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Isolation and identification of α-amylase producing *Bacillus* sp. from dhal industry waste

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A bacterial strain was isolated from dhal industry red gram waste and identified as *Bacillus*. A thermostable extracellular amylase was partially purified from the strain. Optimum temperature and *p*H for the enzyme were found to be 60°C and 6.5, respectively. The maximum amylase production was achieved with maltose as carbon source. Among the nitrogen sources, peptone and yeast extract produced maximum amylase.

Keywords: Bacillus spp., Thermostable amylase, Dhal industry waste.

Amylases are the important enzymes, particularly in the process involving starch hydrolysis. Though they originate from different sources (plants, animals and microorganisms), in industry, they are mainly produced from the microbes, due to their higher yield and thermostability[1]. They find potential applications in industries such as food, fermentation, textile, paper and detergent[2]. Microbial amylases have been proved to be an alternative to the chemical hydrolysis of starch[3].

The bioprocessing of starch into malto-oligosaccharides is gaining importance, due to their uses in food, pharmaceutical and fine chemical industries. A high value is placed for thermostable and thermoactive amylases in the bioprocessing of starch, as they are more economical. At elevated temperature, they improve the solubility of starch, decrease the viscosity, limit microbial contaminants, reduce reaction time[4]. Also, with the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistries[5]. Demand for microbial amylases has increased, due to their specificity of reaction, mild conditions required for reaction, and less energy consumption than the conventional chemical methods[5].

Bacillus spp. are considered to be the most important sources of α-amylase and have been used for its production[6]. Some *Bacillus* strains produce the enzyme in the exponential phase, whereas some others in the mid stationary phase. Though the pattern of growth and the enzyme profiles of *Bacillus* spp. have similarities, the optimized conditions for the enzymes differ widely, depending upon the strain. In the present study, a bacterial strain was isolated from red gram dhal industry waste, which was found to be a potential source of thermostable amylase. The properties of extracellular amylase were also reported.

Materials and Methods

All the chemicals used were of analytical grade and purchased from SD fine Chem (Mumbai, India) and HiMedia (Mumbai, India).

Isolation, media and culture conditions

Bacillus sp. (B_3) was isolated from dhal industrial red gram waste (near dhal industries, Gulbarga, Karnataka State, India) using plate dilution technique[7]. Starch-degrading microbes were isolated using media I (starch, 10.0 g l^{-1}; agar, 20.0 g l^{-1} and adjusted to *p*H 7.0), media II (starch, 1 g l^{-1}; peptone, 0.5 g l^{-1}; $K_2HPO_4.2H_2O$, 0.2 g l^{-1}; $MgSO_4.7H_2O$, 0.05 g l^{-1}; $FeCl_3$ traces; agar, 20 g l^{-1}; glycerol, 1 ml l^{-1} and *p*H adjusted to 7.0) and media III (nutrients are same as in media II, except that in place of peptone, NH_4NO_3 was used). The colonies formed on blue background by iodine solution were selected[7] and identified as described[8].

Soil sample was added to liquid media (media II and III, without agar in Erlenmeyer flask; 250 ml), incubated at 55°C for 6 days and 0.1 ml of this culture media was added to fresh media. The growth was

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observed at 55°C within 2 days and 0.1 ml of culture from this was plated on plates prepared with media II and III. Plates were then incubated at 50°C, white colored colonies were observed on plate after 24 h and were maintained on slants containing media II and III.

Effect of carbon and nitrogen sources on production of amylase

For optimization of cultural conditions, media IV (starch, 10.0 g l^{-1}; yeast extract, 3.0 g l^{-1}; peptone, 5.0 g l^{-1}; NaCl, 3.0 g l^{-1}; $MgSO_4.7H_2O$, 0.05 g l^{-1}; and adjusted to *p*H 7.0) was used. To study the effect of carbon source on production of amylase activity in media IV, 1% starch was replaced by different carbon sources as listed in Table 3. Similarly, for studying the effect of nitrogen source in media IV, peptone and yeast extract were replaced by 1% simple or complex nitrogen sources (Table 4).

Amylase assay

Unless otherwise stated, all experiments were carried out in triplicate. Amylase activity was assayed as described[9], with some modifications. Briefly, the 0.5 ml of 1% starch in 0.1 *M* phosphate buffer (*p*H 6.5) + 0.5 ml of enzyme were incubated for 30 min at room temperature (37ºC). The reaction was arrested by adding 1.0 ml of dinitrosalicysilic acid reagent and kept on boiling water bath for 5 min and 10 ml of distilled water was added. Absorbance was measured at 540 nm against blank. Blank was the same as above without incubation. One unit of the amylase activity was defined as the amount of enzyme that liberated one µmole of reducing sugar (maltose equivalent) under assay conditions.

Partial purification of amylase

Isolate B_3 was selected for partial purification of the enzyme, since it gave the maximum activity among all the isolates. Inoculum was prepared by transferring one loop-full of cells from slant culture to the inoculum media (50 ml/250 ml Erlenmeyer flask) and incubating the flask at room temperature in a rotary shaker at 120 rpm for 48 h. Fermentation medium (total volume 100 ml in 250 ml Erlenmeyer flask) was inoculated with 0.1% inoculum and incubated for 72 h under the same conditions. After 48 h of fermentation, broth was centrifuged at 6000 rpm for 15 min at 4°C. Partial purification of amylase was carried out by ammonium sulphate precipitation (40%). Protein concentration was estimated by Bradford method[10] using bovine serum albumin as standard.

Effect of *p*H and temperature on amylase

Effect of *p*H was studied from *p*H 2.0 to 11.9 (HCl/KCl buffer for *p*H 2.0; glycine/HCl buffer for *p*H 2.5 to 3.5; acetate buffer for *p*H 4.0 to 5.5; phosphate buffer for *p*H 6.0 to 7.5; Tris/HCl buffer for *p*H 8.0 to 9.0; glycine/NaOH buffer for *p*H 9.5 to 10.5 and Na_2HPO_4/NaOH buffer for *p*H 11.0 to 11.9). Effect of temperature was studied from 5 to 80°C.

Results and Discussion

In the present study, seventeen cultures (A_1 and A_2, A_4 to A_9, B_3 to B_9 and T_1 and T_2) were isolated, of which fifteen were mesophiles and two were thermophiles. The cultures A_1 and A_2, A_4 to A_9, and T_1 and T_2 were isolated on media II, whereas B_3 to B_9 were isolated on media III at 55ºC. Only six cultures (A_1 and A_9 and B_3, B_4, B_7 and B_8) could be able to produce amylase, with the cultures B_3, B_4, B_7 and B_8 showing the maximum amylase activity; B_3 produced maximum amylase, while no activity was detected in B_9 (Table 1). The morphological and physiological characteristics of the B_3 culture are shown in Table 2. The strain of *Bacillus* sp. was confirmed as described elsewhere[8].

Table 1—Extracellular amylase activity (µmole/min/ml) from isolated cultures in media IV
[*Data are average of triplicates]

Culture type	After 24 h incubation	After 48 h incubation	After 80 h incubation
A_1	0.05	0.14	0.13
A_2	0.04	0	0
A_4	0.05	0.03	0.03
A_5	0	0	0
A_6	0	0.04	0.03
A_7	0.08	0.05	0.04
A_8	0.03	0.07	0
A_9	0.13	0.02	0.04
B_3	0.15	0.36	0.38
B_4	0.08	0.27	0.36
B_5	0.06	0	0
B_6	0.03	0.1	0.17
B_7	0.04	0.21	0.25
B_8	0.03	0.27	0.2
B_9	0	0	0
T_1	0	0.03	0
T_2	0	0.04	0

"A" and "T" represent cultures isolated on media II at 55ºC; and "B" cultures isolated on media III at 55ºC

Table 2—Morphological and physiological characteristics of culture B_3

Parameters	Characteristics
a) Cellular characteristics	
Morphology	Straight, Rod shape
Staining characteristics	Gram positive, spore-forming
b) Cultural characteristics	
Nutrient agar colonies	Finger-like projections
c) Physiological characteristics	
Growth factor	Optimum growth at 35°C range 25 to 42°C
Ammonia from arginine	Negative
Protein liquefaction (gelatin)	Positive
Catalase reaction	Positive
Indole production	Negative
Litmus milk test	No acid
Hydrolysis of urea	Positive
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Nitrate reduced to nitrite	Negative
Methyl red test	Negative
Deamination	Negative
Citrate utilization	Positive

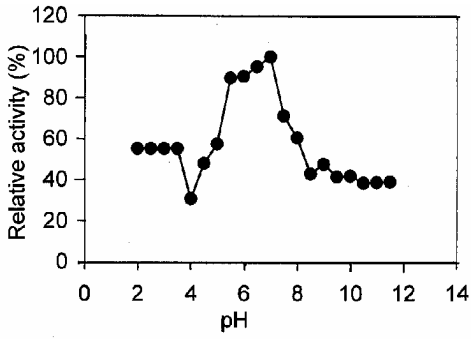

Fig. 1—Effect of *p*H on amylase from *Bacillus* sp.

Amylase production by B_3 strain showed maximum activity at neutral *p*H and high temperature (besides the enzyme was thermostable). These characteristics are important in industrial applications. Amylase was partially purified by ammonium sulphate precipitation; the 40% fraction had amylase activity. Optimum *p*H and temperature were found to be 6.5 and 60°C (Fig. 1 and 2), respectively. Our results were similar to that of optimum *p*H and temperature of amylase reported in a previous study[14]. Earlier, production of thermostable α-amylase was reported from *B. amyloliquifaciens*[11], *B. caldolyticus*[12], and *B. steraothermophilus*[13].

Table 3—Effect of carbon source on the production of amylase from *Bacillus* sp.
[Data are average of triplicates]

Carbon source	Amylase activity (μmole/min/ml)
Arabinose	0.0648
Dextrin	0.1574
D-Mannitol	0.1389
Fructose	0.3889
Galactose	0.2778
Glucose	0.1204
Glycerol	0.0278
Lactose	0.1667
Maltose	0.4631
myo-Inositol	0.1574
Raffinose	0.3426
Ribose	0.2963
Sodium acetate	0.0093
Sodium citrate	0.1204
Starch	0.0648
Sucrose	0.2963
Xylose	0.2778

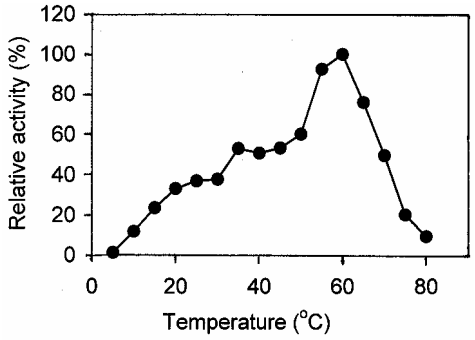

Fig. 2—Effect of incubation temperature on amylase from *Bacillus* sp.

The effect of carbon source on amylase production from *Bacillus* spp. was studied by using various carbon sources. Maltose induced maximum amylase activity (0.464 U), followed by fructose, raffinose, ribose, sucrose and xylose, whereas starch, arabinose and sodium acetate induced very low activity (Table 3). In an earlier study, lactose was found to be good carbon source for *Bacillus* spp. IMD 435 and α-amylase production was not related to biomass production[14]. *Bacillus* spp. NCIB 11203 and IMD 370 produced maximum amylase by using starch. Growth and amylase production was induced by starch in *B. subtilis*[15]. In addition to soluble starch and lactose,

Table 4—Effect of nitrogen source on the production of amylase from *Bacillus* sp. (Incubation 60 h)
[Data are average of triplicates]

Nitrogen source	Amylase activity (μmole/min/ml)	Protein (μg/ml)	Specific activity (U/mg)
Simple nitrogen source			
$(NH_4)_2SO_4$	0	12	0
$(NH_4)_2NO_3$	0	2	0
NH_4Cl	0	16	0
$(NH_4)H_2PO_4$	0	4	0
CH_3COONH_4	0	4	0
L-Glutamic acid	0	4	
KNO_3	0.08	0.002	40
Urea	0.08	0.004	20
Complex nitrogen sources			
Peptone	0.36	0.10	3.6
Yeast extract	0.32	0.11	2.9
Tryptone	0.28	0.34	0.82
Soybean meal	0.23	0.43	0.53
Beef extract	0.27	0.40	0.67
Gelatine	0.11	0.26	0.42
Redgram flour	0.12	0.30	0.4

glucose and dextran were also found suitable for α-amylase production; the highest yield being obtained with glucose[16].

Different patterns of the enzyme induction were obtained when beet pulp, corn cob, rice husk, wheat bran and wheat straw were used to partially replace the nutrients of selected medium; α-amylase was maximally expressed in the presence of corn cob and wheat bran. In another study on the effect of different carbon sources (glucose, maltose, xylose and starch) on α-amylase production, higher cell density and specific growth were obtained with glucose, but both higher enzyme and specific activities were obtained from starch[17]. Hiller *et al* [18] demonstrated the effect of lactose and nitrogen on cell physiology and α-amylase production. Results showed cell growth and α-amylase production patterns were similar, regardless of the limiting nutrients and suggested stationary phase gene control of α-amylase production, as opposed to a direct response to nutrient limitation.

The strain B_3 produced higher amounts of amylase with the complex nitrogen sources, than with simple nitrogen sources (Table 4). Among simple nitrogen sources, only KNO_3 and urea resulted in amylase production, whereas among complex nitrogen sources, peptone produced maximum amylase, followed by yeast extract, tryptone, soybean meal, beef extract, gelatin and red gram flour. Earlier, yeast extract was found to be good nitrogen source for the production of α-amylase in *B. alkalophilus*, *Bacillus* strains NCIN 1120, IMD 37024[19] and MID 435[14] and *B. amyloquefaciens*[20]. Thus, our results were in agreement with these studies.

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