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Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues

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Abstract

Agro industrial residues, cheap sources of energy have high potential in the area of fermentation for the production of lignocellulases. Different agro industrial waste products were evaluated as substrates in submerged and solid-state fermentation for xylanolytic enzyme production by *Bacillus pumilus* and the enzyme titers were compared. Higher titer of endoxylanase was obtained with solid state than with liquid fermentation with feeble amount of cellulase. High proteolytic activity was observed in submerged fermentation where as in solid state the proteolytic activity was lower in all cases except in media supplemented with soy meal due to its high protein content. The endoxylanase produced by solid-state fermentation was stable over a wide range of pH and temperatures. The highest enzyme activity was obtained at pH 6.5–7 and at 50 °C. The enzyme could retain 30% of its activity even after 1 h at 60 °C. Considering the conditions under which Kraft pulps are bleached during the manufacture of paper, xylanase from *B. pumilus* exhibit favorable potential for application in the paper processing industries.

Keywords: Submerged fermentation; Solid-state fermentation; *Bacillus pumilus*; Endoxylanase; Cellulase; Proteases

1. Introduction

Large quantities of agro industrial residues accumulate in the environment, which adversely affect the ecosystem and result in the loss of potentially valuable materials that can be processed such as food, fuel and a variety of chemicals [1]. Effective utilization of these materials for the production of enzymes, biofuels and other metabolites needs extensive studies. Xylan is one of the major heterogeneous hemicelluloses present in agro industrial residues, the hydrolysis of which requires the action of xylanase. Microbial xylanases are advantageous over their counter parts from plant and animal sources because of their easier availability, structural stability and ease of genetic manipulations [2]. Xylanase have been isolated from a diverse range of microorganisms including fungi and bacteria [3] of which endo β-1-4-D-xylanase (E.C. 3.2.1.8) are mainly responsible for the random hydrolysis of xylan [4]. Xylanase as industrial enzymes have potential applications particularly in food, pharmaceuticals, paper-pulp processing and agricultural waste processing [5,6]. Alkaline endoxylanase active at high temperature can hydrolyze the xylan deposits on cellulose fibers and enhance release of lignin from paper pulp [7]. Therefore, xylanase from alkaliphilic bacteria [8], fungi [9,10] and actinomycetes [11] have been studied widely for such applications. Most of the mesophilic xylanase known to date are optimally active at temperature below 50 °C and act in acidic or neutral pH ranges. Only few xylanase are reported to be cellulase-free as well as active and stable at alkaline pH and high temperature [12]. In view of their potential role, cost effective development of enzymes is crucial, as this will significantly benefit the overall economics of biological processes. Agricultural residues make good substrates for fermentation, provided bioreactors are designed with suitable operational control [13]. There are many reports related to production of endoxylanase by submerged fermentation (SmF) using bacteria [14] and solid-state fermentation (SSF) by fungi [10,15]. Only few reports are there related to bacterial SSF system [16,17]. The objective of the present work was to compare overall physiological behavior of *Bacillus pumilus* for endoxylanase production by liquid and solid fermentations and to evaluate the feasibility of agro industrial residues to reduce the cost of enzyme

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production. The enzyme profile of the extract from solid-state fermentation was analyzed. The paper pulp industries are the major target for xylanase application where the use of purified enzyme will be a costly issue, so this study aimed at the characterization of partially purified enzyme after ammonium sulphate precipitation. Enzyme characterization studies were also carried out for its temperature and pH stability as well as activity at different pH and temperatures. Effect of metal ions on enhancement of enzyme activity was also studied.

2. Material and methods

2.1. Microorganism

Bacillus strain, isolated earlier [14] was used for these studies was identified by IMTech Chandigarh, India as *B. pumilus* based on its characteristics and the fatty acid profile. The strain was maintained in xylan agar media containing (g%) peptone 0.5, yeast extracts 0.5, K_2HPO_4 0.1, $MgSO_4$ 0.02, oat spelt xylan 0.5 and agar 2.

2.2. Xylanase production using SmF

The culture maintenance media without agar was used as the fermentation media for the production of enzyme as well as for the preparation of preinoculum. Oat spelt xylan in the media (0.5 g%) was replaced by different agro industrial residues such as wheat bran (WB), rice bran (RB), rice straw (RS), sawdust (SD), coconut pith (CP) and soybean flakes (SBF) at 1 (g%) level. Liquid media were autoclaved and pH was adjusted to 8 using sterilized 10% Na_2CO_3 and inoculated with 5% (v/v) 18 h grown culture. This was maintained at 150 rpm at 35 °C in an environmental shaker and samples were removed at regular intervals. The culture broth was centrifuged at 4 °C for 20 min at 10,000 rpm and the cell free supernatant was used as enzyme source. Experiments were done in triplicate and the average was taken.

2.3. Enzyme production using SSF

For production of endoxylanase by SSF, the strain was grown with different agro industrial residues as substrates such as WB, RS, RB, SW, CP and SBF. Ten gram of substrate plus salt solutions—K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.04 (g%) was taken in 250 ml Erlenmeyer flask. The mineral salt was added in such a way that the final substrate-to-moisture ratio was 1:2.5. Combinations of maximum productive substrates, WB + RS, WB + SBF (5 g each), WB + RS + SBF (3.25 g each) were tried. After sterilization of the media by autoclaving, the flasks were cooled and inoculated with 10% (v/w) 18 h grown inoculum. The enzyme was extracted from the bactobran using distilled water and centrifuged at 4 °C for 20 min at 10,000 rpm and the cell free supernatant was used as crude enzyme for analysis. Experiments were done in triplicate.

2.4. Enzyme assays

Xylanase activity was assayed using oat-spelt xylan [Sigma Chemicals Co., St Louis, MO] of 0.5% as substrate in pH 7 buffer at 50 °C [14]. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of xylose min^{-1}. A similar method was used for the assay of cellulase [18] by incubating filter paper [Whatman No. 1] 50 mg in 0.5 ml phosphate buffer pH 7 with 0.5 ml of crude extract at 50 °C for 1 h. The reaction was terminated by adding 2 ml of dinitrosalicylic acid reagent followed by 5 min of boiling. Absorbance was measured at 540 nm. One unit cellulase activity was defined as the amount of enzyme that release 1 μmol of glucose min^{-1}. Protease was assayed [19] using 2% casein in 0.1 M sodium phosphate buffer pH 7 as substrate. 0.5 ml of casein was incubated at 40 °C for 10 min with 0.5 ml suitably diluted culture filtrate. The reaction was terminated by adding 1 ml of 10% (w/v) TCA. The released tyrosine was estimated by Lowry's method using tyrosine as standard. One unit of protease activity was expressed as 1 μmol of tyrosine min^{-1} ml^{-1} of enzyme under assay conditions. β-Xylosidase activity was assayed by incubating 50 μl of 2 mM *p*-nitrophenyl-β-D-xylopyranoside [Sigma Chemicals Co., St Louis, MO] with 50 ml of suitably diluted enzyme at 50 °C for 20 min. A unit of enzyme was defined as 1 μmol of *p*-nitrophenol min^{-1}.

2.5. Determination of pH and temperature optima, pH and temperature stability

The optimum pH was determined by measuring the activity at 50 °C over a range of pH from 5 to 11 using the following buffer (100 mM): citrate phosphate pH 5 and 6, phosphate buffer pH 6.5, 7, 7.5 and 8, glycine–NaOH buffer pH 8.6, 9, 9.6, 10, 10.5 and 11. The optimum temperature was determined by assaying the enzyme activity at various temperatures ranging from 40 to 65 °C at pH 7. pH stability was determined after incubating the enzyme in the buffers described above for 24 h at 30 °C and measuring the residual activity. The temperature stability was determined by measuring the residual activity at 50 °C, after incubation of the enzyme in different temperatures ranging from 25 to 60 °C and pH 7 in the absence of substrate. The crude enzyme extracted from SSF medium was partially purified by ammonium sulphate precipitation (40–60%) and used for characterization studies.

2.6. Effect of various additives on xylanase activity

The effect of various additives on endoxylanase activity was determined by incubating enzyme at room temperature (30 ± 2 °C). The different monovalent and divalent metals like (10 mM) Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, EDTA (metal chelator), potassium dichromate (oxidizing agent) and SDS (protein disulphide reducing agent) were taken as additives. Activity was monitored at every 15 min for 30 min and activities were determined using oats spelt xylan prepared at pH 7. Residual activity was expressed as the percentage of the activity observed in the absence of any compound.