity, "+" a little activity, "++" higher activity but 3-aminopropanamide was not converted completely; "+++" 3-aminopropanamide was converted completely.

Table 2 Summary of the purification of AMI-pn

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude AMI-pn	457.9	3451.2	7.5	1	100
Purified AMI-pn	95.8	2301.1	24.0	3.2	66.7%

Table 3 Substrate spectrum of purified enzyme AMI-pn

substrate	Relative activity (%)	substrate	Relative activity (%)
3-aminopropanamide hydrochloride	100.0±5.0	D-valinamide hydrochloride	4.8±1.6
butyramide	513.0±6.5	(*S*)-2-amino butyramide hydrochloride	1.9±0.3
3-hydroxypropionamide	97.0±1.2	(*R*)-2-amino butyramide hydrochloride	7.1±0.5
piperidine-2-carboxamid	5.3±0.9	(*R*)-2-amino-4-methyl amyl amide	4.8±0.7
piperidine-3-carboxamide	42.5±2.4	(2*R*)-2-amino-3-phenyl acrylic amide	6.1±0.4
piperidine-4-carboxamide	57.9±6.4	L-leucine amide hydrochloride	8.3±1.9
pyrrolidine-3-carboxamide hydrochloride	48.0±4.7	acrylamide	4.8±0.7
L-phenylalaninamide	11.9±1.1	D-(-)-phenylglycinamid	1.9±0.4
L-phenylglycinamide	10.4±2.3	glycinamide hydrochloride	0.5±0.2
urea	9.8±2.5	L-alaninamide hydrochloride	0
D-prolinamide	6.8±1.4	nicotinamide	0
L-prolinamide	7.8±2.4	benzamide	0
L-valinamide hydrochloride	5.5±1.0		