Bioresource Technology 102 (2011) 6579–6586

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

An improved bioprocess for synthesis of acetohydroxamic acid using DTT (dithiothreitol) treated resting cells of *Bacillus* sp. APB-6

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ARTICLE INFO

Article history:
Received 5 February 2011
Received in revised form 21 March 2011
Accepted 22 March 2011
Available online 12 April 2011

Keywords:
Acyltransferase
Bacillus sp. APB-6
N-methylacetamide
DTT
Acetohydroxamic acid

ABSTRACT

Acyltransferase activity of amidase from *Bacillus* sp. APB-6 was enhanced (24 U) by multiple feedings of N-methylacetamide (70 mM) into the production medium. Hyperinduced whole resting cells of *Bacillus* sp. APB-6 corresponding to 4 g/L (dry cell weight), when treated with 10 mM DTT (dithiothreitol) resulted in 93% molar conversion of acetamide (300 mM) to acetohydroxamic acid in presence of hydroxylamine-HCl (800 mM) after 30 min at 45 °C in a 1 L reaction mixture. After lyophilization, a 62 g powder containing 34% (wt wt^{-1}) acetohydroxamic acid was recovered. This is the first report where DTT has been used to enhance acyltransfer reaction and such high molar conversion (%) of amide to hydroxamates was recorded at 1 L scale.

1. Introduction

Amidase or amidohydrolase (E.C. 3.5.1.4) is an interesting member of nitrilase superfamily which catalyzes the hydrolysis of amides to carboxylic acid and ammonia and is used by prokaryotes in carbon and nitrogen fixation (Pace and Brenner, 2001). In industries amidases are employed in combination with nitrile hydratases for the production of commercially important organic acids (acrylic acid, p-aminobenzoic acid, pyrazinoic acid, nicotinic acid etc.) through biotransformation of nitriles (Banerjee et al., 2002). They are also utilized as industrial catalysts in effluent treatment (Madhavan et al., 2005; Nawaz et al., 1996) and wastewater treatment (Chand et al., 2004).

Different wide spectrum amidases exhibit acyl transfer activity (transfer of R–CO group) in the presence of hydroxylamine (Fournand et al., 1998a) which acts as an acyl acceptor and the amide acts as an acyl donor. Acyl transfer activity of amide-hydrolysing enzymes has been described long since. Grossowicz et al. (1950) reported the formation of hydroxamic acids (RCONHOH) by the enzyme-catalysed replacement of the amide groups of glutamine and asparagine with hydroxylamine. Thiery et al. (1986) have reported a wide spectrum amidase from *Brevibacterium* sp. R312 with acyl transferase, acid transferase and ester transferase activity.

Maestracci et al. (1986) explained the detailed catalytic action of the aliphatic amidase from *Rhodococcus* sp. R312 with acetamide as acyl group donor and hydroxylamine as the acyl group acceptor and suggested the mechanism was of "Ping Pong Bi Bi" type where amides react with the enzyme to give acyl-enzyme complexes, which then transfer acyl groups to the cosubstrate (water or hydroxylamine) which lead to formation of carboxylates or hydroxamates (Fig. 1). In case of group transfer reactions better yield of product is observed when hydrolases with high ratios of transferase to hydrolase activity are used (Kasche, 1986).

Acyltransferase activity of amidase has been used for the biosynthesis of a range of hydroxamic acids, which have a high chelating potential. Several hydroxamic acids are used as drugs and have been reported as tumor inhibitors, anti-HIV and anticancerous (Fournand et al., 1998b; Ramakrishna et al., 1999). Some hydroxamic acids can conjugate with metal ions and thus find their use to eliminate metal ions in wastewater treatment and nuclear technology (Koide et al., 1987). Some other hydroxamic acids (α-aminohydroxamic acid, acetohydroxamic acid etc.) have also been investigated as anti-human immunodeficiency virus agents, antimalarial agents and have also been recommended for treatment of ureaplasma infections and anaemia (Gao et al., 1995; Holmes, 1996). Some fatty hydroxamic acids have been studied as inhibitors of cyclooxygenase and 5-lipooxygenase with a potent anti-inflammatory activity (Hamer et al., 1996).

The unsubstituted aliphatic hydroxamic acids (such as acetohydroxamic acid) are well established as effective inhibitors of plant and bacterial urease in vitro (Fishbein et al., 1965; Kobashi and Hase, 1967) and have been shown to effectively inhibit ureolytic activity and/or to lower blood ammonia levels in mice, rats, sheep, cows, dogs and men (Brent and Adepoju, 1967; Streeter et al., 1969).

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doi:10.1016/j.biortech.2011.03.071

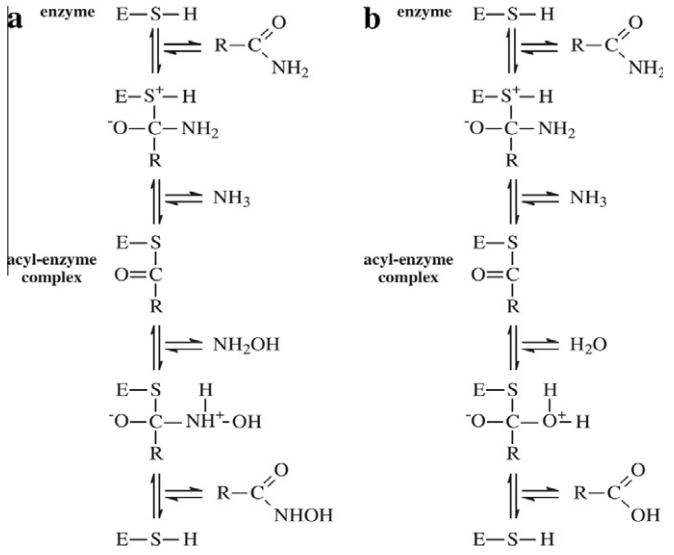

Fig. 1. Mechanism of the acyl transfer reaction from amides to hydroxylamine (a) and the amide hydrolysis reaction (b) catalyzed by the aliphatic amidase from *Rhodococcus* sp. R312 (Maestracci et al. 1986).

Keeping in view the novel characteristics of acyltransferase and medical applications of acetohydroxamic acid (AHA), in the present work a bioprocess has been developed for synthesis of AHA at 1 L scale; using DTT (dithiothreitol) treated whole resting cells of *Bacillus* sp. APB-6. The amidase of *Bacillus* sp. APB-6 shows good acyltransfer activity and the cells were further induced to produce acyltransferase by multiple feedings of N-methylacetamide in the production medium. This is the first report where DTT has been used to enhance acyltransfer reaction to achieve high molar conversion (%) of amide (acyl donor) to hydroxamates in presence of hydroxylamine-HCl (acyl acceptor) at 1L scale.

2. Methods

2.1. Chemicals

All the chemicals were of analytical grade. The nitriles and amides were from Alfa Aesar, A Johnson Matthey Company (earlier Lancaster Synthesis). Media components were from HiMedia (Mumbai) and the inorganic salts were of analytical grades. For high performance liquid chromatography (HPLC), solvents were from Merck, India.

2.2. Microorganism and culture conditions

Bacillus sp. APB-6, a nitrile metabolizing bacterium was isolated from the soil samples of Shimla (Himachal Pradesh, INDIA). This bacterium has been identified and deposited as *Bacillus* sp. APB-6 at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India) with accession number MTCC-7540.

Preculture was prepared by transferring a single colony of *Bacillus* sp. APB-6 grown over nutrient agar for 48 h at 30 °C to 50 ml medium containing peptone (0.5%), beef extract (0.3%), yeast extract (0.1%) and 1% glucose (pH 7.5) in 250 ml Erlenmeyer flask and incubated at 30 °C, 160 rpm in an incubator shaker till $OD_{600} \approx 15$. The preculture (4 ml) was inoculated into 50 ml of medium (pH 9.0) containing peptone (2.6%), NaCl (0.3%), arabinose (0.5%), yeast extract (0.9%), beef extract (0.7%) and $(NH_4)_2HPO_4$ (0.66%) in 250 ml Erlenmeyer flask and incubated at 30 °C, 160 rpm in an incubator shaker. After specific incubation time the cells from the culture were harvested by centrifugation at 8000*g* (4 °C, 5 min) and washed twice with 0.2 M KH_2PO_4/K_2HPO_4 buffer (pH 7.5), finally suspended in the same buffer and were referred to as resting cells.

2.3. Enzyme assays

2.3.1. Acyltransferase assay

Acyltransfer activity was determined spectrophotometrically by a modified version of the method of Brammar and Clarke (1964). The reaction mixture (2 ml) contained (if not otherwise mentioned) 0.1 M glycine-NaOH buffer (pH 7.5), 100 mM acetamide, 200 mM hydroxylamine HCl (both the substrates freshly neutralized with 10.0 N NaOH) and resting cells. This reaction mixture was incubated at 45 °C for 5 min in a water bath shaker and the reaction was stopped by the addition of 4 ml of $FeCl_3$ reagent. The reaction mixture was centrifuged at 5000*g* for 10 min, discarded the pellet and clear supernatant was collected for estimation of hydroxamic acid. The absorbance was read at 500 nm. One unit (U) of acyltransferase activity was defined as that amount of enzyme which catalyzed the release of one micromole of acetohydroxamic (AHA) acid per min under assay conditions.

2.3.2. Amidohydrolase assay

The amidohydrolase assay was performed in a reaction mixture (2.0 ml) containing 0.1 M glycine-NaOH buffer (pH 7.5), 100 mM acetamide and resting cells at 30 °C in a water bath shaker. After 15 min of incubation, reaction was stopped with equal volume of 0.1 N HCl. The amount of ammonia released in the reaction mixture was colorimetrically estimated using phenate-hypochlorite method (Fawcett and Scott, 1960). One unit (U) of amidase activity was defined as that amount of enzyme which catalyzed the release of one micromole of ammonia per min by the hydrolysis of amide under assay conditions.

2.3.3. HPLC analysis

For the direct quantitative estimation of substrate and product in the assay mixture, HPLC was performed using *series 200 Ic pump* (Perkin Elmer) equipped with Reverse phase Lichrosorb C18–5 μm (4 × 125 mm) column (Merck) and *785A Programmable Absorbance Detector* (Applied Biosystem). The standard curves of acetamide (20–200 mM) and acetohydroxamic acid (1–10 mM) were prepared. The absorbance was monitored using NetWin Software (Netel Chromatographs, India). The analysis of acetamide/acetohydroxamic acid was done at a flow rate of 1.0 ml per min at 210 nm using 25 mM Orthophosphoric acid with 1% (v/v) methanol as mobile phase. The volume of sample injected was 5 μl.

2.4. Hyperinduction of amidase for acyltransferase activity

2.4.1. Effect of various nitriles and amides on enzyme induction

Various inducers (nitriles and amides) were added at a concentration of 50 mM in the production medium (Table 1) to select appropriate inducer for hyperinduction of acyltransferase activity in the whole cells of *Bacillus* sp. APB-6. Both amidohydrolase and acyltransferase activities were measured. The efficiency of selected inducer for acyltransferase activity was tested by adding it in various concentrations (10–100 mM).

2.4.2. Time course of acyltransferase induction in presence of inducer

The growth curve and acyltransferase activity profile of *Bacillus* sp. APB-6 was studied in 50 ml production medium supplemented with inducer. In control, inducer was omitted from the production medium. The samples (2.0 ml) were withdrawn after every 6 h up to 72 h. The acyltransferase activity was assayed using acetamide and hydroxylamine–HCl as substrates and the growth [mg dry cell weight (dcw)/ml] of cells was determined turbidometrically (OD_{600}).