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Biotransformation of nicotinamide to nicotinyl hydroxamic acid at bench scale by amidase acyl transfer activity of *Pseudomonas putida* BR-1

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ABSTRACT

Acyl transfer activity of amidase of *Pseudomonas putida* BR-1 has been explored for the conversion of *N*-substituted aromatic amide (nicotinamide) and hydroxylamine to nicotinyl hydroxamic acid. Nicotinyl hydroxamic acid is an important pharmaceutical compound with enormous biomedical applications. *P. putida* BR-1 produces maximum amidase acyl transfer activity 138 U/mg dcm at 50 °C, with highest conversion (95%) of nicotinamide to nicotinyl hydroxamic acid. A bioprocess was developed for production of nicotinyl hydroxamic acid in batch reaction (final volume 1 L) by adding 200 mM nicotinamide and 1000 mM of hydroxylamine in 100 mM sodium phosphate buffer (pH 7.5) at 50 °C, using 20 U/ml acyl transfer activity resting cells of *P. putida* BR-1 in reaction mixture. From 1 L reaction mixture 16 g of nicotinyl hydroxamic acid was recovered with 32 g/L/h volumetric productivity. The amidase acyl transfer activity of *P. putida* BR-1 and the process developed in the present study are of industrial significance for the enzyme mediated production of nicotinyl hydroxamic acid.

1. Introduction

Hydroxamic acid (R—CO—NHOH) and their derivatives have tremendous applications in medicine, agriculture, bioremediation, food additives, antibiotics, antifungal agents, tumor inhibitors [1,2] siderophores, enzyme inhibitors [3] and in bioremediation [4,5]. Most of the hydroxamic acids are synthesized through various chemical routes [6–8]. A number of hydroxamic acids viz. acetohydroxamic acid, butyrohydroxamic acid, benzohydroxamic acid (BHA), succinic hydroxamic acid have been synthesized using biocatalytic routes [9], but very little information is available about *N*-substituted aromatic hydroxamic acids. Among these, nicotinyl hydroxamic acid (NHA) finds application as anti-HIV, antimicrobial and antineoplastic agent. It is also used in the treatment of anemia, and reported as potential inhibitor of leukemia and ureaplasma [10]. Nicotinyl hydroxamic acid has been synthesized at test tube scale using *Rhodococcus* sp. R312 [9,10] and *Bacillus smithii* strain IITR6b2 [11] acyl transfer activity of amidase. However, study of these earlier reports divulged that the acyl transfer activity of the amidases of *Rhodococcus* sp. R312 and *Bacillus smithii* strain IITR6b2 suffer from substrate inhibition in the reactions and also produced by-products [10,12]. In the present study, a bench scale (1 L) process is developed for the synthesis of NHA using the acyl transfer activity of the amidase of *P. putida* BR-1 at higher temperature and with higher purity than reported before.

2. Materials and methods

2.1. Chemicals

All the nitriles and amides used in the present study were purchased from Sigma–Aldrich, USA. The culture media ingredients were procured from Hi Media (Mumbai, India). All the chemicals were of analytical grade.

2.2. Microorganism and culture conditions

P. putida BR-1 (isolated in our laboratory and identified at the Institute of Microbial Technology, Chandigarh, India) is used as a source of acyl transfer activity. This organism was grown aerobically in 250 ml Erlenmeyer flask containing 50 ml salt medium ($Na_2HPO_4{\cdot}12H_2O$: 2.5 g, KH_2PO_4: 2.0 g, $MgSO_4{\cdot}7H_2O$: 0.5 g, $FeSO_4{\cdot}7H_2O$: 0.03 g, $CaCl_2{\cdot}2H_2O$: 0.06 g and yeast extract: 1 g L^{-1} of distilled water, added 1% (v/v) isobutyronitrile) at 30 °C,

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160 rpm in an incubator shaker for 12 h to prepare preculture. Preculture (5%) was used as seed and isobutyronitrile 20 mM as inducer was added at four different intervals to 50 ml of salt medium in 250 ml Erlenmeyer flask and incubated for 56 h at 25 °C and 160 rpm in an incubator shaker for the production of acyl transfer activity. The cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C and washed twice with 100 mM sodium phosphate buffer (pH 7.0). The cells were suspended in the same buffer and stored at 4 °C until further use [11].

2.3. Acyl transfer activity assay

Acyl transfer activity was assayed using the method developed by Brammar and Clarke [13]. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 100 mM nicotinamide, and 500 mM hydroxylamine and acyl transfer activity containing resting cells 4.0 mg dry cell mass (dcm). The reaction mixture was incubated at 50 °C for 60 min and the reaction was stopped by adding 1 ml $FeCl_3$ reagent containing 6% $FeCl_3$ and 2% HCl. It was centrifuged at 10,000 × *g* for 5 min and then absorbance of supernatant was measured at 500 nm. One unit (U) of acyl transfer activity was defined as the amount of resting cells (mg dcm) which catalyzed the release of 1 μmol of nicotinyl-hydroxamic acid per min under the assay conditions.

2.4. Analytical analysis

The concentration of nicotinamide, nicotinyl hydroxamic acid and nicotinic acid in the reaction mixture were quantified by HPLC using series 200 Ic pump (Perkin Elmer) and programmable Absorbance Detector (Applied Biosystem) equipped with a Nucleosil C18 column (25 cm × 4.6 mm, 5 μm particle size; GL Sciences, Japan). The substrate and product was detected at 230 nm, at a flow rate of 1 ml/min of mobile phase comprised of potassium phosphate buffer (0.1 M) and methanol in the ratio of 9:1 in HPLC grade water and pH of the mobile phase adjusted to 3.5 with ortho-phosphoric acid.

2.5. Optimization of reaction conditions for conversion of nicotinamide to nicotinyl hydroxamic acid

Different reaction parameters were varied one by one to determine the optimal reaction conditions. To work out the optimum pH and temperature, reactions were carried out at pH 2.0–11.0 in various buffer systems (borate buffer, potassium phosphate buffer, sodium phosphate buffer, citrate buffer, carbonate buffer of 100 mM), buffer molarity (20–500 mM), biocatalyst (0.06–0.6 mg dcm/ml), and reaction temperature (10–80 °C). Fifty different combinations of substrate and co-substrate were tested in such a manner that the concentration of nicotinamide was varied from 100 to 1000 mM at different hydroxylamine concentrations ranging from 200 to 1000 mM in the reaction mixture to determine the optimum concentration and ratio for highest acyl transfer activity of amidase of *P. putida* sp. BR-1. In order to check the stability of enzyme at different temperatures this enzyme was stored at different temperatures (4 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C) and then acyl transfer activity was measured after 1 h interval at the standard temperature i.e. 50 °C.

2.6. Process development for the production of nicotinyl hydroxamic acid

To develop an efficient bioprocess for the maximum production of nicotinyl hydroxamic acid, different combinations of nicotinamide and resting cell concentrations were made (100 mM: 5.0 U/ml, 200 mM: 10 U/ml, 300 mM: 15 U/ml, 400 mM: 20 U/ml and 500 mM: 25 U/ml). For maximum conversion of 200 mM nicotinamide to nicotinyl hydroxamic acid in shortest time, resting cell concentration of *P. putida* BR-1 was assessed by varying the resting cell amount from 5.0 U/ml to 25.0 U/ml while the concentration of substrates was kept constant. The complete conversion of nicotinamide and hydroxylamine to nicotinyl hydroxamic acid was analyzed by HPLC.

2.6.1. Fed batch reaction at 50 ml scale (200 mM nicotinamide and 1000 mM hydroxylamine per feed)

Fed batch reaction was carried out at 50 ml scale in 100 mM sodium phosphate buffer (pH 7.5) using 200 mM nicotinamide, 1000 mM hydroxylamine and resting cell equivalent to 20 U/ml acyl transfer activity at 50 °C. The same amount of nicotinamide and hydroxylamine were fed after 30 min, so that reaction moves in the forward direction for getting higher yield of the product. Nicotinyl hydroxamic acid formed in the reaction was periodically analyzed by HPLC.

2.6.2. Bench scale production of nicotinyl hydroxamic acid at 1 L scale

On the basis of optimized process parameters the conversion of nicotinamide to nicotinyl hydroxamic acid was scaled up to 1 L using New Brunswick Scientific (NBS) BIOFLO C-32 fermenter.

2.6.3. Recovery of nicotinyl hydroxamic acid

The reaction mixture was centrifuged at 10,000 × *g* for 30 min to remove the cells. The nicotinyl hydroxamic acid was purified by solvent extraction method [14] and analyzed by HPLC.

3. Results

3.1. Optimization of reaction conditions for acyl transfer activity of P. putida BR-1

3.1.1. Effect of buffer system and buffer pH

For the selection of buffer and optimum pH, six different buffers (citrate, sodium phosphate, potassium phosphate, borate and glycine NaOH) of 100 mM concentration having different pH range (2–11) were tested. The acyl transfer activity was higher in sodium phosphate buffer (104.44 ± 0.03 U/mg dcm, pH 7.5) in comparison to potassium phosphate buffer (79.95 ± 0.02 U/mg dcm, pH 7.5) (Fig. 1). Sodium phosphate buffer (0.1 M, pH 7.5) was used in subsequent experiments. In citrate buffer, the acyl transfer activity increased only when its pH reached beyond 5.0 whereas at low pH no activity was observed. In borate and glycine NaOH buffers, *P. putida* BR-1 cells showed very small acyl transfer activity.

3.1.2. Effect of buffer strength

The enzyme activity was increased as the concentration of sodium phosphate buffer increased from 20 to 100 mM with a maximum at 100 mM (110.21 ± 0.03 U/mg dcm) (Fig. S1). However, above 100 mM sodium phosphate buffers the acyl transfer activity of *P. putida* BR-1 experienced inhibitory effect and at 500 mM of sodium phosphate buffer only 34.85 ± 0.01 U/mg dcm acyl transfer activity was observed.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2014.07.001.

3.1.3. Amount of biocatalyst

Maximum acyl transfer activity 114.42 ± 0.01 U/mg dcm of amidase of *P. putida* BR-1 was observed with 0.12 mg dcm/ml resting cells. Above 0.12 mg dcm/ml cell concentration, no increase in activity was recorded (Fig. 2). At 0.60 mg dcm/ml cell concentration a decrease in acyl transfer activity 62.24 ± 0.02 U/mg dcm of amidase was observed.