Jahangirnagar University J. Biol. Sci. **5**(2): 1-10, 2016 (December)

Screening of α-amylase producing bacteria from tannery wastes of Hazaribag, Bangladesh

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Abstract

Alpha amylases (α-amylases) are one of the most imperative enzymes for producing simple sugar units from complex sugar molecules. Attempts were made to isolate amylolytic bacterial strains from soil samples of tannery wastes collected from Hazaribagh, Dhaka and subsequent partial characterization was performed. Bacterial isolates were primarily screened for α-amylase activity on starch agar medium. Based on microscopic and biochemical properties of isolates, α-amylase activity of bacterial isolates were determined to find out two best producers of the enzyme. Subsequent molecular identification of these two α-amylase producing bacterial isolates using 16s rRNA sequence analysis showed that isolates were *Bacillus amyloliquefaciens* and *B. subtilis* respectively. In submerged fermentation the *B. amyloliquefaciens* showed the highest activity (2.13 U/ml) while *B. subtilis* showed the second highest activity (1.89 U/ml). Characterization of the enzyme produced by *B. amyloliquefaciens* revealed that the maximum activity demonstrated at incubation time 25 min, pH 7.0 and temperature 50^0C. This newly isolated *B. amyloliquefaciens* could be exploited for the industrial production of α-amylase with commercial implications.

Key words: α-amylase producing bacteria, 16s rRNA sequence, tannery wastes.

INTRODUCTION

Now-a-days industrial production and use of enzyme is increasing due to the increase of number of industries especially in food, beverage, textile, leather and paper industries and the issues are related to environmental safety. The current world market for industrial enzymes is estimated to be US $1.6 billion which are distributed among food enzymes (29%), feed enzymes (15%) and general technical enzymes (56%) (Outtrup *et al.*, 2002). Alpha-amylases are one of the main enzymes used in these industries (Swain *et al*., 2006). These enzymes are of great significance in biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (Rajagopalan & Krisnan, 2008). In particular, α-amylase catalyzes the hydrolysis of internal α-1, 4-glycosidic linkages in starch to produce low molecular weight products, such as glucose, maltose and maltotriose units (Gupta *et al.*, 2003). They can be obtained from several sources, such as plants, animals and microorganisms. Microbial origin of α-amylases could be ideal source for production of the enzyme in a cost effective manner than while compared to plant and animal sources (Tanyildizi *et al.*, 2005). Several fungi, yeasts and

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bacteria have reported to produce α-amylases (Das *et al.*, 2011). The members of the genus *Bacillus sp.* producing extracellular α-amylases are of particular significance in industrial applications. Scientists are on continuous effort to isolate and identify the best producer strains of different extracellular enzymes as microbial prospecting offers significant advantages in discovering novel strains. Though microbes are versatile in their natural habitats, screening of non-conventional sources could offer microbes with unique potential. Conventional approaches of microbial screening coupled with molecular techniques serve as confirmatory basis of identification and subsequent modification. The present research work is focused on presumptive identification of α-amylases producing bacteria from uncommon source, i.e. tannery wastes based on morphological and biochemical properties and subsequent molecular characterization of the selected α-amylases producing strain. Furthermore, partial characterization of crude α-amylase enzymes of the identified bacterial strains was conducted based on a number of properties influencing the optimum activity of the extracellular enzymes.

MATERIALS AND METHODS

Isolation of bacterial cultures: All the samples were collected from soil near tannery wastes found at East Asia Tannery, Hazaribagh, Dhaka, Bangladesh. Samples were kept in sterilized glass vials for further analysis. For the isolation of bacteria, soil samples were serially diluted and spread on skim milk agar plate. At first, samples were mixed with sterile 0.85% NaCl solution and vortexed. Ten-fold serial dilutions were then carried out. It was followed by spreading 0.1 mL from each dilution aseptically on skim milk agar plates by using a glass spreader. The plates were incubated at 37^0C in the incubator for 24 h. After 24 h incubation, clear zones were observed and differential growth patterns of bacteria were found in each skim milk agar plate. In this process, a small amount of inoculums from each morphologically different single colony were streaked onto the starch agar plate and incubated at 37^0C for 24 h.

Primary screening of α-amylase producing bacteria and comparative analysis of α-amylase activity: Bacterial isolates were screened for amylolytic properties by starch hydrolysis test on starch agar plate (Atlas *et al.*, 1995). The microbial isolates were streaked as a line on the starch agar plate and then the plates were incubated at 37^0C for 24 h. After incubation, the plates were flooded with freshly prepared 1% Lugol's iodine solution. Presence of the dark blue color in the plates indicated negative result while a clear zone of hydrolysis surrounding the growth indicated positive result. Thus, the isolates which produced clear zones of hydrolysis were considered as α-amylase producers and the diameters of activity zones and colonies were measured in mm and recorded. Specific zones were calculated as the ratio of the diameter of the clear zones and diameter of colonies.

Assessment of extracellular activity of enzymes: Isolated bacterial strains were cultured in 50 ml starch liquid medium into a 250 ml flask at 37°C for 24 h at 120 rpm agitation. Cells were harvested by centrifuging at 8000 rpm for 15 min and the supernatant was transferred to a fresh tube. Then, 20 µL of supernatant was transferred to starch agar plates with antibiotics. After 24 hours the activity zones were measured in mm and recorded (Table 1.1).

Identification of potent α-amylolytic bacterial isolates: A series of conventional biochemical tests were carried out for the identification of the genus of bacterial strains according to the Bergey's Manual of systemic bacteriology (Sneath *et al.*, 1986).

The molecular identification was also done by 16S rRNA gene sequencing. Two universal primer for 16s rRNA gene amplification were used for the purpose of PCR. 27F (AGAGTTTGATCMTGGCTCAG) was used as the forward primer while U1492R (GGTTACCTTGTTACGACTT) was used as the reverse primer. The 16s rRNA gene was sequenced using ABI 3700 Genetic Analyzer in the 1st Base Laboratory SdnBhd, Malaysia. The 16s rRNA gene sequences were aligned using BioEdit7.2 software. The sample sequences were analyzed using BLAST-NCBI.

Inoculum preparation and submerged fermentation for α-amylase production: For inoculum preparation, 50 ml of nutrient broth medium was transferred to each of 250 ml cotton plugged Erlenmeyer flask and sterilized in autoclave for 15 min. After cooling at room temperature, 1 ml of bacterial cultures was aseptically transferred to each flask. The flasks were loaded on a rotary shaking incubator at 150 rpm 37°C for 24 h.

Amylase production was carried out in sterile basal (Asgher *et al.*, 2007) medium (0.1% KH_2PO_4, 0.25% Na_2HPO_4, 0.1% NaCl, 0.2% $(NH_4)_2SO_4$, 0.005% $MgSO_4$.$7H_2O$, 0.005% $CaCl_2$) containing 0.2% tryptone and 1% soluble starch. The initial pH of the medium was adjusted to 7.0. Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with 1 ml of an overnight culture and incubated at 37°C in a rotary shaker incubator at 150 rpm for 48 h.

Separation of cells from culture media: After incubation, fermented broth was centrifuged at 4°C, 8000 rpm for 15 min in a cooling centrifuge. Supernatant was collected and preserved or used for enzyme assay.

Determination of α-amylase activity: Assay for α-amylase was performed the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959) and α-amylase activity was measured (Bernfeld, 1955). At first, a fresh solution of 0.5% soluble starch solution was prepared by dissolving 0.5g soluble starch in 100 mL 0.02 M sodium phosphate buffer (pH 6.9) (Gomes *et al.*, 2001). Then 0.5 mL enzyme solution was diluted into 1 mL distilled water and 0.2 mL starch solution was added and incubated at 37^0C for 5 min. Then, 3 mL DNS reagent was added subsequently. The solution was kept in a boiling water bath at 90^0C or 15 min and then under running tap-water to cool down at room temperature. Next, 10mL distilled water was added into it. The absorbance was measured at 540 nm with UV-Vis

spectrophotometer (Optizen pop, Mecasys Co., Ltd., Taiwan). One unit (U/mL) of α-amylase activity is defined as the amount of α-amylase required to liberate 1 mg of reducing sugar (maltose) from starch/min, under the assay conditions.

Effect of cultivation period for α-amylase production: The effect of cultivation period on the production of α-amylase was investigated by carrying out fermentation for 72 h at 37^0C as a measure of optimization of culture conditions. The sample was collected every 24 h to observe the production of enzymes. The pH and volume of the medium were 7.0 and 50 ml respectively and the assay was carried out with reaction period of 5 min.

Partial characterization of crude α-amylase: The activity of α-amylase was determined by conversion of starch into simple sugars. A fresh solution of 0.5% starch was prepared by dissolving 0.5 g soluble starch in 0.02 M sodium phosphate buffer (pH 6.9) to obtain a 100 mL solution. 0.2 mL of the prepared starch was transferred into test tubes along with 0.5 ml of the α-amylase and incubated accordingly. The amount of reducing sugar was determined by DNS method (Miller, 1959). To investigate the optimum incubation time of the α-amylase, reaction was carried at different time intervals (5, 10, 15, 25, 30 and 60 min) and the enzyme activities were recorded. In addition, for determination of optimum temperature for maximum activity of the crude α-amylase, the enzyme samples were incubated for 5 min at temperature ranging from 30° to 90°C. Again, optimum pH on activity of crude α-amylase was determined using different buffer systems in the pH 6.0-10.0 (0.2M glycine-NaOH buffer and 0.2 M sodium phosphate buffer). Then the enzyme activity was determined and the assay was carried out for an incubation period of 5 min.

RESULTS AND DISCUSSION

Isolation of bacterial strains and primary screening of α-amylase producer: In industrial production of enzymes and their biotechnological applications, the key is always the choice of the appropriate microorganisms. Isolation and selection of suitable organisms are very essential for the production of extracellular amylases. Members of genus *Bacillus* were found to be better producer of different types of α-amylases (Nusrat and Rahman, 2007). In this connection, 36 bacterial isolates were found in initial screening from soil samples of tannery wastes. Among them, 8 of the isolates were found to be protease producing while 12 of them were capable of producing α-amylases. Interestingly, all of the protease producers possessed the ability of producing α-amylases and produced α-amylases into the media. The comparison of α-amylase activity the isolates were demonstrated on starch agar plate and clear zone were observed after treatment with Lugol's iodine solution in a method described earlier (Suman & Ramesh, 2010). The diameters for the clear zones were measured in millimeters (mm) and presented in Table 1.

Among 8 tested bacterial isolates, 2 amylase producing bacteria IBLR204 (top amylase producer), IBLR82 (top amylase producer among proteolytic strains) were selected for further biochemical studies and subsequent molecular identification.

Table 1. The diameter of clear zones by different bacterial isolates on starch agar medium

ID No.	Diameter (mm, Mean±SE; n=3)	Specific zone
IBLR201	26 ± 1.125	1.40
IBLR202	26.43± 1.435	**1**.57
IBLR203	27.19± 1.745	1.17
IBLR204	28.16 ± 1.086	1.59
IBLR31	23.26 ± 1.003	1.27
IBLR71	22.90± 0.984	1.21
IBLR82	24.56 ± 1.235	1.41
IBLR122	24.08± 2.996	1.38

IBL stands for Industrial Biotechnology Laboratory while R for the person conducting experiments

Assessment of extracellular activity of enzyme: Submerged fermentation was carried out for α-amylase production as reported earlier (Riaz *et al.*, 2009). Among 2 isolates, IBLR204 was found to be better producer of α-amylase than IBLR82 (Table 2). Therefore, the results indicated that despite collected from same source, IBLR204 strain only produced amylase while IBLR82 strain produced both the enzymes (α-amylase and protease). However, the proteolytic activity of IBLR82 strain was also found to be high. As IBLR204 was the best α amylase producer, it was selected for further investigation for partial characterization of α-amylase. However, both the strains were subjected to molecular identification.

Table 2. Assessment of extracellular activity of enzyme produced by two isolates

ID No.	Diameter (mm, Mean±SE; n=3)
R204	24.91 ±1.535
R82	22.53 ±1.238

Biochemical and molecular identification of selected isolates: The biochemical identification of the selected isolates was performed according to the Bergey's manual of systemic bacteriology (Sneath *et al.*, 1986) and both of the isolates were presumptively identified as *Bacillus sp*. The biochemical test results are presented in Table 3.16S rRNA gene amplification and sequencing was previously described as means of identifying as well as characterizing *Bacillus sp.* (Gomma & Momtaz, 2007). The use of molecular techniques along with the microscopic and biochmical methods, adds more precision and accuracy to the phylogenic identification and also to the true reflection of microbial diversity.

Table 3. Results of different morphological and biochemical tests of the isolates

Name of the test	IBLR204	IBLR82
Gram reaction	+	+
Shape of the cell	Rod	Rod
Catalase /Gelatin /Starch hydrolysis / Voges–Proskauer/ Nitrate reduction/ 10% NaCl	+	+
Methyl Red / Oxidase / Indole / Growth on MacConkey agar	-	-
Carbohydrate fermentation test (acid production)		
D-Glucose	-	+
D-Fructose	-	+
Sucrose	-	+
Mannitol	-	-
Maltose	-	-
Lactose	-	-
Carbohydrate fermentation test (Gas production)		
D-Glucose/ D-Fructose/ Sucrose/ Mannitol/ Maltose	-	-

'+' indicates positive result and '-'indicates negative result

According to 16S rRNA gene sequence analysis, bacterial strains R204 and R82 were identified as *B. amyloliquefaciens* and *B. subtilis*, respectively.

Effect of incubation period on enzyme production: Optimization of culture conditions is very important for maximum microbial growth and enzyme production by microorganisms (Kathiresan & Manivannan, 2006). From the time course study in shake culture it was found that the rate of enzyme increased with the duration of fermentation and reached its maximum activity after incubation of 48 h (Fig. 1). A prolonged incubation time beyond 48 h did not increase the enzyme production. These finding are similar to the result reported by (Haq *et al.*, 2010). The reducing sugars were measured by adding 3, 5-dinitro salicylic acid reagent, using maltose as standard (Miller, 1959) and the enzyme activity of crude enzyme was calculated as 2.13 U/ml/min. This enzyme fermentation was not optimized properly and showed less activity in comparison to previous studies on *B. subtilis* (Riaz *et al.*, 2003).

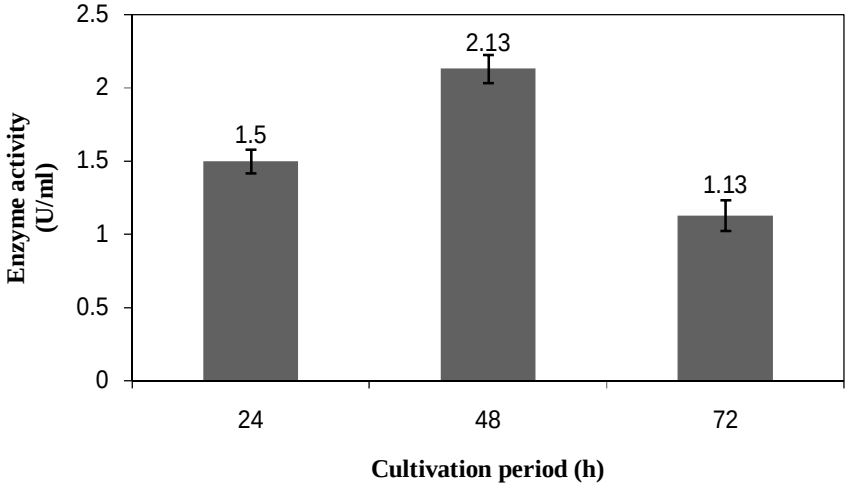

Fig. 1. Effect of cultivation period on α-amylase production by IBLR204

Effect of incubation time on activity of crude α-amylase: The maximum activity for the extracellular enzyme produced by IBLR204 was observed at 25 min which was recorded approximately as 2.34 unit/ml/min (Fig. 2). However, activity appeared to decrease gradually if the incubation period was further extended.

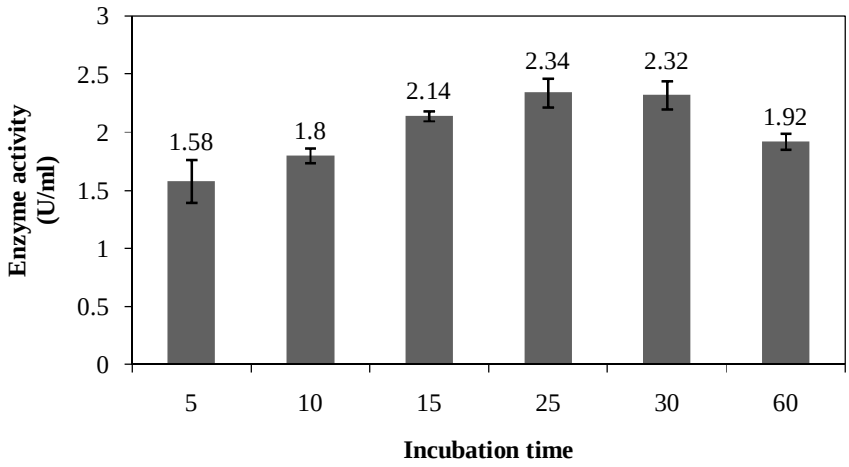

Fig. 2. Effect of incubation time on activity of crude α-amylase

Effect of temperature on activity of crude α-amylase: The optimum temperature for α-amylase activity is usually related to the growth temperature of the microorganisms. However, it was also reported that extracellular enzymes were optimally active at temperature above and beyond the host organism's optimum growth temperature (Vieille and Zeikus, 2001). A temperature range of 40 to 60^0C was found promising with 50^0C

optimum for activity of α-amylase produced by *B. amyloliquefaciens* (Fig. 3) which was comparable previous findings (Demirkan *et al.*, 2005).

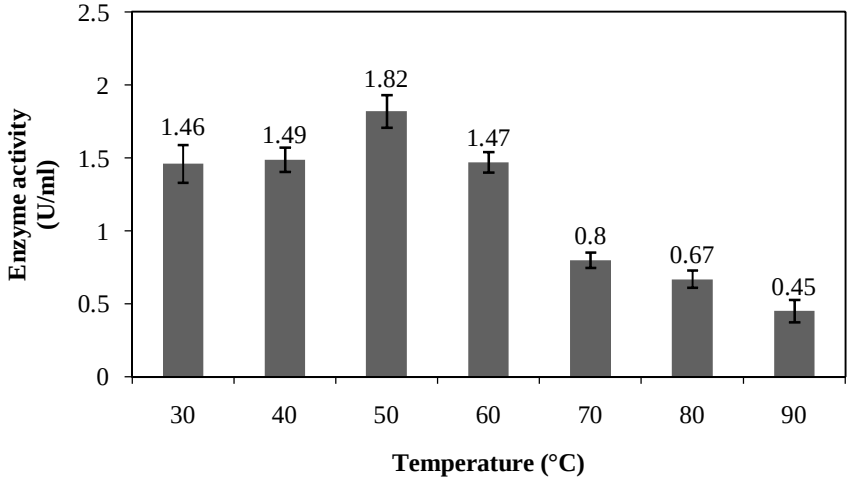

Fig. 3. Effect of Temperature on activity of crude α-amylase

Effect of pH on activity of crude α-amylase: The enzyme exhibited high activity in acidic or neutral conditions with the optimal pH 7.0 (Fig. 4) which is within the range of values for most starch degrading bacterial strains (Gupta *et al.*, 2003). Regarding to *Bacillus* genus, α-amylase enzymes with optimum activities at pH values was as low as 3.5 or as high as 10.6 as previously reported (Hayashi *et al.*, 1988).

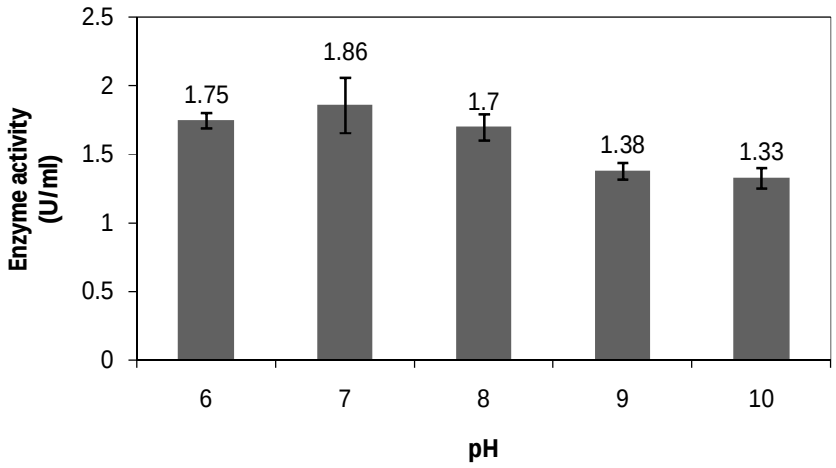

Fig. 4. Effect of pH on activity of crude α-amylase

Bacterial species could be a good source for the production of α-amylases and *B. amyloliquefaciens* (R204) could be a good candidate for α-amylase production. The present study showed that α-amylase from *B. amyloliquefaciens* had optimum pH 7.0 and temperature 50^0C. Future work should employ the complete purification of α-amylase so that higher specific activity could be achieved. Again, further characterization of the enzyme could provide information structure function relationship and elucidate the mechanism of action. This would open up a possibility for genetic improvement of α-amylase producing strains, the enzyme and their subsequent utilization in the related industries.

Acknowledgement: The authors would like to acknowledge the funding from INSPIRE project supported by British Council, Bangladesh.

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