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Isolation and Partial Characterization of Amylase Producing *Bacillus sp.* from Soil

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Abstract : Isolated *Bacillus* strains BA1 and BA2 were a good source of amylase as they also showed high amylolytic activity. Both showed a clear zone on starch agar plate of 4.0 & 4.2mm in diameter respectively. The strain BA1 and BA2 produce amount of amylolytic enzyme 0.908µ/ml and 0.802µ/ml after the incubation of 24 hours at 37±2^{0}C respectively. As the time of incubation increase the amylolytic activity decrease. Both the isolated *Bacillus* strains were producing amylase was novel and makes it potential for industrial application.
Keywords: Bacillus, Amylase, Amylolytic activity, Reducing sugar, TLC.

Introduction:

Enzyme is a biocatalyst which accelerates biological reactions, source of enzyme used in commerce is plant and animal cell. The sources of enzymes are microorganism, higher plants and animals. Animals enzymes used currently are lipases, trypsin, rennets etc.most prevalent plant enzyme are protease, amylase, soyabean lipooxygenase. At present more than 2000 enzyme have been isolated and characterized, out of which about 1000 enzymes are recommended for various applications. Amylase are the extracellular enzymes that breakdown starch or glycogen, a polysaccharide (a molecule which consist of eight or more monosaccharides molecules) in to maltose, a disaccharide (double sugars, i.e. composed of two monosaccharide molecules) and some monosaccharides such as glucose.

Starch is a complex carbohydrate (polysaccharides) composed of two constituents: amylose and amylopectin the relative content of amylose and amylopectin varies with the source of starch. Amylase is enzymes which hydrolyse the starch molecules in to polymers consists of glucose units. α- amylase is ubiquitous in distribution, with plants, bacteria and fungi being the major sources. α- amylase acts on starch and break them up into sugars (saccrification). The microbial enzymes meet the industrial demands a large number of them are available commercially and have almost replaced chemical hydrolysis of starch processing industry[1]. Amylase can be derived from a variety of sources. Many microorganism used in α- amylase and β-amylase production including *Bacillus subtilis, B. cereus, B. polmyxa, B. amyloliquefaciens, B. coagulans, Lactobacillus, Escherichia, proteus, B. lincheniformis, B. steriothermophilus B. megaterium, Strepotmyces sp., Pseudomonas sp.* etc. *Bacillus sp.* And the related genera produce a large variety of exacellular enzymes, of which amylase are of particular significance to the industry e.g., *B. cereus, B. subtilis, B. lincheniformis, B. circulans,* and *Closteridium thermosulfurogenes.* Bacteria belonging mainly to the *Bacillus* sp. have been widely used for the commercial production of thermostable α- amylase. α- amylase play a significance role in various industries for different task. α- amylase are used in bread and chapatti industry for the improvement of the quality, taste, aroma and porosity of the bread. It is also used in the textile industries for increasing the stiffness of the finished products and desizing agent for removing starch from the grey cloths before its further processing, sugar and glucose industries alcohol industries for the production of glucose from the starch, paper industry for the hydrolyzing of the raw starch that is used for sizing and coating the paper, detergent, building product and feed industries for improvement of detergency of laundry bleach composition and bleaching

without color darkening. Hence present study deals with the isolation and identification of the amylase producing *Bacillus* strains and their growth pattern and amylolytic activity as amylase producers.

Experimental

Sampling

The test soil samples were collected from the different sites of Kanya Gurukul Campus with the help of sterile spatula from 4-5 cm depth in to sterile plastic bags. Soil samples were air dried at room temperature.

Isolation and purification

Isolation of bacteria from soil carried out by serial dilution method[2] and isolated bacterial colonies were purified by sub culturing and stored in slants[3].

Characterization of bacterial culture

Various biochemical tests were performed for the identification and characterization of isolated bacteria viz- Gram staining, Catalase test, Starch hydrolysis, Casein hydrolysis, Fermentation test, IMViC test, **U**rease test, Nitrate test. Morphological, cultural, physiological and biochemical properties of the isolated strains were studied according to the methods given in Bergey's manual of systemic bacteriology[4].

Growth curve study

For plotting the growth curve 150 ml of nutrient broth was prepared and divided into 3 flasks, all containing 50 ml of media. All flasks were autoclaved and cooled at room temperature. Two of them were inoculated with bacterial culture and incubated at 37 ± 2^0C/200rpm. One flask containing 50 ml media was stored as blank. After every 2 hours absorbance of inoculated flask was read at 570 nm against the uninoculated blank.

Enzymatic study

a. Qualitative screening of bacteria (for amylase)

The bacteria were streaked on starch agar plate and plates were incubated at 37^0C for 24 hours. After incubation the starch agar plate was flooded with Gram's iodine solution and kept for 30 min. A clear zone around the growth in blue background indicate amylolytic activity of the strains and visible difference in the extent of zone of clearing was recorded for amylolytic activity

b. Estimation of reducing sugar (glucose) by DNSA method (standard curve of glucose)

Prepared different concentration of glucose (10^1- 10^{10}) in different test tubes and volume of each test tube was made up to 2ml with distilled water. Added 2ml 3, 5 dinitrosalicyclic acid in each test tube and place all the test tubes at water bath for 10 min at 100^0C. Tubes were cooled at room temperature and absorbance was measured at 540 nm.

c. amylolytic activity (quantitative determination)

For quantitative determination of amylolytic activity (1%) starch containing amylase production medium was prepared. Mixed and distributed in 40-50ml volumes in to 100ml Erlenmeyer flask sterilized by autoclaving at 121^0C for 15 min. after cooling media inoculates bacteria and incubated. The flask was loaded on rotary shaker incubator at a speed of 200 rpm at 37^0C for 24 hours. After incubation, fermented broth was centrifuged in a cooling centrifuge. Supernatant was collected and used for the estimation of amylase. The amylase saccrilytic activity was determined by dinitrosalicyclic acid method[5].

d. Amylase assay

The amylase was assayed by adding 0.2ml of enzyme (crued extract/fermented broth supernatant) to 0.5ml of 1% soluble starch and incubated for 30 min at 37^0C. The reaction was stopped by adding 1ml of 3, 5dinitrosalicyclic acid, followed by boiling for 10min and to develop brown colour. The final volume was made up to 4.0 ml with distilled water and the absorbance measured at 540nm with a spectrophotometer.

One saccharolytic amylase unit is defined as the amount of enzyme required for the liberation of 1ml reducing sugars per minute under the assayed condition.

e. Thin layer chromatographic analysis of amylase activity

Detection of reducing sugar in culture extract carried out by making slurry of silica gel and transferred to the applicator for spreading on clean glass slide. Dried the plate and kept it at 100^0C for 2 hours for activation before use. Took sample (starch digested) and loaded it on a line 2.5cm away from one end of the plate with the help of capillary tube. kept the plate in solvent system containing Butanol: Ethanol: water ratio as (5:3:2) for 30 min and dried overnight at room temperature. The individual sugars were visualized by spraying with anilinediphenylamine reagent[6].

Results

Table-1 Culture morphology and Biochemical Characterization of *Bacillus* sp. BA1 & BA2

Characteristics	**BA1**	**BA2**
Culture characteristic		
Size	Big	Small
Shape	Irregular	Irregular
Margin	Uneven	Even
Pigmentation	Nill	Nill
Morphological characteristic		
Shape	Rod	Rod
Arrangement	Chain	Chain
Gram's reaction	Positive	Positive
Biochemical Tests		
Lactose Fermentation	-	±
Mannitol Fermentation	+	+
Sucrose Fermentation	AG	AG
Dextrose Fermentation	A	AG
Catalase Test·	+	+
Starch Hydrolysis	+	+
Casein Hydrolysis	+	+
Indole test	-	-
Methyl Red	+	+
Voges proskauer test	-	-
Citrate Utilization Test	+	+
Urease test	-	-
Nitrate Reduction	+	+

± Variation Reaction, + Positive, - Negative, AG Acid and Gas production, A- Acid production

In the present study for both the isolates BA1 and BA2 Bacterial cultures were identified as *Bacillus* strains by Gram staining and various biochemical tests viz., casein hydrolysis, catalases test etc (Table 1). Growth curve for both the isolates were plotted between optical density vs incubation time up to 48 hours and sigmoid curve (figure 1) were obtained. It involves lag, log, stationary and decline phase. The strain BA1 showed 4.0 mm zone of clearing around the colony whereas BA2 showed 4.2 mm zone of clearing around the colony as qualitative amylolytic activity (figure 2). The strain BA1 and BA2 produce amount of amylolytic enzyme 0.908µ/ml and 0.802µ/ml after the incubation of 24 hours at 37 ± 2^0C respectively as quantitative (figure 2 & 3). As the time of incubation increase the amylolytic activity decrease. Different spots of solute were appeared on the TLC plates (table 2& 3) which were travelled by the help of solvent in chromatographic assembly. In strains BA1 and BA2 the reducing sugar spots were identified as ribose and fructose respectively.

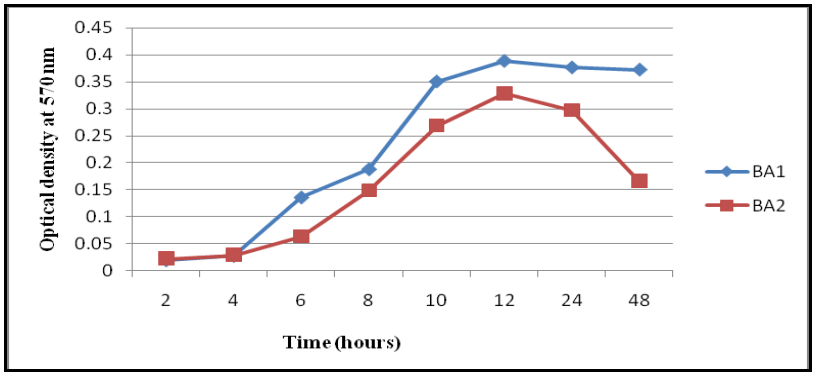

Figure 1 Growth curve of *Baciilus* sp. BA1 & BA2

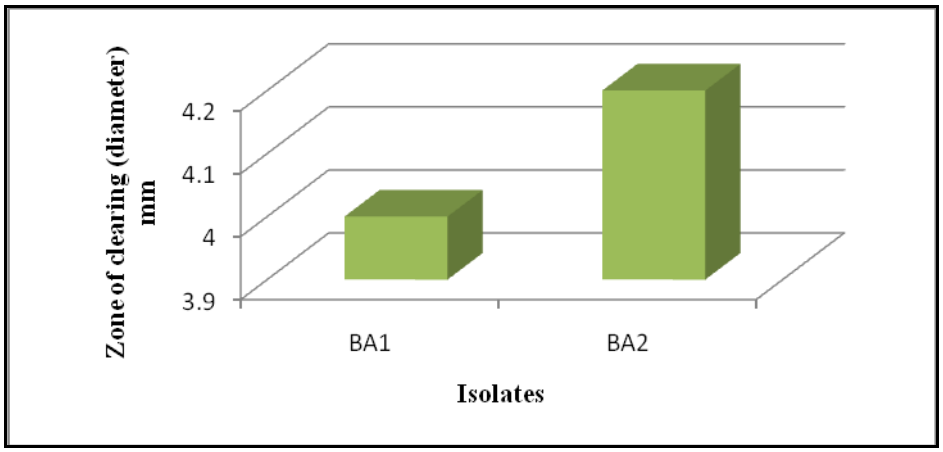

Figure 2 Qualitative screening of *Bacillus* sp. BA1 & BA2 for amylolytic activity

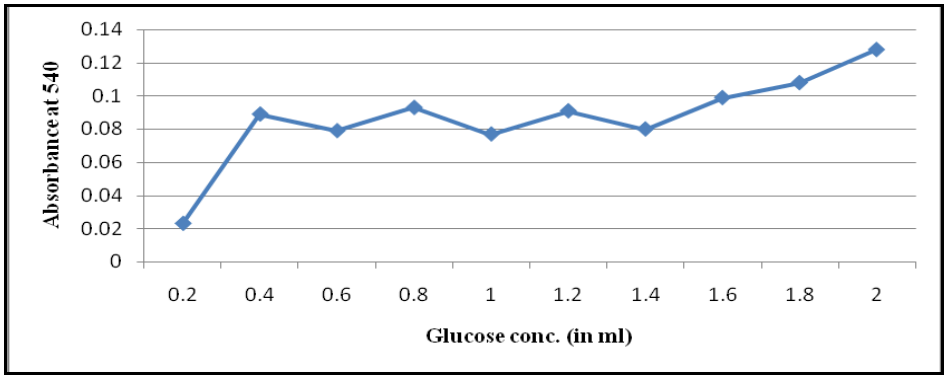

Figure 3 Showing standard curve of glucose concentration

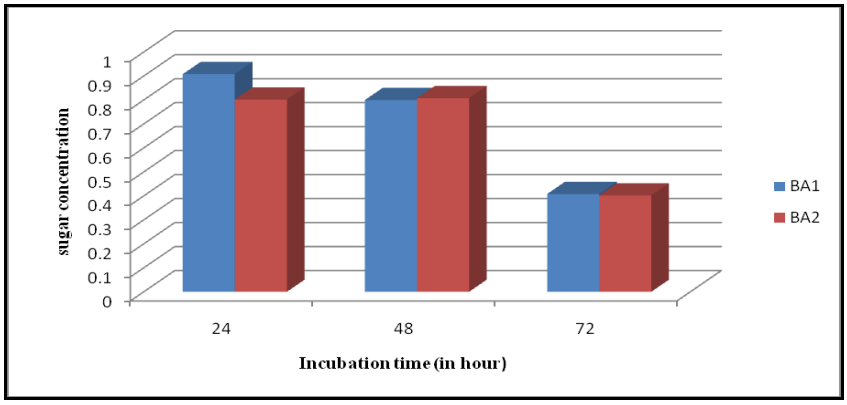

Figure 4 Quantitative screening of *Bacillus* sp, BAI & BA2 for amylolytic activity

Table -2 Standard table for Rf value of sugars

Sugars	Rf (Ratio of fraction)
Lactose	0.17
Maltose	0.26
Sucrose	0.42
Galactose	0.38
Glucose	0.44
Mannose	0.47
Sorbose	0.54
Fructose	0.51
Arabinose	0.53
Xylose	0.66
Ribose	0.69
Rhamnose	0.74

Table-3 Different Sugars present in enzymatic crude of *Bacillus* sp. BAt & BA2

S.No.	Sugars	BA1	BA2
1.	Fructose	+	-
2.	Ribose	-	+

(+) Positive (-) Negative

Discussion

Amylase producing organism like fungi and bacteria are genrally isolated from soil and most of the work in focused on amylase. Therefore the present study deals with isolation of amylase producing bacteria from soil. Isolation of amylase producing bacteria was perform by the serial dilution spread plate technique. Similar method has been used by Clark., et al 1958 and Abe, 1988. Identification of selected *Bacillus* strain was on the basis of standard morphological and biochemical test according to the method described in Bergey's manual of determinative Bacteriology. The isolated *Bacillus* strains were primarily screened for the production of amylase was done by starch agar plates method. Further screening of amylase producing bacteria was carried out in 1% starch medium containing (g/l) of distilled water NaCl (0.4), peptone (2.0), yeast extract by the starch hydrolysis test on the basis of zone of clearance appeared as 4.0 &4.2mm for BA1 and BA2 respectively. Similar method of characterization for protease were carried out by Uehara et al., 1979.

Bacterial isolates from soil were tested for production of amylase by the starch hydrolysis test. On the bases of area of clearance two bacterial isolates were selected for further study. *Bacillus* sp. BA2 showed maximum zone of clearance in comparison of *Bacillus* sp. BA2 on starch agar medium. The estimation of amylase activity was carried out according to the dinitrosalycyclic method of Miller, 1959 and Bernfield, 1955. Both bacteria BA1 and BA2 were found to be effective in releasing high amount of reducing sugars. The amylase activity decrease from 0.908 to 0.408µ/ml as the incubation time increase from 24-72 hours at 35 ± 2^0C. similar findings were obtained by Aguilar et al., 2000. The detection of reducing sugars was done by TLC[6].The reduced products of the enzyme were ribose and fructose. However, it was not able to produce glucose. Similar findings have been reported by Mezghani et al., 1999 and Prieto 1995 but present strain BA1 was found to be more competent for the production of amylase as compared to previous findings.

Conclusion

Hence it has been concluded that both the isolated *Bacillus* strains produce amylase was novel and make it potential for industrial applications. Significant use of amylase enzymes includes hydrolysis of starch to yield glucose syrup, amylase rich flour and dextrin during baking in food industries. Furthermore, in the textile industry, amylases are used for removal of starch sizing and as additives in detergents. However, the cost of producing this enzyme is high and the cost of procurement by developing countries can be even higher, hence present potential *Bacillus* strain BA 1 could be best alternative for industrial production of amylase.

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