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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.

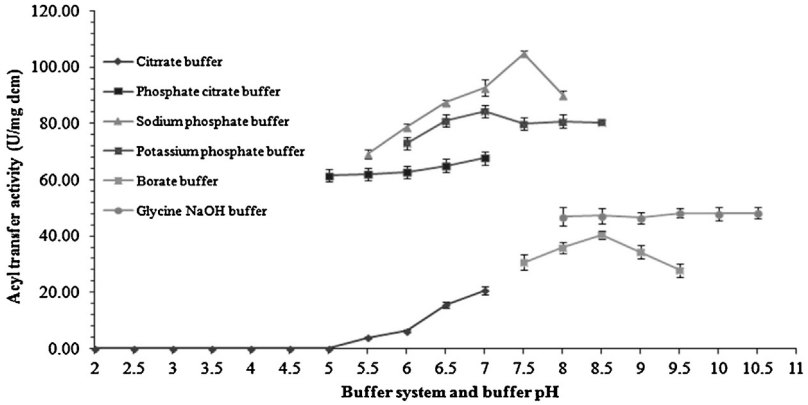

Fig. 1. Effect of different buffer system and pH on acyl transfer activity of *P. putida* BR-1.

3.1.4. Incubation temperature

Amidase of this organism exhibited very little acyl transfer activity 6.74 ± 0.02 U/mg dcm at 10 °C. Above 10 °C, a steady increase in the activity was observed with the rise in temperature up to 50 °C. Maximum acyl transfer activity 124.67 ± 0.02 U/mg dcm of *P. putida* BR-1 was recorded at 50 °C (Fig. 3). At higher temperature i.e. 80 °C there was drastically decrease in acyl transfer activity and only 5.10 ± 0.03 U/mg dcm activity could be observed.

3.1.5. Effect of substrate combinations

Enzymatic synthesis of hydroxamic acid is a bi-substrate reaction, therefore our main aim was to get the best combination of both substrates for maximum activity and bioconversion.

It has been observed that when substrate and co-substrate were used in 1:5 ratio (200 mM nicotinamide and 1000 mM hydroxylamine), maximum acyl transfer activity 128.34 ± 0.05 U/mg dcm was achieved and this combination proved to be best of all the combinations tested (Fig. 4).

3.1.6. Stability profile of acyl transfer activity of P. putida BR-1 stored at different temperatures

It has been found that the resting cells of *P. putida* BR-1 stored at 4 °C and 15 °C retained acyl transfer activity for a long time (71% after 10 h), but the cells at 45 °C retained only 50% activity after 10 h. Higher temperature exhibited fast decrease in acyl transfer activity as compared to the cells stored at 4 °C and 15 °C. The cells exposed to 60 °C exhibited very rapid loss in acyl transfer activity. The half life of the enzyme at 50 °C was 3 h and at 60 °C it was 1 h (Fig. 5).

3.2. Bioprocess development for the conversion of nicotinamide to nicotinyl hydroxamic acid

On the basis of result of the preceding optimization of reaction parameters, the acyl transfer activity of amidase from *P. putida* BR-1 was used for the synthesis of nicotinyl hydroxamic acid.

3.2.1. Time course of nicotinamide conversion to nicotinyl hydroxamic acid with increase in substrate and resting cell concentrations

Substrate and resting cell concentrations were varied from 100 mM: 5.0 U/ml to 500 mM: 25 U/ml, whereas the concentration of hydroxylamine was kept constant i.e. 1000 mM. The reaction mixture containing 10 U/ml and 200 mM nicotinamide as substrate showed highest conversion rate and yield of nicotinyl hydroxamic acid (186 mM) in 1 h and 20 min. On varying the substrate and cell concentration the percentage yield of NHA was observed i.e. 100 mM – 70% yield, 200 mM – 93% yield, 300 mM – 80% yield, 400 mM – 78% yield, 500 mM – 75% yield (Fig. 6). The high concentration of substrate had inhibitory effect on the formation of nicotinyl hydroxamic acid, though the enzyme concentration in the reaction was increased proportionally.

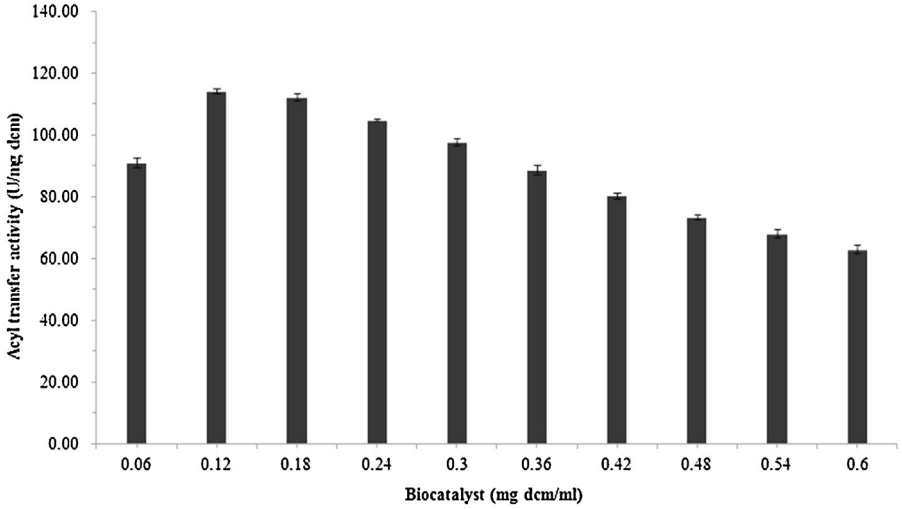

Fig. 2. Effect of biocatalyst concentrations on acyl transfer activity of amidase from *P. putida* BR-1.

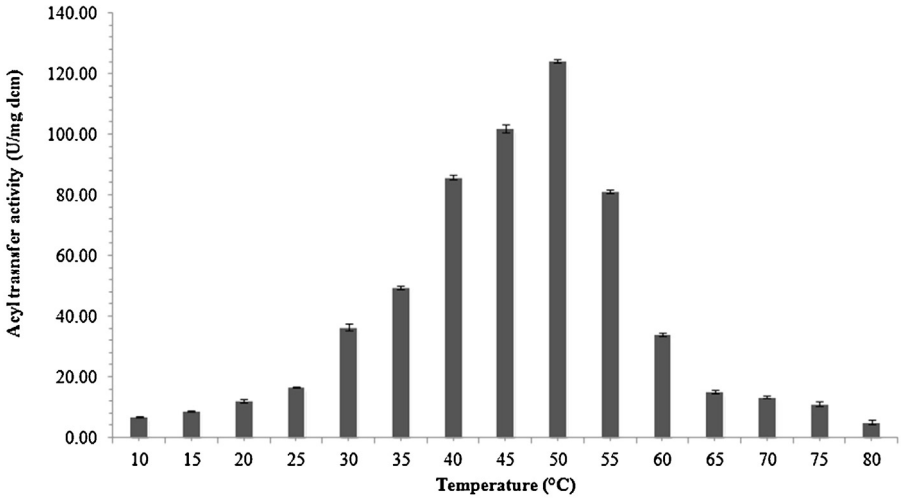

Fig. 3. Effect of different temperatures on acyl transfer activity of *P. putida* BR-1.

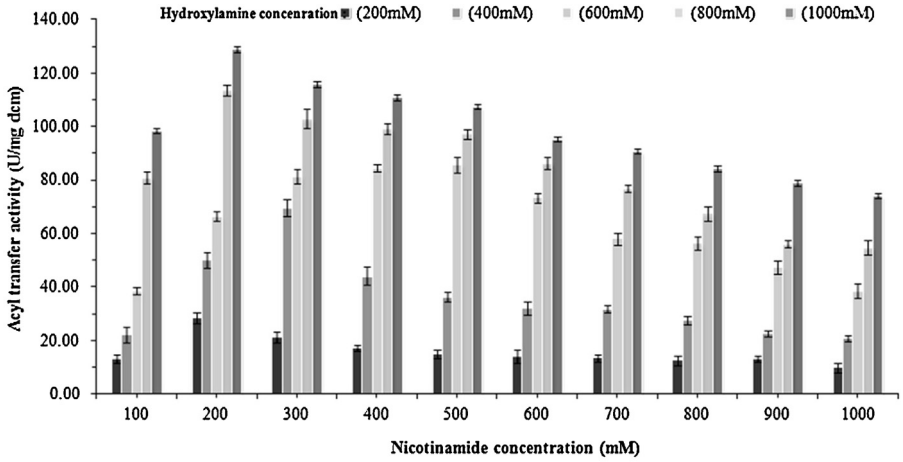

Fig. 4. Effect of different combinations of nicotinamide and hydroxylamine concentration on acyl transfer activity of *P. putida* BR-1.

3.2.2. Effect of resting cell concentration on conversion of 200 mM of nicotinamide to nicotinyl hydroxamic acid

Optimum resting cell concentration of *P. putida* BR-1 for maximum conversion of 200 mM nicotinamide and 1000 mM hydroxylamine was assessed by varying the resting cell amount equivalent to 5.0–25.0 U/ml of acyl transfer activity. On increasing the amount of biocatalyst (resting cell), conversion rate of substrate to product also increased. Highest conversion of nicotinamide and hydroxylamine into nicotinyl hydroxamic acid (188 mM) in 30 min was observed with resting cells having 20 U/ml acyl transfer activity. While, resting cells having (15 U/ml) acyl transfer activity took 50 min for the production of NHA. When there is almost full

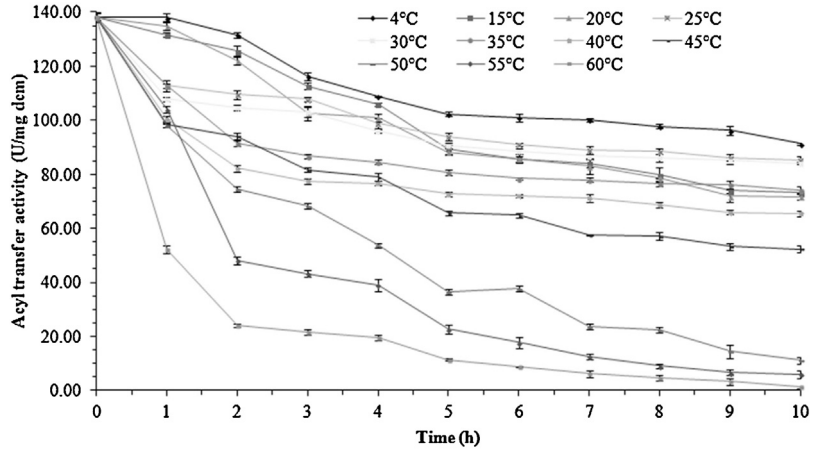

Fig. 5. Stability profile of acyl transfer activity of *P. putida* BR-1 stored at different temperatures.

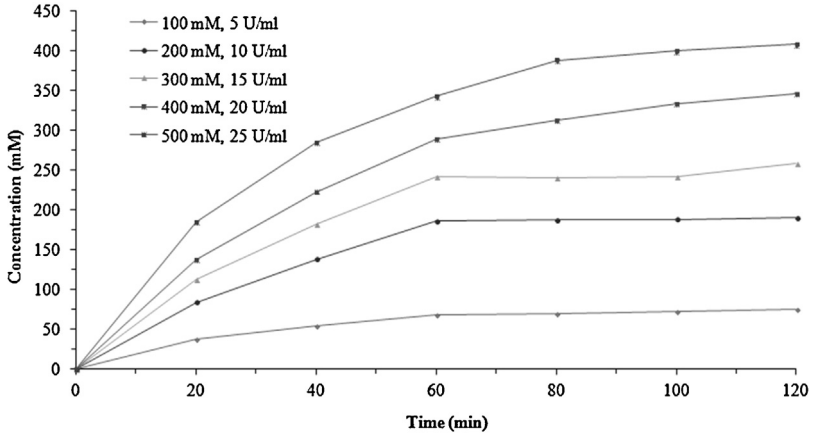

Fig. 6. Effect of increasing cell concentration on varied substrate concentration.

conversion or almost no substrate left, it seems that undesirable reactions become operational. Therefore, resting cell having 20 U/ml acyl transfer activity was used for conversion of nicotinamide into nicotinyl hydroxamic acid (Fig. 7).

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

In the fed batch reaction after 30 min (i.e. before second feeding of substrates) 188 mM nicotinyl hydroxamic acid was formed from 200 mM nicotinamide with 94% conversion without any side product but after 1 h (i.e. before third feeding of substrates) out of 400 mM of nicotinamide, 256 mM of NHA and 2 mM of nicotinic acid (side product) was formed. Further feeding did not resulted in significant enhancement of nicotinyl hydroxamic acid production (data not shown). This might be due to product inhibition, when more enzyme gets saturated and is unable to convert the substrate into product, resulting in longer reaction times and unwanted side reactions.

3.2.4. Conversion of nicotinamide and hydroxylamine to nicotinyl hydroxamic acid at bench scale (1 L)

Batch reaction was carried out at 1 L scale for 30 min. The reaction mixture consisted of 200 mM nicotinamide, 1000 mM hydroxylamine, 100 mM sodium phosphate buffer (pH 7.5), and resting cell equivalent to 20 U/ml. The temperature and impeller speed of the fermenter was maintained at 50 °C and 160 rpm respectively. At the end of the reaction 190 mM nicotinyl hydroxamic acid (95% molar conversion yield) was produced, it was 1% more than that achieved at 50 ml scale and this gain due to the change in kinetics with the change in reaction volume moreover enzyme and substrate has more interaction surface area at 1 L.

3.2.5. Recovery of nicotinyl hydroxamic acid

After completion of the reaction, the resting cells were separated from the reaction by centrifugation at 10,000 × *g* for 15 min. In 1 L batch mode reaction mixture 16 g of nicotinyl hydroxamic acid was recovered after solvent extraction. This powder was found 98% pure without any side product in HPLC analysis (Fig. 8). *P. putida* BR-1 biotransformed nicotinamide into nicotinyl hydroxamic acid with 13 g/h/dcm catalytic productivity and 32 g/L/h volumetric productivity.

3.2.6. Recycling of recovered cells

After bench scale reaction, 80% of resting cells were recovered, which showed 92% residual acyl transfer activity 126.14 ± 0.03 U/mg dcm. When these cells were again used in 50 ml

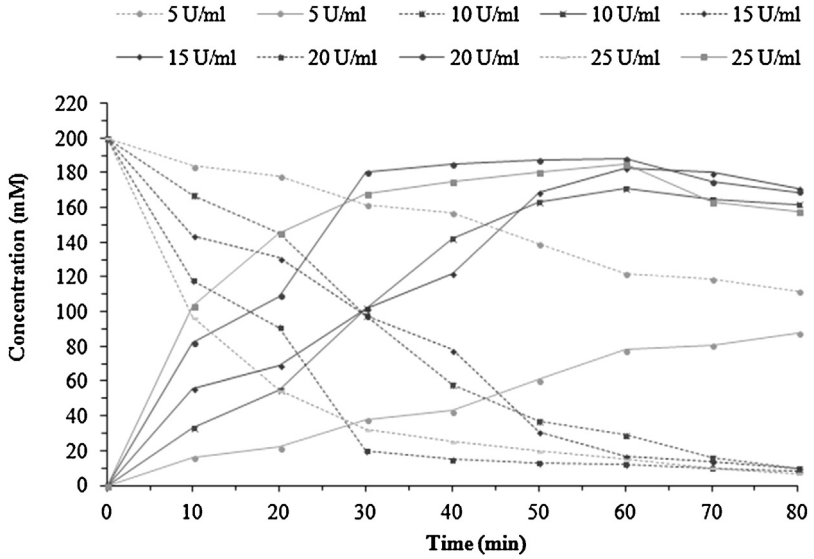

Fig. 7. Effect of resting cell concentration on 200 mM nicotinamide conversion into nicotinyl hydroxamic acid (solid line = nicotinyl hydroxamic acid and dotted line = nicotinamide).

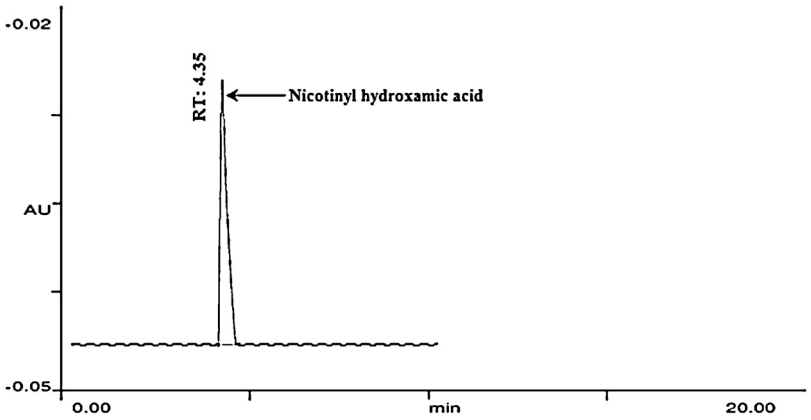

Fig. 8. HPLC analysis of purified nicotinyl hydroxamic acid RT: 4.35 (nicotinamide RT: 3.60 min, and nicotinic acid RT: 6.45 min).

reaction mixture in the next experiment, 84% conversion of 200 mM nicotinamide to nicotinyl hydroxamic acid was achieved.

4. Discussion

The amidase of *P. putida* BR-1 showed acyl transfer activity through a wide range (5.0–10.0) of pH in different buffer system, but maximum acyl transfer activity was recorded in 100 mM sodium phosphate buffer of pH 7.5. Acyl transfer activity in citrate buffer below pH 5.0 was not observed, which might be due to denaturation of enzyme in acidic pH. Beyond optimum pH (7.5), and at higher buffer molarity (>100 mM) a decline in acyl transfer activity was observed because these elevated buffers molarity and pH are not favoring the ionic conditions responsible for the formation of enzyme-substrate complex at faster rate which is quite necessary for the product formation. Most of the amidases reported so far, are active around neutral pH. A comparative analysis of acyl transfer activity of amidases from different organisms against *P. putida* BR-1 showed that this amidase is more or less similar to earlier reported amidases (Table 1).

P. putida BR-1 exhibited highest acyl transfer activity of amidase at 50 °C for the production of nicotinyl hydroxamic acid similar to other mesophilic enzymatic systems. This temperature supported the effective collisions between the enzyme and substrate, which will lead to higher acyl transfer activity, but as the temperature increased above from its optimum value (50 °C), the non-covalent bonds holding the amino acids at active site of enzyme starts breaking due to high kinetic energy. This changes the 3D structure of the enzyme, which ultimately leads to loss of activity [17]. This organism showed highest acyl transfer activity at 50 °C similar to *G. pallidus* RAPc8 [18] reported from thermal habitat.

In all the different combinations when the co-substrate hydroxylamine is present in lower concentration in the reaction mixture the conversion rate is very slow and hydrolysis of amide takes place at a faster rate due to the lack of an adequate amount of a strong nucleophile (hydroxylamine). However, as the concentration of co-substrate increased in comparison to substrate, the rate of formation of nicotinyl hydroxamic acid also increased and undesirable reactions not observed. Fournand et al. [10] have also reported that the increase in concentration of hydroxylamine (10–500 mM) considerably reduced the undesirable hydrolysis of amide as well as hydroxamic acid. As it was clear from all earlier studies (Table 1) that hydroxylamine is required in excess, so that acyl group of amide transferred more rapidly on hydroxylamine for the formation of respective hydroxamic acid [9]. Moreover, instant use of multiple ratio of nicotinamide (400 mM) and co-substrate hydroxylamine (2000 mM) might cause substrate inhibition and reduced the conversion rate.

The half life of amidase of *P. putida* BR-1 was 3 h at 50 °C and 1 h at 60 °C. The amidase of *Geobacillus pallidus* RAPc8 showed half life 5 h at 50 °C whereas *G. subterraneus* RL-2a exhibited $t^{1/2}$ for 9 h at 60 °C [18,19]. The amidase from this organism have lesser stability at higher temperature than thermophilic strains that might be due to absence of charged amino acids (Arg, Lys, His, Asp, Glu) in the polypeptide chain [20].

It was observed that fed batch mode of reaction was not so efficient, as there was no absolute biotransformation in each feeding this might be due to product inhibition or enzyme get saturated and unable to convert the substrate into product moreover hydrolysis of amide and hydroxamic acid to nicotinic acid also get operational in

Table 1
Comparative analysis of different properties of *P. putida* BR-1 with earlier studies.

S. no.	Parameter	*P. putida* BR-1	*Rhodococcus* sp. R312 [10]	*Alcaligenes* sp. MTCC 10674 [11]	*Bacillus smithii* strain IITR6b2 [12]	*Bacillus* sp. APB-6 [14]	*Rhodococcus erythropolis* [15]	*G. pallidus* BTP-5x MTCC 9225 [16]
1	Buffer and pH	Sodium phosphate, 100 mM, pH 7.5	Sodium phosphate, 20 mM, 7.0	Potassium phosphate, 100 mM, pH 7.0	Potassium phosphate, 100 mM, pH 7.0	Glycine-NaOH 100 mM, pH 7.5	Potassium phosphate, 50 mM, pH 7.0	Sodium phosphate, 100 mM, pH 7.0
2	Reaction temperature	50 °C	30 °C	50 °C	30 °C	45 °C	30 °C	65 °C
3	Substrate	Nicotinamide	Nicotinamide	Benzamide	Nicotinamide	Acetamide	Acetamide	Acetamide
4	Amide and hydroxyl amine	200 mM 1000 mM	50 mM 100 mM	100 mM 200 mM	200 mM 250 mM	300 mM 800 mM	100 mM 200 mM	100 mM 500 mM
5	Conversion rate	95%	40%	66%	94.5%	94%	–	93%
6	Scale up	1 L	Test tube scale	500 ml	50 ml	1 L	Test tube scale	1 L

long duration reaction. Agarwal et al. [12] reported the production of nicotinic acid hydroxamate using acyl transfer activity of *Bacillus smithii* strain IITR6b2 at moderate temperature (30 °C), and only at 50 ml scale with 86.4% purity along with side product i.e. nicotinic acid but the present study resulted into production of nicotinyl hydroxamic acid significantly with high purity and at elevated temperature. *P. putida* BR-1 with 20 U/ml activity cells in 1 L batch mode reaction produced 16 g of nicotinyl hydroxamic acid with 32 g/L/h volumetric productivity.

5. Conclusion

P. putida BR-1 has emerged as a novel source for transformation of *N*-substituted aromatic amide (nicotinamide) and hydroxylamine to nicotinyl hydroxamic acid as it exhibited acyl transfer activity at high temperature (50 °C) with substrate and product tolerance among the until now reported bacterial systems in batch mode till date. The bench scale (1 L scale) production of nicotinyl hydroxamic acid using resting cells of *P. putida* BR-1 (containing acyl transfer activity) showed 13 g/h/dcm catalytic productivity and produced 16 g of nicotinyl hydroxamic acid with 98% purity without any side product. The bioprocess developed in the present study is of potential application in industry for production of highly pure nicotinyl hydroxamic acid.

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