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Production of a thermostable α-amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production

Harmeet K. Sodhi, Kanupriya Sharma, Jugal K. Gupta, Sanjeev K. Soni*

Department of Microbiology, Punjab University, Chandigarh 160014, India

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Abstract

Bacillus sp. PS-7, isolated from the hot springs of Manikaran (H.P.), India, produced very high levels of thermostable α-amylase by solid state fermentation (SSF) in Erlenmeyer flasks and enamel coated metallic trays. Productivity was affected by the nature of the solid substrate, nature of the moistening agent, level of moisture content, incubation temperature, presence or absence of surfactant, carbon, nitrogen, mineral, amino acid and vitamin supplements. Maximum enzyme production of 4,64,000 U/g dry bacterial bran was obtained on wheat bran supplemented with glycerol (1.0%, w/w), soyabean meal (1.0%, w/w), L-proline (0.1%, w/w), vitamin B-complex (0.01%) and moistened with tap water containing 1% Tween-40 and 1 mM $MgSO_4 \cdot 7H_2O$ at ratio of 1:1.5 after 48 h incubation at 37 °C. The enzyme was partially purified to 12.7-fold by ammonium sulphate precipitation, gel filtration on Sephadex G-75 column followed by Phenyl agarose hydrophobic interaction chromatography. The kinetic characterisation of the partially purified enzyme exhibited maximum activity at 60 °C and pH 6.5. The thermal stability profile revealed the half-life of more than 6 h at 60° and 5(1/2) h at 70 °C respectively in presence of Ca^{2+}. The enzyme did not exhibit marked increase in activity in presence of any metal ion. It could effectively act in synergism with the commercial amyloglucosidase during direct malt starch hydrolysis for ethanol fermentation.

Keywords: *Bacillus* sp.; Thermostable; α-Amylase; Solid state fermentation; Malt; Starch; Hydrolysis

1. Introduction

Starchy residues appear to be the cheapest future raw material of the Indian alcohol industry in view of the rising cost and deteriorating quality of fermentable molasses due to the Government's decontrol policy and technological advancements in the sugar recovery processes. Thus a significant interest exists in research in amylolytic enzymes as the conventional production of ethanol from starch requires an initial pretreatment step whereby the action of amylolytic enzymes on the starch molecules produces low molecular weight sugars. The initial step of starch processing is an energy demanding process, where raw starch is heated to a temperature of ∼100 °C, a process known as gelatinization. Gelatinized starch is highly viscous thus posing serious problems of mixing and pumping. To overcome these problems, gelatinization is coupled with liquefaction, which is brought about by the action of thermostable α-amylases. Thermostable α-amylases have been reported from several bacterial strains and have been produced using submerged (SmF) [1–5] as well as solid state fermentations (SSF) [6–8]. However the use of SSF has been found to be more advantageous than SmF and allows the cheaper production of enzymes [9,10].

In this study we report the production of a thermostable α-amylase from a *Bacillus* sp. PS-7 under solid state fermentation and its properties.

2. Materials and methods

2.1. Microorganism

The starch degrading amylolytic bacterial strains were isolated from a hot spring of Manikaran, HP, India. Based on the levels of α-amylase produced by solid state cultures and

* Corresponding author. Tel.: +91-172-534143; fax: +91-172-541770.
E-mail address: sonisk@pu.ac.in (S.K. Soni).

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the properties of the enzyme, one isolate designated as PS-7 was selected for further studies and identified as *Bacillus* sp. on the basis of various morphological, physicochemical and biochemical characteristics following the criteria laid down in Bergey's Manual of Systematic Bacteriology [11].

2.2. Solid state fermentation

Five grams of wheat bran in 250 ml Erlenmeyer flask, moistened with 5 ml distilled water and autoclaved at 15 psi for 30 min, was taken as the basal medium for SSF studies and had a pH of 5.8–6.0. It was cooled, inoculated with 2.5 ml of the inoculum (A_{600}; 0.8), having 1.5×10^6 cells/ml, from 12 h old shake culture and incubated at 37 °C for 96 h. Visual observations regarding growth were made each day and the bran with bacterial growth was mixed with 50 ml of tap water and filtered through a metallic sieve. The extracted filtrate was centrifuged (10,000 × *g*; 4 °C) for 10 min and cell free supernatant used as the source of α-amylase.

2.3. Enzyme assay

The activity of α-amylase was determined at 60 °C by mixing 0.25 ml of appropriately diluted enzyme source with 0.25 ml of 0.2% (w/v) soluble starch dissolved in 0.1 M Phosphate buffer, pH 6.5. The residual starch was determined after 10 min according to the method of Fuwa [12]. One Unit of α-amylase activity was defined as the amount of enzyme that caused 10% reduction in the starch-iodine colour, under the assay conditions while enzyme productivity has been expressed as U/g dry bacterial bran (DBB). All values given are averages of three determinations.

2.4. Optimization of fermentation conditions for enzyme production

α-Amylase production was optimized in SSF of wheat bran, unless otherwise stated, by altering various physicochemical and cultural conditions and observing the effect after 48 h of incubation at 37 °C, unless otherwise stated.

(1) The effect of substrates in SSF for α-amylase production was studied using a variety of solid substrates and their combinations (in equal proportions) in the basal medium.
(2) The effect of the nature of the moistening agent was studied using various moistening agents including tap water, distilled water and different buffers (0.1 M) with variable pH.
(3) The effect of the ratio of the moistening agent was studied by altering the level of distilled water in the basal medium for SSF. Since the substrate and tap water ratio of 1:1.5 yielded the highest enzyme productivity, all the subsequent optimization studies for enzyme production were carried out with this moisture level in the basal medium.
(4) The effect of incubation temperature on enzyme production was studied by incubating the seeded standard basal medium at different temperatures.
(5) The effect of various supplements on α-amylase production was studied by adding different carbon sources (1% (w/w) of dry substrate), nitrogen sources (1% (w/w) of dry substrate), metal ions (1 mM in the moistening agent), surfactants (1% (v/v) in the moistening agent), amino acids (0.1% (w/w) of dry substrate) and vitamins (0.01% (w/w) of dry substrate) in the basal medium.

2.5. Enzyme production using SSF in different production vessels

Enzyme production was studied in various sized Erlenmeyer flasks and enamel coated metallic trays using wheat bran, moistened with distilled water as the solid medium. Flask fermentations involved 250, 500 and 1000 ml Erlenmeyer flasks while the tray fermentations involved the use of enamel trays (27.5 cm × 22.5 cm × 4.5 cm, 38.5 cm × 30 cm × 4.2 cm) covered with aluminium foil containing different quantities of wheat bran as the substrate in the basal media, with a bed depth ranging from 1 to 3 cm, without and with the supplementation of 0.5% (w/w) glucose, 0.5% (w/w) soyabean meal and 0.1 mM $CaCl_2 \cdot 2H_2O$. The flasks and trays were autoclaved, cooled and inoculated with appropriate volume of standard inoculum size (50 ml/100 g of dry substrate; A_{600}, 0.8), having 1.5×10^6 cells/ml so as to give a final moisture level of 1:1.5 and incubated at 37 °C for 48 h in case of the flasks and 72 h in case of the trays.

2.6. Scanning electron microscopy

The SSF bran was fixed to an aluminium stub and sputter coated with gold to improve the conductivity of the material. The coated samples were scanned at an operating voltage of 10 kV.

2.7. Enzyme purification

To the cell free supernatant, ammonium sulphate was added to 60% saturation and centrifuged (10,000 rpm for 10 min) after 2 h of incubation at 4 °C. The precipitates were dissolved in a minimum volume of 10 mM phosphate buffer, pH 6.5, dialyzed overnight against the same buffer and retained for further purification by Sephadex G-75 gel filtration followed by phenyl agarose 4XL hydrophobic interaction chromatography.

2.7.1. Sephadex G-75 column chromatography

The supernatant after dialysis, containing enzyme protein, was applied to a Sephadex G-75 column (34 cm × 1.4 cm) pre-equilibrated with 10 mM phosphate buffer, pH 6.5. Fractions (2.5 ml each) were collected at the flow rate of 15 ml/h and those showing protein were analyzed for