Batch and fed-batch synthesis of butyrohydroxamic acid using alginate gel entrapped *Bacillus* sp. APB-6

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INTRODUCTION AND OBJECTIVES

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. 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). In addition, derivatives of α-aminohydroxamic acid, acetohydroxamic acids as well as butyrohydroxamic acid (BHA) 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 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). 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-

$$R\text{–}CONH_2 + NH_2OH \xrightarrow{\text{Acyltransferase}} R\text{–}CONHOH + NH_3$$

Here, we describe, a laboratory scale synthesis of BHA using acyltransferase activity of resting cells of Bacillus sp. APB-6 entrapped in alginate gel beads.

MATERIALS AND METHODS

Microorganism, culture conditions and preparation of resting cells The bacterial isolate Bacillus sp. APB-6 NHB-2 has been procured from the culture collection of the Department of Biotechnology, H. P. University, Shimla-5, India. Precultures were 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. These 24 h precultures were added to 50 ml of production medium (Piotraschke *et al.* 1994) and 0.25% butyronitrile (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' which were assayed for acyltransferase activity and used for further investigations.

Acyltransferase assay The acyltransferase activity was determined spectrophotometrically by the method described by Brammar and Clarke (1964). If not stated otherwise, the assay was performed 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 butyrohydroxamic formed in reaction by comparison with a standard. Retention times of hydroxylamine hydrochloride, butyramide and butyrohydroxamic acid were 1.416, 1.936 and 1.782 respectively. Generally, the standard deviations of the retention times were below 0.002 min.

HPLC analysis Butyramide, hydroxylamine hydrochloride and BHA 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* Bacillus sp. APB-6 *in alginate beads Whole resting cells were immobilized by the method as described by Kierstan and Bucke (1977).

Optimization of reaction parameters for gel entrapped resting cells The biotransformation reaction was carried out using alginate gel entrapped resting cells of Bacillus sp. APB-6 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 butyramide from 0.1M to1.0 M as well as hydroxylamine hydrochloride from 0.1 M to1.0 M.

Butyrohydroxamic acid synthesis by batch and fed batch mode supply of substrate In order to synthesize BHA, the biotransformation of butyramide and hydroxylamine hydrochloride to BHA was performed in a batch and fed-batch mode in 1.5 litre compact New Brunswick Scientific Fermenter using a 500 ml reaction mixture at 45 ^{0}C**.** In batch mode a single feed of 62.5 ml of 4 M butyramide, 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 butyramide to BHA 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 butyramide and hydroxylamine HCL were 500 mM and 1000 mM respectively. The maximum BHA production was observed at 50 ^{0}C in 2 h of reaction.

Butyrohydroxamic acid synthesis by batch and fed batch mode supply of substrate In batch mode the rate of formation of butyrohydroxamic acid gradually increased up to 2 h of incubation time and thereafter no further increase occurred. Maximum synthesis of BHA recorded was 8.46 g g^{-1} dry cell weight h^{-1}, in case of batch mode supply of substrates whereas when the substrates were added in fed batch manner, the amount of BHA produced was 14.46 g g^{-1} dry cell weight h^{-1}.

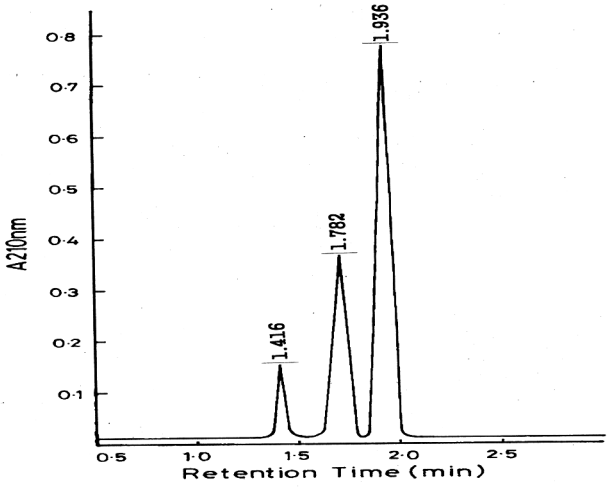

Fig. 1 HPLC chromatogram of a sample from batch reaction

As evident from HPLC chromatograms (Fig. 1 and 2), fed-batch mode shows higher conversion yield as compared to batch mode.

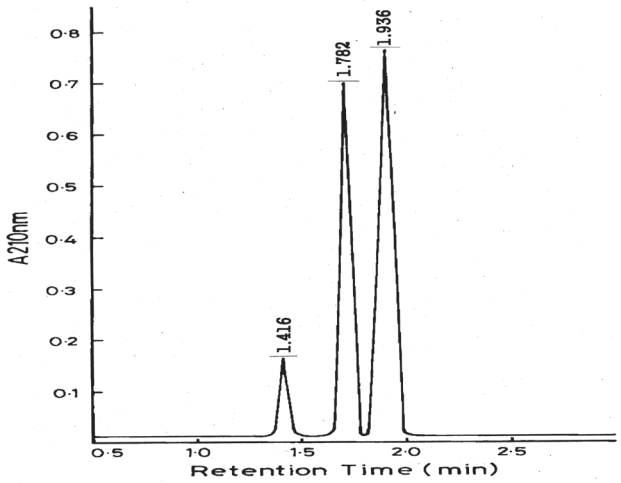

Fig. 2 HPLC chromatogram of a sample from fed–batch reaction

CONCLUSIONS

The results obtained in this investigation show that Bacillus sp. APB-6 entrapped in alginate gel beads express thermostable acyltransferase activity that may be used for the synthesis of BHA in batch or fed-batch mode. Biotransformation at commercial scale has a very high potential in contrast to chemical processes for the synthesis of BHA, 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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