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Characterization of Alkaline α-Amylase from *Bacillus sp.* AB 04

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ABSTRACT

A medium containing meat extract and fructose (1%) has been optimized for production of extra-cellular and alkaline α-amylase from *Bacillus sp.* AB 04. The crude enzyme showed maximum activity at pH 8 with optimum temperature of 40°C with more than 75% activity in range of 50 - 80°C. The enzyme retained more than 94% of activity after 5 h of incubation at optimum pH and stable in the pH range of 7 - 10. Ca^{+2} and Co^{+2} act as strong activator of this enzyme. AB04 α-amylase rapidly hydrolyzed starch resulting in the formation of glucose, maltose and other oligosaccharides.

Key Words: α-Amylase; Alkaline; Starch hydrolysis; Starch-converting enzyme

INTRODUCTION

Enzymatic hydrolysis of starch by α-Amylase (1, 4 - α-D-glucan glucanohydrolase; EC 3.2.1.1), which catalyzes the hydrolysis of internal (endoglycosidase) α-1, 4-glucan links in polysaccharides containing 3 or more α-1, 4-linked D-glucose units (amylose & amylopectin) yielding a mixture of maltose and glucose, has wide applications in industry. It is used in brewing and fermentation industries for the conversion of starch to fermentable sugars (Chi *et al.*, 1995; Farid *et al.*, 2002), in the textile industry for designing textiles and in the laundry industry in a mixture with protease and lipase to launder clothes (Achle, 1997; UpaDek & Kottwitz, 1997; Ito *et al.* 1998), in the paper industry for sizing (Whistler *et al.*, 1984; Van der Maarel *et al.*, 2002), and in the food industry for preparation of sweet syrups (Bello-Perez, 2002; Rao & Satyanarayana, 2003), to increase diastase content of flour, and for the removal of starch in jelly production. After the addition of the α-amylase in the bread-baking process, the bread's volume increased and kept its softness longer (Ammar *et al.*, 2002). This enzyme is widely distributed in various bacteria, fungi, plants and animals and has a major role in the utilization of polysaccharides (Shaw *et al.*, 1984; Reddy *et al.*, 1987; Tomita *et al.*, 1990; Ilori *et al.*, 1997; Ribeiro, 2000; Hagihara *et al.*, 2001; Zoltowska, 2001; Bassinello, 2002; Haq *et al.*, 2003). Wide ranges of microorganisms producing amylases having different specificities, properties and action patterns have been reported (Hansen *et al.*, 1994; Bibel, 1998; Talamond *et al.*, 2002). Alkaline α-amylases is more useful as working pH of detergents is between 8 - 11 (Ito *et al.*, 1998). Taking into consideration the extensive applications of the enzyme, a novel α-amylase producing *Bacillus sp.* AB 04 has been isolated in our laboratory having optimum pH in both acidic and alkaline pH range.

MATERIALS AND METHODS

All media components were of bacteriological grade and purchased from HiMedia (Mumbai, India). Ammonium sulphate was purchased from E-Merck (Mumbai, India). All other chemicals were of analytical grade and procured from Qualigens Fine Chemicals (Mumbai, India) and SD Fine Chemicals (Mumbai, India).

Collection of samples, enrichment and screening of ∝-amylase producing microorganisms. Various soil samples were collected from the area in and around Jalandhar Distt. Punjab (India). Soil samples were suspended in sterilized water and thoroughly mixed. Suspension was used to inoculate medium having Nutrient Broth containing 1% (w/v) starch as carbon source. Flasks were incubated with constant shaking (150 rpm) at 40°C for 24-36 h.

A plate of Nutrient Agar containing 1.0% soluble starch was formed. The test strain was streaked in the center and plate was incubated overnight at 40°C and flooded with Lugol's iodine. The clear zone indicates the extent of starch degradation. The selected strains were maintained on a medium (pH 6.8 - 7.0) containing per liter: Yeast Extract: 5.0 g; Starch 10.0 g; $MgSO_4.7H_2O$ 0.5 g; K_2HPO_4 1.0 g; Agar 15.0 g. The identification and taxonomical studies of the isolate AB 04 were carried out according to Bergey's Manual of Systematic Bacteriology (Sneath, 1994).

Amylase assay. The enzyme activity was studied by following the technique of Shaw *et al.* (1995) with certain modifications. Assay mixture (500 µL) having 250 µL of starch (1%) in 0.2 M sodium phosphate buffer of appropriate pH and 50 µL of appropriately diluted enzyme was incubated at 40°C (or otherwise stated) for 10 min. After incubation, 750 µL of DNS reagent was added and mixture was boiled for 10 min followed by addition of 1.25 mL of distilled water. The absorbance was read at 540 nm in Spectronic $20D^+$ spectrophotometer with appropriate substrate and enzyme blank. Glucose was used as standard. One unit (U) of α-amylase is described as amount, which produces 1 nmol of glucose per mL per min.

Estimation of extra cellular protein. Concentration of

protein was estimated by the method of Lowry *et al.* (1951). Routine calibration curve from standard BSA solution (0.02 - 0.2 mg) was used to measure protein concentration.

Optimization of α-amylase production parameters. The selected strain AB 04 was used for the production of α-amylase in the media having starch (1%), yeast extract (0.5%), $MgSO_4.7H_2O$ (0.05%) and K_2HPO_4 (0.1%). The pH of the medium was adjusted to 7.0. The inoculum was prepared by incubating the flasks in a rotary shaker at 40°C for overnight at 150 rpm. Different sets (in duplicate) of 100 mL Erlenmeyer flasks each containing 25 mL medium was inoculated with 2% inoculum. After specific intervals of time, samples were taken out to determine cell density, enzyme activity, sugar concentration and pH.

Optimization of nitrogen sources. Different organic nitrogen sources (yeast, meat, beef extracts, tryptone, peptone, biopeptone *etc.*) at a concentration of 1.0% (w/v) were used. pH of the medium was adjusted to 7.0. The nitrogen sources showing better activity were optimized at different concentrations.

Optimization of carbon sources. Different carbon sources (lactose, glucose, maltose, fructose, sucrose, starch mannitol *etc.*) were used at a concentration of 10 g L^{-1}. These were used to replace starch in standard growth medium.

Characterization of soluble α-amylase

Effect of incubation temperature and pH on α-amylase activity. The effect of temperature on soluble α-amylase was measured in the range of 30 to 80°C. The reaction mixture was incubated for 10 min at different temperatures and the residual activity was determined.

The effect of pH from 4.0 to 10.0 on the α-amylase activities was measured in 0.2 M citrate buffer (pH 4 - 5), 0.2 M sodium phosphate buffer (pH 6 - 8) and 0.2 M glycine-NaOH buffer (pH 9 - 10). The relative α-amylase activity was determined after carrying out the reactions in different pH.

Stability characterization. To estimate the thermal stability, the appropriate dilution of enzyme were made in 0.2 M phosphate buffer (pH 5.0 & 8.0) and incubated at 40°C. Samples were with-drawn at different intervals of time and immediately placed in ice before measuring α-amylase activity.

To determine the stability at different pH, the enzyme was incubated for 12/24 h at different pH values before the residual activity was determined at pH 4.0 and 10.0.

Effect of cations/inhibitors/activators on α-amylase activity. Cations/activators/inhibitors (final concentration 5 mM-10 mM) were added to reaction medium. The enzyme activity was assayed under standard conditions and inhibition/activation was expressed as a percentage of the activity of control (without the effectors).

Starch hydrolysis studies. The enzymatic reaction was stopped at different interval of time and reaction mixture was precipitated with acetone. The supernatant with standard sugars was spotted on Kieselgel 60 P_{254} plates (Merck, Germany). The spots were developed in solvent mixture of chloroform: acetone: water (6:7:1). The plates were dried and sprayed with aniline: diphenylamine: phosphoric acid {1% (v/v): 1% (w/v): 1% (v/v) in acetone} and incubated at 100°C for 10 min.

Fig. 1. Effect of nitrogen sources on the production of α-amylase from *Bacillus sp.* AB 04

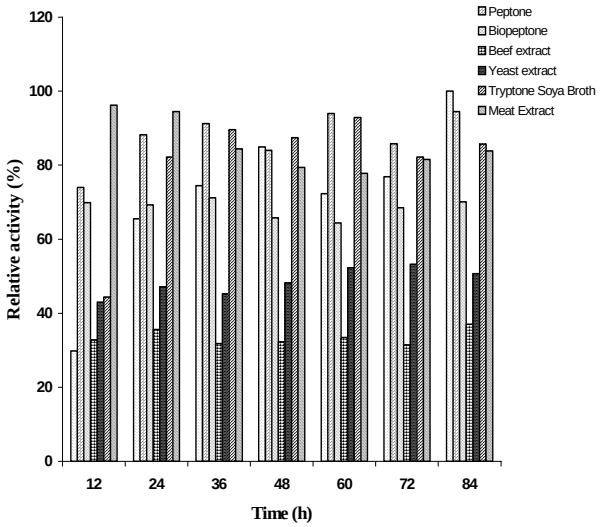


Fig. 2. Effect of different carbon sources on the production of α-amylase from *Bacillus sp.* AB 04

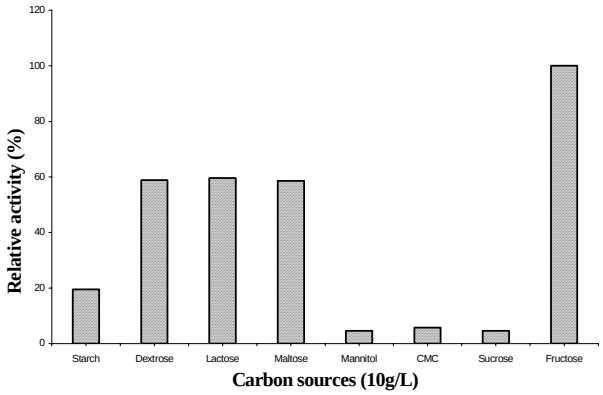
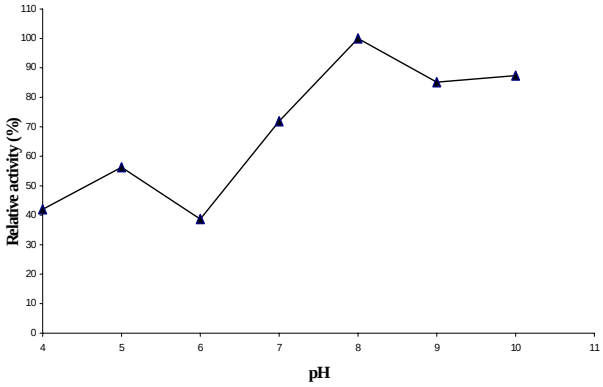


Fig. 3. Effect of pH on the activity of α- amylase from *Bacillus sp.* AB 04



RESULTS AND DISCUSSION

The morphological, physiological and biochemical characteristics of the isolate AB 04 studied according to Bergey's Manual of Systematic Bacteriology (Sneath, 1994) showed irregular margins and was Gram positive rod shaped. The characteristics of the isolated strain confirm the *Bacillus sp.* The enzyme production was initiated within 12 h and increase in exponential phase. Effect of the nitrogen source (Fig. 1) on the α-amylase productivity from strain AB 04 was studied using various complex nitrogen sources having starch (1%) as the carbon and energy sources. Enhancement of 350% was achieved with Meat Extract (1%) as compared to Luria Broth within 12 h. For production of enzyme, beef extract was shown to be less effective as a nitrogen source.

Fig. 4. Effect of temperature on the α-amylase activity from *Bacillus sp.* AB 04

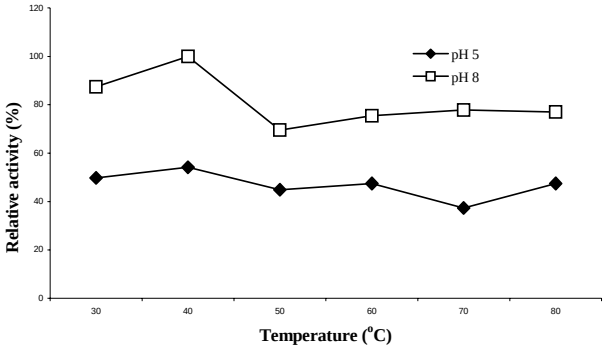


Fig. 5. Effect of pH on the stability (12 h) of enzyme α-amylase

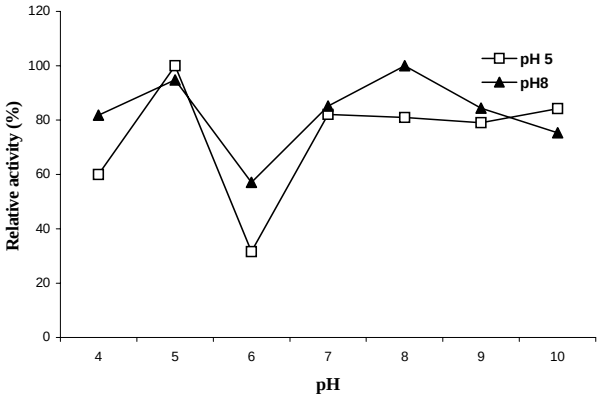


Fig. 6. Studies of thermal stability of α-amylase (40°C)

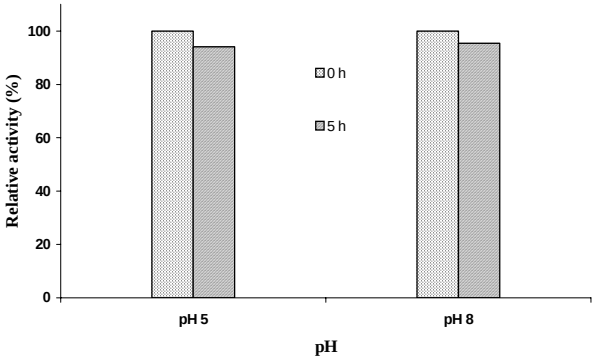


Table I. Effect of metal ions on the activity of α-amylase

Metal ions	Relative Enzyme Activity (%)	
	5 mM	10 mM
[a]Na^{+}	85.4	73.8
[a]K^{+}	97.0	87.4
[a]Mg^{+2}	86.4	66.0
[a]Co^{+2}	145.6	171.8
[a]Fe^{+3}	88.3	91.3
[a]Ca^{+2}	163.1	202.9
[a]Hg^{+2}	35.0	52.4
[b]Mg^{+2}	73.8	102.4
[b]Cu^{+2}	48.5	56.3
Control	100	100

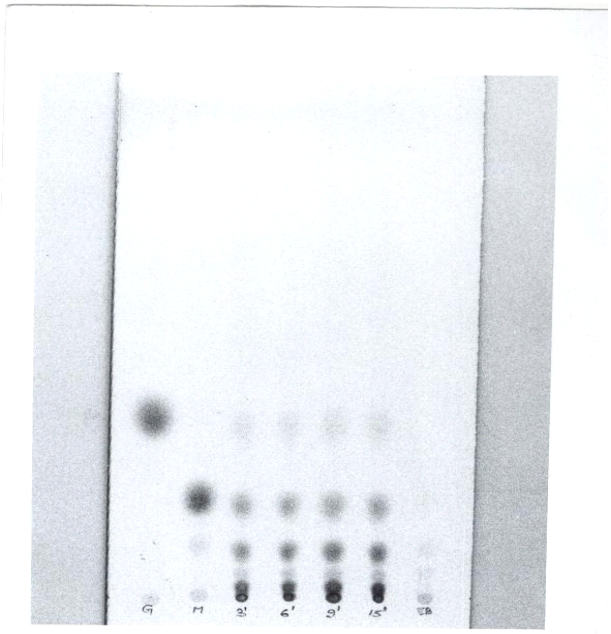
a : as chloride; b: as sulphate

The influence of the carbon source on α-amylase production by *Bacillus* sp. AB 04 was investigated using different carbon sources as shown in Fig. 2. Strain AB 04 did not grow on fructose as the sole carbon source. Although maximum growth observed with mannitol and sucrose, but with little enzyme production. Carboxymethyl cellulose and lactose support very less growth of Strain AB 04. Starch the main substrate for α-amylase hydrolytic activity showed 20% of the enzyme production as compared to fructose. During growth on Luria Broth without any external carbon sources very less (9%) activity was observed.

Characterization of enzyme. The optimum pH for AB 04 α-amylase was 8.0 (Fig. 3). While the activity declined sharply on acidic side as compared to alkaline pH side of its optimal range, a sudden increase (45% as compared to pH 6.0) was observed at pH 5.0, suggesting a change in conformation. These results are quite distinct from most bacterial α- amylase which are active at slight acidic to neutral pH (Hamilton, 1999). The enzyme obtained from AB 04 strain retain more than 75% activity even after 12 h of incubation in pH range of 7-10 *i.e.* alkaline environment (Fig. 5). Table I shows the effect of different monovalent and divalent cations like Hg^{+2} and Cu^{+2} acted as strong inhibitors. In present study, K^{+} and Fe^{+3} had almost no effect on enzyme activity. α-amylase activity showed dramatic increase in the activity to 171 and 202% with Co^{+2} and Ca^{+2} as compared to control.

The activity of AB 04 α-amylase was measured at various temperatures at pH 5 and 8. The enzyme showed activity between 40 and 80°C and the optimum activity was at 40°C with more than 70% activity is observed up to 80°C at pH 8 (Fig. 4). To study the thermal stability of the enzyme, α-amylase was incubated at 40°C and residual activity was measured. As shown in Fig. 6, more than 94% activity was retained even after 5 h of incubation. The

Fig. 7. TLC showing hydrolysis of starch using α-amylase from *Bacillus sp.* AB 04 Row 1: Glucose; Row 2: Maltose; Row 3 –6: Reaction products after 3, 6, 9, 15 min of incubation; Row 7: Enzyme blank.



optimal performance of the detergents require α-amylase with optimum temperature 40-60°C and alkaline pH (Bisgaard-Frantzen *et al.*, 1999).

Effectiveness of enzyme in hydrolyzing starch is examined in Fig. 7. Hydrolysis of starch was indicated by the formation of large amount of maltose and other oligosaccharide the initial stage followed by the increase in the amount of glucose and maltose.

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