Biotransformation of Acetamide to Acetohydroxamic Acid at Bench Scale Using Acyl Transferase Activity of Amidase of *Geobacillus pallidus* BTP-5x MTCC 9225

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Abstract The bioprocess employing acyl transferase activity of intracellular amidase of *Geobacillus pallidus* BTP-5x MTCC 9225 was harnessed for the synthesis of pharmaceutically important acetohydroxamic acid. *G. pallidus* BTP-5x exhibited highest acyl transferase activity with acetamide: hydroxylamine in ratio of 1:5 in 0.1 M NaH₂PO₄/Na₂HPO₄ buffer (pH 7.5) at 65°C. In one liter fed-batch reaction containing 1:5 ratio of two substrates total of eight feedings of 0.05 M/20 min of acetamide were made and it was found that maximum acetohydroxamic production was achieved at 3:5 ratios of substrate and cosubstrate. In 1 l bench scale batch reaction containing 0.3 M acetamide, 0.5 M hydroxylamine in 0.1 M NaH₂PO₄/Na₂HPO₄ buffer (pH 7.5, 50°C, 400 rpm) and 0.5 mg/ml (dry cell weight) of whole cells of *G. pallidus* BTP-5x (as biocatalyst) resulted in an yield of 0.28 M of acetohydroxamic acid after 20 min reaction time at 50°C. The acetamide bioconversion rate was 90–95% (mol mol⁻¹) and 51 g powder containing 40% (w/w) acetohydroxamic acid was recovered after lyophilization.

Keywords *Geobacillus pallidus* BTP-5x MTCC 9225 · Acetohydroxamic acid · Thermophilic amidase · Acyl transferase activity · Hydroxamic acid

Introduction

Hydroxamic acids are weak organic acids of general formula R-CO-NHOH and find applications in biology and medicine. They are key pharmacophore in chemotherapeutic agents and possess a wide spectrum of activities as growth factors, food additives, tumor inhibitors, and antimicrobial agents, anti-tuberculosis and antileukemic agents [1]. In nature, microbes exploit the chelating properties of hydroxamic acids to obtain iron especially in iron-deficient environments. Acetohydroxamic acid is a potent and irreversible inhibitor of bacterial and plant urease and also used as adjunctive therapy in chronic urinary infection [2]. It selectively inhibits arachidonate 5-lipoxygenase and thus has potential use in the treatment of asthma [3]. Gao et al. [4] have reported anti-HIV activity of aminohydroxamic acid and acetohydroxamic acids.

Hydroxamic acids are either chemically synthesized through Angeli-Rimini reaction or Lossen rearrangement or by the method of hydrochloric hydroxylamine reaction with acetic ether in alcohol water medium [5] or enzymatically (Fig. 1) using acyl transferase activity of amidases. Four-nand et al. [6] used immobilized *Rhodococcus* sp. R312 for bench scale production of acetohydroxamic acid and only 55–60% of conversion was achieved. In recent years Fourier transform infrared (FTIR) spectroscopy was used as a simple and rapid real-time assay for detection of acetohydroxamic synthesis or acetamide conversion amidase catalysed reactions catalysed by a recombinant amidase (EC 3.5.1.4) from *Pseudomonas aeruginosa* [7]. Pacheco et al. [8] studied the acyl transferase activity of amidase in non-conventional media in reversed micellar system composed of the cationic surfactant tetradecyltrimethyl ammonium bromide (TTAB) in heptane/octanol (80/20%) for production of hydroxamic acids. Commercialisation of acetohydroxamic

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Fig. 1 Enzymatic synthesis of hydroxamic acid

acid production could not be achieved because of lower yield i.e. 50–60% and presence of high concentration of hydroxylamine.

In the present paper the optimization of process parameters for bench scale production of acetohydroxamic acid is described using acyl transferase activity of amidase of *G. pallidus* BTP-5x which resulted in increased product yield at bench scale in short duration of time.

Materials and Methods

All the amides were from Lancaster, England. The medium components were procured from HiMedia, Mumbai (India). All other reagents were of analytical/HPLC grade from the commercial vendors.

Source of Microorganism and Culture Conditions

Geobacillus pallidus BTP-5x MTCC 9225 was isolated from Tatapani thermal spring (Himachal Pradesh, India). It was cultured in the medium containing (g/L) peptone 12.5 g; yeast extract 3 g; beef extract 5 g and NaCl 5 g [9] under the following culture conditions: 160 rpm shaking; 1.5% (v/v) of preculture, 0.3% (v/v) formamide (inducer), 55°C, pH 7.0. Cells were harvested after 20 h by centrifugation (10,000 × g at 4°C for 10 min). Cell pellet was washed in 0.1 M NaH₂PO₄/Na₂HPO₄ buffer (pH 7.0) and resuspended in the same buffer.

Acyl Transferase Assay

The acyl transferase activity was assayed (if not otherwise mentioned) in 1 ml reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5) using 50 µl cell suspension and 0.1 M acetamide (2 M acetamide stock freshly neutralized with 10 N NaOH) as substrate and 0.2 M hydroxylamine (4 M hydroxylamine stock freshly neutralized with 10 N NaOH) as co-substrate incubated at 50°C for 5 min. The resulting hydroxamic acid was assayed using the method developed by Brammar and Clarke [10], based on colorimetric determination of the

red-brown complexes with Fe(III). The reaction was stopped by adding 2 ml of iron chloride solution (21 ml FeCl₃, 27.5% w/v, 5.3 ml 12.5 M HCl, distilled water to 100 ml) and absorbance was measured at 500 nm and each reaction was catalysed in a set of triplicate. One unit of acyl transferase activity was defined as the amount of enzyme which catalysed the formation of one µ mol of acetohydroxamic acid/min/mg dcw under the assay conditions.

HPLC Analysis

The amount of acetohydroxamic acid produced in the reaction mixture was also determined as described by Fournand et al. using Perkin Elmer HPLC system equipped with C-18 Nucleosil reverse phase column (mm) at a flow rate of 1.0 ml/min, ambient temperature (20–25°C) and detection wavelength 210 nm with mobile phase 0.025 M orthophosphoric acid with 1% (v/v) methanol. To check the purity of the product, 2 g of the powder was resuspended in 20 ml acetone after acidification with 0.6 ml of 12.5 N HCl. The solution was filtered to remove salts and evaporation of the filtrate resulted in recovery of 0.8 g of residual slightly stained oil [6]. The injected sample volume was 5 µl. HPLC calibration curves were prepared each for acetamide (10–100 mM) and acetohydroxamic acid (0.1–1.0 mM).

Optimization of Reaction Conditions for Assay of Acyl Transferase Activity of *G. pallidus* BTP-5x MTCC 9225

To work out the optimum pH and temperature enzyme reactions were carried out at different pH range (4.0–10.5) in various buffer systems (citrate buffer, potassium phosphate buffer, sodium phosphate buffer, borax buffer, glycine NaOH buffer, tris HCl buffer and sodium carbonate buffer) and at different temperatures ranging from 30 to 70°C.

Effect of Substrate and/or Co-Substrate Concentration on Acyl Transferase Activity

The concentrations of amide/hydroxylamine were varied to study the effect of substrate and co-substrate concentration on acyltransferase activity of *G. pallidus* BTP-5x.

Operational Stability of the Acyl Transferase Activity at Various Temperatures

The operational stability of acyl transferase activity of *G. pallidus* BTP-5x was evaluated by following the time course of enzyme reaction at different temperatures (40, 50, 65°C) under the optimized reaction conditions using

0.11 mg resting cells (dry cell weight)/ml reaction volume and 0.1 M acetamide and 0.5 M hydroxylamine.

Fed Batch Reaction for Production of Acetohydroxamic Acid

To achieve maximum product formation, 0.1 M of substrate (acetamide) was added at zero min and eight feedings of 0.05 M of substrate were added after every 20 min under optimised reaction conditions (50°C, 0.18 mg dcw/ml reaction and 0.5 M hydroxylamine) and accumulation of acetohydroxamic acid during the reaction was assessed by the HPLC.

Batch Reaction for Production of Acetohydroxamic Acid

To obtain higher acetohydroxamic acid yield, the batch reaction was carried out in 0.1 M sodium phosphate buffer containing 0.3 M of substrate (acetamide) and 0.5 M of co-substrate (hydroxylamine) under the optimized reaction conditions (50°C, pH 7.5, resting cells 0.18 mg dcw/ml reaction volume). To investigate optimum concentration of biocatalyst for maximum conversion of substrate to product, different amounts of resting cells (0.1–0.5 mg dcw/ml reaction volume) were used in the reaction and the acetohydroxamic acid accumulation was analyzed by HPLC.

Bench Scale Production of Acetohydroxamic Acid

On the basis of information of the preceding experiments, the conversion of acetamide to acetohydroxamic acid was

scaled up to 1 l and carried out in a BioFlow C-32 fermenter (New Brunswick Scientific, U.S.A.). The reaction mixture comprised 0.3 M acetamide, 0.5 M of hydroxylamine, 0.5 g resting cells (dcw) of *G. pallidus* BTP-5x in 0.1 M sodium phosphate buffer, pH 7.5 at 50°C. Cells were separated from the reaction mixture by centrifugation at $10,000 \times g$ for 10 min at 30°C. Supernatant was lyophilized (Flexi-Dry MP™, FTS USA) and white powder was recovered and analyzed by HPLC.

Results

Optimization of Reaction Condition for Assay of Acyl Transferase Activity

The amidase of *G. pallidus* BTP-5x exhibited acyl transferring activity in broad pH range with its maximum at pH 7.5 in sodium phosphate buffer (Table 1) and at 65°C.

Effect of Substrate and/or Co-Substrate Concentration on Acyl Transferase Activity

Effect of substrate (acetamide) and cosubstrate (hydroxylamine) on acyl transferase activity of BTP-5x was studied by varying concentration acetamide (0.02–0.2 M) and keeping the concentration of hydroxylamine constant (0.5 M). There was an increase in activity with the increase in substrate (acetamide) concentration and maximum acetohydroxamic acid production was observed with 1:5 ratios of substrate and cosubstrate. Above this ratio a decrease in the acyl transferase activity was observed, which may be

Table 1 Effect of various buffer systems on acyltransferase activity of thermostable amidase of *G. pallidus* BTP-5x

Buffer pH	Specific activity (U/mg dcw)						
	Citrate buffer	Potassium phosphate buffer	Borate buffer	Tris–HCl buffer	Glycine NaOH buffer	Sodium phosphate buffer	Sodium carbonate buffer
4.0	3.228						
4.5	4.749						
5.0	4.815						
5.5	5.476						
6.0	6.534	6.984					
6.5		6.878					
7.0		6.971		7.5	7.672	8.386	
7.5		6.812	8.122	8.095	7.698	8.558	
8.0		7.116	7.897	7.989	8.016	8.161	
8.5			7.989				
9.0			7.91				
9.5							5.966
10.0							3.915
10.5							3.558

Fig. 2 Effect of acetamide concentration (20–200 mM) on acyl transferase activity of BTP-5x (hydroxylamine concentration was kept constant i.e. 500 mM)

due to substrate inhibition (Fig. 2). The kinetic constant for this set of experiment (up to 0.1 M concentration) was $K_{\text{Acetamide}} = 3.987 \text{ mM}$ and $V_{\text{max}} = 8.576 \mu\text{mol min}^{-1} \text{mg}^{-1}$ resting cells of *G. pallidus* BTP-5x.

In second set of experiment, keeping the concentration of the acetamide constant (0.1 M) the concentration of hydroxylamine was varied from 0.025 to 0.5 M. It was observed that with the increase in the concentration of hydroxylamine, acyl transferase activity of *G. pallidus* BTP-5x was also increased up to 0.5 M (Fig. 3). For this set of reaction the kinetic constant was $K_{\text{Hydroxylamine}} = 68.59 \text{ mM}$ and $V_{\text{max}} = 15.24 \mu\text{mol min}^{-1} \text{mg}^{-1}$ dwt resting cells of *G. pallidus* BTP-5x.

To obtain maximum acetohydroxamic acid production, acyl transferase activity with several combinations of amide (0.025–0.5 M) and hydroxylamine ratio (0.1, 0.3 and 0.5 M) were performed. The results showed that slight inhibition of acyl transfer activity occurred when the [amide]/[hydroxylamine] ratio was higher than 3:5 (Fig. 4).

Fig. 3 Effect of hydroxylamine concentration (25–500 mM) on acyltransferase activity of BTP-5x (acetamide concentration was kept constant i.e. 100 mM)

Fig. 4 Effect of relative amide excess on acyltransferase activity

This means that a relative excess of amide inhibited the acyl transfer reaction but this phenomenon was a function of the [amide]/[hydroxylamine] ratio.

Effect of Temperature on Time Course of Enzyme Reaction

The operational stability of acyl transferase was studied by preincubating resting cells of *G. pallidus* BTP-5x suspended in 0.1 M sodium phosphate buffer (pH 7.5) at 40, 50 and 65°C for 150 min (Fig. 5). At 40°C, a maximum production of 0.076 M of acetohydroxamic acid was achieved. At 50 and 65°C, 0.1 M acetamide was completely converted to 0.1 M acetohydroxamic acid after 60 and 50 min respectively.

Fed Batch Reaction

Fed batch reaction was designed with the aim to maximize the yield of acetohydroxamic acid. It was observed that when 0.1 M acetamide was used in 1:5 ratio with

Fig. 5 Time course of acyltransferase catalysed production of acetohydroxamic acid (AHA) at various temperatures

Fig. 6 Accumulation of acetohydroxamic acid in fed batch reaction design (↓ Feed of 50 mM acetamide)

hydroxylamine, complete conversion of acetamide to acetohydroxamic acid was obtained at 50 and 65°C after 60 and 50 min respectively. An excess amount of co-substrate was remained unutilized in the reaction. In order to maintain the substrate ratio; acetamide was fed after 20 min when the half of the substrate was converted into acetohydroxamic acid. The addition of acetamide substrate (acetamide) in fed batch mode (0.05 M after 20 min) showed that acetamide concentration up to 0.3 M has no inhibitory effect on acyl transferase activity of amidase of *G. pallidus* BTP-5x and an accumulation of 0.22 M of acetohydroxamic acid was achieved in 100 min with 5 feeding of acetamide (Fig. 6), and further feeding of acetamide in the reaction didn't raise the product accumulation rather it reverse the reaction. With further feeding of substrate, the product formed in the reaction started decreasing and after 8th feed (i.e. 0.5 M acetamide concentration) only 0.15 M of the acetohydroxamic acid was detected in the reaction due to amidase activity of the enzyme.

Batch Reaction for Production of Acetohydroxamic Acid

Acetamide bioconversion was studied with amidase of *G. pallidus* BTP-5x in batch reaction under optimized reaction conditions (50 ml; pH 7.5, 50°C, 0.3 M acetamide, 0.5 M hydroxylamine and 0.18 mg/ml biocatalyst concentration in 0.1 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer pH 7.5). A yield of 0.26 M of acetohydroxamic acid was achieved after 80 min (Fig. 7). The amount of resting cells in the reaction mixture was varied from 0.1 to 0.5 mg/ml (dry cell weigh basis). In the presence of 0.1 to 0.3 mg/ml concentration of biocatalyst, maximum conversion achieved was 0.20 M, 0.22 mmoles and 0.24 M respectively and after 20 min reverse reaction was observed at all the concentrations, but at 0.4 (mg/ml) and 0.5 (mg/ml) concentration of

Fig. 7 Acetohydroxamic acid accumulation in batch reaction

G. pallidus BTP-5x complete conversion of the acetamide to acetohydroxamic acid was observed in 20 min and the rate of reverse reaction was lower in 0.5 mg/ml concentration in comparison to the 0.4 mg/ml. Hence for bench scale production of acetohydroxamic acid, 0.5 mg/ml concentration of the biocatalyst was selected (Fig. 8).

Bench Scale Production of Acetohydroxamic Acid and its Downstream Processing

The bioconversion of acetamide to acetohydroxamic acid was scaled up to one litre and carried out in a BioFlow C-32 fermenter (New Brunswick Scientific, U.S.A.). The one litre reaction mixture comprised 0.3 M acetamide, 0.5 M hydroxylamine and 0.5 mg/ml resting cells (dcw) of *G. pallidus* BTP-5x in 0.1 M sodium phosphate buffer, pH 7.5 and reaction was performed at 50°C for 20 min. The cells were separated from reaction mixture by centrifugation at $10,000 \times g$ for 10 min at 4°C. Freeze drying of the supernatant yielded 51 g white powder. The extraction of the 2 g of this powder with acidified acetone vis-à-vis

Fig. 8 Effect of biocatalyst concentration on acetohydroxamic acid production

filtration and evaporation of the extract yielded 0.8 g viscous liquid. The HPLC analysis of the latter revealed the presence of acetohydroxamic acid. The colorimetric assay of acetohydroxamic acid exhibited the 40% (wt/wt) acetohydroxamic acid in the powder. The HPLC analysis showed the yield of acetohydroxamic acid was 0.28 M (i.e. 95%).

Discussion

Most of the amidases so far reported possess acyl transferase activity and this exhibit diversity for substrate specificity. Like other purified and immobilized amidases, *G. pallidus* BTP-5x MTCC 9225 exhibit maximum acyl transferase activity at neutral pH [11–16]. The immobilized purified amidase of *Rhodococcus* sp. R312 exhibited maximum acyl transferase activity at pH 7.0 with neutral amides as substrate and at pH 8.0 with α -amino amides because this pH was close both to the pKa values (about pH 8–9) and the optimum pH value (pH 7) [11]. *G. pallidus* RAPc8 amidase immobilized in Eupergit C beads displayed wide pH optima and maximum acyl transferase activity was reported at pH 7.0 [17].

Fournand et al. [16] reported maximum acyl transferase activity in sodium phosphate buffer for *Rhodococcus* sp. as in *G. pallidus* BTP-5x, while potassium phosphate buffer was found to be suitable buffer for acyl transferase of *Brevibacterium* sp. [15]. The apparent optimal temperature was 55°C for the soluble amidase and around 80°C for the enzyme-carrier complex for amidase of *Rhodococcus* sp. R312 [6]. Optimum temperatures for acyl transferase activity in *Rhodococcus erythropolis* MP50 [14] and *G. pallidus* RAPc8 [17] were 30 and 50°C respectively. Maximum acetohydroxamic acid accumulation was reported with 1:2 ratios of acetamide and hydroxylamine and only 55–60% of conversion was reported [6].

In *Rhodococcus* sp. R312 and *G. pallidus* BTP-5x, inhibition of acyl transferase activity was observed when amide was present in relative excess. This could be due to competition between amide and hydroxylamine for the acyl-enzyme complex, resulting in an inactive complex when two amide molecules are fixed on the enzyme. Amidase of *Rhodococcus* sp. R312 showed higher affinity for hydroxylamine and lower inhibition was observed in the presence of relatively excess amount of substrate (acrylamide).

The operational stability of acyl transferase activity of *G. pallidus* BTP-5x amidases at various temperatures and time was followed and at 50°C complete conversion of acetamide to acetohydroxamic acid was observed in 1 h. At higher temperature (i.e. 65°C) rate of reverse reaction was found to be higher than reaction at 50°C. To increase the

yield of the product, acetamide was fed after 20 min when the half substrate was utilized. The feeding up to 300 mM acetamide concentration showed no inhibitory effect on acyl transferase activity of amidase of *G. pallidus* BTP-5x. Further feeding of substrate resulted in product saturation and caused amidase catalyzed reverse reaction.

The study on the acyl transfer activity of the amidase from *G. pallidus* BTP-5x and recombinant *Rhodococcus* sp. R312 showed that hydroxamic acid can be produced in aqueous medium and large amounts of final product can be obtained quite rapidly with a few milligrams of enzyme per litre of reaction. Ten gram of insoluble biocatalyst (recombinant *Rhodococcus* sp. R312 amidase) produced 1 litre of acetohydroxamic acid (at least 4.1 g l⁻¹) solution after 90 min reaction time. The acetamide bioconversion rate was 55–60% (mol mol⁻¹). After lyophilization, 10 g powder containing 40% (w/w) acetohydroxamic acid was recovered [6]. The 0.5 mg/ml (dcw) free cells of *G. pallidus* BTP-5x produced 95% (mol mol⁻¹) of acetohydroxamic acid in a batch reaction at 50°C after 20 min. Freeze drying yielded 51 g white powder containing 40% (w/w) of acetohydroxamic acid in the powder.

Conclusion

The present studies resulted in comparable acetohydroxamic acid production using whole cell biocatalyst in short time course. Similar process can be scaled up for the synthesis of other pharmaceutically relevant hydroxamic acids which find wide application in different industries.

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