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Short Communication

Partial Characterization of Extracellular α-Amylase from Three *Bacillus* Isolates

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α-Amylase produced by three *Bacillus* isolates had been compared on the basis of the following criteria: heat and pH effects on activity and stability, effect of metal ions on enzyme activity and kinetic parameters. The culture filtrates obtained by growing the organisms in starch medium were fractionated by ammonium sulphate precipitation technique, and the highest enzyme activities were recovered from 70% saturation fraction. The enzyme from *Bacillus amyloliquefaciens* showed optimum activity at 60°C, while *B. subtilis* and another *Bacillus* isolate at lower (55°C) temperature. The pH optima of the enzymes from all sources were between 6.5 and 7.0 with an optimum reaction time of 10 to 15 min. α-Amylases were moderately thermostable exhibiting almost full activities at 50°C for at least 20 min. The enzyme from all sources showed stability over a wide range of pH (4.0-8.5). The apparent K_m values on soluble starch varied between 1.6 mg/ml in case of *B. amyloliquefaciens* and 2.5 mg/ml in case of *B. subtilis*. Metal ions like Mg^{2+} and Mn^{2+} seemed to have positive influence on the enzyme activities of *B. subtilis* and *Bacillus* sp. The enzyme activities from three isolates were strongly inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+}.

Key words: Extracellular α-amylase, *Bacillus* spp., Enzyme characterization

α-Amylases are group of enzymes classified as hydrolases that catalyze the hydrolysis of ortho-glycosyl compounds of starch and glycogen. They catalyze the hydrolysis of starchy materials into smaller glucose subunits that in turn are acted upon by other amylases to produce glucose[1]. Amylases, starch-degrading enzymes, have numerous biotechnological applications. These enzymes are used in textile and garments, paper industries, starch liquefaction, food, adhesive and sugar production and pharmaceuticals[2].

Enzymatic degradation of starch on an industrial scale has been practiced for many years and has replaced to a considerable extent the traditional acid-catalyzed processes[3]. Enzymatic processes now produce, over 75% of syrup and solid dextrose in the USA. New developments have taken place in the area of starch-degrading enzymes. Enzymes have several advantages. First, the specificity of enzymes allows the production of sugar syrups with well-defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in few side reactions and less browning[4].

Because of the commercial and industrial uses, α-amylases from many sources has been studied in great detail. The genus *Bacillus* is the single most important bacterial source of this enzyme. Due to the thermostability of the enzyme produced by genus *Bacillus*, they have commercial significance[4]. We have previously reported that three *Bacillus* isolates could produce appreciable amount of α-amylase in submerged culture[5]. The present article reports the partial purification and characterization of α-amylases of the three *Bacillus* isolates.

B. subtilis, *B. amyloliquefaciens* and another *Bacillus* isolate were grown in the medium containing 1.0% starch, 0.5% peptone, 0.5% corn steep liquor, 0.8% $(NH_4)_2SO_4$, 0.2% $MgSO_4.7H_2O$, 0.05% $CaCl_2.2H_2O$, 1.4% K_2HPO_4 and 0.6% KH_2PO_4 with an initial pH 7.0[6]. Shake-flask cultures were carried out in 250-ml Erlenmeyer flasks at 37°C for at least 72 h with continuous shaking (150 rpm) in an orbital shaker incubator. Extracellular α-amylases were removed by centrifugation and the enzyme activity was measured in the cell-free supernatant essentially as described by Gomes *et al*.[7]. The reducing sugar released was determined by the method of Miller[8]. One unit (U) of enzyme activity was defined in all cases as the amount of enzyme releasing 1 μmol of glucose or glucose equivalents from the substrate per min under the assay conditions.

For partial purification of the enzyme, the soluble protein contents of the culture supernatants were precipitated by stepwise addition of solid ammonium sulphate to 100% saturation. For temperature optima, the partially purified enzymes were assayed at different temperatures between 30 and 80°C with standard incubation time. The activity *vs*. pH profile was determined according to standard assay procedure at various pH values using 0.05 *M* citrate buffer (pH 4.0-6.0), 0.05 *M* phosphate buffer (pH 6.5-8.5) and 0.05 *M* Tris-HCl buffer (pH 9.0-10.0). Thermostability experiments were

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performed by incubating the enzyme preparation in 0.5 *M* phosphate buffer (pH 7.0) at various temperatures (4-100°) for 20 min. For determination of pH stability of α-amylase, the enzyme preparation in various pH buffers were incubated at 4°C for 24 h, and thereafter the residual activities were measured by standard procedure. For kinetic analysis, the α-amylase activity was assayed using various substrate (soluble starch) concentrations (0.5-6.0 mg/ml) under standard assay conditions. The enzyme kinetic parameters, namely K_m and V_{max}, were derived by the method of Lineweaver and Burk[9] using double reciprocal plot. The apparent K_m of α-amylase was expressed as mg/ml and the V_{max} of the enzyme as U/mg protein. The effect of various metal ions on the enzyme activity was measured by incubation the enzyme preparation at 4°C and pH 7.0 for 1 h in the presence of various chemicals at 5 m*M* concentration. The residual activities were measured by standard procedure.

α-Amylase produced by the three *Bacillus* isolates was partially purified by ammonium sulphate fractionation. Total soluble protein as well as α-amylase activity were obtained in 70% ammonium sulphate saturation fraction. Similar result was also reported for α-amylase produced by *Streptomyces chattanoogensis*[10]. The enzyme was assayed at different temperatures (30-80°C), pH (4.0-10.0) and reaction time (5-60 min) and the results are summarized in Table 1. The optimum temperatures for α-amylase were 60°C in case of *B. amyloliquefaciens* and 55°C in case of *B. subtilis* and *Bacillus* sp. The enzyme activity drastically decreased with the increase of temperature from their optimum temperatures that indicates a rapid inactivation of enzyme. These findings were in accordance to that reported by other investigators on α-amylases from different *Bacillus* strains[4]. In another study, the optimum temperature of α-amylase from yeast, *Saccharomyces cerevisiae*, was found to be 50ºC[11]. All the assayed activity had an optimum near neural pH (6.5-7.0). The maximum α-amylase activity of *B. subtitis* and *Bacillus* sp. was obtained after 15 min incubation, while the optimum reaction time for the enzyme from *B. amyloliquefaciens* was shorter (10 min). Sarikaya[6] also reported maximum α-amylase activity of *Bacillus subtilis* and *Bacillus amyloliquefaciens* after 10 min incubation.

Table 1. *Optimum temperature, pH and reaction time of extracellular α-amylase from three Bacillus isolates*

Reaction condition	Optimum activity		
	B. subtilis	*B. amyloliquefaciens*	*Bacillus* sp.
Temperature (°C)	55	60	55
pH	7.0	6.5	6.5
Time (min)	15	10	15

High thermal stability of enzymes is a desired property for the use an enzyme in industrial processes. α-Amylase from *B. subtilis* and *Bacillus* sp. was completely stable at 50°C for at least 20 min (Figure 1). The enzyme from *B. amyloliquefaciens* was more stable than the enzyme from two other isolates, exhibiting 100% of the original activity after exposure at 60°C for 20 min. These indicate that α-amylases from the *Bacillus* isolates was fairly thermostable, as was also reported by other investigators[12].

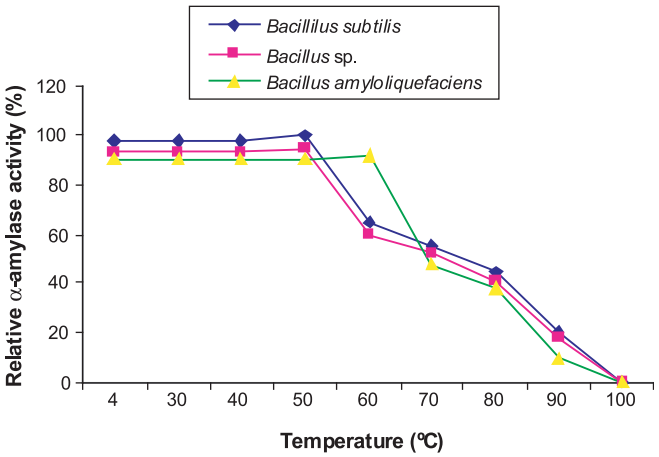

Figure 1. *Thermal stability of extracellular α- amylase from three Bacillus isolates.*

The wide range of pH stability might be an advantage of handling and preserving the enzyme in industrial and commercial purposes. In the present study, the extracellular α-amylase from the three *Bacillus* isolates inhibited nearly or more than 80% of the original activity over a wide range of pH (4.0-8.5) (Figure 2). Similar pH stability for α-amylase from *Bacillus licheniformis* was repoted by Saito[13].

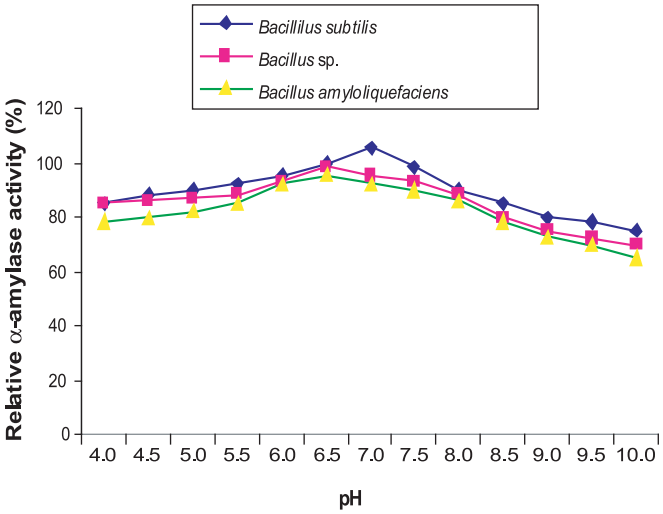

Figure 2. *pH stability of extracellular α-amylase from three Bacillus isolates.*

For kinetic analysis, α-amylase activity was measured at various starch concentrations (0.5-6.0 mg/ml). The enzyme kinetic parameters, namely K_m (Michaelis-Menten constant) and V_{max}, were derived from Lineweaver-Burk double reciprocal plot. The V_{max} values of partially purified α-amylase from *B. subtilis*, *B. amyloliquefaciens* and *Bacillus* sp. were 20.00, 14.28 and 16.66 U/mg protein respectively. The apparent K_m of α-amylases from

B. subtilis, *B. amyloliquefaciens* and *Bacillus* sp. were 2.5, 1.6 and 2.0 mg/ml respectively. In a study, K_m and V_{max} values of α-amylase from *Saccharomycopsis fibuliger* have been reported to be 1.37 mg/ml and 1.2 mg/ml/min using starch[14].

Effect of different metal ions (5 m*M*) on α-amylase activity was tested and the results are depicted in Figure 3. α-Amylase activity from *B. subtilis* was enhanced to some degree in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} ions, while the activity was remarkable decreased in the presence of Cu^{2+}, Ag^{2+} and Zn^{2+} ions. The enzyme activity from *Bacillus* sp. was slightly increased in the presence of Mg^{2+} and Mn^{2+} ions, while the activity was highly inhibited in the presence of Cu^{2+}, Ag^{2+} and Zn^{2+} ions. All the metal ions, except Mn^{2+}, exhibited severe inhibitory effect on the enzyme from *B. amyloliquefaciens*.

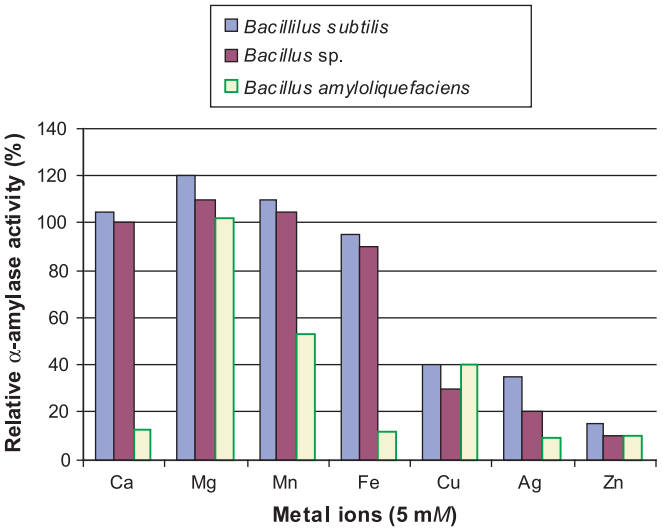

Figure 3. *Effects of metal ions on extracellular α-amylase activity from three Bacillus isolates.*

In view of the results obtained in this study, *Bacillus* isolates appear to be good sources of α-amylase with regard to temperature and pH effects. Little differences in pH stability were found among the α-amylases produced by the three isolates. However, a remarkable thermal stability of the enzyme was found for *B. amyloliquefaciens*. Metal ions such as Ca^{2+}, Mn^{2+} and Fe^{2+} had little or no influence on the enzymes from *B. subtilis* and *Bacillus* sp., but they exhibited strong inhibitory effect on the enzyme from *B. amyloliquefaciens.*

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