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ORIGINAL PAPER

Bench-scale biosynthesis of isonicotinic acid from 4-cyanopyridine by *Pseudomonas putida*

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Pseudomonas putida CGMCC3830 harboring nitrilase was used in isonicotinic acid production from 4-cyanopyridine. This nitrilase showed optimum activities towards 4-cyanopyridine at pH 7.5 and 45 °C. The half-life of *P. putida* nitrilase was 93.3 h, 33.9 h, and 9.5 h at 30 °C, 38 °C, and 45 °C, respectively. 4-Cyanopyridine (100 mM) was fully converted into isonicotinic acid within 20 min. The bench-scale production of isonicotinic acid was carried out using 3 mg of resting cells per mL in a 1 L system at 30 °C and finally, 123 g L^{-1} of isonicotinic acid were obtained within 200 min without any by-products. The conversion reaction suffered from the product inhibition effect after the tenth feeding. The volumetric productivity was 36.9 g L^{-1} h^{-1}. *P. putida* shows significant potential in nitrile hydrolysis for isonicotinic acid production. This paper is the first report on isonicotinic acid biosynthesis using *Pseudomonas putida* and it represents the highest isonicotinic acid production reported so far.

Keywords: biocatalysis, 4-cyanopyridine, isonicotinic acid, nitrilase, *Pseudomonas putida*

Introduction

Among several examples of nitrilase-mediated biocatalysis, pathways for the biosynthesis of isonicotinic acid from 4-cyanopyridine have not been studied extensively. The effort has been focused on the application of this enzyme for the production of its isomer, nicotinic acid, and other carboxylic acids, such as glycolic acid, acrylic acid, *p*-hydroxybenzoic acid, *N*-acylamino acids, and (*R*)-(−)-mandelic acid (Kumar & Bhalla, 2013; Chaplin et al., 2004; Gong et al., 2012). Thus, the exploration of its practical potential in isonicotinic acid production is of great significance since it plays a functional role in the synthesis of plant growth regulator inabenfide, antituberculostatic drug isoniazid, and antidepressant nialamide in agricultural and pharmaceutical industry (Arai et al., 2004; Sharma et al., 2012).

Conventionally, isonicotinic acid was produced through chemical approaches, while the enzymatic hydrolysis approach has attracted much attention in recent few years due to its mild reaction conditions, high specificity, high selectivity and eco-friendliness. However, only very few organisms including *Nocardia globerula* NHB-2, *Pseudomonas fluorescens* C2, *Fusarium solani* O1, and *Aspergillus niger* K10 have been reported for isonicotinic acid production. On the other hand, *Nocardia globerula* NHB-2 nitrilase has been already used in 4-cyanopyridine biotransformation (Sharma et al., 2012). The authors applied fed-batch reaction to continuously transform 4-cyanopyridine at 35 °C and 117.9 g (958 mM) of isonicotinic acid were finally obtained within 400 min with an addition of 100 mM of 4-cyanopyridine per 20 min. The catalyst suffered from substrate inhibition after the 7th feeding. Very recently, Maksimova and coworkers isolated a strain of *P. fluorescens* C2 containing nitrilase and found that it showed certain specific

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activity toward 4-cyanopyridine. About 16 g L^{-1} of 4-cyanopyridine were converted within 300 min by this strain (Maksimova et al., 2013).

In addition, it has been proved that fungal nitrilases from the *Aspergillus*, *Fusarium*, and *Gibberella* genera have potential in 3-, 4-cyanopyridine hydrolysis (Gong et al., 2012). These nitrilases show high specificity towards this sort of nitriles. However, amide compounds were simultaneously generated in the conversion reaction. Researchers from the Czech republic (Vejvoda et al., 2006) prepared isonicotinic acid using *Aspergillus niger* K10 nitrilase, which was highly specific towards 4-cyanopyridine, as the catalyst. The enzyme was found to generate large amounts of amide from nitrile at an amide/acid molar ratio of 1/3. For this reason, the authors had to use the co-immobilization method to remove the by-product; then, *Rhodococcus erythropolis* A4 amidase was co-immobilized on a butyl sepharose column with nitrilase. The mole ratio of amide was decreased to approximately 5 %. Another fungal nitrilase from *Fusarium solani* O1 afforded < 0.2 mole % of amide when using the same approach from 2 %. Afterwards, continuous-stirred membrane reactors were developed using cross linked *F. solani* O1 nitrilase and *R. erythropolis* A4 amidase to further improve the conversion process (Malandra et al., 2009). Interestingly, the purity of isonicotinic acid was further increased to 99.9 %. However, the by-product, isonicotinamide, still could not be fully removed while the cost increased and the procedure became complicated.

In this work, *Pseudomonas putida* was used as the biocatalyst for the biotransformation of 4-cyanopyridine to isonicotinic acid (Zhu et al., 2013a). This strain was previously isolated from soil in our laboratory. It showed significant catalytic efficiency on 3- and 4-cyanopyridine hydrolysis, while no amide was formed in the reaction mixture. Its nitrilase gene was identified by our group and the conversion reaction was proved to be mediated by nitrilase (Zhu et al., 2013b). Reaction conditions of the 4-cyanopyridine conversion were investigated and a bench-scale process was carried out applying the fed-batch method.

Experimental

4-Cyanopyridine, isonicotinic acid, and isonicotinamide were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other analytical-grade reagents and chemicals were obtained from commercial sources and they were used in the present study without further purification.

In all investigations, *P. putida* CGMCC3830 was employed. This strain was deposited in the China General Microbiological Culture Collection Center (Beijing, China). It was precultivated at 30 ℃ in a basal medium (pH 6.0) containing 12.5 g of glycerol, 12.5 g of tryptone, 5 g of yeast extract, 1 g of KH_2PO_4, and 1 g of NaCl per liter for 24 h. This preculture (1 vol. %,) was inoculated to a 250 mL flask containing 55 mL of basal culture medium additionally supplemented with 1 g L^{-1} of urea as the inducer. The incubation was routinely performed at 30 ℃ for 36 h under shaking at 120 min^{-1}. The harvested cells were collected by centrifugation (10000g, 10 min) and washed twice with 100 mM of potassium phosphate buffer (pH 7.5). The mass of dry cells (DCW) of the resting cells was determined after drying at 115 ℃ for 2 h.

Unless otherwise stated, the standard reaction for 4-cyanopyridine conversion to isonicotinic acid was performed in 10 mL of 100 mM potassium phosphate buffer (pH 7.5) at 30 ℃ with 5 mg of cells and 50 mM of 4-cyanopyridine. The samples were withdrawn at regular intervals, and the reaction was terminated by adding 10 vol. % of 1 M HCl. Subsequently, the samples were centrifuged at 10000g for 5 min. 4-Cyanopyridine and isonicotinic acid in the supernatant were analyzed by high performance liquid chromatography (HPLC). One unit of nitrilase activity was defined as the dry cell of 1 mg catalyzing the formation of isonicotinic acid at the rate of 1 μmol min^{-1} under the standard assay conditions. The optical density of culture broth was spectrophotometrically determined at 600 nm (Mapada UV-1800, Shanghai, China).

The effects of pH, temperature, substrate concentration, and resting cell concentration on the conversion reaction were studied. The pH range of the reaction was from 5.0 to 9.0 with sodium acetate buffer (pH 5.0–6.0), potassium phosphate buffer (pH 6.0–7.5), or Tris–HCl buffer (pH 7.5–9.0). The optimum reaction temperature was determined by carrying out the reaction at various temperatures, from 20 ℃ to 60 ℃. The cells were pre-incubated at 30 ℃, 38 ℃, and 45 ℃ to determine the thermostability of the enzyme. The cell suspension was sampled at regular intervals to measure the residual activity which was determined by comparison with the initial nitrilase activity of unincubated cells. The reaction systems, including 4-cyanopyridine in different concentrations (50 mM to 300 mM), were used to evaluate the effect of substrate concentration. To obtain the optimal cell concentration for substrate conversion, various amounts of resting cells (1 mg of DCW per mL to 3 mg of DCW per mL) were tested for the complete conversion of 100 mM of 4-cyanopyridine to isonicotinic acid.

The bioconversion reaction was performed using the resting cell concentration of 3.0 mg of DCW per mL at 30 ℃ under constant stirring. 4-Cyanopyridine was added into the 1 L reaction mixture in the fed-batch mode and the conversion reaction was monitored by HPLC. It was carried out in a 3 L fermentor (Baoxing Bio-Engineering Equipment Co., Shanghai, China). The agitation speed of the fer-

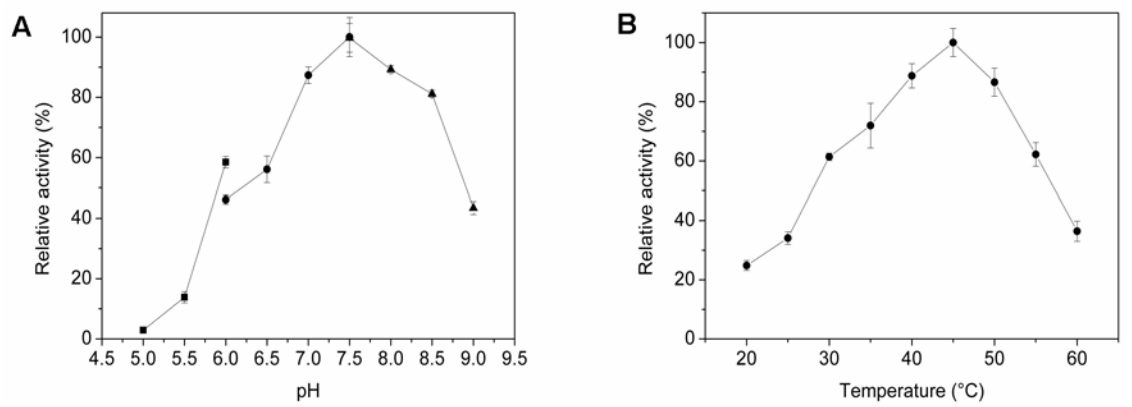

Fig. 1. Effect of pH (A) on nitrilase acitivity of *P. putida*: relative activity was expressed as the percentage of the activity at pH 7.5 (specific activity of 5.2 U mg^{-1}). Symbols: sodium acetate buffer (■), potassium phosphate buffer (●), Tris–HCl buffer (▲). Effect of temperature (B) on nitrilase acitivity of *P. putida*: relative activity was expressed as the percentage of the activity at 45 ℃ (specific activity 8.2 U mg^{-1}).

mentor was maintained at 400 min^{-1}, at a constant temperature of 30 ℃.

The amounts of 4-cyanopyridine and isonicotinic acid in the reaction mixture were determined by HPLC (UltiMate 3000 liquid chromatography system; Dionex, Sunnyvale, CA, USA). HPLC analysis was performed employing a Chromeleon chromatography data system, using an Atlantis dC18 column (particle size of 5.0 μm; dimensions of 150 mm × 4.6 mm; Waters, Milford, MA, USA) at the wavelength of 268 nm. The mobile phase was acetonitrile: 0.025 vol. % phosphoric acid ($\varphi_r = 6/4$) at the flow rate of 0.5 mL min^{-1} at 30 ℃.

Results

Effect of reaction conditions on bioconversion

P. putida nitrilase is able to hydrolyze 4-cyanopyridine in a relative wide pH range and it shows the maximal activity at pH 7.5 with the potassium phosphate or Tris–HCl buffer, while almost identical activities were observed using these two buffer solutions (Fig. 1A). The activity decreased sharply at pH below 6.0, and only 14 % of the activity was determined at pH 5.5. Interestingly, this nitrilase preferred alkaline conditions and 43 % of activity was retained at pH 9.0. No spontaneous degradation of 4-cyanopyridine was detected under acidic or alkalic conditions.

P. putida nitrilase showed higher catalytic activity for 4-cyanopyridine conversion in the temperature range of 40–50 ℃ (Fig. 1B); the maximum activity was achieved at 45 ℃. The nitrilase activity of *P. putida* increased from 20 ℃ to 45 ℃ with the reaction temperature, while the activity decreased rapidly when the temperature exceeded 45 ℃. It can be attributed to the inactivation of intracellular nitrilase at higher temperatures. Furthermore, there is a certain difference in the optimum conditions for wild and recombinant nitrilase, where the reaction temperature and pH of purified recombinant nitrilase with 3-cyanopyridine as substrate were 40 ℃ and pH 7.5, respectively (Zhu et al., 2013b).

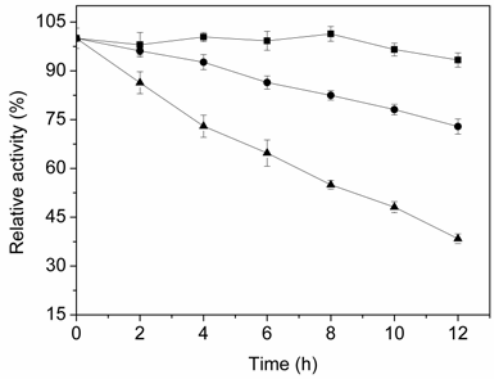

Fig. 2. Thermostability of intracellular nitrilase of *P. putida*. Variation of nitrilase relative activity with time at different temperatures: 30 ℃ (■), 38 ℃ (●), and 45 ℃ (▲).

In order to investigate the thermostability of *P. putida* nitrilase, resting cells were preincubated at 30 ℃, 38 ℃, and 45 ℃, respectively. Fig. 2 shows that the intracellular enzyme was stable at 30 ℃ and retained 93 % of its activity after a 12 h incubation. The half-life ($t_{1/2}$) of this intracellular nitrilase was calculated to be 93.3 h (30 ℃), 33.9 h (38 ℃), and 9.5 h (45 ℃), according to the residual activity plotted against time, while the recombinant nitrilase of this strain showed poorer stability than the wild one (Zhu et al., 2013b). Taking into account the enzyme activity and thermostability, the temperature of 30 ℃ was chosen as the reaction temperature in further experiments.

To obtain the optimal substrate concentration, the reaction was carried out at different concentra-

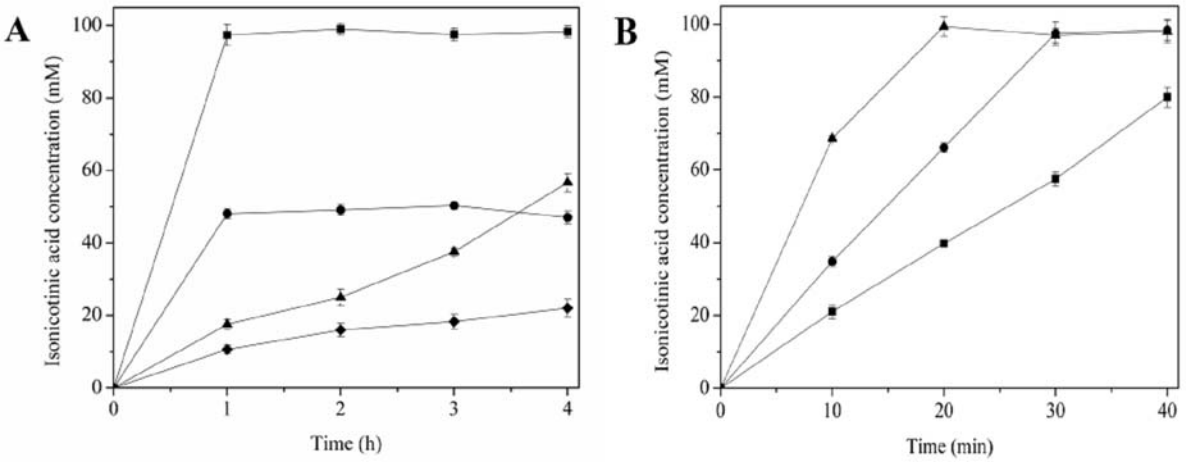

Fig. 3. Effect of 4-cyanopyridine concentration (A) on its bioconversion. Initial 4-cyanopyridine concentration: 50 mM (●), 100 mM (■), 200 mM (▲), and 300 mM (◆). Effect of resting cells concentration (B) on 4-cyanopyridine bioconversion. Resting cell concentration in mg of DCW per mL: 1 (■), 2 (●), and 3 (▲).

tions of 4-cyanopyridine ranging from 50 mM to 300 mM (Fig. 3A). Complete conversion of 100 mM of 4-cyanopyridine was achieved within 1 h. The rate of isonicotinic acid synthesis decreased when 4-cyanopyridine concentration was higher than 100 mM. This could be caused by the toxicity and inhibition due to high 4-cyanopyridine concentration. Finally, 100 mM of 4-cyanopyridine were chosen as the optimal substrate concentration.

Fig. 3B shows that an increased production of isonicotinic acid was observed with the cell concentration increase from 1 mg of DCW per mL to 3 mg of DCW per mL. A 66 % conversion was observed for 100 mM of 4-cyanopyridine within 20 min when using 2 mg of DCW per mL; while 3 mg of DCW per mL afforded a 100 % 4-cyanopyridine conversion. Therefore, the concentration of 3 mg of DCW per mL was chosen as the optimal cell concentration in further experiments.

Fed-batch reaction for isonicotinic acid production

Complete conversion of 100 mM of 4-cyanopyridine into isonicotinic acid was achieved within 20 min when using *P. putida* resting cells as the catalyst. The fed-batch reaction was thus carried out at 30 ℃ in 1 L of potassium phosphate buffer solution (100 mM, pH 7.5). The feeding rate of 4-cyanopyridine in the fed-batch reaction was 100 mM per 20 min. The product inhibition (isonicotinic acid) effect began to occur after the tenth feeding. The concentration of 123 g L^{-1} (1000 mM) of isonicotinic acid was obtained from the corresponding 4-cyanopyridine within 200 min (Fig. 4). The volumetric productivity was calculated to be 36.9 g L^{-1} h^{-1}.

However, the addition of substrate was still carried out in order to fully explore the potential of this catalyst. After the thirteenth and fourteenth feeding

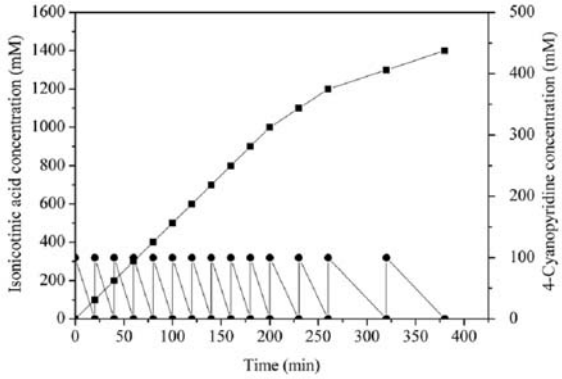

Fig. 4. Fed-batch reaction profile of 4-cyanopyridine bioconversion by *P. putida* nitrilase. Concentration of: isonicotinic acid (■), 4-cyanopyridine (●).

of 100 mM of 4-cyanopyridine to the reaction mixture, the conversion process efficiency decreased due to product inhibition and enzyme inactivation. The 100 % conversion of the newly added 4-cyanopyridine was observed after 60 min. Finally, 172 g L^{-1} of isonicotinic acid (1400 mM) were accumulated within 380 min by 14 substrate feedings (Fig. 4). No isonicotinamide was detected in the reaction mixture.

Discussion

In recent years, nitrilase-mediated biocatalysis using whole microbial cells or purified enzymes as the catalysts has been paid substantial attention by many academic institutions and industrial companies (Martínková & Křen, 2010). However, their applications were hindered by several limitations including relatively low catalytic activity, poor operational stability, by-products generation, etc. (Gong et al., 2012). A strain of *P. putida* CGMCC3830 harbor-

ing outstanding nitrilase activity while no amide was by-produced has been isolated, and the application studies showed its significant potential in enzymatic conversion of 3- and 4-cyanopyridine into the corresponding acids (Zhu et al., 2013a). The current research work is aimed at the exploration of the potential of *P. putida* for isonicotinic acid synthesis from 4-cyanopyridine. Although there are a number of studies on *Pseudomonas* nitrilase available (Layh et al., 1998; Kiziak et al., 2005; Banerjee et al., 2006), it is the first report on employing *P. putida* as a biocatalyst for isonicotinic acid biosynthesis. This green process is an attractive alternative to the chemical process owing to the mild reaction conditions and high conversion rate. In addition, this strain has been proved as promising for its catalytic efficiency.

Product concentration is one of the limiting factors of biotransformation process (Gong et al., 2012). In the present study, this nitrilase showed good product tolerance. The reaction suffered from product inhibition after the tenth feeding (1000 mM). With regard to *N. globerula* NHB-2, the rate of 4-cyanopyridine hydrolyzation decreased to 60 % when 700 mM of isonicotinic acid were accumulated (Sharma et al., 2012).

Moreover, in the conversion process of *P. putida,* the formation of the by-product isonicotinamide was not detected. This was superior to nitrilases of *A. niger* K10 and *F. solani* O1, which generated 25 % and 2 % of the by-product isonicotinamide in the total products, respectively (Vejvoda et al., 2006). Here, the downstream process for by-product removal could be avoided and the purity of the acid product improved.

The resting cells harboring nitrilase showed a moderate thermostability at 30 ℃, which enabled the operation of the fed-batch process for continuous production of isonicotinic acid in practical applications. The concentration of 123 g L^{-1} of isonicotinic acid from the corresponding 4-cyanopyridine was achieved through ten substrate feedings within 200 min. The volumetric productivity was 36.9 g L^{-1} h^{-1}. Maksimova et al. (2013) isolated a *P. fluorescens* C2 harboring nitrilase which was used for 4-cyanopyridine hydrolysis. The results showed that about 16 g L^{-1} of 4-cyanopyridine were converted within 300 min; however, there are no further application studies on isonicotinic acid production by this strain. On the other hand, Sharma et al. (2012) observed substrate inhibition after the seventh feeding using *N. globerula* NHB-2 and finally obtained a total of 958 mM of isonicotinic acid (117.9 g L^{-1}) within 400 min. Their fed-batch reaction was scaled up to 1 L and complete conversion of 700 mM of 4-cyanopyridine to the corresponding isonicotinic acid (86.2 g L^{-1}) was obtained in 140 min after seven feedings. On the other hand, Sharma and co-workers simultaneously attempted the conversion of 200 mM of 4-cyanopyridine using resting cell in the reaction; they found that complete conversion into isonicotinic acid (24.6 g L^{-1}) was achieved within 40 min. The conversion suffered from substrate inhibition in the fed-batch reaction (200 mM of substrate feed per 40 min) and ultimately resulted in the formation of 729 mM of isonicotinic acid (89.8 g L^{-1}) (Sharma et al., 2012). Furthermore, a subsequent substrate feeding was performed in this study, and 172 g L^{-1} of isonicotinic acid were obtained within 380 min. The high isonicotinic acid production can be derived from the good product tolerance as well as its moderate thermostability.

Conclusions

The present study introduces a new biocatalyst that can be used for the production of isonicotinic acid through bioconversion of 4-cyanopyridine. This is the first report about isonicotinic acid biosynthesis by *P. putida*. Moreover, the aforementioned properties indicate that *P. putida* CGMCC3830 is a promising biocatalyst for enzymatic production of isonicotinic acid. *P. putida* nitrilase displayed outstanding catalytic activity towards 4-cyanopyridine. Also, this catalyst supported high isonicotinic acid production due to the attractive feeding batches, which can be derived from the product tolerance as well as its thermostability. Subsequent gene expression and protein engineering strategy studies are undergoing to further improve the stability of this nitrilase and thus enhance its catalytic efficiency towards nitriles.

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