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PARTIAL PURIFICATION AND CHARACTERIZATION OF AN ACIDOPHILIC EXTRACELLULAR α-AMYLASE FROM *BACILLUS LICHENIFORMIS* JAR-26

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ABSTRACT:

In the present investigations, a thermostable amylase producing *Bacillus licheniformis* JAR-26 was isolated from spoiled tomato and its enzyme productivity and activity evaluated. The enzyme synthesis was optimum at 45 ^{0}C when initial pH of fermentation medium was 5.5. The enzyme remained stable in a range of temperature and pH between 30 ^{0}C-85 ^{0}C and 3.5-8.5 pH, respectively. The optimum enzyme activity was displayed at 85 ^{0}C and pH 5.5. The enzyme was concentrated by isopropanol precipitation and further purified by ion-exchange chromatography on DEAE cellulose DE-52 and gel chromatography on Sephadex G-100, respectively. The elution profile showed two peaks on DEAE cellulose DE-52 which was resolved into single peak when passed through Sephadex G-100. The purified amylase migrated on SDS-PAGE as a single band corresponding to molecular mass of 38 KDa. The thermostability of the enzyme was also studied and it was found that the amylase was thermostable with 55% activity retained at 100 ^{0}C and 48 % at 104 ^{0}C (30 min). The purified amylase shows interesting properties useful for industrial applications.

Key Words: Amylase, *Bacillus licheniformis* JAR-26, acidophilic, thermostable.

[I] INTRODUCTION

Amylases are extracellular enzymes that hydrolyze starch molecules to yield diverse products like dextrins and progressively smaller polymers composed of glucose (maltooligosaccharides) and/or maltose and glucose units [1, 2]. Amylases can be divided into endoamylases and exoamylases. Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from non-reducing end successively resulting in short end products. Amylases find potential application in a number of industrial processes such as in food, textile and paper industries. α-Amylase (EC 3.2.1.1; α-4-glucan glucano-hydrolase) is an endo-type enzyme and holds the maximum market share of the enzymes sales with major industrial applications in starch processing, brewing and sugar production, food and paper

industry, designing in textile industries and in detergent manufacturing processes [1, 3]. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medical and analytical chemistry [4]. α-Amylase holds the maximum market share of the enzymes sales with major industrial applications. Although amylases can be obtained from several sources, such as plants, animals, and microorganisms, the enzymes from microbial sources particularly grown in habitat characterized by extreme environments, prove to be useful for industrial processes/demand.

Gelatinization and liquefaction of the starch are the key processes involved in the manufacture of dextrose and syrups from native starch [5]. Liquefaction of the starch at high temperature using heat stable amylases has definite advantages over the other methods of thinning starch. Use of heat stable amylase can reduce the retrogradation of starch. It also prevents the formation of undesirable byproducts inherent to acid thinning process. Thermostable amylases find wide applications in a number of industrial processes e.g. in starch-glucose, textile, pharmaceutical and brewing industries [6, 7]. Amylases used commercially for starch processing are thermostable but recently the interest has been focused on thermophilic amylase capable of working at low pH range of 4.5-5.5 [8]. The present communication deals with the production of thermostable amylase capable of working at low pH range. Relationship between growth and amylase production, amylase production and thermostable characteristics of extracellular amylase, partial purification and characterization/nature of enzyme has been studied.

[II] MATERIALS AND METHODS

2.1 Isolation and Characterization

A starch hydrolyzing bacteria (*Bacillus licheniformis* JAR 26) was isolated from spoiled tomatoes and screened for extracellular acidophilic amylase production. The starch medium [9] used for the isolation of bacteria contained (g/L): Starch (Merck, Germany), 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 5.5. One gram of each sample was suspended in 9.0 ml of sterile water and 0.1 ml of suitably diluted suspension was spread on the agar plates. The plates were incubated at 45 ^{0}C, 50 ^{0}C, 55 ^{0}C and 60 ^{0}C for 24 to 48 h. The isolated colonies were flooded with iodine solution. Colonies with good colorless halos around them were picked and maintained on starch agar slants at 4 ^{0}C and further assessed for enzyme production in liquid medium.

The characterization and identification of the isolate was made following Bergey's Manual of Systemic Bacteriology [10]. The method of identification was as given by Collee *et al.* [11].

2.2 Amylase production

The medium for enzyme production comprised (gl^{-1}): starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02. Initial pH of the medium was adjusted at 5.5 and 50 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker (Remi) at 170 rpm at 45 ^{0}C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 x g for 10 minutes and cell-free culture supernatant fluid used as enzyme source.

2.3 Assay of amylase

Culture filtrates (supernatant) was assayed for enzyme activity. For determining amylase activity, the method of Srivastava and Baruah [6] was followed. One ml of 1% (w/V) starch (Merck, Germany) solution was taken in test tube and 0.2 ml of 0.2 M phosphate buffer (pH 5.5) and 0.2 ml of deionized water was added to it. The mixture was equilibrated at 70 ^{0}C for 10

minutes in a water bath. 0.1 ml of supernatant was added and then reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS). The mixture was heated and the color intensity was measured at 540 nm [12] using a spectrophotometer (Systronics Spectrophotometer 169). One unit of amylase activity was defined as the amount of amylase that liberates 1.0 mg of glucose per minute under assay conditions.

In all the above experiments the enzyme activity was calculated as the average of three independent sets of experiments and the standard deviation in all cases was found negligible.

2.4 Thermostability of the enzyme

The thermostability of the enzyme produced by *Bacillus* sp. JAR-26 was studied by the method given by Srivastava and Baruah [6]. The supernatant was kept at various temperatures ranging between 70 ^{0}C to 110 ^{0}C in the absence of the substrate and at different time intervals. Enzyme (0.1 ml) was withdrawn and amylase activity was estimated as mentioned above. For optimum temperature the reaction mixture was heated at different temperature i.e. 40 ^{0}C-90 ^{0}C for 10 minutes and amylase activity was determined same as above.

2.5 Enzyme purification

All operations, unless stated otherwise, were carried out on ice or at 4 ^{0}C in a cold chamber. The crude enzyme solution, used for purification, was obtained by growing *Bacillus licheniformis* JAR-26 for 36 h. The supernatant was collected by centrifugation at 10,000x g for 10 min at 4 ^{0}C. Two volumes of cold isopropanol were added slowly to the cold supernatant fluid with constant stirring, maintaining the temperature at 4-8 ^{0}C for 40 min. The precipitate was collected by centrifugation at 10,000x g for 10 min at 4 ^{0}C, dissolved in minimal volume of 0.01 M citrate phosphate buffer (pH 5.5) and undissolved materials were removed by centrifugation at 10,000x g for 10 min at 4 ^{0}C. The clear solution was dialysed against the same buffer for 18 h at 5-8 ^{0}C. The dialysate was applied to a column (1.6 x 45 cm) of CM-Cellulose and DEAE cellulose DE 52 (Whatman) equilibrated with the dialysis buffer, ph 5.5 containing 0.02% sodium azide as preservative. After washing the column with 50 ml of the same buffer, elution was performed with a linear gradient of NaCl concentrations from 0.1 to 1.0 M in 0.01 M citrate phosphate buffer (pH 5.5). The volume of NaCl gradient was kept 100 ml. The flow rate was adjusted to 20 ml h^{-1}, 2.5 ml fractions were collected and analyzed for protein and amylase activity. The active fractions were pooled, concentrated by isopropanol precipitation and redissolved in minimum quantity of same buffer, pH 5.5. This was applied on Sephadex G-100 (Sigma, Germany) column (1.6 x 50 cm) and fractioned as above.

2.6 SDS-PAGE

The homogeneity of the purified amylase and determination of approximate molecular weight was ascertained by the SDS-PAGE carried out in Tris-glycine buffer (pH-8.3) using 10 % (w/V) polyacrylamide slab gel (1 mm thick) according to the method of Laemmli [13] along with standard protein molecular weight markers. After electrophoresis, protein bands were visualized by staining with Coomassie blue R-250 staining method.

[III] RESULTS

Bacillus licheniformis JAR-26 grew well on the starch medium (Bajpai et al., 1991) and showed a good amount of α-Amylase production. The enzyme production was improved when the bacteria were grown on modified starch medium (starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02, ph 5.5) and inoculum was taken from 24 hour old starch medium (Bajpai et al., 1991) grown cultures having attained an optical density 0.5. Maximum bacterial growth and enzyme production was observed at 36 hours (2.82 units/mL). Further

increase in incubation period resulted in decreased enzyme production (2.426 units/mL), however, growth stayed almost constant up to 48 h.

3.1 Purification of amylase

The purification scheme and the results obtained in various purified fractions from 530 mg protein in the crude enzyme extract are summarized in Table 1. The crude enzyme solution could be precipitated with cold isopropanol. This precipitate was dissolved in 0.01 M citrate phosphate buffer (pH 5.5) and dialyzed overnight against 250 volume of the same buffer at 4 ^{0}C. The dialyzed fraction accounted for a total activity of 1150 U and a specific activity of 127.7 U/mg protein and 39.2 fold purity (Table 1).

Crude enzymes concentrated by isopropanol were separated by ion exchange chromatography, firstly on CM cellulose followed by DEAE cellulose DE-52, pH 5.5, using a molarity gradient elution with 0.1 to 1.0 M NaCl in 0.01 M citrate phosphate buffer (pH 5.5) into two distinct amylolytic peaks, A-I and A-II (Figure 1, 2). The peak II (fractions 26-30) showed maximum amylase activity. The peak II of amylase showed a total activity of 596 U and specific activity of 248.33. The overall purification increased to 76.41 fold. The results appear to indicate the anionic (acidic) nature of amylase.

Table 1. Summary of Enzyme Purification.

Enzyme	Total volume (ml)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Overall Yield %	Purity (fold)
Cell free supernatant	**100**	**1725**	**530**	**3.25**	**100**	**1**
Isopropanol precipitate	**1**	**1150**	**9.0**	**127.77**	**67**	**39.31**
CM-Cellulose column	**25**	**684**	**4.4**	**155.45**	**40**	**47.83**
DEAE-Cellulose column	**25**	**596**	**2.4**	**248.33**	**35**	**76.41**
Sephadex G-100 column	**20**	**248**	**0.78**	**317.9**	**15**	**97.8**

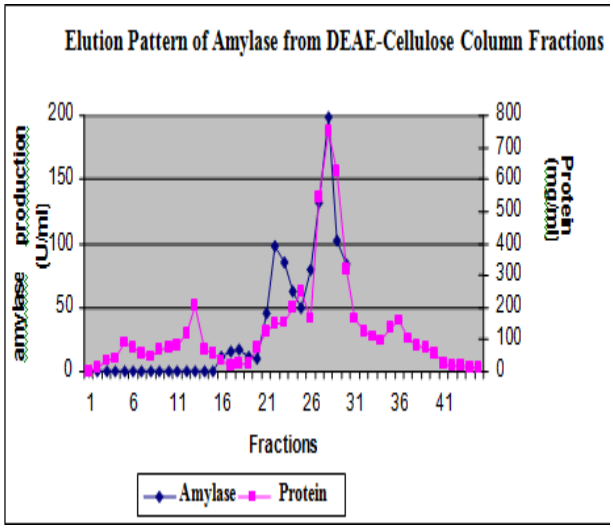

Figure 1 - Elution Pattern of Amylase from DEAE-Cellulose Column fractions.

This peak II was pooled and concentrated and applied on to Sephadex G-100 column. Active fractions were separated on a Sephadex G-100 column where they revealed only single peak of amylase activity. At this stage, the specific activity of the purified enzyme increased by 97.8 fold with respect to original filtrate. A single band of protein was observed (Figure 3) following SDS-PAGE, indicating that the amylase exists as a monomer.

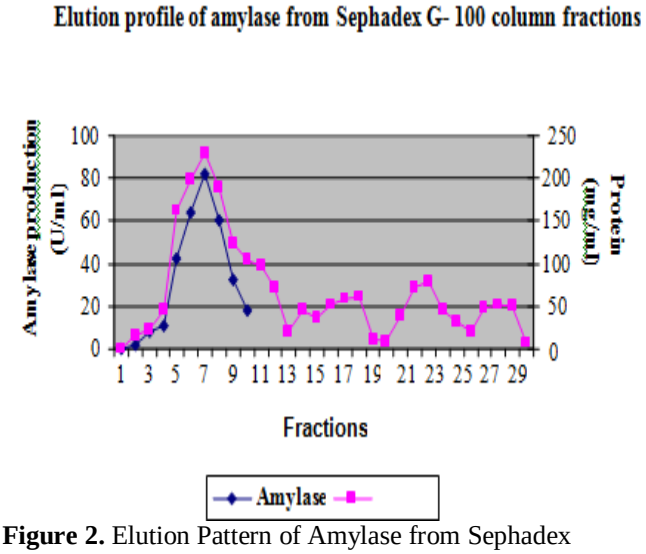

Figure 2. Elution Pattern of Amylase from Sephadex G-100 Column fractions.

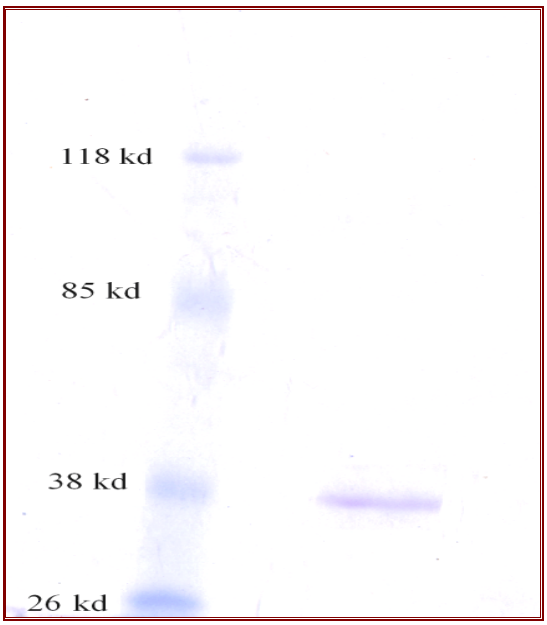

Figure 3. SDS-PAGE of the thermostable purified amylase
A: Molecular weight markers B: α-amylase from *Bacillus licheniformis* JAR-26

3.2 Thermostability of the enzyme

The optimum temperature for enzyme assay was 85^0C at pH 5.5. When the thermostability was checked, it was found that the enzyme was highly thermostable with 55 % activity retained at 100 ^{0}C which further decreased to 48 % at 104 ^{0}C (30 minutes).

3.3 Homogeneity of Sephadex G-100 purified amylase

Sephadex G–100 purified α-amylase was tested for its homogeneity and molecular weight by SDS-PAGE electrophoresis which revealed that the purified amylase migrated as a single band corresponding to molecular mass of 38 KDa.

[IV] DISCUSSION

Amylases secreted by thermophilic and hyperthermophilic microorganisms are more heat stable [8, 14] and preferred for use in starch processing industry. The thermostable α-amylases that have been used on commercial scale, have been obtained from *Bacillus amyloliquefaciens, B. stearothermophilus* and *B. licheniformis* which are thermophilic with optimum temperature of growth in the mesophilic range of 30-40^{0}C [8, 15]. The amylase procedure described here resulted in the purification of the major acidophilic extracellular amylolytic fraction with an enzyme yield of 15 % and purity of 97.8 which is much higher in comparison to previous reports [16, 17]. De *et al*. [18] have reported 88 fold purification of α-*amylase* from *Bacillus amyloliquefaciens* NCIM 2829 by ammonium sulphate purification, DEAE-Sephadex elution and gel filtration through Sephadex G-75 with an enzyme yield of 6.8 %. In the present study, the enzyme preparation obtained at the end of purification process had a specific activity of 317.9 and in SDS-PAGE a single band of protein with a molecular mass of 38 KDa was obtained, thus indicating that it was a homogeneous protein (Figure 3). Molecular weights of α-amylases are usually between 50-60 KDa but variations in molecular weights ranging from 10 KDa-210 KDa are reported in the literature [2]. These differences in molecular weights of enzyme molecule(s) result from the gene corresponding to the source organism [19]. Comparatively lower molecular masses of α-amylases from other *Bacillus* species have been reported in literature. These include *Bacillus amyloliquefaciens* (49.1 KDa), *Bacillus stearothermophilus (*48.0 KDa*)* and *Bacillus subtilis* strains (47.0 to 48.0 KDa) [20]. In a recent study, De *et al.* [18] have reported a molecular weight of 67500 for α-*amylase* produced by *Bacillus amyloliquefaciens.* The α-amylase reported in present study appears to be distinct from previously reported enzymes in terms of molecular weight, subunit enzyme purity, enzyme yield and suitability for extreme conditions and holds promise for application in industrial processes.

[V] CONCLUSION

In starch industry large amount of salts have to be removed by ion exchangers in second step because of unavailability of thermostable

amylases capable of working at low pH. This study indicates that *Bacillus licheniformis* JAR-26 produces thermostable and acidophilic amylase, which can find industrial use in starch saccharification.

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