BIOCATALYSIS

Bioprocess development for nicotinic acid hydroxamate synthesis by acyltransferase activity of *Bacillus smithii* strain IITR6b2

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Abstract In this work, acyltransferase activity of a new bacterial isolate *Bacillus smithii* strain IITR6b2 was utilized for the synthesis of nicotinic acid hydroxamate (NAH), a heterocyclic class of hydroxamic acid. NAH is an important pyridine derivative and has found its role as bioligand, urease inhibitor, antityrosinase, antioxidant, antimetastatic, and vasodilating agents. Amidase having acyltransferase activity with nicotinamide is suitable for nicotinic acid hydroxamate production. However, amidase can also simultaneously hydrolyze nicotinamide and nicotinic acid hydroxamate to nicotinic acid. Nicotinic acid is an undesirable by-product and thus any biocatalytic process involving amidase for nicotinic acid hydroxamate production needs to have high ratios of acyltransferase to amide hydrolase and acyltransferase to nicotinic acid hydroxamate hydrolase activity. Isolate *Bacillus smithii* strain IITR6b2 was found to have 28- and 12.3-fold higher acyltransferase to amide and hydroxamic acid hydrolase activities, respectively. This higher ratio resulted in a limited undesirable by-product, nicotinic acid (NA) synthesis. The optimal substrate/co-substrate ratio, pH, temperature, incubation time, and resting cells concentration were 200/250 mM, 7, 30 °C, 40 min, and 0.7 mg_{DCW} ml^{-1}, respectively, and 94.5 % molar conversion of nicotinamide to nicotinic acid hydroxamate was achieved under these reaction conditions. To avoid substrate inhibition effect, a fed-batch process based on the optimized parameters with two feedings of substrates (200/200 mM) at 40-min intervals was developed and a molar conversion yield of 89.4 % with the productivity of 52.9 g h^{-1} g_{DCW}^{-1} was achieved at laboratory scale. Finally, 6.4 g of powder containing 58.5 % (w/w) nicotinic acid hydroxamate was recovered after lyophilization and further purification resulted in 95 % pure product.

Introduction

Nicotinic acid derivatives are attractive compounds due to their pharmaceutical and analytical applications [16]. Nicotinic acid hydroxamate (NAH), also known as 3-pyridine hydroxamic acid, is one of the nicotinic acid derivatives in which the hydroxamic acid moiety (–CONHOH) is present at the third carbon of the pyridine ring [1]. The NAH can serve as whitening agent and antioxidant in the cosmetic, medicine, and food-processing industries [3]. Lin et al. [14] reported NAH as a more effective in vitro inhibitor of monophenolase and diphenolase activities of mushroom tyrosinase as compared to other structural analogues of nicotinic acid derivatives. NAH was found to exhibit dose-dependent diphenylpicrylhydrazyl (DPPH), hydroxyl radical scavenging activities, and anti-low density lipoprotein peroxidation ability [15]. Nicotinic acid hydroxamate is a ligand with two co-ordination sites and it was utilized in the design of a heterobimetallic novel wave-like co-ordination polymer using NAH as bridging scaffolds [18]. Similarly, ruthenium (III) dimethyl sulphoxide pyridine hydroxamic acid complex was developed as a potential antimetastatic agent [10]. Development of a new

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class of bifunctional metallodrug based on 3-pyridine hydroxamic acid was achieved by exploiting the well-established anticancer properties of Pt and the NO releasing ability of hydroxamic acid [9]. NAH is also reported to be used for the treatment of hyperammonemia, as urease inhibitor and vasodilating agent [1, 13, 22].

In recent years, increased interest in nicotinic acid hydroxamate resulted in higher attention on development of improved production methods with high yield and productivity. Chemical methods of nicotinic acid hydroxamate synthesis involve reaction of carboxylic acid or ester with O/N-protected hydroxylamine such as NH_2–O–Bn under strong basic conditions in solvents like methanol, ethanol, tetrahydrofuran, and dimethylformamide [8, 12]. Sometimes carboxylic acids found to be reluctant in the activation step that results in poor yield of product during the coupling reaction. After reaction, O-alkylated derivative of nicotinic acid hydroxamate (NAH) is obtained, and this needs further catalytic hydrogenation with palladium to release the free hyroxamic acids. Development of a single-step biotransformation process for NAH synthesis will provide a simple, convenient, and environmentally friendly economic route. In the recent past, acyltransferase activity of amidases has been utilized for hydroxamic acids and acid hydrazides syntheses [4, 6]. Unlike the chemical method of synthesis, amidase-catalyzed hydroxamic acid synthesis avoids post-reaction acidification steps, coupling reaction, expensive O/N-protected hydroxylamine, and omits an extra step of catalytic hydrogenation of hydroxamic acid derivatives with palladium. Biocatalytic synthesis also utilizes cheaper source of substrates like corresponding amides and reaction generally takes place in organic solvent-free medium. Although bioprocesses for aceto [5, 20], benzo [2, 23], and 2-phenylpropiono [11] hydroxamic acids syntheses using amidase enzyme have been reported, there is no report on the biotransformation process for synthesis of heterocyclic class of hydroxamic acids.

To achieve higher yield of hydroxamic acid by minimizing the undesirable by-product (acid) formation from amide, it is desirable to have a biocatalyst with high acyltransferase activity and minimum amide and hydroxamic acid hydrolase activities (Fig. 1). The aim of the present study is to develop a process for synthesis of nicotinic acid hydroxamate from nicotinamide using acyltransferase activity of *Bacillus smithii* strain IITR6b2. In this work, special emphasis was placed on process development for NAH production with minimum formation of the by-product nicotinic acid. Different process parameters were optimized for efficient production of NAH by fed-batch biotransformation in order to achieve high product concentration.
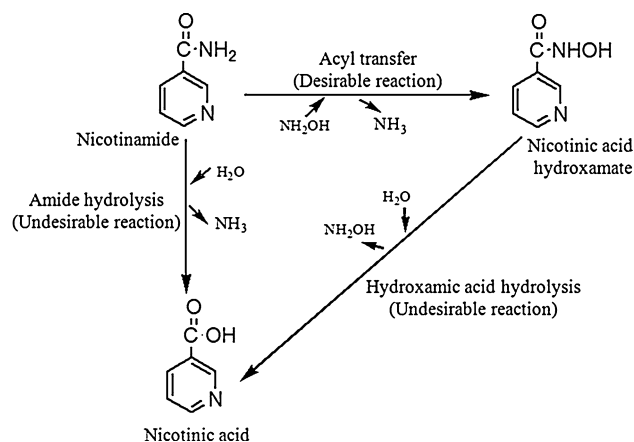

Fig. 1 Enzymatic reactions during nicotinic acid hydroxamate synthesis

Materials and methods

Chemicals

The nicotinamide and hydroxylamine-HCl were obtained from Himedia (Mumbai, India). Nicotinic acid hydroxamate was purchased from Sigma-Aldrich (St. Louis, MO, USA). The culture media components were obtained from S.D. Fine Chem (Mumbai, India). All other chemicals were of analytical or HPLC grade as per requirement, procured from various commercial sources.

Culture media and growth conditions

Bacillus smithii strain IITR6b2 was cultured in a mineral base medium having the following composition: 10 g l^{-1} glycerol, 0.2 g l^{-1} tri-sodium citrate, 1.35 g l^{-1} KH_2PO_4, 0.87 g l^{-1} K_2HPO_4; 10× mineral base (10 g l^{-1} NaCl, 2 g l^{-1} $MgSO_4·7H_2O$, 0.1 g l^{-1} $CaCl_2$) and 1 ml l^{-1} trace elements solution (0.3 g l^{-1} H_3BO_3, 0.2 g l^{-1} $CoCl_2·6H_2O$, 0.2 g l^{-1} $ZnSO_4·7H_2O$, 0.1 g l^{-1} $MnCl_2·4H_2O$, 0.03 g l^{-1} $Na_2MoO_4·H_2O$, 0.03 g l^{-1} $NiCl_4·6H_2O$ and 0.01 g l^{-1} $CuCl_2·2H_2O$). Then, 10 mM phenylacetonitrile was added in the sterilized mineral base medium (pH 7) as the sole source of nitrogen. To prepare inoculums, the organism was grown aerobically in 50 ml of the sterile medium in a 250-ml Erlenmeyer flask for 36 h at 45 °C and 200 rpm in an incubator shaker. The inoculums (500 μl) was added into 100 ml of the same medium in a 500-ml Erlenmeyer flask and incubated under similar conditions. The bacterial cells were harvested at mid-exponential phase of growth ($OD_{600=}$ 0.6–0.9) by centrifugation at 10,000×g for 12 min at 4 °C and washed twice with 100 mM phosphate buffer (pH 7.0). Bacterial cells were suspended in the same buffer and referred to as resting cells.

Acyltransferase activity assay

The acyltransferase activity assay was performed in a reaction mixture (1 ml) having the following composition: 400 μl of nicotinamide solution (250 mM in 100 mM phosphate buffer, pH 7), 500 μl of hydroxylamine-HCl solution (1,000 mM in distilled water, freshly neutralized with 10.0 N NaOH) and 100 μl of resting cells (0.5 mg_{DCW} ml^{-1}) in phosphate buffer (100 mM, pH 7). After incubation of the reaction mixture at 45 °C for 10 min, the reaction was stopped by adding 1 ml of acidic $FeCl_3$ solution (356 mM $FeCl_3$ in 0.65 N HCl) in 500 μl of reaction mixture. The mixture was centrifuged at 10,000×g for 8 min, and the clear supernatant was collected for spectroscopic estimation of nicotinic acid hydroxamate at $\lambda = 500$ nm. Reaction mixture without resting cells was also tested for any possible spontaneous chemical synthesis of NAH. One unit of acyltransferase activity was defined as that amount of resting cells (mg dry cell = mg_{DCW}) that catalyzed the formation of 1 μmol of nicotinic acid hydroxamate in 1 min under the assay conditions.

Amide hydrolase activity assay

The amide hydrolase activity assay was performed in a reaction mixture (1 ml) of the following composition: 800 μl of nicotinamide solution (125 mM in 50 mM phosphate buffer, pH 7), 100 μl of 50 mM phosphate buffer, and 100 μl of resting cells (0.5 mg_{DCW} ml^{-1}) in phosphate buffer (50 mM, pH 7). The control reaction was conducted in the absence of enzyme. After incubation of the reaction mixture at 45 °C for 10 min, the reaction was terminated by the addition of 10 μl of 1 N HCl and cells were removed by centrifugation at 10,000×g for 8 min. Supernatant was collected for HPLC analysis. One unit of amide hydrolase activity was defined as the amount of resting cells (mg_{DCW}) that catalyzed the formation of 1 μmol of nicotinic acid in 1 min under the assay conditions.

Hydroxamic acid hydrolase activity assay

The hydroxamic acid hydrolase activity assay was performed in a reaction mixture (1 ml) of the following composition: 800 μl of nicotinic acid hydroxamate solution (125 mM in 50 mM phosphate buffer, pH 7), 100 μl of 50 mM phosphate buffer, and 100 μl of resting cell (0.5 mg_{DCW} ml^{-1}) in phosphate buffer (50 mM, pH 7). Control reaction was conducted in the absence of enzymes. After incubation of the reaction mixture at 45 °C for 10 min, the reaction was terminated by the addition of 10 μl of 1 N HCl and cells were removed by centrifugation at 10,000×g for 8 min. Supernatant was collected for HPLC analysis. One unit of hydroxamic acid hydrolase activity was defined as the amount of resting cells (mg_{DCW}) that catalyzed the formation of 1 μmol of nicotinic acid in 1 min under the assay conditions.

Analytical methods

The nicotinic acid hydroxamate (NAH) formed was quantified spectrophotometrically by determining the absorbance of red brown complexes of hydroxamic acid with Fe(III) [7]. The molar extinction coefficient of the nicotinic acid hydroxamate/Fe(III) complex was determined at $\lambda = 500$ nm. A solution of NAH was prepared in the concentration range of 0.5–4.0 mM in phosphate buffer (50 mM, pH 7) and 1 ml of freshly prepared $FeCl_3$ solution was added into each sample. Absorbance was read at $\lambda = 500$ nm and a standard curve was plotted to obtain the mean extinction coefficient value ($\in m$). To validate the spectrophotometer method of nicotinic acid hydroxamate determination, HPLC analysis of NAH was also carried out with three samples (Supplementary Table S1). HPLC analysis of nicotinic acid hydroxamate, nicotinic acid and nicotinamide were done by Varian Prostar HPLC with waters spherisorb® 10 μm ODS2, 4.6 × 250 mm analytical column. The analysis was carried out at a flow rate of 1.0 ml/min at 210 nm using 10 % (v/v) acetonitrile in milliQ water adjusted to pH 2.8 by H_2SO_4 as mobile phase. Then, 20 μl of the sample was injected.

Identification of 6b2 isolate

The identification of the strain was carried out on the basis of the biochemical characteristics and nucleotide sequence analysis of amplified 16S rDNA. Carbon substrate utilization by isolate was determined using the standardized Biolog Phenotype GEN III plates. After incubation at 37 °C for 24 h, the microplate was read on a Biolog system (MicroStation System/MicroLog, Hayward, CA, USA). For the 16S rDNA sequence analysis, extraction of the chromosomal DNA was done by DNA extraction kit (Himedia). The 16S rDNA was amplified by polymerase chain reaction with the universal primers pair p16s-8 (5′-AGAGTT TGATCCTGGCTCAG-3′) and p16S-1541 (5′-AAGGA GGTGATCCA GCCGCA-3′) in a thermal cycler under the following conditions: 5 min at 95 °C, 30 cycles of 40 s at 95 °C, 1 min at 55 °C, 2 min at 72 °C, and a final extension was performed for 10 min at 72 °C. The PCR product was analyzed on 0.8 % agarose gel and size of the amplified fragment was approximately 1.4 kb. The amplified PCR product was sequenced by Ocimum Biosolutions (Hyderabad, India). The sequence obtained was compared with sequences in GenBank databases using the BLAST

program to determine phylogenetic position. Multiple alignments of sequences were done by CLUSTALW software. The construction of phylogenetic tree by the neighbor-joining method and a bootstrap analysis to evaluate the tree topology were performed by Phylip version 3.69.

Thermal stability profiles of three activities of *Bacillus smithii* strain IITR6b2

Thermal stability profile of acyltransferase, amide hydrolase, and hydroxamic acid hydrolase activities of whole-cell enzyme were determined at 45 °C. Whole-cell suspension (0.5 mg_{DCW} ml^{-1}) in phosphate buffer (100 mM, pH 7) was incubated at 45 °C. Samples were withdrawn at regular intervals of time to determine the residual activities.

Determination of effects of temperature and pH on acyltransferase activity

The effect of temperature on acyltransferase activity was determined for a temperature range of 25–70 °C under standard assay conditions (100 mM nicotinamide, 500 mM hydroxylamine-HCl, 50 mM phosphate buffer, pH 7) for 10 min. The optimum pH for the reaction was determined for a pH range of (4.0–10) in the following buffers (50 mM): acetate buffer (pH 4.0–5.8), potassium phosphate buffer (pH 5.8–8.0), borate buffer (pH 8.0–9.2), and sodium carbonate buffer (pH 9.2–10.0) under standard assay conditions. Reactions were performed at 55 °C for 10 min.

Optimization of substrate and co-substrate concentrations

This experiment was performed in such a manner that the concentration of nicotinamide was varied from 50–1,000 mM at different hydroxylamine-HCl concentrations ranging from 100–1,000 mM in the reaction mixture. A total of 55 combinations were used to determine the optimum concentration with highest acyltransferase activity.

Thermal stability of acyltransferase of *Bacillus smithii* strain IITR6b2

Thermal stability of whole-cell enzyme with acyltransferase activity was determined at 30, 45, and 55 °C. Whole-cell suspensions in phosphate buffer (100 mM, pH 7) were incubated at 30, 45, and 55 °C. Samples were withdrawn at regular intervals of time to determine the residual acyltransferase activity.

Fed-batch biotransformation at 50-ml scale

Fed-batch biotransformation was carried out in a 250-ml Erlenmeyer flask containing 50 ml of reaction mixture with initial nicotinamide and hydroxylamine-HCl concentrations of 200 and 250 mM, respectively, and 0.7 mg_{DCW} ml^{-1} cells in phosphate buffer (50 mM, pH 7) at 30 °C. Powdered nicotinamide (1.22 g) and highly concentrated solution (2 ml, 5 M, pH 7) of hydroxylamine-HCl were fed in two subsequent feeds after 40 and 80 min to maintain the residual nicotinamide and hydroxylamine concentrations around 200 and 250 mM, respectively. A total of 500 µl of sample was withdrawn at every 10 min during the reaction and monitored for nicotinic acid hydroxamate, nicotinic acid, and nicotinamide concentrations. Effort was made to maintain the reaction volume constant at around 50 ml. A control experiment was also conducted with the same parameters without enzyme for any spontaneous chemical reaction.

Effect of nicotinic acid hydroxamate concentration on acyltransferase activity

The effect of NAH on acyltransferase activity was determined for an NAH concentration range of 0–500 mM under assay conditions (200 mM nicotinamide, 250 mM hydroxylamine-HCl, 50 mM phosphate buffer, pH 7, 45 °C, cell concentration—0.7 mg $_{DCW}$ ml^{-1}).

Results and discussion

Determination of molar extinction coefficient for nicotinic acid hydroxamate/Fe(III) complex

Molar extinction coefficient ($\in_M$) of the NAH/Fe(III) complex was determined to quantify NAH formed in the acyltransferase reaction. The $\in_M$ value of 4.14×10^2 l mol^{-1} cm^{-1} was obtained from the slope of a standard curve (Supplementary Fig. S1). Fournand et al. [7] determined the $\in_M$ values of aliphatic saturated, aliphatic unsaturated and α, β, γ-amino hydroxamic acids but this is the first report on determination of molar extinction coefficient ($\in_M$) for heterocyclic class of hydroxamic acids. Comparison of both spectrophotometer and HPLC methods for nicotinic acid hydroxamate determination was also carried out and analyzed statistically (Supplementary Fig. S2 and Supplementary Table S1). It was observed that both methods are comparable for determination of nicotinic acid hydroxamate concentration in reaction mixture.
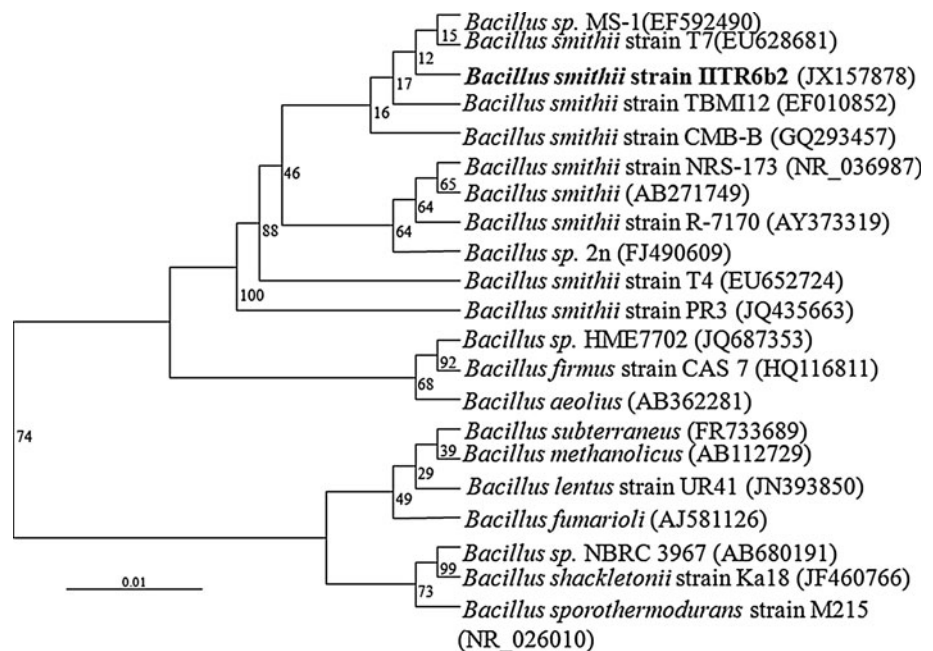
Screening of bacterial isolate for nicotinic acid hydroxamate synthesis

Various previously isolated nitrile metabolizing strains containing amidase were used in the present study. The screening for acyltransferase activity of these isolates was carried out with nicotinamide. Among these isolates, strain 6b2 was selected for further studies because it posses 28-fold higher acyltransferase activity as compared to amide hydrolase activity while the mole ratio of product (NAH) and by-product (NA) formed was 28.4 (data not shown).

Identification of 6b2 isolate

A biochemical analysis of strain 6b2 was carried out using the Biolog system to determine its carbon substrate utilization profile. According to the data (data not shown) from the Biolog system, the strain was identified as the *Bacillus* genus. Further 16S rDNA sequence of the isolate 6b2 was also determined. Based on the partial 16S rDNA sequence of strain 6b2 and 20 similar strains, a phylogenetic tree was constructed by the neighbor-joining method (Fig. 2). The sequence was deposited in the GenBank database with the accession no. JX157878. Comparative 16S rDNA sequence analysis suggested a strong similarity between strain 6b2 and the *Bacillus smithii* strains. Strain 6b2 showed 99 % similarity with the *Bacillus smithii* strain TBMI12 (GenBank accession no. EF010852) and *Bacillus smithii* strain T7 (GenBank accession no. EU628681). Based on biochemical characteristics and 16S rDNA sequence analysis, the strain was identified as *Bacillus smithii* and referred to as *Bacillus smithii* strain IITR6b2.

Bioprocess development for nicotinic acid hydroxamate synthesis

Enzymatic reactions during nicotinic acid hydroxamate synthesis

The amidase from *Rhodococcus* sp. strain R312 catalyzed amide hydrolysis, amide acyltransferase, acid acyltransferase, and hydroxamic acid hydrolase reactions [6]. It was suggested that during amidase-catalyzed hydroxamic acid synthesis, hydroxamic acid can be formed by transfer of acyl groups of both amide and acid to hydroxylamine. At the same time by-product (acid) can also be synthesized by hydrolysis of amide and hydroxamic acid. Whole-cell biocatalyst of *Bacillus smithii* strain IITR6b2 was found to possess amide acyltransferase, amide hydrolase, and hydroxamic acid hydrolase activities but not acid acyltransferase activity (Table 1). Amide acyltransferase activity was 28- and 12.3-fold higher as compared to amide hydrolase and hydroxamic acid hydrolase activities, respectively. Although whole cells exhibited all of these activities, to confirm that these reactions are mediated by amidase enzyme system of the strain, a known amidase inhibitor (Di-ethyl phosphoramidate) was used at 1-, 5-, and 10-mM concentrations. The results (Table 1) indicate that the extent of inhibition of whole-cell acyltransferase and hydrolase activities were nearly similar at different concentrations of inhibitor. Thus, the inhibition profile of these activities suggested that amidase enzymes of *Bacillus smithii* strain IITR6b2 possess amide acyltransferase, amide hydrolase, and hydroxamic acid hydrolase activities. Further confirmation was carried out by performing the

Fig. 2 Phylogenetic tree for *Bacillus smithii* strain IITR6b2 and related strains based on the 16S rDNA sequences. *Numbers* after the name of organisms are accession numbers of published sequences. Bootstrap values are based on 1,000 iterations

Table 1 Comparison of *Bacillus smithii* strain IITR6b2-catalyzed reactions and effect of amidase inhibitor on them

Reactions catalyzed	Enzyme activity (U mg$_{DCW}^{-1}$)	Inhibitor[a] (residual enzyme activity %)		
		Di-ethyl phosphoramidate		
		1 mM	5 mM	10 mM
Amide acyltransferase	16.1	79.5	46.6	41.3
Amide hydrolase	0.57	72	41	36.1
Hydroxamic acid hydrolase	1.3	66.2	38.6	27.7
Acid acyltransferase	ND			

[a] Reaction conditions: Inhibitor was preincubated with whole-cell suspensions at 30 °C for 30 min and then whole cells were tested for residual enzyme activity

ND not detected

thermal stability study of these three activities at identical conditions (pH 7 and 45 °C). From the results (Supplementary Fig. S3), it can be observed that all three activities followed similar deactivation profiles. Thus, it confirmed that these three activities belong to the same protein. The advantage of using this whole cell of *Bacillus smithii* strain IITR6b2 is that hydrolase activities were significantly lower than acyltransferase activity, hence synthesis of by-product, nicotinic acid is limited.

Effects of temperature and pH on acyltransferase activity

Acyltransferase activity increased with increasing reaction temperature from 25 to 55 °C and showed maximum activity at 55 °C with marginally lower activities at 45 and 50 °C (Supplementary Fig. S4). At 25 and 70 °C, acyltransferase activities reduced to 63.8 and 62 % of its maximum activity, respectively. *Rhodococcus* sp. N-771 amidase also showed an optimum temperature of 55 °C but above 60 °C, its activity decreased sharply [19]. The effect of initial reaction pH on the acyltransferase activity was studied for a pH range of (4.0–10). These results indicate that pH considerably affected the acyltransferase activity with optimum at pH 7. Nearly 52 % of the maximum activity was retained at pH 5.8 and 10. Most of the reported amidases in the literature are active at neutral or alkaline pH. Amidases of *Rhodococcus* sp. R312 [4] and *Geobacillus pallidus* [17] are reported to have optimum activity at pH 7.

Optimization of substrate and co-substrate concentrations for acyltransferase activity

In a bi-substrate reaction, mole ratio of reactants (substrate/co-substrate) is one of the most effective parameters for desired higher molar conversion and better yield. To get the optimum mole ratio of substrates (nicotinamide and hydroxylamine-HCl), reactions were needed to be performed with several possible ratios of amide and
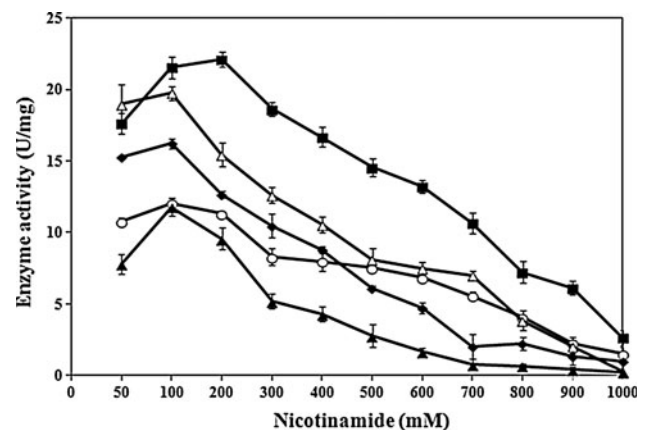

Fig. 3 Optimization of substrate/co-substrate concentration. Hydroxylamine-HCl concentration: 100 mM (*open circles*), 250 mM (*filled squares*), 500 mM (*open triangles*), 750 mM (*filled diamonds*) and 1,000 mM (*filled triangles*). The data are mean ± SD, where $n = 3$

hydroxylamine-HCl. Initially, acyltransferase activity of whole cells increased with an increase in nicotinamide concentration and thereafter it continuously decreased with amide concentration for all hydroxylamine-HCl concentrations studied. The highest acyltransferase activity was obtained with 250 mM hydroxylamine-HCl and 200 mM nicotinamide followed by 500 mM hydroxylamine-HCl and 100 mM nicotinamide. It was found that increased concentration of hydroxylamine-HCl was also inhibitory to the enzyme activity (Fig. 3). At the optimum concentration of 200/250 mM, nicotinic acid production was also reduced (33 %) (Supplementary Fig. S5). Fournand et al. [4] reported that an increase in concentration of hydroxylamine-HCl reduced the undesirable hydrolysis of amide. From these results, 200 mM of nicotinamide and 250 mM of hydroxylamine-HCl was chosen for further work.

Thermal stability of acyltransferase of Bacillus smithii strain IITR6b2

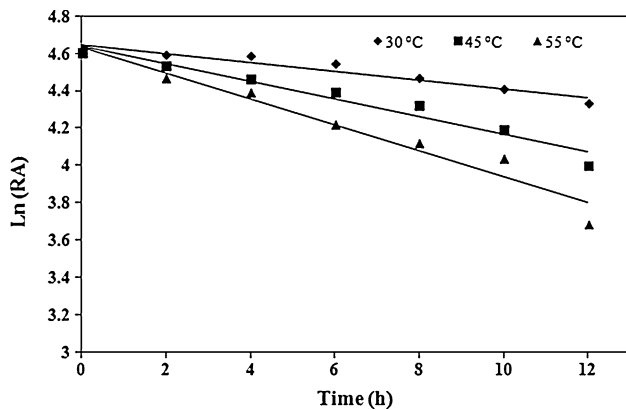Thermal stability of whole-cell enzyme with acyltransferase activity was determined at 30, 45, and 55 °C. The

Fig. 4 Thermostability profile of acyltransferase activity of *Bacillus smithii* strain IIT6b2. 30 °C (*filled diamonds*), 45 °C (*filled squares*) and 55 °C (*filled triangles*). The data are mean ± SD, where $n = 3$

Fig. 5 Effect of reaction temperature on nicotinic acid hydroxamate synthesis. Reaction conditions: 25 ml phosphate buffer (50 mM, pH 7), nicotinamide (200 mM), hydroxylamine-HCl (250 mM), 0.5 mg_{DCW} ml^{-1} resting cells, temperatures (30, 45, and 55 °C). Nicotinic acid hydroxamate (mM) at 30 °C (*filled circles*), 45 °C (*filled triangles*), and 55 °C (*filled squares*), nicotinic acid (mM) at 30 °C (*open circles*), 45 °C (*open triangles*), and 55 °C (*open squares*). The data are mean ± SD, where $n = 3$

natural logarithm of residual acyltransferase (lnRA) activity was plotted against time (Fig. 4). The half-lives of whole-cell enzyme at specific temperatures were determined. The results indicated that the whole-cell enzyme was relatively stable at 30 °C with a half-life of 29 h and at 45 and 55 °C half-lives were 14 and 10 h, respectively.

Time course of bioconversions at different temperatures

It was important to decide the operating temperature for bioconversion by simultaneously considering the optimum temperature (55 °C) and stability of enzyme. Time course of bioconversions at 30, 45, and 55 °C were investigated for 90 min to choose the optimum temperature of bioprocess for nicotinic acid hydroxamate synthesis. From the results (Fig. 5) it can be observed that initial conversion of substrate to product was higher at 55 °C. However, after 30 min, the concentration of product was higher at 30 °C. It was also observed that at 45 and 55 °C, the concentration of product decreased after 30 min whereas at 30 °C, the product concentration decreased after 40 min. The rate of decrease of product concentration with time was higher at 55 °C as compared to 30 °C. To explain this discrepancy in conversion at later stage, the by-product (nicotinic acid) concentration was also determined at these two temperatures. It was observed that the by-product (nicotinic acid) formation was 3.4 times higher at 55 °C as compared to 30 °C. Thus, this lower conversion of substrate to NAH at 55 °C was due to the higher by-product formation. Pandey et al. [19] also reported a decrease in concentration of acetohydroxamic acid with time at all temperatures after achieving maximum conversion. This study suggests that better thermal stability and higher conversion (90.4 %) of nicotinamide to nicotinic acid hydroxamate along with only 1.4 % conversion to by-product at 30 °C made it more suitable to choose 30 °C as the operating temperature. A
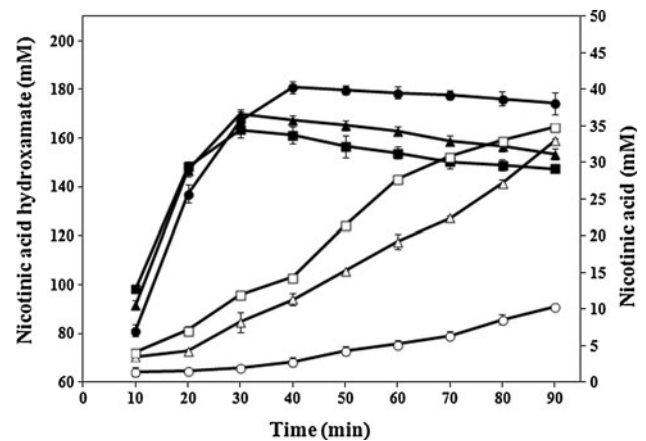
similar trend of higher by-product formation was also observed at 45 °C.

Effect of resting cells concentration

The nicotinamide bioconversion to nicotinic acid hydroxamate in the reaction mixture containing 200/250 mM substrate/co-substrate at 30 °C with different cell concentrations are shown in Fig. 6. Molar conversion of nicotinamide to NAH increased with increase in resting cells concentration from 0.3 to 0.7 mg_{DCW} ml^{-1} due to availability of more acyl-enzyme complex. However, at 0.9 mg_{DCW} ml^{-1} concentration, maximum conversion was 96.3 %, which is marginally higher than the 94.5 % conversion achieved at 0.7 mg_{DCW} ml^{-1} in 40 min. This illustrates that the additional cells did not improve the conversion significantly. This may be due to mass transfer limitations or lower substrate-to-biocatalyst ratio. To reduce both cost and possibility of hydroxamic acid hydrolysis (due to higher enzyme availability) during the biotransformation process, 0.7 mg_{DCW} ml^{-1} resting cells with 200/250 mM initial substrates (nicotinamide and hydroxylamine-HCl) concentration at 30 °C was used for fed-batch reaction.

Fed-batch process development at 50-ml scale

Substrate inhibition is a common problem in enzyme-mediated processes that can be partially overcome by the fed-batch method [2, 21]. As it was observed that higher initial concentration of the substrate/co-substrate
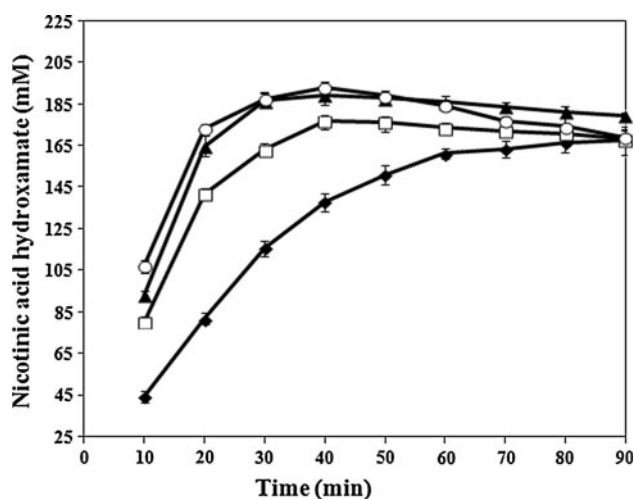
Fig. 6 Effect of resting cell (DCW) concentration on biocatalytic synthesis of nicotinic acid hydroxamate. Reaction conditions: 25 ml phosphate buffer (50 mM, pH 7), 30 °C, nicotinamide (200 mM), hydroxylamine-HCl (250 mM), cell concentration—0.3 mg ml^{-1} (*filled diamonds*), 0.5 mg ml^{-1} (*open squares*), 0.7 mg ml^{-1} (*filled triangles*), 0.9 mg ml^{-1} (*open circles*). The data are mean ± SD, where $n = 3$

(nicotinamide and hydroxylamine-HCl) had inhibitory effects on molar conversion, a fed-batch process was developed. The investigation of biotransformation performance of whole cells of *Bacillus smithii* strain IITR6b2 showed that when the reaction pH, concentration of substrate/co-substrate, temperature, and resting cells concentrations were 7, 200/250 mM, 30 °C, and 0.7 mg$_{DCW}$ ml^{-1}, respectively, excellent molar bioconversion yield (94.5 %) was achieved within 40 min. Hence, the optimum feeding strategy for production of NAH was pulse feedings of nicotinamide and hydroxylamine-HCl (200/200 mM) at 40-min intervals. It was predicted (from the nicotinamide-consumption profile) that in the first 40 min, nearly 50 mM of hydroxylamine-HCl remain unutilized, hence only 200 mM of it was fed during the subsequent addition of substrates. Periodic analysis of the reaction mixture revealed that after two feedings of substrates (200/200 mM), an accumulation of 536 mM of nicotinic acid hydroxamate was obtained in 120 min (Fig. 7a). Further addition of a third feeding resulted in no significant enhancement of NAH production (data not shown).

This process resulted in 89.4 % molar conversion of 600 mM nicotinamide to NAH in 120 min at a production rate of 52.9 gh^{-1} g$_{DCW}^{-1}$ with 4.5 % undesirable by-product, nicotinic acid. During this whole process, a 36.5 % decrease in resting cells concentration (measured by optical density at 600 nm) due to lysis and 40.6 % loss in acyltransferase activity was observed. The effect of NAH concentration on acyltransferase activity was also studied (Fig. 7b) and a 76.4 % decrease in
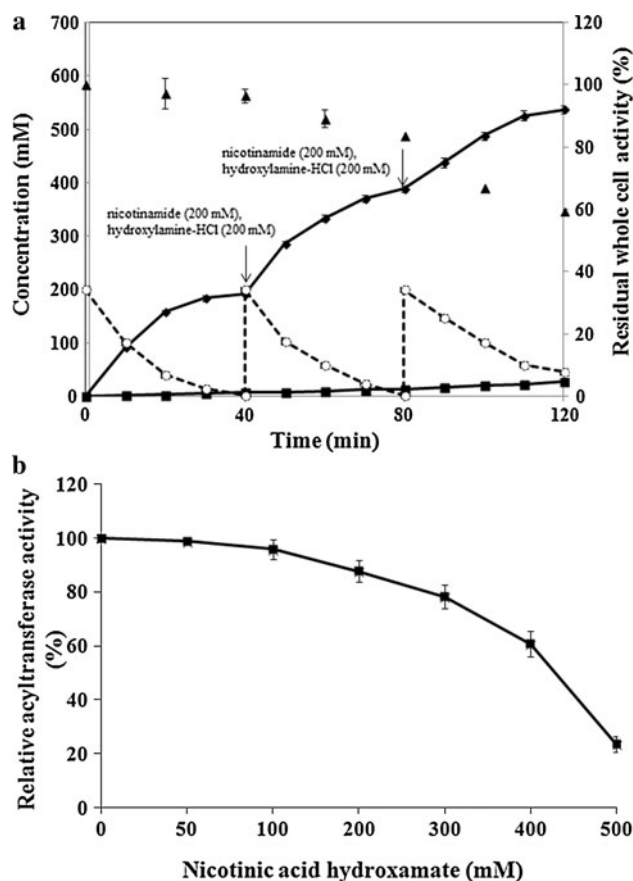

Fig. 7 **a** Time course of production of nicotinic acid hydroxamate during fed-batch biotransformation process (*down arrow* feeding of nicotinamide and hydroxylamine-HCl). Reaction conditions: 50 ml phosphate buffer (50 mM, pH 7), 30 °C, resting cells concentration (0.7 mg$_{DCW}$ ml^{-1}). Nicotinic acid hydroxamate (mM) (*filled diamonds*), nicotinic acid (mM) (*filled squares*), and nicotinamide (mM) (*open circles*), residual whole-cell activity (%) (*filled triangles*). The data are mean ± SD, where $n = 3$. **b** Effect of nicotinic acid hydroxamate concentration on acyltransferase activity. Reaction conditions: nicotinamide (200 mM), hydroxylamine-HCl (250 mM), cell concentration—0.7 mg$_{DCW}$ ml^{-1}, 55 °C, phosphate buffer pH 7 (50 mM). The data are mean ± SD, where $n = 3$

acyltransferase activity was observed at 500 mM concentration of NAH. Thus, this confirms that product inhibition along with cell lysis and low enzyme stability are responsible for no further conversion of substrate beyond the second feeding of substrates. This is the first report on biocatalytic synthesis of any hydroxamic acid at this molar concentration with high conversion rate. Previously, Fournand et al. [5], used amidase from *Rhodococcus* sp. R312, immobilized on Duolite A-378 resin for bench-scale production of acetohydroxamic acid and achieved only a 55–61 % (mol mol^{-1}) conversion while Pandey et al. [20] reported an improved bioprocess for acetohydroxamic acid synthesis using DTT-treated resting cell of *Bacillus* sp. ABP-6 and observed a molar conversion of 93 % of acetamide (300 mM) to

acetohydroxamic acid. The productivity obtained in the present study was significantly higher than the previously reported productivity for acetohydroxamic acid and benzohydroxamic acid synthesis [1–3].

Recovery of nicotinic acid hydroxamate

After completion of the reaction, the reaction mixture was centrifuged ($10{,}000 \times g$ for 20 min) to separate the cells. The supernatant obtained was freeze-dried to recover 6.4 g of pinkish white powder. HPLC analysis of this powder was carried out to determine nicotinic acid hydroxamate and nicotinic acid concentrations. It was found that powder contains 58.5 % (w/w) NAH and 2.6 % nicotinic acid. The product was further purified by the extraction of 200 mg of powder with 5 ml of acidified acetone (with 11.5 N HCl). The extract after filtration and evaporation yielded 121 mg of viscous liquid. The HPLC analysis of this liquid confirms the presence of nicotinic acid hydroxamate and purity level of 95 % was obtained.

Conclusions

An efficient biotransformation process for NAH production has been developed where high molar conversion with limited side reaction was achieved in a short period of time. This is the first report that describes the process development for the synthesis of nicotinic acid hydroxamate from nicotinamide using acyltransferase activity of *Bacillus smithii* strain IITR6b2. The starting material of this bioprocess is nicotinamide, which is cheaper than acid and ester of nicotinic acid, which are commonly used in chemical methods for NAH synthesis. This reaction also takes place in aqueous medium leading to reduced cost of bioprocess. Since the whole cells exhibited a high ratio of acyltransferase-to-hydrolase activities, a molar conversion (89.4 %) of nicotinamide (600 mM) to nicotinic acid hydroxamate (536 mM) was achieved in 120 min with only 4.5 % by-product, nicotinic acid. During this fed-batch process, after two subsequent feedings, further conversion to product is almost stopped, which is probably due to cell lysis, low enzyme stability, and product inhibition of acyltransferase activity. This process can be extended beyond second feedings by using an immobilized reactor along with simultaneous removal of product. The data presented here suggest that the isolate *Bacillus smithii* strain IITR6b2 has potential for commercial production of heterocyclic class of hydroxamic acids.

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