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# Nitrilase-catalyzed hydrolysis of 3-aminopropionitrile at high concentration with a tandem reaction strategy for shifting the reaction to β-alanine formation

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ABSTRACT

Given the importance of β-alanine, the nitrilase BjNIT3397 from *Bradyrhizobium japonicum* strain USDA110 was examined toward the hydrolysis of 3-aminopropionitrile. It has been found that nitrilase BjNIT3397 effectively hydrolyzed 3-aminopropionitrile with substrate concentration up to 3 M (210 g/L) at the pH 7.3 and temperature 30 °C. With the increase of substrate concentration from 0.6 to 3 M, 3-aminopropanamide was formed and its percentage in the products was increased up to 33%. In order to reduce the formation of 3-aminopropanamide, aspartate ammonia-lyase and fumaric acid were added into the reaction system to consume the byproduct ammonia. As expected, the reaction was shifted toward the formation of β-alanine, resulting in the decrease of 3-aminopropanamide from 33% to 3%. Therefore, a tandem reaction strategy was developed to effectively prevent the formation of 3-aminopropanamide. This might also offer a possibility of producing β-alanine and L-aspartic acid in one process.

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## 1. Introduction

β-Alanine is a naturally occurring non-essential amino acid which is widely used in pharmaceuticals, food & feed additives, polymeric materials and electroplating industry[1–6]. For example, β-alanine serves as a principal component of several commercially available supplements which are used to increase athletic performance by delaying accumulation of lactate during exercise [7–9]. It has been used as a building block for the synthesis of pharmaceutical intermediates including balsalazide and pamidronate disodium. β-Alanine was found to suppress malignant breast epithelial cell aggressiveness through alterations in metabolism and cellular acidity [9–11], showing potential as a co-therapeutic agent in the treatment of breast tumors [12,13]. β-Alanine is also involved in the biosynthesis of pantothenic acid.

There are a number of chemical and biocatalytic methods for the preparation of β-alanine [14–16]. The hydrolysis of nitriles should offer an effective approach for the synthesis of carboxylic acid because nitriles are synthetically more accessible than the corresponding carboxylic acid. However, the chemical hydrolysis of nitrile typically requires strongly basic or acidic conditions and high temperature, and usually produces unwanted byproducts. On the other hand, biocatalytic hydrolysis of nitriles to carboxylic acids offers a greener alternative, allowing clean and mild synthesis with high yield and selectivity [16–23].

It has been reported that β-alanine was prepared from β-aminopropionitrile by microorganisms, *Alcaligenes* sp. OMT-MY14, *Aminobacter aminobrance* ATCC 23314 [24] and *Rhodococcus* sp. G20 [25]. However, the low substrate concentration (1.29%, v/v) limits their industrial application. In order to search for the nitrilases with high activity for the hydrolysis of β-aminopropionitrile at high substrate concentration, the nitrilases available in our laboratory were screened. The screening results suggested that a nitrilase from *Bradyrhizobium japonicum* USDA110 (BjNIT3397) [26] showed high promise for the hydrolysis of 3-aminopropionitrile to β-alanine. Therefore, the reaction conditions for the hydrolysis of 3-aminopropionitrile by BjNIT3397 have been optimized to explore its application potential for the production of β-alanine.

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**Table 1**
Information of nitrilases from our laboratory and the results of screening.

| Nitrilase | GenBank accession number and origin[b] | Host/vector | Activity[c] |
|---|---|---|---|
| AtNIT1 | NP_851011; *Arabidopsis thaliana* | *E. coli* BL21(DE3)plysS/pST7 | — |
| AtNIT2 | NP_190016; *Arabidopsis thaliana* | *E. coli* BL21(DE3)plysS/pST7 | — |
| AtNIT3 | NP_190018; *Arabidopsis thaliana* | *E. coli* BL21(DE3)plysS/pST7 | — |
| AfNIT | AEP34036; *Alcaligenes faecalis* ZJUTB10 | *E. coli* BL21(DE3)plysS/pST7 | ++ |
| NIT190 | AAR97489 (Ala190His); Environmental sample | *E. coli* BL21(DE3)/pET32a(+) | — |
| SsNIT | BAA10717; *Synechocystis* sp. PCC 6803 | *E. coli* Rosetta2(DE3)/pET15b | — |
| BjNIT3397 | NP_770037; *Bradyrhizobium japonicum* USDA 110 | *E. coli* Rosetta2(DE3)/pTXB1 | +++ |
| BjNIT6402 | NP_773042; *Bradyrhizobium japonicum* USDA 110 | *E. coli* BL21(DE3)/pTXB1 | — |
| ZmNIT2 | NP_001105196; *Zea mays* | *E. coli* BL21(DE3)/pET3a | + |
| RxNIT[a] | YP_643025; *Rubrobacter xylanophilus* DSM 9941 | *E. coli* BL21(DE3)/pET32a(+) | — |
| RpNIT[a] | YP_001990754; *Rhodopseudomonas palustris* TIE-1 | *E. coli* BL21(DE3)/pET32a(+) | — |
| HsNIT[a] | ZP_11253146.1; *Herbaspirillum* sp. GW103 | *E. coli* BL21(DE3)/pET32a(+) | — |
| AsNIT[a] | ZP_10388817.1; *Acidovorax* sp. CF316 | *E. coli* BL21(DE3)/pET32a(+) | — |
| AlNIT[a] | XP_002875656; *Arabidopsis lyrata* subsp. *lyrata* | *E. coli* BL21(DE3)/pET32a(+) | — |

"—" having no activity; "+" having activity, but 3-aminopropionitrile was not converted completely, "++" converted more; "+++" 3-aminopropionitrile was converted completely.

[a] Previously unreported nitrilases.

[b] The genes were synthesized and cloned into pET32a (+) by Shanghai Xuguan Biotechnological Development Co., Ltd. (Shanghai, China), or the plasmids were available in our laboratory.

[c] The activity of nitrilase towards 3-aminopropionitrile (5 mM) at pH 7.2 for 12 h.

## 2. Materials and methods

### 2.1. Materials

The microbial strains used for expression of nitrilases were maintained in our laboratory. 3-Aminopropionitrile was purchased from TCI (Tokyo Chemical Industry Co. Ltd.) and 3-aminopropanamide was synthesized via a similar method of literature [27]. The other chemicals were obtained from commercial sources.

### 2.2. Selection of the nitrilase

The nitrilase genes were expressed in appropriate *Escherichia coli* cells and the recombinant strains were cultured in the LB medium at the optimized induction conditions (Table 1). The cells were harvested by centrifugation. The biotransformation was performed with the whole cells. Wet cells (100 mg) and 3-aminopropionitrile (5 or 50 mM) were mixed in 1 mL of potassium phosphate buffer (100 mM, pH 7.3). The resulting mixture was incubated at 30 °C and 200 rpm for 24 h. The reaction was monitored by TLC analysis at 4, 8 and 24 h intervals.

### 2.3. Preparation of whole cell biocatalysts

*E. coli* Rosetta2(DE3) strain expressing nitrilase BjNIT3397 gene was cultured in Luria–Bertani (LB) medium (containing 100 μg $mL^{-1}$ ampicillin) at 37 °C, and induced by adding of 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 12 h at 25 °C when the optical density at 600 nm (OD600) was 0.6–0.8. The cells were harvested by centrifugation, washed once with stroke-physiological saline solution (0.9%, w/v) and stored for use.

*E. coli* K-12 MG1655 harboring L-aspartate ammonia-lyase gene was cultured in medium (pH 7.3 adjusted by $NH_3{\cdot}H_2O$) containing 1.5% corn powder, 0.145% NaCl, 0.1% $MgSO_4{\cdot}7H_2O$ and 0.5% fumaric acid at 37 °C for 12 h. The cells were harvested by centrifugation, washed once with stroke-physiological saline solution (0.9%, w/v) and stored for use.

### 2.4. Analytical procedure

Aliquots (200 μl) were taken at different time intervals, and the reaction was quenched by equal volume of 10% sodium carbonate. The thin-layer chromatography (TLC) was performed by developing in solvent mixture of butanol, water and glacial acetic acid (4/1/1, v/v), and visualized by 2,2-dihdroxyindane-1,3-dione. The conversion was determined by HPLC analysis performed on an Agilent 1200 series system with an Eclipse XDB-C18 column and a UV detector at wavelength of 340 nm. The substrate and product were derivatized with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA, 0.3%) by mixing FDAA with sodium bicarbonate (1 M) and suitably diluted reaction mixture (10/5/2, v/v) at 40°C for 60 min. Equal volume of HCl solution (1 M) as sodium bicarbonate was added. The mobile phase was a mixture of 0.05 M triethylamine phosphate (pH 3.0) and acetonitrile in the volume ratio of 62.5:32.5. The flow rate of the mobile phase was 0.8 mL/min. For the sample with aspartic acid in the reaction mixture, the volume ratio of triethylamine phosphate (pH 3.0) to acetonitrile was 80:20, and the flow rate was 1.0 mL/min.

### 2.5. Effects of pH and temperature on the hydrolysis of 3-aminopropionitrile

The effect of pH on the hydrolysis of 3-aminopropionitrile by BjNIT3397 were performed in the pH range from 5.0 to 10.0. Because of the strongly alkaline of substrate, it was difficult to adjust the pH at high substrate concentration by using buffers such as sodium citrate buffer, potassium phosphate buffer and their like. Concentrated hydrochloric acid (12 M) was used to adjust the pH to the range from 5.0 to 10.0 and the final substrate concentration was 600 mM. The wet cells (100 mg) were added into the substrate solution (1 mL) and the biotransformation was implemented at 25 °C, 200 rpm for 2 h. The reaction was terminated by addition of 10% sodium carbonate. The conversion of 3-aminopropionitrile was measured by HPLC analysis as described in Section 2.4.

Similarly, the effect of temperature was carried out at different temperatures ranging from 20 to 60 °C and the optimal pH obtained above for 2 h. The reaction mixture was treated and analyzed by HPLC as described above.

### 2.6. Effect of substrate concentration on the hydrolysis of 3-aminopropionitrile

Similar to the procedure in Section 2.5, the hydrolysis of 3-aminopropionitrile was carried out at substrate concentration ranging from 0.6 to 3.5 M and the optimal pH and temperature for 12 h. The reaction mixture was treated and analyzed by TLC and HPLC as described above.

### 2.7. Reducing the formation of 3-aminopropanamide by removal of ammonia

The reaction was carried out at various substrate concentration ranging from 0.6 to 3.0 M which was adjusted to pH 7.3 by fumaric acid. The wet cells of BjNIT3397 (100 mg) and aspartate ammonia-lyase (150 mg) were added into the substrate solution (1 mL). The bioconversion was implemented at 25 °C, 200 rpm for 12 h. The reaction was terminated by addition of 10% sodium carbonate. The conversion of 3-aminopropionitrile was measured by HPLC analysis as described in Section 2.4.

### 2.8. Time course for the biotransformation and product characterization

The biotransformation of 3-aminopropionitrile was performed by following the same procedure in Section 2.4. The reaction volume was 20 mL with final concentration of substrate being 3 M and 2.0 g (1000 U) of wet cells. The reaction was implemented at 30 °C, pH 7.3 and 200 rpm for 8 h. At every half hour interval in the first 4 h, and then every hour interval, a sample was withdrawn from the reaction mixture, and analyzed by HPLC analysis as described above. A quarter of the reaction mixture was derivatized by following a literature method [28]. Di-*tert*-butyldicarbonate (9.0 g, 41 mmol) and NaOH (5.0 g, 125 mmol) was added to the stirred reaction solution (50 mL). The mixture was stirred overnight at room temperature. The *N*-Boc 3-aminopropanamide **(3a)** was extracted by dichloromethane (4 × 100 mL). The combined organic extracts was dried over anhydrous sodium sulfate, the solvent was evaporated *in vacuo* and the residue was purified by silica gel column chromatography to afford *N*-Boc 3-aminopropanamide **(2a,** 0.42 g, 15%**)** as a white solid. The water layer was acidified by saturated citric acid solution to pH 3.0 and extracted with ethyl acetate (4 × 100 mL). The combined organic extract was dried over anhydrous sodium sulfate, the solvent was evaporated *in vacuo* and the residue was purified by silica gel column chromatography to afford *N*-Boc β-alanine (**1a**, 1.89 g, 67%) as a white solid. *N*-Boc β-alanine **(1a)** $^1$H NMR (400 MHz, $CD_3OD$): $\delta$ 3.31 (t, $J$ = 6.8 Hz, 2 H, –$CH_2$NH–), 2.49 (t, $J$ = 6.8 Hz, 2 H, –$CH_2CO_2H$), 1.45 ppm (s, 9H, –Boc). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta$ = 174.07, 156.89, 78.76, 35.98, 33.89, 27.38. *N*-Boc 3-aminopropanamide **(2a)** $^1$H NMR (400 MHz, $CD_3OD$): $\delta$ 3.28–3.36 (m, 2H, –$CH_2$NH–), 2.40 (t, $J$ = 6.8 Hz, 2H, –$CH_2CONH_2$), 1.45 ppm (s, 9 H, -Boc). $^{13}$C NMR (100 MHz, $CD_3OD$): $\delta$ 175.33, 156.92, 78.73, 36.49, 35.23, 27.35.

The biotransformation of 3-aminopropionitrile by nitrilase and aspartate ammonia-lyase followed the same procedure as described above. The pH of a solution of 3-aminopropionitrile (4.2 g, final concentration of 3 M) was adjusted to 7.3 by adding fumaric acid (4.0 g, final concentration 1.8 M) and the final volume was 20 mL. The wet cells of BjNIT3397 (2.0 g, 1000 U) and aspartate ammonia-lyase (3.0 g, 3297 U) were added into the reaction mixture and the reaction was implemented at 30 °C, 200 rpm for 8 h. The samples were withdrawn from the reaction mixture at different time intervals, and analyzed by HPLC analysis as described above. A quarter of the reaction mixture was purified by cation exchange resin and a mixture of ammonium salts of β-alanine and L-aspartic acid (**1** + **3,** 1.91 g). $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.69 (dd, $J$ = 8.7, 3.6 Hz, $^1$H, L-aspartic acid, –C*H*–), 3.01 (t, $J$ = 6.7 Hz, 12 H, β-alanine, –$CH_2NH_2$), 2.60 - 2.68 (m, 1H, L-aspartic acid, –$CH_2$–), 2.47 (dd, $J$ = 17.1, 8.7 Hz, 1H, L-aspartic acid, –$CH_2$–), 2.40 ppm (t, $J$ = 6.7 Hz, 12H, β-alanine, –$CH_2CO_2H$). $^{13}$C NMR (100 MHz, $D_2O$): $\delta$ 178.54 (β-alanine, –$CO_2H$), 177.98 (L-aspartic acid, –$CH_2CO_2H$), 175.65 (L-aspartic acid, –$CHCO_2H$), 52.44 (L-aspartic acid, –C*H*–), 37.70 (L-aspartic acid, –$CH_2$–), 36.57 (β-alanine, –$CH_2NH_2$), 33.94 (β-alanine, –$CH_2CO_2H$). The product mixture (**1** + **3)** was derivatized with 1-fluoro-2-4-dinitrophenyl-5-l-alanine amide and analyzed by LC–MS. The mobile phase was a mixture of water (0.5% formic acid) and methanol in the volume ratio of 64:36 and the flow rate was 1.0 mL/min (see Supplementary Information). The high-resolution MS (ESI) of FDAA derivatized L-Aspartic acid (**3b**): *m*/*z*: calcd for $C_{13}H_{16}N_5O_9^+$: 386.0948 $[M+H]^+$, found: 386.0942. The high-resolution MS (ESI) of FDAA derivatized β-alanine (**1b**): *m*/*z*: calcd for $C_{12}H_{16}N_5O_7^+$: 342.1050 $[M+H]^+$, found: 342.1049.

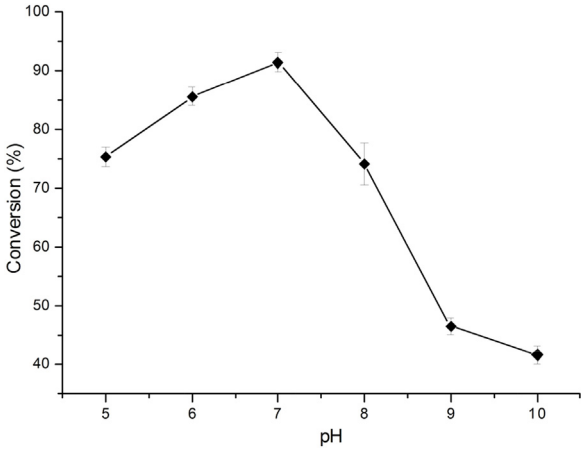


**Fig. 1.** pH dependence of the enzyme activity of nitrilase BjNIT3397 towards 3-aminopropionitrile (600 mM substrate, 25 °C for 2 h).

## 3. Results and discussion

### 3.1. Nitrilases selection

The biotransformation of 3-aminopropionitrile by the nitrilases available in our laboratory were carried out at the substrate concentration of 5 mM and TLC analysis showed that a few nitrilases catalyzed the hydrolysis of 3-aminopropionitrile to β-alanine (Table 1). However, when the substrate concentration increased to 50 mM, only BjNIT3397 remained the activity. As such, BjNIT3397 was selected for the further studies.

### 3.2. Effect of reaction pH and temperature

The nitrilase activity of the whole cell biocatalyst toward 3-aminopropionitrile at different pH were studied by measuring the conversion of the substrate. Concentrated hydrochloric acid (12 M) was used to adjust the pH of the aqueous substrate solution to the desired value. It had been found that the enzyme was active in this pH range and the optimal pH was around 7.0 (Fig. 1). This is slightly different from the pH profile of the purified BjNIT3397 toward phenylacetonitrile, which possesses a relatively broad working pH range with optimum from pH 7.0 to 8.0 [26].

The nitrilase activity of the whole cell biocatalyst toward 3-aminopropionitrile was measured at different temperatures ranging from 20 to 70 °C. The optimal reaction temperature was about 40 °C (Fig. 2), slightly lower than that (45 °C) of the purified enzyme toward phenylacetonitrile [26].

### 3.3. The formation of 3-aminopropanamide

The nitrilase BjNIT3397 catalyzed the hydrolysis of 3-aminopropionitrile at the substrate concentration up to 3.0 M under the optimal reaction conditions. However, as the substrate concentration increased, a significant amount of 3-aminopropanamide (about one third of the products at 3 M substrate concentration) was observed in the product mixture, demolishing the production of β-alanine (Fig. 3). The by-product

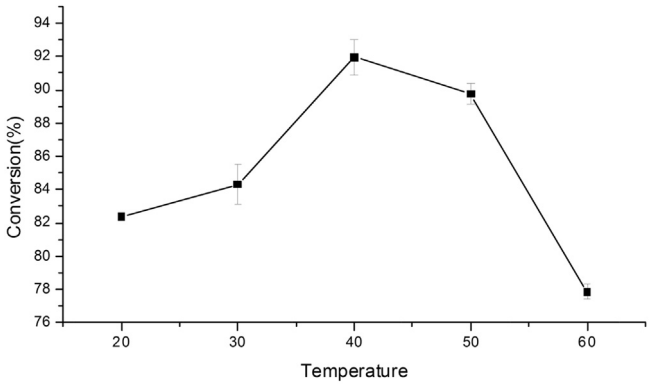


**Fig. 2.** Temperature dependence of the enzyme activity of nitrilase BjNIT3397 towards 3-aminopropionitrile (600 mM substrate, pH 7.3 for 2 h).

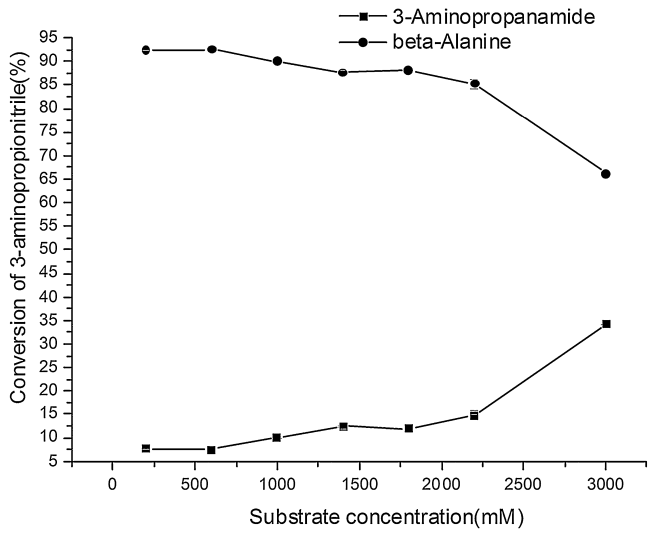


**Fig. 3.** The percentage of 3-aminopropanamide with the increase of substrate concentration (pH 7.3, 30 °C for 12 h).

3-aminopropanamide was isolated and characterized as *N*-Boc 3-aminopropanamide.

Some nitrilases are known to hydrolyze nitrile to the corresponding amide. Amide compounds might be formed because of the belated delivery of the second $H_2O$ [29,30]. The addition of the first water to the nitrile group leads to a thiomidate intermediate, which then releases ammonia to form the acylenzyme complex (Fig. 4). The thiomidate intermediate may also break down to produce the corresponding amide product, causing some nitrilases having the same character as nitrile hydratase [31]. The amide by-products were also detected in the hydrolysis of some other nitriles catalyzed by nitrilase BjNIT3397 [26]. The hydrolysis of

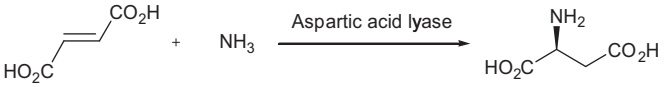


**Fig. 5.** L-Aspartate ammonia-lyase catalyzed formation of aspartic acid.

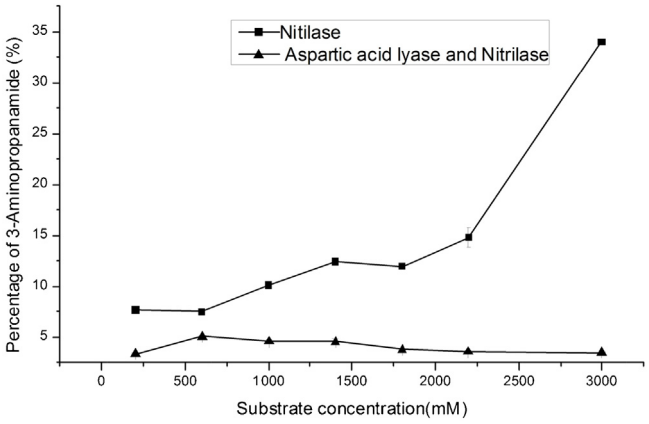


**Fig. 6.** Effect of ammonia removal on the formation of 3-aminopropanamide at different substrate concentrations (pH 7.3, 30 °C for 12 h).

3-aminopropionitrile by purified nitrilase BjNIT3397 and whole cells harboring this enzyme were compared. The purified enzyme was prepared according to the previously reported procedure [26]. The results showed that nearly the same percentages of 3-aminopropanamide were formed in both cases, implying that formation of the amide may not involve the endogenous nitrile hydratase of the host *E. coli* cells. It has been reported that lower reaction temperature and higher pH prompted the reaction toward amide formation [32]. For the biotransformation of 3-aminopropionitrile by BjNIT3397 at different temperatures and pHs, the percentage of 3-aminopropanamide in the products were almost the same. When the concentration of 3-aminopropionitrile was 1 M, the percentages of 3-aminopropanamide were 9.7% for 20 °C and 10.1% for 50 °C. And those were 7.4% and 7.6% for pH 5.0 and pH 9.0, respectively, at the substrate concentration of 600 mM.

### 3.4. Reducing the formation of 3-aminopropanamide

As shown in Fig. 4, removal of $NH_3$ from the reaction system may facilitate the cleavage of C–N bond, thus shifting the reaction toward the formation of β-alanine. Therefore, the whole cells of *E. coli* K-12 MG1655 harboring aspartate ammonia-lyase gene and fumaric acid were added into the reaction system to consume $NH_3$. Aspartate ammonia-lyase catalyzed the amino addition to the C=C bond of fumaric acid, forming L-aspartic acid (Fig. 5). The results are presented in Fig. 6. It can be seen that the percentage of 3-aminopropanamide in the product decreased from 33% to 3%.

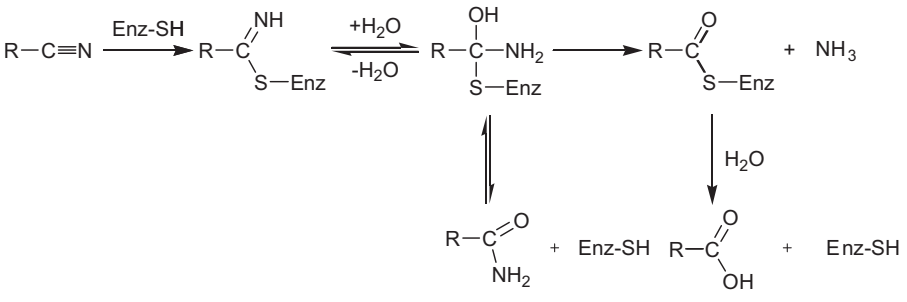


**Fig. 4.** Reaction mechanism for nitrilase.

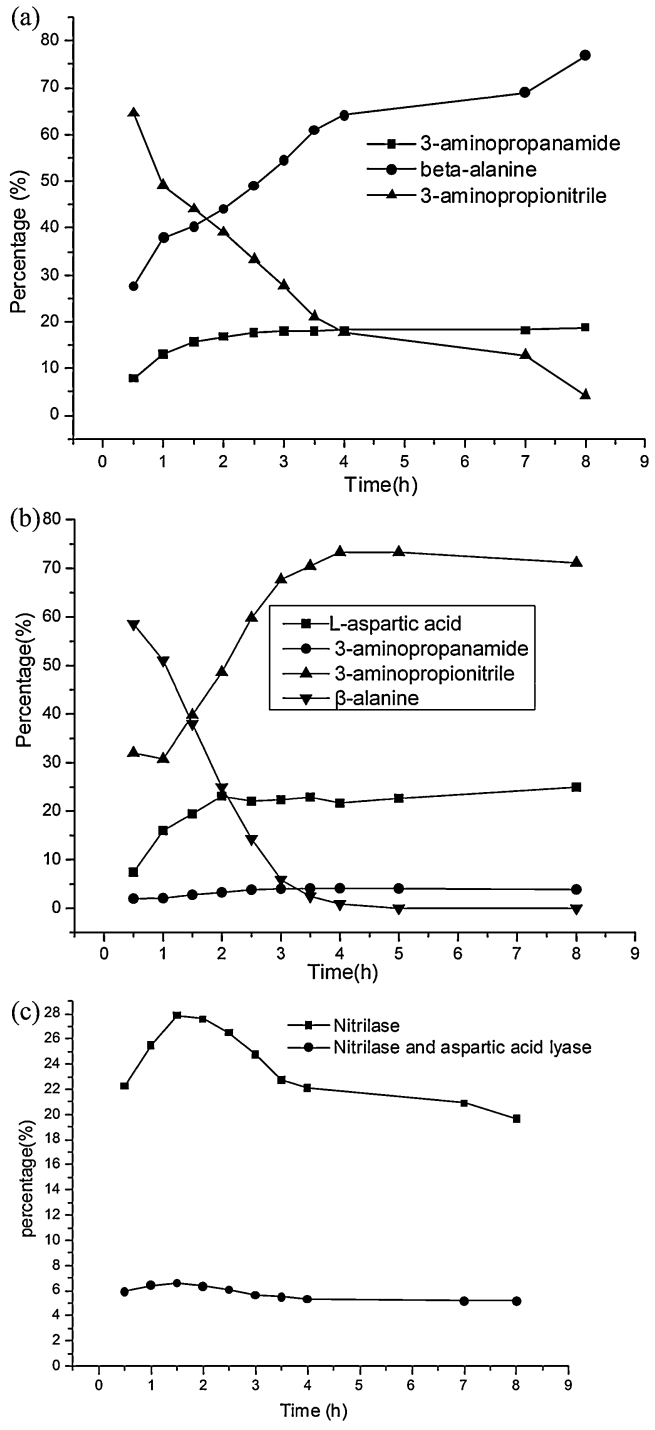


**Fig. 7.** Time course for the biotransformation of 3-aminopropionitrile by BjNIT3397 with (b) and without (a) addition of L-aspartate ammonia-lyase/fumaric acid (initial pH 7.3, 30 °C). (c) The percentage of 3-aminopropanamide in the products of biotransformations with and without addition of L-aspartate ammonia-lyase/fumaric acid at different time intervals.

### 3.5. Time course for the biotransformation

The hydrolytic reactions of 3-aminopropionitrile at substrate concentration of 3 M by BjNIT3397 with and without addition of aspartate ammonia-lyase and fumaric acid were followed, respectively, by quantifying the contents of 3-aminopropionitrile, 3-aminopropanamide, β-alanine and L-aspartic acid. The results indicated that the formation of 3-aminopropanamide occurred during the first 2 h (Fig. 7(a)), resulting in the increase/decrease pattern for the percentage of 3-aminopropanamide in the products (Fig. 7(c)). The formation of 3-aminopropanamide was inhibited at the beginning of the reaction by adding aspartate ammonia-lyase and fumaric acid (Fig. 7(b)), suggesting that the amino addition to the C=C bond of fumaric acid effectively removed $NH_3$ and shifted the reaction toward the acid formation. Formation of L-aspartic acid in the reaction was confirmed by the isolation of a mixture of the ammonium salts of β-alanine and L-aspartic acid, which were identified by NMR and LC–MS. The tandem reaction strategy of removing the byproduct ammonia effectively prevented the formation of 3-aminopropanamide.

## 4. Conclusion

Recombinant *E. coli* cells harboring the nitrilase BjNIT3397 from *B. japonicum* strain USDA110, which was classified as an aliphatic nitrilase in terms of its activity, catalyzed the hydrolysis of 3-aminopropionitrile at high substrate concentration up to 3 M, and optimal pH 7.3 and temperature 30 °C. However, with the increase of substrate concentration, 3-aminopropanamide was formed, reaching 33% at the substrate concentration of 3 M. A tandem reaction strategy was developed by introducing the aspartate ammonia-lyase-catalyzed amination of fumarate, which utilized the by-product ammonia as the amino donor in the formation of aspartic acid. As such, formation of 3-aminopropanamide was significantly inhibited with its amount being reduced from 33% to 3%. The tandem reaction strategy of removing the byproduct ammonia might offer a possibility of producing β-alanine and L-aspartic acid in one process if the problem in the separation of these two products was solved.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.02.007.

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