Synthesis of acetohydroxamic acid in batch and fed-batch mode using acyltransferase activity of resting cells of *Nocardia globerula* entrapped in alginate gel beads

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INTRODUCTION

Hydroxamic acids (HAs) are compounds that have been reported to be useful for a variety of application e.g. in wastewater treatment, nuclear technology and pharmacology (Fournand *et al*. 1998). HAs can conjugate and eliminate contaminating metal ions and thus may be used for wastewater treatment and in nuclear technology (Heitner *et al.* 1992). Pharmaceutically active HAs may be used as anti-tumor drugs, for treatment of anaemia and infectious diseases (Brown *et al.* 1978). Derivatives of α-aminohydroxamic acid are potent inhibitors of several metalloproteases and the zinc-dependent endopeptidases involved in the tissue remodeling considered to be important in tumor progression and metastasis (Cawston 1996, Ramakrishna *et al.* 1999). In addition, derivatives of α-aminohydroxamic acid as well as acetohydroxamic acid (AHA) among other HAs, have also been investigated as anti-human immunodeficiency virus agents or antimalarial agents (Holmes 1996, Fournand *et al*. 1998).

Various chemical methods for synthesis of HAs have been described (Miller *et al.* 1983) but, these methods requires many solvents and sometimes high temperatures, nitrogen atmosphere, tricky and cumbersome steps and may yield an unwanted byproduct (Fournand *et al.* 1998). As an alternative to the chemical synthesis, enzymatic transformations needs less severe pH and temperature conditions and are stereo-selective and produce pure products and also facilitate otherwise difficult reactions under mild conditions (Kobayashi *et al.* 1993).

The use of acyltransferase or bacteria with microbial enzymes with acyltransferase activity may be used to convert amides to HAs (as shown in reaction below) (Fournand *et al.* 1998). In acyltransferase catalyzed biotransformation, amides acts as acyl-group donors and hydroxylamine as acyl-group acceptors-

$$\underset{\text{(amide)}}{R{-}CONH_2} + \underset{\text{(hydroxylamine)}}{NH_2OH} \xrightarrow{\text{Acyltransferase}} \underset{\text{(hydroxamic acid)}}{R{-}CONHOH} + \underset{\text{(ammonia)}}{NH_3}$$

Here, we describe, a laboratory scale batch and fed-batch mode synthesis of AHA using acyltransferase activity of resting cells of *Nocardia globerula* entrapped in alginate gel beads.

Materials and methods

Chemicals

The nitriles, amides, acetohydroxamic acid and hydroxylamine hydrochloride used in the present study were purchased from Lancaster Synthesis, England. The media components were obtained from HiMedia, Mumbai, India. All other reagents and chemicals used were of highest analytical grade available.

Microorganism, culture conditions and preparation of resting cells

The bacterial isolate *Nocardia globerula* NHB-2 has been procured from the culture collection of the Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-5, India. It had been isolated earlier from the soils of Himachal Pradesh as a nitrile-metabolizing bacterium (Bhalla *et al.* 2005). The bacterium was routinely subcultured on nutrient agar slants at 30 ^{0}C and maintained at 4 ^{0}C. Preculture was prepared by inoculating a loop full of culture from the slant to 2 ml of seed medium containing 0.5% peptone, 0.3% beef extract, 0.1% yeast extract and 1% glucose (pH 7.5) at 30 ^{0}C, 160 rpm for 24 h. This 2 ml of seed culture was added to 50 ml of seed medium and grown under the same conditions. These 24 h preculture were added to 50 ml of production medium containing 3% Tryptone, 1.5% yeast extract, 0.5% NaCl, pH 8.5 (Piotraschke *et al.* 1994) and 0.2% acetonitrile (v/v) as an inducer, followed by incubation at 30 ^{0}C for 24 h in an incubator shaker at 180 rpm.

Cells were harvested by centrifuging the cultures at 5000 x g for 20 min at 0-4 ^{0}C. The pellets were suspended and washed twice with glycine-NaOH buffer (pH 8.5) and finally re-suspended in the same buffer and were referred to as 'whole resting cells'. The whole resting cells were assayed for acyltransferase activity and used for further investigations.

Acyltransferase assay

The acyltransferase activity was determined spectrophotometrically measured by the method described by Brammar and Clarke (1964). If not stated otherwise, the assay was performed in 2 ml of reaction mixture containing resting cells, acetamide solution and hydroxylamine hydrochloride solutions (adjusted to pH required with 10N NaOH) in 100mM glycine-NaOH buffer (pH 8.5) at 55^O C for 15 min. The absorption was measured at 500 nm and related to the amount of acetohydroxamate formed in reaction by comparison with a standard.

HPLC analysis

Acetamide, hydroxylamine hydrochloride and AHA present in the reaction mixture were quantitatively analyzed by high performance liquid chromatography (HPLC), as described by Fournand *et al.* (1998) using a Perkin Elmer HPLC system equipped with an C-18 reverse phase column (4.6 X 250 mm) at a flow rate of 1 ml min^{-1}, at an ambient temperature (20 to 25 ^{0}C) with 25 mM orthophosphoric acid and 1 % (v/v) methanol as an mobile phase. Spectrophotometric detection was performed at a wavelength of 210 nm. The volume injected was 5µl.

Immobilization of Nocardia globerula in alginate gel beads

Whole resting cells were immobilized by the method as described by Kierstan and Bucke (1977). Known amount of whole resting cells of *Nocardia globerula* NHB-2 were added to the 2% sodium alginate solution and mixed well. The mixture was extruded drop wise via a 10 ml syringe from a height of about 20 cm into 1 L of chilled, stirred 0.2 M calcium chloride ($CaCl_2$) solution. The generated beads were kept in 0.2M $CaCl_2$ solution for 1 h for proper hardening and then the beads were placed in 10mM $CaCl_2$ solution till further use.

Optimization of reaction parameters for gel entrapped resting cells

The conversion of acetamide to acetohydroxamic acid was carried out using alginate gel entrapped resting cells of *Nocardia globerula* in selected 100 mM glycine-NaOH buffer at different pH value from 5.0 to 11, at temperature between 30 to 70 ^{0}C and varied concentrations of acetamide from

0.1M to1.0 M as well as hydroxylamine hydrochloride from 0.1 M to1.0 M. Substrate affinity of acyltransferase activity of immobilized cells was tested using a number of substrates.

Acetohydroxamic acid synthesis by batch and fed batch mode supply of substrate

In order to synthesize AHA, the biotransformation of acetamide and hydroxylamine hydrochloride to AHA was performed in a batch and fed-batch mode in 1.5 litre compact New Brunswick Scientific Fermenter (Fig. 1) using a 500 ml reaction mixture at 45 ^{0}C**.** In batch mode a single feed of 62.5 ml of 4 M acetamide, 62.5 ml of 8 M hydroxylamine hydrochloride in 100mM glycine-NaOH buffer pH 8.5 and alginate gel beads (containing 50 mg dry cell wt.) were added, whereas in fed-batch mode the substrates were added in 5 feedings, 12.5 ml of each at an interval of 2 hour. The reaction was allowed to proceed for 10 h.

Results and Discussion

Optimization of reaction parameters for gel entrapped resting cells

The maximum turnover of acetamide to AHA was obtained in 100 mM glycine-NaOH buffer at pH 8.5 and a temperature of 55 ^{0}C. Gel beads with 0.8 mg resting cells (dcw) showed a maximal acyltransferase activity when concentrations of acetamide and hydroxylamine HCL were 500 mM and 1000 mM respectively. The maximum AHA production was observed at 50 ^{0}C in 2 h of reaction (Fig. 2).

Fig. 1 Setting of batch synthesis of AHA using resting cells of *Nocardia globerula* entrapped in alginate gel beads.

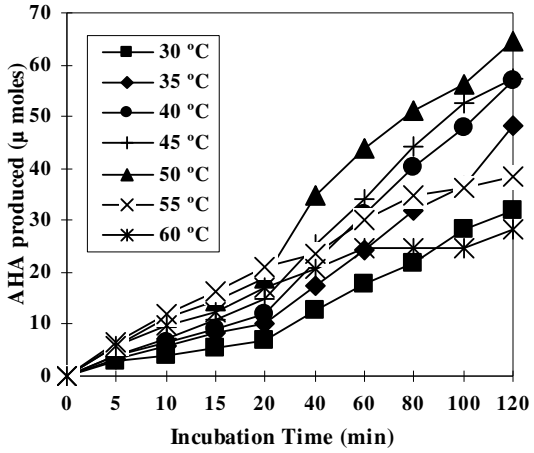

Fig. 2 Time course of acyltransferase reaction at different temperatures.

Gel beads entrapped cells exhibited broad substrate affinity with greater turnover of aliphatic amides as compared to aromatic amides to their respective hydroxamic acids. Similar broad-spectrum amide specificity was reported for acyltransferase activity of amidase of *Rhodococcus* sp. R312 (Fournand *et al*. 1998) and *Rhodococcus rhodochrous* NHB-2 (Chand *et al.* 2004).

Acetohydroxamic acid synthesis by batch and fed batch mode supply of substrate

In batch mode the rate of formation of acetohydroxamic acid gradually increased up to 2 h of incubation time and thereafter no further increase occurred. Maximum synthesis of AHA recorded was 6.496 g g^{-1} dry cell weight h^{-1}, whereas when the substrates were added in fed batch manner, the amount of AHA produced was 12.12 g g^{-1} dry cell weight h^{-1}. As evident from HPLC chromatograms (Fig. 3 and 4), fed-batch mode shows higher conversion yield as compared to batch mode.

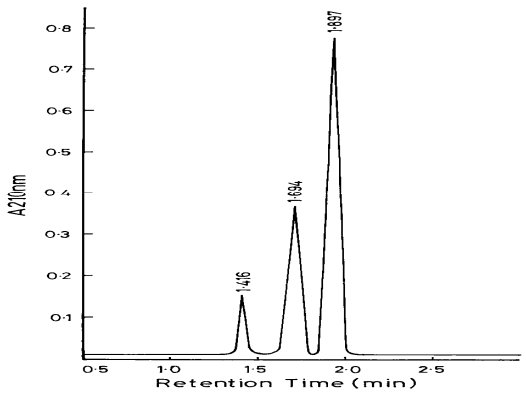

Fig. 3 HPLC chromatogram of a sample from batch reaction

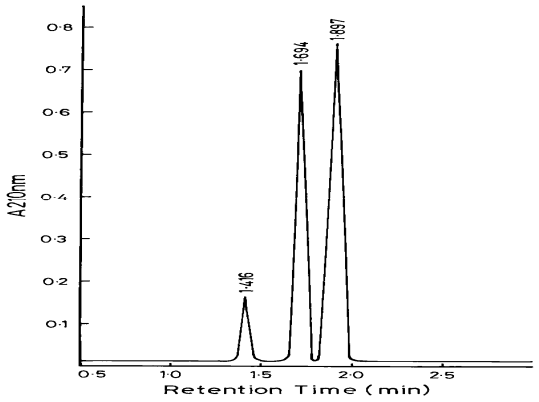

Fig. 4 HPLC chromatogram of a sample from fed-batch reaction

Conclusions

The results obtained in this investigation show that *Nocardia globerula* entrapped in alginate gel beads express thermostable acyltransferase activity that may be used for the synthesis of AHA in batch or fed-batch mode. Biotransformation at commercial scale has a very high potential for the in contrast to chemical processes for the synthesis of AHA, which is a key compound for a variety of applications including the use as pharmaceutical, e.g., in the treatment of tumors, HIV and other health threats.

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