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Green synthesis of acetohydroxamic acid by thermophilic amidase of *Bacillus smithii* IIIMB2907

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Acetohydroxamic acid is a pharmaceutically active metal chelating agent which has various applications in the field of medicine. Current study focuses on the enzymatic synthesis of acetohydroxamic acid catalysed by thermophilic amidase from *Bacillus smithii* IIIMB2907. Bacterial cells were grown in 7 L fermenter for amidase production and effect of pH, temperature and substrate concentration for the biotransformation of acetamide to acetohydroxamic acid was studied. Batch reaction was also successfully optimized at bench scale with the recovery of ≈ 81% acetohydroxamic acid (purified).

Keywords: Acetohydroxamic acid, *Bacillus smithii* IIIMB2907, Thermophilic amidase

Hydroxamic acids have been reported for the excellent medicinal properties against many diseases[1]. Among hydroxamic acids, acetohydroxamic acid is a potent hydroxamic acid drug molecule which is used in the treatment of chronic urinary tract infections and selectively inhibits arachidonate 5-lipoxygenase that can be used in the treatment of asthma[2-4]. It is also reported for exhibiting anti-HIV activity[5]. Currently, acetohydroxamic acid is being synthesized by chemical methods which are expensive, involve complex reactions, generate hazardous and non-degradable compounds[6]. To avoid these complicated processes, an alternative green synthesis route has been explored to synthesize acetohydroxamic acid using acyltransferase activity of amidases. Acetohydroxamic acid production using amidase has been reported previously by immobilized cells of *Rhodococcus* sp. R312[7], *Pseudomonas aeruginosa*[8] *etc*. Pacheco *et al.* have studied acyltransferase activity of amidase in non-conventional media for the production of acetohydroxamic acid[9]. Due to non-availability of efficient method for acetohydroxamic acid production and purification process, present study was carried out to develop an efficient process for the synthesis of acetohydroxamic acid on bench scale using amidase of *Bacillus smithii* IIIMB2907.

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Materials and Methods

Chemicals and media components

All amides and nitriles were purchased from Sigma-Aldrich, USA, Merk (Germany) and Himedia (India). Media components were purchased from Sigma-Aldrich and Himedia.

Microorganism and culture conditions

In the present study, amide hydrolyzing bacterium was isolated by our group from soil samples of hot springs (80±2°C) of Manikaran, Himachal Pradesh, India. This bacterium was characterized as *Bacillus smithii* IIIMB2907 by 16s rDNA sequencing and deposited at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India) with accession number MTCC-7540. Isolated strain was inoculated in a 250 mL conical flask containing 50 mL mineral base medium and supplemented with 1 mL/L trace elements and 20 mM ε-caprolactam as nitrogen source. The flask was kept for incubation at 50°C and 200 rpm for 20 h and further used as a seed culture (1%) for 7 L fermenter containing 5 L mineral base medium. The cultivation of strain *Bacillus smithii* IIIMB2907 was performed in a 7 L fermenter (Scigenics India Pvt. Ltd.) with mineral base (MB) medium using 20 mM ε-caprolactam as a nitrogen source. The composition of mineral base medium was as follows: 5 g/L^{-1} glycerol, 0.2 g/L^{-1} citric acid, 0.27 g/L^{-1} KH_2PO_4, 0.174 g/L^{-1} K_2HPO_4, 5g/L^{-1} NaCl, 0.2 g/L^{-1}

$MgSO_4.7H_2O$, 0.01 g/L^{-1} $CaCl_2$, and trace elements solution (0.3 g/L^{-1} H_3BO_3, 0.2 g/L^{-1} $CoCl_2.6H_2O$, 0.1 g/L^{-1} $ZnSO_4$. $7H_2O$, 0.03 g/L^{-1} $MnCl_2.4H_2O$, 0.03 g/L^{-1} $Na_2MoO_4.H_2O$, 0.02 g/L^{-1} $NiCl_2.6H_2O$ and 0.01 g/L^{-1} $CuCl_2.2H_2O$)[10,11]. Fermentation conditions with 200 rpm agitation, 50°C temperature, 0.6 BAR air pressure and 1% inoculum of *Bacillus smithii* IIIMB2907 were used for the growth in fermenter and sampling was done with an interval of 3 h. The samples were further analyzed for acyltransferase activity. After batch completion, cell biomass was separated from broth by centrifugation at 10000 g for 10 min and lyophilized, which was further stored at –20°C for further studies.

Biotransformation of acetamide to acetohydroxamic acid through acyltransferase assay

For biotransformation of acetamide to acetohydroxamic acid, only lyophilized cells were used. Reaction mixture (750 µL) was prepared containing 0.5 mg dry cell mass of *Bacillus smithii* IIIMB2907, 500 µL of acetamide as a substrate (10 mM in 100 mM phosphate potassium phosphate buffer, pH-7) and 250 µL of hydroxylamine as a co-substrate (1.5 M, pH 7.0). A control was also prepared containing buffer and cells (without any substrate) and a reagent blank containing substrate and buffer (without cell biomass) were checked for any possible spontaneous reaction and incubated at 50°C for 30 min. Reaction was terminated by the addition of 10 µL of 1N HCl followed by centrifugation at 10000 g for 5 min and 750 µL supernatant was taken and 1 mL of ferric chloride was added in the reaction. Hydroxamic acid was quantified using the method developed by Brammar and Clarke based on colorimetric determination of the red-brown complexes with Fe (III) and absorbance was measured at 500 nm[12]. One unit of acyltransferase activity was defined as the amount of enzyme which catalyzed the formation of 1.0 µM of acetohydroxamic acid per min per mg dry cell mass under standard assay conditions. Acetohydroxamic acid formation was confirmed by thin layer chromatography (TLC) using chloroform and methanol (95:5) as mobile phase. TLC was developed in 335 mM $FeCl_3$ solution in 0.65N HCl.

Determination of effects of temperature and pH on production of acetohydroxamic acid

The effect of temperature on acetohydroxamic acid production was determined. Reaction mixture (500 µL of 10 mM acetamide, 250 µL of 1.5 M, hydroxylamine and 0.5 mg cells) was prepared and incubated at different temperature (from 20 to 60°C) with an interval of 5°C for 90 min. The optimum pH for the reaction was determined for a pH range of 4.0-9.2 in the following buffers (100 mM): sodium acetate buffer (pH 4.0-5.8), potassium phosphate buffer (pH 5.8-8.0) and borate buffer (pH 8.0-9.2).

Effect of substrate concentrations on production of acetohydroxamic acid

To study the effect of substrate concentration on acyltransferase activity, varied concentrations of acetamide (10 to 400 mM) at constant hydroxylamine concentration (1.5 M) were used under standard assay conditions (phosphate buffer 100 mM, pH-7.0 and incubation at 50°C for 90 min). After incubation, reaction mixtures were centrifuged at 10000 g for 5 min. Supernatant was collected in fresh test tube and acetohydroxamic acid was quantified by Brammar and Clarke method[12].

Batch reaction for production of acetohydroxamic acid

To obtain higher acetohydroxamic acid yield, the batch reaction (150 mL scale) was carried with 100 mL of acetamide (400 mM in 0.1 M phosphate buffer) and 50 mL of co-substrate hydroxylamine (1.5 M) under the optimized reaction conditions (50°C, pH 7.0, resting cells 100 mg dcw) and sampling was done with 15 min time interval.

Product recovery and purification

After incubation of reaction mixture for 90 min, reaction was terminated and centrifuged at 10000 g for 10 min. The supernatant was concentrated under the reduced pressure vacuum in rota-vapor. Acetohydroxamic acid from obtained mixture powder was purified by silica gel (60-120 mesh) column chromatography with chloroform. Fraction was collected and dried under the reduced pressure vacuum and characterized by colorimetric assay, TLC and mass spectrometry.

Results and Discussion

Cultivation of strain *Bacillus smithii* IIIMB2907

Bacillus smithii IIIMB2907 was cultured in MB medium (4.5 L) supplemented with trace elements (1 mL/L) and ε-caprolactam (20 mM) as an inducer in 7 L fermenter (Scigenics India Pvt. Ltd). After 12 h of incubation, when acyltransferase activity reached its maximum, (data not shown) fermenter was terminated and cell biomass was separated from broth by centrifugation at 10000 g for 10 min at 4°C. Wet biomass obtained was further lyophilized at −20°C for storage. The total dry cell mass obtained was 2.5 g.

Biotransformation of acetamide to acetohydroxamic acid

Biotransformation of acetamide to acetohydroxamic acid using *Bacillus smithii* was confirmed by colorimetric assay and product was analyzed by TLC (Fig. 1). This is the first report in which acetohydroxamic acid was synthesized using amidase of any theromophilic *Bacillus smithii* strain. In reported literature, *Bacillus smithii* strain IITR6b2 was reported for the synthesis of nicotinic acid hydroxamate and isoniazid[13,14]. However, *Rhodococcus pyridinivorans* NIT-36 was reported for the synthesis of acetohydroxamic acid[15].

Determination of effects of pH and temperature on production of acetohydroxamic acid

To determine the effect of pH on acetohydroxamic acid production, pH range was varied from 4.0-9.2. The optimum pH for the acetohydroxamic production (28.19 U/mg ± 0.14) was 7.5 (Fig. 2A) in potassium phosphate buffer (100 mM). In previous literature, most of the amidase producing microorganisms *i.e. Bacillus smithii* strain IITR6b2[13], *Geobacillus pallidus* BTP-5x MTCC 9225[16] and *Rhodococcus pyridinivorans* NIT-36[15] exhibited maximum acyltransferase activity at neutral pH.

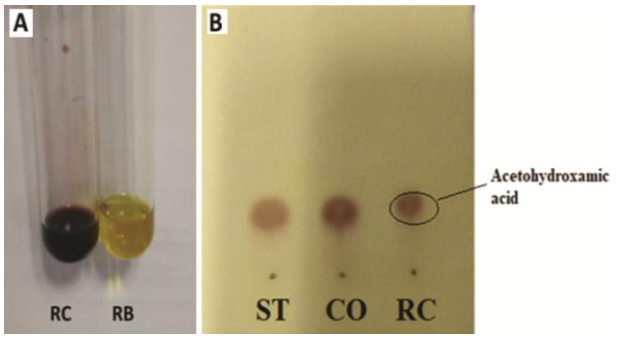

Fig. 1 — Confirmation of biotransformation (A) by colorimetric assay: RC-reaction, RB-Reagent blank; and (B) TLC analysis of biotransformation reaction (ST-standard acetohydroxamic acid, CO-Spot of reaction and standard acetohydroxamic acid, RC-biotransformation reaction)

Strain *Bacillus smithii* IIIMB2907 was isolated from hot spring soil sample of Himachal Pradesh, therefore to determine the optimum temperature for maximum production of acetohydroxamic acid, the reactions were carried out from 20 to 60°C with an interval of 5°C. It was observed (Fig. 2B) that acyltransferase activity was significant from a range of 40 to 55°C with maximum (26.83 U/mg ± 1.34) at 50°C. This trend may be due to the fact that increase in temperature causes random movement of substrate molecules in different regions of the enzyme, thus destabilizing the weak bonds and causing a conformational change in shape of the enzyme resulting in decrease in activity. In literature, *Bacillus smithii* strain IITR6b2 has been reported for maximum acyltransferase activity at 55°C[13]. Amidase from *Rhodococcus* sp. N-771 also showed an optimum temperature of 55°C but above 60°C, its activity decreased significantly[17].

Effect of substrate concentrations on production of acetohydroxamic acid

The tolerance of enzyme towards high substrate concentration is an industrially viable factor for the

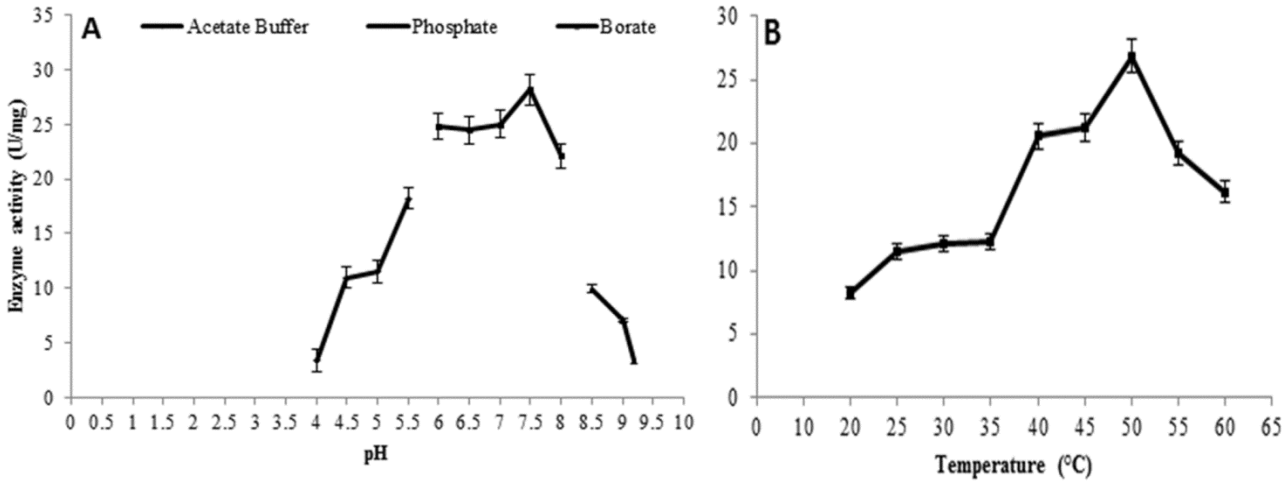

Fig. 2 — (A) Effect of pH on production of acetohydroxamic acid. {Enzyme assay conditions: 10 mM acetamide, three different buffer-Acetate Buffer (*filled diamond with solid line*), Phosphate Buffer (*filled square with solid line*) and Borate Buffer (*filled triangle with solid line*) with pH range of 4 to 9.2, Concentration-0.1 M, Reaction temperature 50°C, Incubation time-90min. Enzymatic assay O.D. at 500 nm}; and (B) Effect of temperature on production of acetohydroxamic acid. {Enzyme assay conditions: 10 mM acetamide, potassium phosphate buffer (100 mM, pH-7.0), 20-60°C (interval of 10°C), incubation time-90 min}

synthesis of products. Therefore, reactions containing different acetamide concentrations (10 to 400 mM) were assessed for acetohydroxamic acid production. It was observed that there were no enzyme inhibition upto 400 mM (Fig. 3) substrate concentrations and maximum conversion of acetamide to acetohydroxamic acid was observed at 400 mM (about 85%) which is a significant result. In literature, *G. pallidus* BTP-5x, have been reported for inhibition of acyltransferase activity when concentration above 100mM of acetamide was used[16].

Batch reaction for the production of acetohydroxamic acid

The bioconversion of acetamide to acetohydroxamic acid was scaled up to 150 mL. Acetohydroxamic acid was quantified after every 15 min and reaction was terminated after 120 min (Fig. 4) Cells were separated from reaction mixture by centrifugation at 10000 g for 10 min at 4°C. The colorimetric assay indicated the presence of acetohydroxamic acid in the reaction mixture. Supernatant yielded 5.2 g of white powder containing acetohydroxamic acid after lyophilization. Further, silica gel column chromatography technique was used for the purification of acetohydroxamic acid from the crude mixture powder and after obtaining fractions of the solvent mixture passed through the column containing acetohydroxamic acid, all the positive fractions were pooled together and vacuum dried in rota-vapor which yielded 1.9 g of acetohydroxamic acid (≈81% recovery).

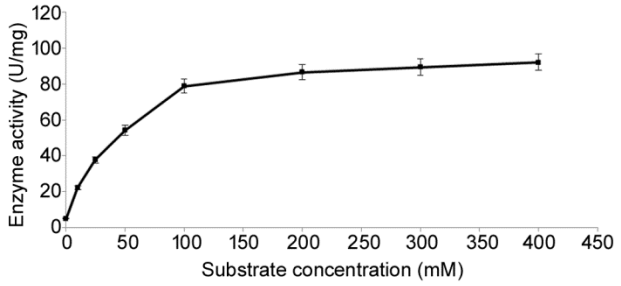

Fig. 3 — Effect of substrate concentrations on production of acetohydroxamic acid. {Enzyme assay conditions: different substrate concentration (10 to 400 mM) acetamide, potassium phosphate buffer (0.1 M, pH-7.0), 50°C, incubation time-90 min.} O.D. at 500 nm

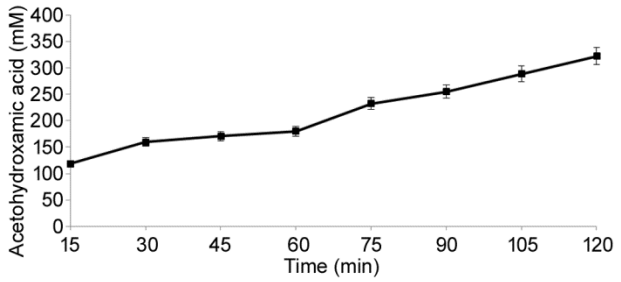

Fig. 4 — Batch reaction for the production of acetohydroxamic acid. {Substrate concentration (400 mM) acetamide, 100 mg resting cells (dcw), potassium phosphate buffer (0.1 M, pH-7.0), 50°C, incubation time-120 min. O.D. at 500 nm}

In the reported literature, Recombinant amidase of *Rhodococcus* sp. R312 reported for 55-60% conversion of acetamide to acetohydroxamic acid with 40% (*w/w*) recovery[7]. However, 90% conversion was achieved from *Bacillus* sp.[18]. In case of *G. pallidus* BTP-5x, 90-95% conversion of acetamide to acetohydroxamic acid was observed in 1 h at 50°C but only 40% (*w/w*) purified acetohydroxamic acid was obtained[16]. In the present study, 85% conversion of acetamide to hydroxamic acid was observed. From which, 81% purified acetohydroxamic acid was obtained.

Conclusion

The present studies concluded that acetohydroxamic acid production using whole cells of *Bacillus smithii* IIIMB2907 is an efficient process. Amidase from strain *Bacillus smithii* IIIMB2907 doesn't show any substrate inhibition during the biotransformation of acetamide to acetohydroxamic acid which can be of great value for large scale production of acetohydroxamic acid at industrial scale. Reported green process may be a milestone for the synthesis of acetohydroxamic acid.

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