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ORIGINAL PAPER

Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis*

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Abstract Extracellular amylase production by a newly isolated alkali-thermotolerant strain *Streptomyces gulbargensis* DAS 131 was optimized and characterized. The highest amylase production was achieved by growing *S. gulbargensis* DAS 131 in media with 1% starch. Strain exhibited maximal activity at pH 9.0 and 45°C and relatively stable in alkaline conditions (pH 11). Starch and peptone were found to be the good source of carbon and nitrogen with a yield of 2,216.6 and 2,156.1 U, respectively. Maltose and maltotriose were the main end products of starch hydrolysis, indicating α-amylase activity. SDS-PAGE analysis revealed a monomeric form with a molecular weight of 55 kDa.

Keywords *Streptomyces* · Amylolytic enzymes · Purification · pH and temperature effect

Introduction

Starch is an important renewable biological resource and the most important industrial enzymes in use today include protease, carbohydrate-hydrolyzing enzymes and ester cleavage fat hydrolyzing enzymes. The specific application of such technical enzymes are in major areas of food processing, beverages production, animal nutrition, leather, paper and pulp, textile and detergents, etc. To meet the current largely increased demand, studies on the cost effective production of industrially important enzymes have become the need of today. Microorganisms are the most important sources for enzyme production; they made significant contribution to the production of foods and beverages in the last three decades. Selection of the right organism plays a key role in high yield of desirable enzymes [1].

Amylases are enzymes, which hydrolyze starch molecule to give diverse products including dextrin and progressively smaller polymers composed of glucose units. Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market [2]. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [3].The possibility of using actinomycetes specially, *Streptomyces* for enzyme production has recently been investigated [4, 5]. *Streptomyces* species are heterotrophic feeders and they can utilize both simple and complex molecules as nutrients. Starch hydrolyzing activity was widely distributed in species of *Streptomyces* and some of them can attack and hydrolyze raw starch granules with the release of maltose as the predominant product, such enzymes are used for the industrial conversion of raw starch into sugar for fermentation [6, 7]. The present study deals with the characterization of the extracellular amylolytic activity found in newly isolated *Streptomyces gulbargensis* species and its optimization.

Materials and methods

Bacterial strain, growth and amylolytic enzyme production

The strain of *S. gulbargensis* was isolated from soil sample collected from Gulbarga, Karnataka, India [8], using the

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methodology described by Goodfellow [9]. Stock cultures were maintained on medium ISP-2 [10], supplemented with 1% (w/v) agar starch slants at 4°C. The medium for enzyme production was starch urea with the following composition: (g l^{-1}): starch, 10.0; urea, 2.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.1 and pH 7.0. The inocula standardization was performed accordingly [11]. Pre-inoculum was prepared in Erlenmeyer flasks, containing 50 ml of starch-urea medium, previously sterilized at 121°C for 15 min, inoculated with 2.0-ml spore suspension containing 3–4 × 10^8 CFU ml^{-1} and cultivated under agitation at 180 rpm, for 24 h. Five milliliters of the pre-inocula were added to 50 ml of starch-urea medium incubated at 28°C for 96 h. At the 12th hour of incubation, samples (5 ml) were taken from each of three replicate flasks. The cells were harvested by centrifugation at 4,000 rpm for 15 min, at 4°C. The supernatant containing amylase was used as the starting material to evaluate enzymatic activity, protein and pH.

Biomass production

Three replicate flasks maintained in the same conditions described for enzyme assays were harvested at 12–h intervals to determine the total amount of growth (biomass). The mycelial mass was removed by vacuum filtration and dried in an oven at 80°C to measure dry biomass weight expressed in terms of mg dry mass per 50 ml of culture medium.

Analytical procedures

The α-amylase activity was evaluated by adding 1.0 ml of supernatant sample in starch solution 1% (w/v), previously gelatinized in 0.1 M citrate-phosphate buffer pH 6.5, at 37°C for 30 min. The reaction was stopped by the addition of 2 ml of dinitrosalicylic acid according to Bernfeld [12]. One unit of α-amylase was defined as the amount of enzyme necessary to produce reducing sugars equivalent to 1 μmol of glucose min^{-1}, at 30°C. The specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry et al. [13], using bovine serum albumin in the standard curve.

Determination of pH and temperature of growth medium for optimal amylolytic activity

Cultures were incubated at 28°C for 24 h in starch-urea medium supplemented with 1.0% starch. The amylolytic activity was determined at different pH values by varying the buffer of the substrate solution in the standard assay: 100 mM acetate buffer for pH range 4.0–6.0, succinate buffer for pH 7.0, Tris-Cl buffer for pH 8.0–9.0, and glycine-NaOH buffer for 10.0–12.0. The optimal temperature for activity was determined by assaying activity at 30, 35, 40, 45, 50, 55 and 60°C. The incubation period during the assay was 30 min at each temperature.

Effect of metal ions and other reagents

The effects of different metal ions on α-amylase activity was determined by the addition of the corresponding ion at a final concentration of 1 mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of Ca^{2+}, Cs^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, Ag^{1+} chlorides.

Purification of α-amylase

Fractionation with ammonium sulphate

The various steps of enzyme purification were carried out at 4°C. In the initial purification step, the supernatant fluid containing the extracellular enzyme was lyophilized to prepare the final solution. This lyophilized supernatant fluid was treated with solid ammonium sulphate as described by Green and Hughens [14], with continuous overnight stirring and separated into the following saturation ranges: 0–20, 20–40, 40–60 and 60–80%. The precipitates collected by centrifugation (12,000*g* for 15 min) were dissolved in 0.1 M citrate-phosphate buffer, pH 5.0. The enzyme solution was dialyzed against the same buffer for 12 h with several changes to remove the salt and assayed by the method described by Plumer [15].

Polyacrylamide gel electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed according to the method of Laemmli [16]. Molecular weights were measured by the method proposed by Weber and Osborne [17], with the aid of an electrophoresis calibration kit.

Effect of carbon and nitrogen sources on amylase activity

The effects of different carbon and nitrogen compounds on α-amylase formation by *S. gulbargensis* sp. DAS 131 was investigated in the cultivation medium containing 1% of (w/v) nitrogen and carbon sources, and incubated for 72 h. Samples were analyzed every 6 h, from 12 to 72 h. The result of maximum amylase activity of strain DAS 131 at various intervals was recorded.

Results and discussion

The results obtained on growth of *S. gulbargensis* sp. DAS 131 showed that the isolate reached the end of its logarithmic phase after incubation for 48 h (Fig. 1).

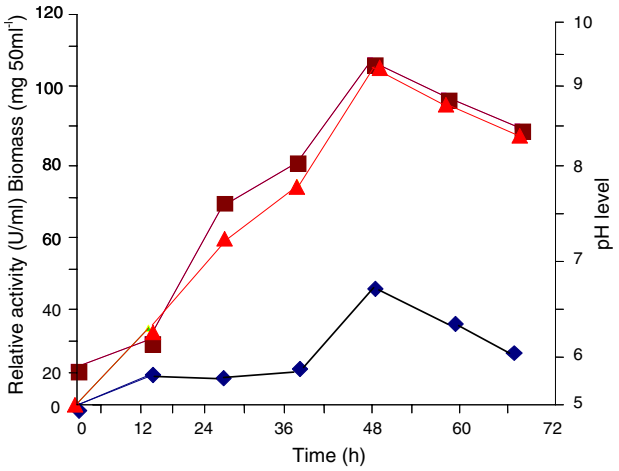

Fig. 1 Production of α-amylase by *Streptomyces gulbargensis* DAS 131 in starch-urea medium. *Dash with filled square* biomass (mg 50 ml^{-1}), *dash with filled triangle* medium pH, *dash with filled diamond* enzyme activity(U ml^{-1}) Each value is an average of three parallel replicates

Effect of pH and temperature on amylase activity

The enzyme exhibited an optimal activity at pH 9.0 (1,341.3 U), and was relatively stable in alkaline condition with at least 70% of the activity at pH 11.0 (Fig. 2). The amylase showed optimal activity at 45°C (1,312.1 U), and was unstable at higher temperature. Thus, more than 40% of the activity was lost between 45–50°C and more than 75–85% at 60°C (Fig. 3). This result is in good agreement with *Halomonas meridiana* [18] in which 80% activity was

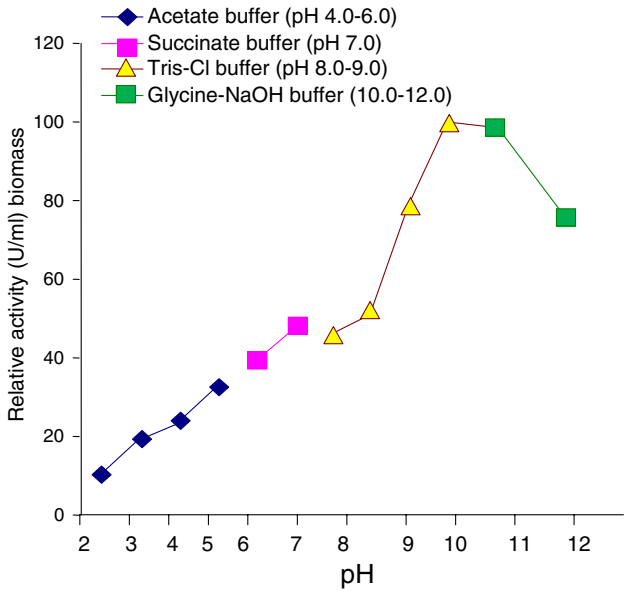

Fig. 2 Effect of pH on α-amylase activity after 30 min of exposure. Acetate buffer (pH 4.0–6.0), succinate buffer (pH 7.0), Tris-Cl buffer (pH 8.0–9.0) and glycine-NaOH buffer (pH 10.0–12.0). Data are the average of three experiments

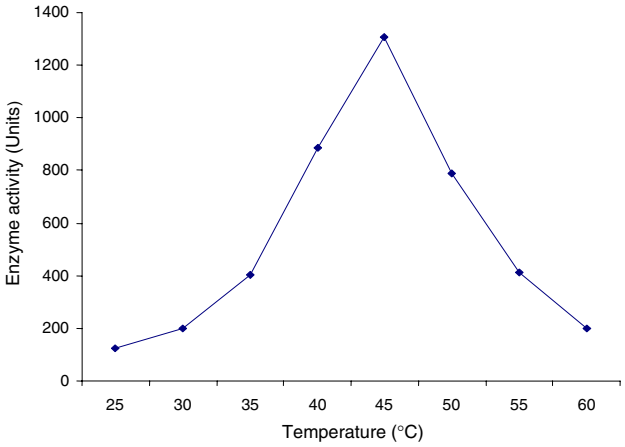

Fig. 3 Effect of temperature on α-amylase activity after 15 min of exposure. Each value is an average of three parallel replicates

lost at 65°C. Regarding the stability of the enzyme at different pH, *Streptomyces praecox* [19] and *Streptomyces limosus* [20] showed the optimum activity at pH 7.0 and sharply decreases as pH rises above pH 11.0. The alkaliphilic nature of the enzyme excreted by DAS 131 was much more remarkable in the stability of the enzyme than in its activity, being a highly interesting feature for possible industrial application.

Effect of carbon and nitrogen sources on enzyme activity

Different carbon and nitrogen sources were tested to obtain the best results for amylase activity. Among carbon sources tested, the optimum yield was obtained when the cells were grown in a minimal medium containing starch at a concentration of 1% (w/v) with an yield of 2,216.6 U, while peptone found to be an good source of nitrogen with a yield of 2,156.1 U, respectively, after three repeated experiments (Fig. 4).

Hydrolysis products of amylase

Streptomyces gulbargensis sp DAS 131 amylase hydrolyzed starch to form maltose and maltotriose as major products (Fig. 5). These products were readily apparent even during the early stages of the reaction and increased in concentration along the time course of the reaction. Maltose was not hydrolyzed by the enzyme; the enzyme may, therefore, preferentially cleave at the α-1,4-linkage adjacent to non-reducing ends, releasing maltose and maltotriose. This behavior might be due to stationary phase regulation on one side and catabolic repression by the glucose released from maltose and maltotriose on the other side. Similar observations were made with those of α-amylase produced by *Streptomyces lividans* TK 24 [21], *Nocardia halobia* [22] and *H. meridiana* [18].

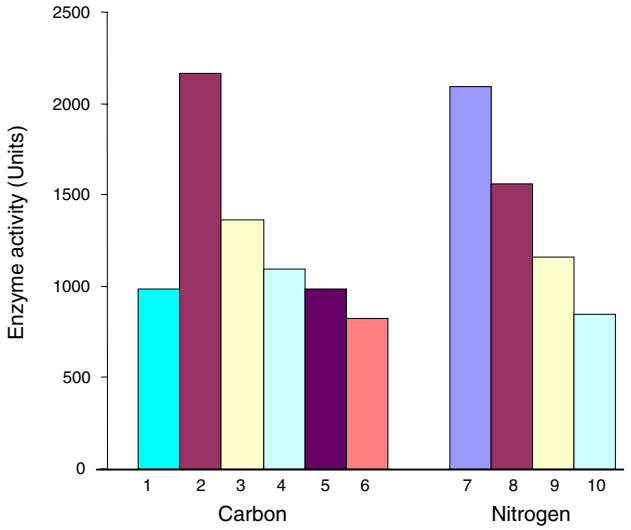

Fig. 4 Effect of carbon and nitrogen sources on α-amylase activity by *Streptomyces gulbargensis* DAS 131. Data are the average of three experiments (*1* sucrose, *2* starch, *3* lactose, *4* xylose, *5* fructose, *6* glucose, *7* peptone, *8* yeast extract, *9* meat extract, *10* protease peptone)

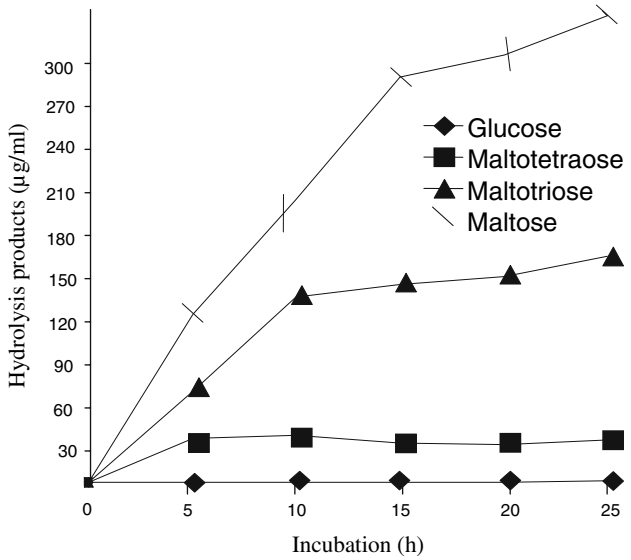

Fig. 5 Hydrolysis products of the α-amylase using soluble starch as substrate, after different incubation times. Data are the average of three experiments

Partial purification of amylase

The partial purification of α-amylase DAS 131 was achieved by ammonium sulphate precipitation at 40–60% concentration. Purified enzyme molecular mass has been determined on SDS-PAGE. The purified enzyme appeared as a single band on SDS-PAGE, corresponding to a molecular mass of 55 kDa (Fig. 6). Using gel filtration chromatography, the native molecular mass of purified enzyme was estimated to be 57.9. The apparent relative molecular mass for the α-amylase by gel filtration was very similar to that found by SDS-PAGE; hence, the enzyme seems to be a monomeric enzyme. Similar observations were made from *Thermus filiformis Ork* A2 with 60 kDa [23], *Thermococcus hydrothermalis*, 53.6 kDa [24] and from *Halothermothrix orenii*, 56 kDa [25].

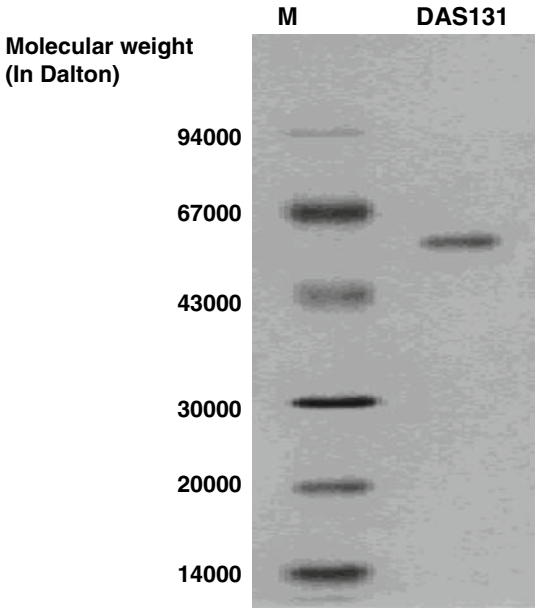

Fig. 6 Protein bands of α-amylase from strain DAS 131 on SDS-polyacrylamide gel electrophoresis. Lane1, *M* marker (Phosporilase B, albumin, ovalbumin, carbonic anhydrase, Tripsin inhibitor, α-lactalbumin), Lane 2 band of *DAS 131*

Table 1 Effect of various metal ions on the activity of α-amylase from *Streptomyces gulbargensis* sp. DAS 131

Metal ions	Concentration (mM)	Relative activity of amylase (%) DAS 131
Control (no addition)	1	100
Ca^{2+}	1	148
Mg^{2+}	1	98
Fe^{2+}	1	81
Fe^{3+}	1	57
Co^{2+}	1	23
Zn^{2+}	1	83
Mn^{2+}	1	96
Hg^{2+}	1	44
Cs^{2+}	1	89
Ni^{2+}	1	100
Sr^{2+}	1	92
Pb^{2+}	1	91
Cu^{2+}	1	100
Ba^{2+}	1	29
Ag^{1+}	1	36

Data are the average of three experiments

Table 2 Comparison of alkaline amylase producing *Bacillus* strains of commercial value with *S. gulbargensis* DAS 131

Organism	Optimum temperature (°C)	Optimum pH	K_m (mg/ml)	Activity (U/mg)	References
S. gulbargensis	45	8.5–11	5.0	1,341.3	This study
Bacillus sp. ANT-6	80	10.5	3.85	195	[28]
Bacillus sp. NRRL B 3881	50	9.2	1.9	18.5	[29]
Bacillus subtilis	50	6.5		5,000	[30]
Bacillus sp. L1711	35–40, 45	9.5–10.0, 7–7.5		1,483	[31]
Bacillus sp. KSM-1378	55	8.0–8.5		157.5	[32]
Bacillus sp. WN11	75–80	8.0–8.5		4221	[33]
Bacillus sp. GM8901	60	11.0–12.0		921	[34]
Bacillus KSM-K38	55–60	8.0–9.5			[35]
Bacillus sp. TS 23	70	9.0	2.7		[36]
Bacillus sp A-40-2	55	10.5			[37]
Bacillus sp. 17.1		4.5–10			[37]
Bacillus sp. 38-2		4.5–7, 8–9			[37]

Effect of metal ions on amylase activity

Many metal ions often influence the activity of α-amylase [26, 27]. The effect of 15 kinds of metal ions on the α-amylase activity was studied (Table 1). The activity of α-amylase was increased by 48% in presence of 1 mM Ca^{2+}. This was similar to the results from *Streptomyces* sp [26], 100% activity was recovered with Ni^{2+} and Cu^{2+} were as Mg^{2+}, Mn^{2+}, Sr^{2+}, Pb^{2+}, Zn^{2+}, Cs^{2+}, Fe^{2+}. Fe^{3+} and Hg^{2+} inhibit the almost 50% of the enzyme activity and nearly 70% or more enzyme activity were inhibited by Co^{2+}, Ba^{2+} and Ag^{1+} (Table 1).

Most of the *Bacillus* strains used commercially for the production of alpha-amylases by submerged fermentation have an optimum pH between 6.0 and 9.0 for growth and enzyme production [28, 29]. Growth of *S. gulbargensis* and amylase production was higher at 45°C and pH 9.0 (Fig. 1). Similarly, temperature and pH for the optimum growth and amylase production in different *Bacillus* sp. were presented in Table 2. Partially purified *S. gulbargensis* amylase was stimulated by Ca^{2+} and Mg^{2+} ions and inhibited partially by Fe^{3+}, Co^{2+}, Ba^{2+} and Ag^{1+} and completely by Hg^{2+}, suggesting its calcium-dependent nature. Most of the alpha-amylases are calcium-dependent metalloenzymes [30]. *Bacillus* sp. ANT-6 also showed increased activity in presence of Ca^{2+} ions [28]. The hydrolysis pattern presented by *S. gulbargensis* amylase showed similarity with that of *H. meridiana* [18] producing glucose, maltose, and malto-oligosaccharides as the main products; hence, it is an alpha-amylase. In all results obtained on growth of *S. gulbargensis* the isolate reached the end of its logarithmic phase after incubation for 48 h (Fig. 1). The maximum production of α-amylase occurred at pH 9.0 and 45°C. The use of α-amylases in detergents for medium-temperature laundering demands enzymes with high stability, and activity in washing environments needs high alkalinity. The enzyme produced by DAS 131, remains active at very high alkaline pH (8.5–11), a characteristic that distinguishes it from most of the other α-amylases from bacterial sources (Table 2). Due to the high alkaline nature of α-amylase produced by *S. gulbargensis* DAS 131, it has shown potentiality for its application in detergent and textile industries.

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