ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation and optimization of amylase producing bacteria from different soils of Jammu province

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ABSTRACT

The enzymes from microbial sources are more stable and obtained cheaply. Amylases are among the most important enzymes and are of great significance in present day industry. The present study describes isolation of amylase producing bacteria from the wheat field soil. Crude enzyme characterization revealed the optimum pH at 7.0. The highest activity was observed at 45°C whereas the considerable activity was at 55°C - 65°C. The enzyme is highly Ca^{2+}dependent metalloenzyme. The isolated strain B15 shows promise in degrading starch and can have practical applications in starch industry.

Keywords: amylase, media optimization, amylase activity, characterization

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ISSN: 0975-8585

INTRODUCTION

Soil bacteria and fungi play pivotal roles in various biogeochemical cycles and are responsible for the cycling of organic compounds [1, 2]. Microorganisms constitute a huge and almost unexplained reservoir of resources likely to provide innovative applications useful to man. They represent by far the richest repertoire of molecular and chemical diversity in nature. Many bacteria produce enzymes which they secrete into the world around them. These enzymes breakdown the target molecules outside the bacterial cell and which can then be absorbed by them. The enzymes from microbial sources generally meet industrial demand and are more stable than with plant and animal amylases and obtained cheaply [3]. Enzymes are the proteins capable of catalyzing biochemical processes. Some of these are capable of catalyzing hydrolytic cleavage (digestion) of such biological polymers as proteins, carbohydrates and fats. These are known as hydrolases. Amylase is the name given to glycoside hydrolase enzymes that break down starch into glucose molecules. Payen and Persoz [4] were the first to become aware of enzymatic starch hydrolysis where they found that the malt extract converted starch to sugar. Enzymes are mostly proteins with a labile nature and there catalytic activity is inactivated by certain agents like temperature, pH, chemicals, etc. which impairs the native conformation of an enzyme. The utility of an enzyme depends mainly on its operational and storage stability [5]. Starch, which is the substrate of amylase, is the most abundant form of storage polysaccharides is of great significant in biotechnology, in various starch processing industries. In recent a years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms [6]. Amylase has one of the most important industrial enzymes applications. It is used in brewing and fermentation industries for, in the textile industry, in the paper industry and in the food industry [7-9].

In the present study, we report the isolation and optimization of novel amylase producing bacteria from the soil samples collected from different field conditions of Jammu district, India. Production conditions were optimized (temperature, pH, metal ions) to achieve high enzyme production and better enzyme activity.

MATERIALS AND METHODS

Soil samples were collected from different environment sources. The samples were taken from potato field, garden soil (bating technique), wheat field and maize field from different Jammu areas. Amylolytic microorganisms were enriched by inoculating 5 grams of soil in a flask containing 100 ml of MEB (Mineral Enrichment Broth) medium composed of Yeast extract - 0.5%, Peptone- 0.5%, Starch- 0.5%, KH2PO4- 0.5%, pH- 7.0. The flasks were incubated for 24 hrs at 37 ºC in an incubator shaker. After 24 hrs the culture broth was serially diluted up to 10^{-3} to 10^{-7}. Then 0.5 ml of the diluted suspension from each tube was transferred to MEB plates. The plates were gently rotated clockwise and anticlockwise for uniform spreading of diluted suspension and were incubated at 37ºC for 24 hrs in an incubator. After 24 hrs, independent colonies were picked and again transferred to fresh MEB plates for getting pure cultures. Then colonies with different morphologies were streaked on plates containing MEM

medium (Yeast extract- 0.5%, Starch- 0.5%, Peptone - 0.5%, KH2PO4 - 0.5%, Agar - 2.0%, pH - 7.0) and incubated at 37 ºC to identify their starch utilizing potential. Pure isolates were maintained on nutrient agar plates.

Screening for Amylase Activity (Starch Iodine Test)

Isolated colonies were picked up from each plate containing pure culture and streaked in straight lines in starch agar plates with starch as the only carbon source. After incubation at 37ºC for 24-48 hrs., individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue color forms, which is the basis of the detection and screening of an amylolytic strain. The colonies which were showing zone of clearance in starch agar plates, were maintained on to MEM agar plate as well as on to nutrient agar slants.

Determination of amylase activity at different time interval

Isolates showing high zone of clearance were used for further studies. They were inoculated in production medium (MEB) and allowed to ferment for 24, 48, 72, 96, 120 hrs under shaking condition at 37°C. A suitable volume of fermentation broth was centrifuged at 8000xg for 20 min at 4°C. Cell free supernatant recovered by centrifugation was used for amylase activity and considered equivalent to crude enzyme.

Amylase activity was measured by the release of glucose from starch by DNS reagent using glucose as standard For determining amylase activity 1ml of crude enzyme was taken and was added in a mixture of 1ml of standard 1% starch solution (1% w/v starch solution- added 1g of starch in 100ml of distilled water and to be stored at 4 ºC) and 0.1 ml of phosphate buffer (pH 7.0). This mixture was vortexed and kept in a water bath at 60^{o}C for 60 minutes. After incubation the stand was removed and reaction was stopped by keeping the reaction tubes in boiling water bath at 100ºC for 2 minutes. The mixture was brought to the room temperature and 3 ml of DNS reagent was added to it and the mixture was vortexed, capped and kept in a pre-heated water bath at 90ºC for 15 minutes. This mixture was cooled to room temperature and absorbance of solution was measured with respect to blank (Glucose solution) at 575nm.

Optimization of temperature, pH and metal ions for amylase activity

The optimization was carried by using enzyme extract to study the effect of temperature, pH and metal ions. For temperature optimization the temperature optimum of the enzyme was evaluated by measuring the amylase activity at different temperatures (25-85°C) and the enzyme extract (supernatant) was incubated at different temperatures for 30 min. The activity was checked using DNSA method. The pH optimum of the enzyme was determined by varying the pH of the assay reaction mixture using the following buffers (0.1 M): sodium acetate (pH 5.0-5.5), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 7.5-8) and glycine-

NaOH buffer (pH 9-10). To determine the stability of amylase, the enzyme was pre-incubated in different buffers (pH 5-10) for 24 h. The enzyme activity was determined. For temperature optimization, the initial pH was kept at 7 while for pH optimization the temperature was kept constant at 37 ºC (incubation temperature). The enzyme activity was studied using different metal salts viz. potassium chloride, magnesium chloride, calcium chloride, Zinc chloride and sodium chloride (1mM concentration).

Medium was inoculated with 1 ml of overnight grown bacterial isolate (1% inoculum level) in each case. Then from each culture amylase activity was estimated by measuring the reducing sugars using DNS reagent after 24 hrs. One unit (U) of amylase activity was defined as the amount of enzyme that releases 1 micro mol of reducing sugar as glucose per ml per minute.

RESULT AND DISCUSSION

Isolation

Isolation was carried out using soil samples taken from different sources on MEB and MEM agar plates. Enrichment method was used and single colonies were purified by repeated streaking and incubating at 37^{o}C for 24 hrs. In our study a total of 68 isolates were isolated of which 40 isolates were pure (Table 1).

Table 1: Soil samples taken from different sources along with their isolate names

Sample	Number of Isolates	Isolate name	Pure Isolates
Potato Field	19	G1 toG19	G4, G7, G9, G12, G13, G15, G17
Garden (Baiting)	19	K1 to K19	K1 to K19
Wheat Field	16	B1 to B16	B3, B9, B11,B13, B14, B15, B16
Maize field	14	M1 to M14	M2, M3, M5, M8 M10, M12, M13

Starch Iodine Test

There were only twelve isolates which gave a strong positive test (high zone of clearance). Based on the maximum zone size, one isolate from each source (B15 (Wheat field), K17 (Baiting), M8 (Maize field) and G12 (Potato field) was considered further for studying its amylase activity.

Amylase production at different time intervals

All the four isolates chosen on basis of zone size of clearance were incubated in MEB medium for different intervals of time (24, 48, 72, 96, 120 hrs) and the maximum activity was found by isolate B15 (wheat field) after 24 hrs interval which was 30.0 U/ml (Fig 1). Thus isolate B15 was chosen for further optimization studies.

ISSN: 0975-8585

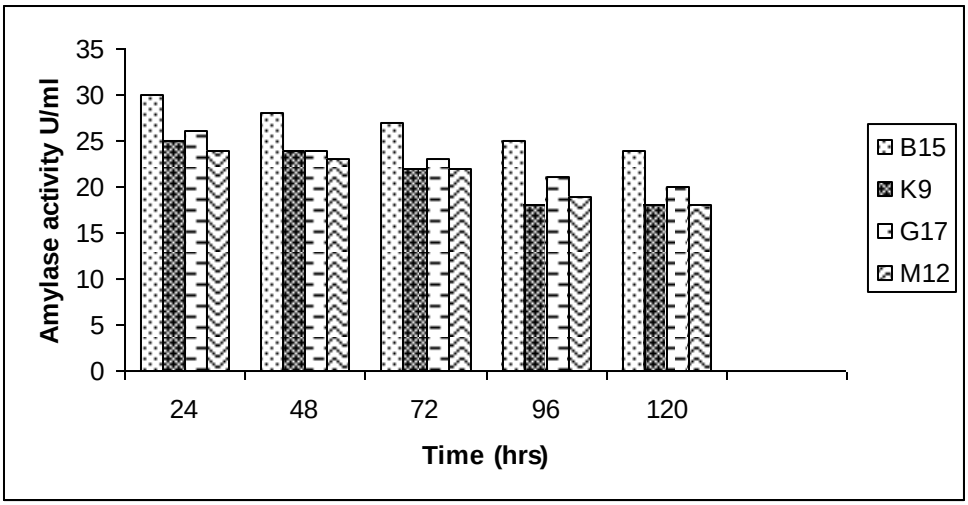

Fig 1: Amylase activity by four different isolates at different time intervals

Culture character of B15 isolate

B15 isolate was isolated from soil sample collected from the wheat field. The colony was small, round and cream in color. On gram staining the organism appeared violet in color thus indicating that it is gram positive. It was *coccus* type and in chains.

Effect of temperature on amylase activity

Enzyme was isolated from B15 after 24 hrs of inoculation and the assay was carried out at different temperatures from 25°C to 85°C for 30 min. The highest activity was observed at 45°C whereas the considerable activity was at 55°C and 65°C (Fig 2). It is desirable that amylases should be active at high temperatures of gelatinization (100–110 °C) and liquefaction (80–90°C) to economize the process; therefore, there has been a need and continual search for more thermophilic and thermostable amylase [10]. Amund and Ogunsina [11] studied three different strains of bacteria, *Bacillus subtilis, Bacillus licheniformis* and *Bacillus cereus* with temperature optima of 30, 37 and 80°C respectively whereas it was 55°C for *Bacillus cereus* using solid state fermentation [12]. Mishra and Behera [13] found maximum amylase activity at 37°C from the bacteria isolated from kitchen wastes. Afiukwa *et al* [14] studied an optimum temperature of 60°C for amylase activity in partially germinated mango seeds.

ISSN: 0975-8585

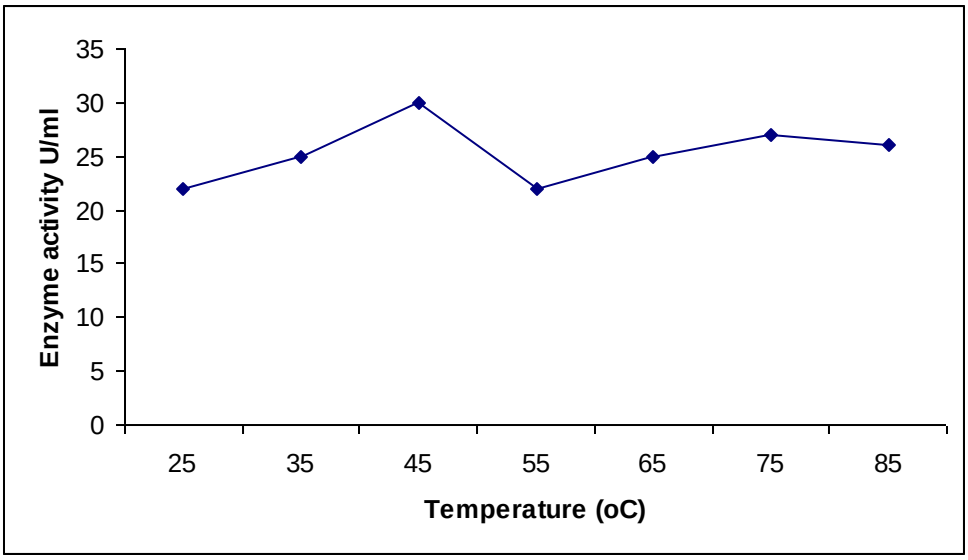

Fig 2: Optimum temperature for amylase activity

Thermal stability of enzyme

The enzyme activity was examined at different temperatures ranging from 20°C to 90°C. It was found that the enzyme was stable at 40°C-50°C upto 60 min and then the stability decreased (Fig 3). Mohamed *et al* [15] reported that the thermal stability of some wheat α -amylases were stable up to 50°C and some at 40°C after incubation for 15 min whereas in *P. erosus* tubers α -amylase was stable at temperature up to 40°C for 30 min incubation followed by rapid inactivation above 40°C [16].

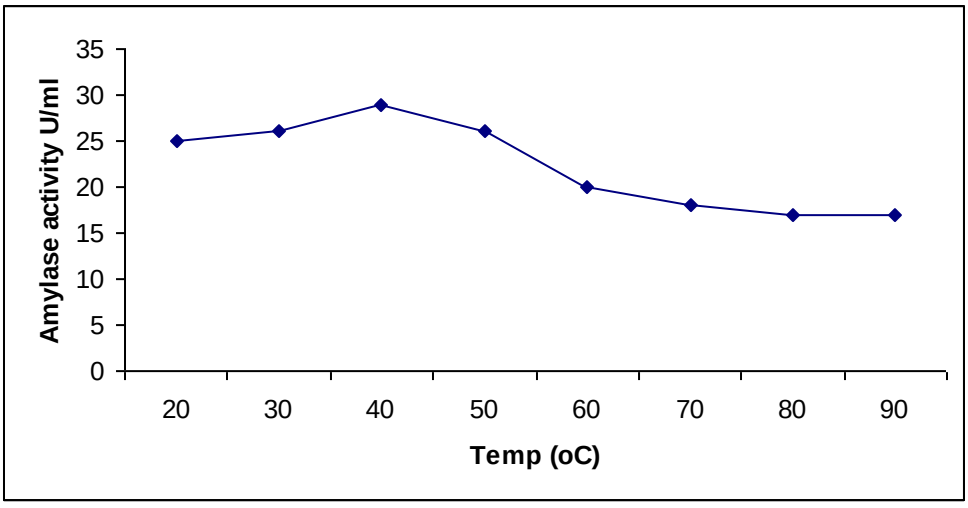

Fig 3: Thermal stability of the enzyme

Effect of pH on amylase activity:

The isolate B15 was inoculated in MEB (production medium) for 24 hrs at 37°C in a shaker. The medium was centrifuged and the supernatant was collected for amylase assay.

ISSN: 0975-8585

Assay was done at pH 3 to 9 using different buffers. Highest activity of amylase was found to be at pH 7 which was 22U/ml whereas lowest activity was at pH 3 and 9. At pH 8 the enzyme activity was considerable (Fig 4). Behal *et al* [17] studied thermostable amylase producing *Bacillus* sp. that revealed an optimum enzyme activity at pH 8.0 whereas in other species the optimum activity was at pH 7.0 [18].

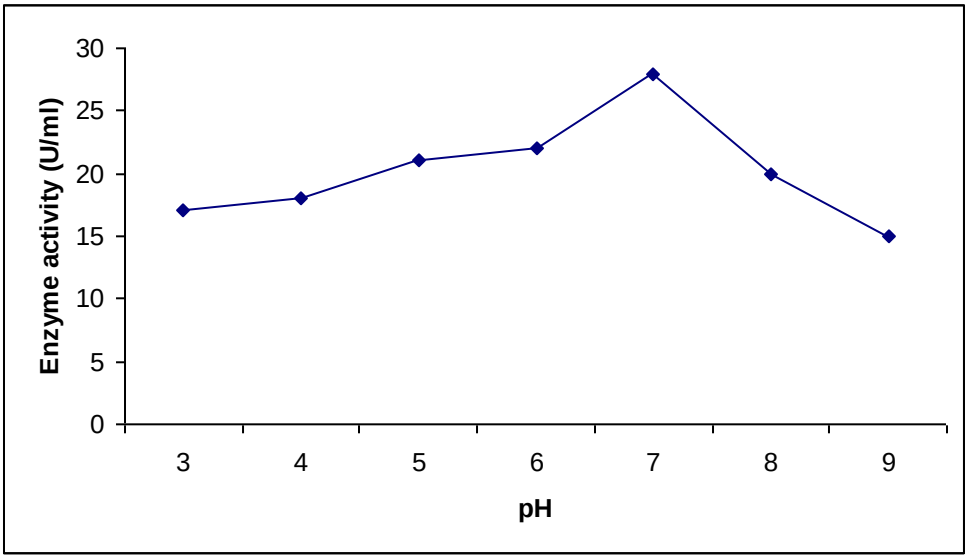

Fig 4: Optimum pH for the enzyme activity

Effect of metal ions on amylase activity

Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, etc. [6]. The relative activity of isolate B15 was studied with different metal salts with enzyme extract in phosphate buffer (pH 7.0) at 37°C for 1hour. The maximum relative activity was found with Ca^{2+} (100% with the control) followed by Mg^{2+}ions whereas it was lowest for Zn^{2+}ions (27%), thereby showing that the enzyme is highly Ca^{2+}dependent metalloenzyme (Fig 5).

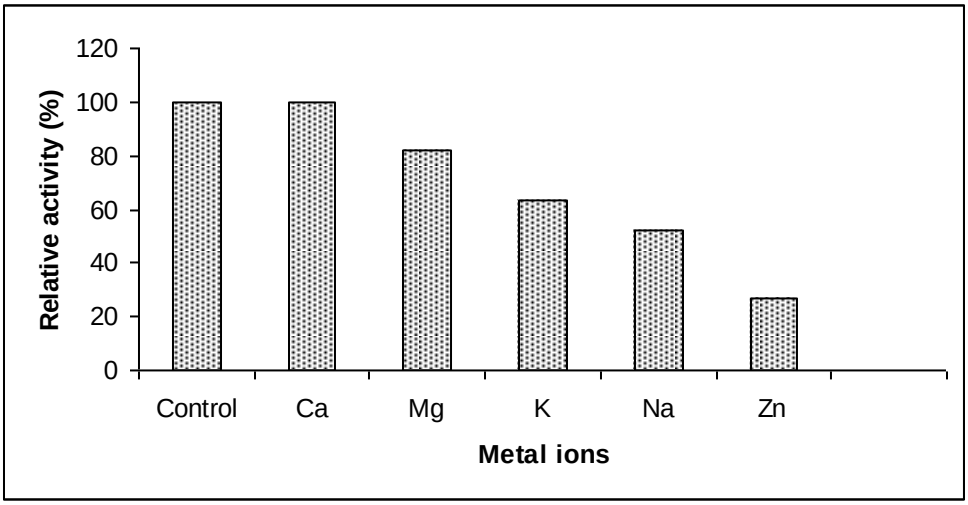

Fig 5: Relative activity of enzyme with different metal ions

$Ca^{2+}$was reported to increase α -amylase activity of an alkaliphilic *Bacillus* sp. ANT-6 [10]. The stabilizing effect of Ca^{2+}on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+}in the protein, thus, causing the adoption of a compact structure [19]. $Zn^{2+}$was reported to inhibit thermostable α -amylases from a thermophilic *Bacillus* sp. suggesting that the inhibition with the ion determined the thermostability of enzyme [20].

CONCLUSION

From initial observations this study has shown that the isolated strain B15 shows promise in degrading starch and may have practical applications in starch industry on account of stability at high temperature and pH. It can be used in agro processing waste containing starch. However in order to achieve industrial scale value addition process further experiments are required.

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