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Original Article

Isolation of Amylase Producing Bacteria from Soil, Identification by 16S rRNA Gene Sequencing and Characterization of Amylase

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The use of amylase enzyme has been extensive in different industrial sectors. Unfortunately, Bangladesh lacks any local production of amylase. This research was conducted to isolate and identify a local amylase producing bacteria from soil and characterize the crude amylase enzyme so that the information acquired can be used to eliminate the issue of amylase scarcity in Bangladeshi industries such as food, pharmaceuticals, garments, and textiles. Bacteria from soil were primarily screened on starch agar medium and out of 48 isolates, 17 were found to be amylase producers. The isolates were screened based on their clear zone ratio, enzyme activities and specific enzyme activities. The best isolate demonstrated an enzyme activity of 0.123 U/ml and specific enzyme activity of 0.173 U/mg. It was then identified by a combination of biochemical tests, morphological and microscopic characteristics along with 16S rRNA gene sequencing. A phylogenetic tree was also constructed which determined that this isolate was a strain of *Bacillus cereus*. Characterization of the crude amylase enzyme revealed that the optimum temperature of the enzyme was 75°C while the optimum pH was 7. Hence this amylase enzyme can be used in industrial processes that require high temperature and neutral pH conditions.

Keywords: Amylase, 16S rRNA, *Bacillus*

Introduction

The enzyme amylase, also addressed as 'glycoside hydrolases' breaks down the carbohydrate molecules into smaller products[1]. Their main substrate is starch which is split into its smaller components such as dextrin, maltose, maltotriose, and glucose. The enzyme basically hydrolyses the α-1, 4 - glycosidic bond that hold the glucose units together[2].

Amylase is produced most widely by microorganisms such as bacteria and fungi compared to plants and animals[3]. Fungi such as *Aspergillus niger*, *Aspergillus oryzea, Thermomyces lanuginosus* and *Penicillium expansum* are known to synthesize amylase[4]. Among bacteria, the *Bacillus* spp. is well recognized as amylase producer. Some of these include *Bacillus subtilis, Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus coagulans* and *Lactobacillus plantarum*[3-5]. Other bacteria such as *Clostridium thermosulphurogenes*, *Proteus* and *Pseudomonas s*pp. are also acknowledged as amylase producers[4,5].

There has been a wide range of applications of amylase in food industries, textile mills and paper industries, in the production of alcohol, detergents, bread, glucose and fructose syrup, fruit juices and manufacture of ethanol as the biofuel. Clinical, medical and analytical chemistry also use bacterial α-amylases[2]. Approximately 25% of the enzyme market is comprised of amylase enzyme[6]. Fungi and bacteria are the best choices of the source because they are very economical with high production rate and can be genetically engineered for the desired quality and quantity of amylase production[1,6].

In Bangladesh, various industries use α- amylase to yield a wide range of products such as high glucose and maltose syrups used in food and pharmaceutical industries. Amylases are also extensively used to remove starch from clothes in garments and textile industries[7]. Due to the lack of any local production of the enzyme, the industries are forced to import amylases which are very costly or use expensive starch degrading chemicals which pose a massive threat to the environment due to their non-biodegradable and toxic properties[3]. Considering these facts, therefore, the aim of the study was to isolate and identify a potent amylase producing bacterial strain from soil and to characterize its crude amylase enzyme.

Materials and Methods

Sample collection

A handful of soil containing decomposed cow manure was collected from a dairy farm in Keraniganj, Bangladesh, in a plastic bag and transported to the laboratory. The soil was stored at 4°C.

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Screening and selecting potent amylase producing bacteria

From the soil sample, 1 g of sample underwent serial dilution from 10^{-1} to 10^{-5} and 0.1 ml was spread on nutrient agar media. Bacterial colonies were subcultured in starch agar plates from the nutrient agar plates and incubated at 37 °C for 48 hours. The plates were then flooded with Gram's iodine and any formation of the clear zone around the colonies were observed and the clear zone ratio was determined. The isolates having the largest clear zone ratio were selected.

Enzyme production in shake flask

The bacterial isolates were inoculated into separate 250 ml Erlenmeyer flasks containing 50 ml fermentation media with the following composition: 5 g/L soluble starch, 5 g/L yeast extract, 2.5µ g/L $(NH_4)_2SO_4$, 0.2µg/L $MgSO_4 \cdot 7H_2O$, 3µg/L KH_2PO_4 and 0.25 µg/L $CaCl_2.2H_2O$ [8]. The flasks were incubated in a shaking incubator for 48 hours at 37°C at 150 rpm. After incubation, the culture fluid was centrifuged at 6000 rpm for 10 minutes. The supernatant obtained was the crude enzyme which was stored at 4°C until use.

Enzyme assay to determine enzyme activity

The DNS method was used to determine the amylase activity of each bacterial isolate[9]. The substrate solution was made by dissolving 1% starch solution in citrate buffer (pH 4.8). To each screw cap test tube, 1 ml substrate solution was added, followed by 1 ml citrate buffer (pH 4.8) and 1 ml of 10 folds diluted crude enzyme. The test tubes were incubated at 50°C for 30 minutes in a water bath. One and half milliliter of DNS solution was added to each test tube to stop the reaction and left undisturbed at room temperature for 10 minutes. The test tubes were then incubated at 100°C for 10 minutes in the water bath and then cooled down to room temperature under running tap water. The colour intensity of the solution was observed by measuring the optical density (OD) using a spectrophotometer at 540 nm. The reading was compared to a prepared blank solution. The concentration of glucose produced for each solution was obtained from a glucose standard curve. The activity of amylase was calculated. One unit of amylase activity is defined as the amount of amylase required to catalyze the formation of reducing sugar, which is equal to µmole of D-glucose per minute under assay conditions[1].

Determination of specific enzyme activity

To a test tube, 0.2 ml crude enzyme (supernatant) and 2 ml alkaline copper sulfate (reagent C) were added and incubated in the dark for 10 minutes. After that, 200 µl of Lowry reagent was added to the test tube and it was incubated again for 30 minutes in the dark. The absorbance of the solution was then measured by using a spectrophotometer at 650 nm. The unknown protein concentration was determined from a standard curve following Folin-Lowry method[10-12]. This was used to calculate the specific enzyme activity.

The isolate showing the highest value for enzyme activity and specific enzyme activity was chosen for identification and further characterization.

Presumptive identification

The selected bacterial isolate was identified presumptively through morphological, phenotypical and biochemical tests (catalase test, oxidase test, MRVP test, TSI test, citrate utilization test, MIU test, cellulose degradation test, gelatin hydrolysis test, nitrate reduction test, MSA plate test, casein hydrolysis test, blood hemolysis test, growth in anaerobic condition, growth at 45°C and 65°C, growth in 7% NaCl, fermentation of sugar: xylose, maltose, sucrose, glycerol and fructose).

16S rRNA gene sequencing

The total genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA). The universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG- 3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T- 3') and the following thermal cycle condition described in a study by Elijah *et al*[13] with slight modification (initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 5 minutes) were used to amplify the 16S rRNA gene via the Polymerase Chain Reaction (PCR). The PCR product was electrophoresed in 1% agarose gel. Afterward, the DNA was purified from the PCR amplicon using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and was sent to the '1st BASE Laboratories' in Malaysia via Invent Technologies Ltd. for sequencing.

Bioinformatics analysis

The genus of the bacteria was determined by comparing the 16S rRNA gene sequence with other bacterial gene sequences in the NCBI Nucleotide database using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The query sequence was converted to FASTA format using the EMBOSS SEQRET website (http://www.ebi.ac.uk/Tools/sfc/emboss_seqret/) and was then used to create a phylogenetic tree using Mega6 software to identify the species of the chosen isolate.

Determination of optimum temperature and pH

The optimum temperature and pH of the crude enzyme were determined using the DNS method as described previously. The optimum temperature was determined by incubating the enzyme at different temperatures starting from 25°C to 85°C at a pH of 6.5 for 30 minutes in a water bath. The optimum pH was determined by incubating the enzyme in the respective buffer solutions: 0.05 M citrate buffer (pH 3 to 5), 0.05 M sodium phosphate buffer (pH 6 and 7), 0.05 M Tris- HCl (pH 8 and 9) and 0.05 M glycine-NaOH (pH 10).

Result and Discussion

After spread plating the diluted sample on nutrient agar medium, 48 colonies were obtained and out of this 17 isolates were found to be amylase producers. In a study by Peltier and Beckord[14], 6.4% isolates from soil and 9.8% isolates from compost were

obtained which produced amylase. Since the sample used here was a mixture of the two separate samples mentioned in the article, it had a larger load of amylase producing bacteria of 36%. Isolates 14 and 28 were initially selected based on the largest clear zone ratio for further study (Figure 1).

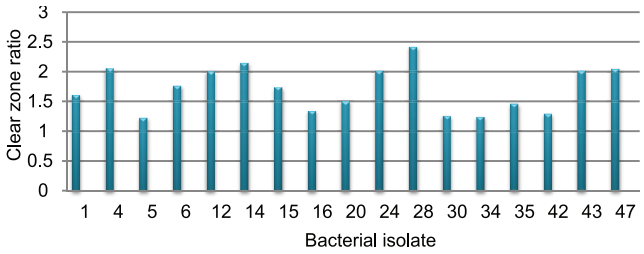

Figure 1. *Comparison among the clear zone ratio of the amylase producing bacteria on starch agar plates*

The isolate 14 showed an enzyme activity of 0.123 U/ml and specific activity of 0.173 U/mg while isolate 28 showed an activity of 0.076 U/ml and specific activity of 0.128 U/mg (Figures 2). Isolate 14 was chosen as the final bacteria to be identified and characterized. In a study by Vaseekaran *et al*[6], the amylase activity of three isolates was found to be within the range from 5 to 8 U/ml which is much greater compared to that found in this study. This might be because the optimum fermentation conditions of isolates 14 and 28 were not known during the initial period of the study. But it can be concluded that isolate 14 is a better amylase producer than isolate 28.

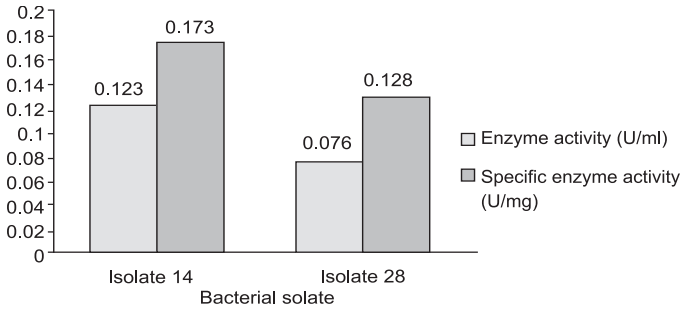

Figure 2. *Enzyme activities and specific enzyme activities of isolates 14 and 28*

The next steps of the study were based on the identification of the bacterial strain. Isolate 14 was observed to be Gram-positive, rod-shaped bacterium, arranged in chains and endospore formers when visualized under the microscope after Gram and spore staining, respectively (Figures 3 and 4). The colony morphology of bacteria was checked after 24 hours incubation at 37°C in nutrient agar medium along with the results of the biochemical profile. According to these presumptive identification results which were checked using the second edition of 'Bergey's Manual of Systematic Bacteriology'[15], it was assumed that isolate 14 might belong to the genus *'Bacillus'*. Table 1 illustrates the biochemical profile of isolate 14.

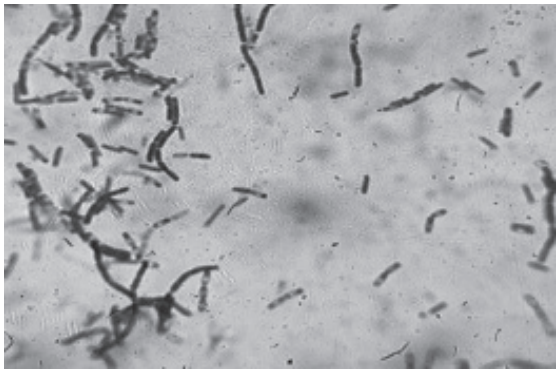

Figure 3. *Appearance of the selected isolate under the microscope after Gram staining*

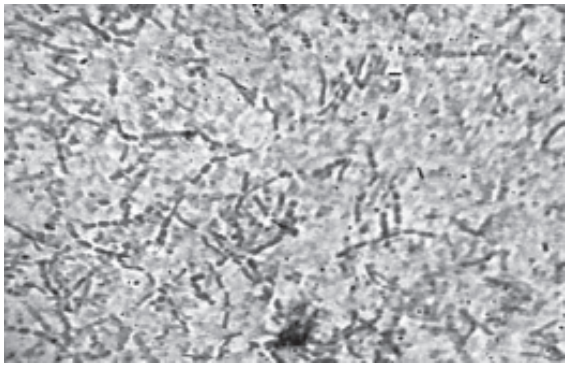

Figure 4. *Appearance of the selected isolate after spore staining under the microscope. The spores are stained green, whereas the bacterial cells are stained red*

Table 1. *Biochemical tests performed and results obtained for isolate 14*

Biochemical test	Result	Biochemical test	Result	Biochemical test	Result
Catalase	+	Growth at 45°C	+	Lactose fermentation	-
Oxidase	-	Growth at 65°C	-	Urease production	-
Voges Proskauer (VP)	-	Growth in 7% NaCl	-	Motility	-
Methyl red	-	Hemolysis of blood	+, ß hemolysis	Gas production	-
Nitrate reduction	+	Xylose fermentation	-	Hydrogen sulphide produciton	-
Citrate	-	Maltose fermentation	+	Casein hydrolysis	+
Gelatin hydrolysis	+	Sucrose fermentation	+	Anaerobic growth	+
Cellulose degradation	+	Glycerol fermentation	+		
MSA plate test	-	Fructose fermentation	+		
Indole production	+	Glucose fermentation	+		

+: for positive results; -: for negative results

The isolate was identified at the genetic level for a complete recognition of the genus and species. After the extraction of the total genome and amplification of the 16S rRNA gene of the bacterial isolate 14, its presence in the amplicon was checked using agarose gel electrophoresis, which indicated that the size of the gene is just below 1,650 base pairs. This is illustrated in Figure 5.

BLAST analysis of the sequence indicated that isolate 14 is from the '*Bacillus*' genus (99% identical). From the phylogenetic tree shown in Figure 6, it was observed that the query sequence shares the same node with a strain of *Bacillus cereus* and the bootstrap value is 70%, confirming that isolate 14 is a strain of *Bacillus cereus*. Among the various types of *Bacillus* species, *Bacillus cereus* is usually isolated at a moderate

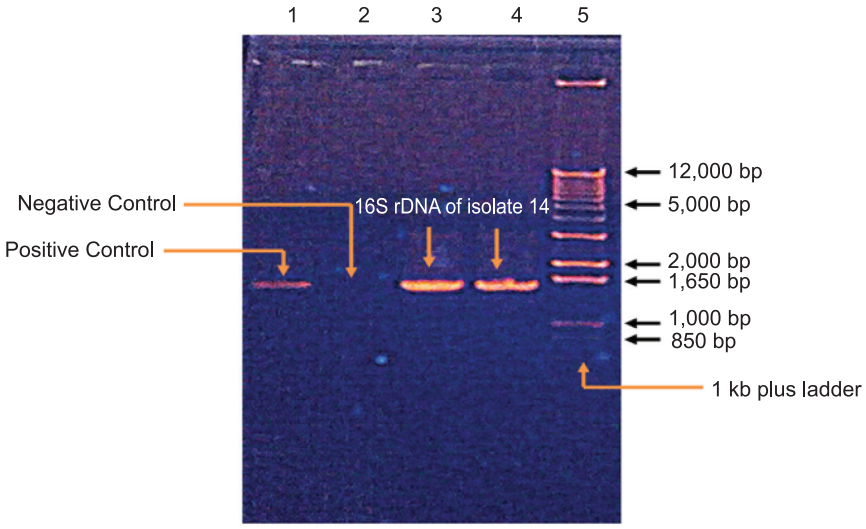

Figure 5. *Visible DNA bands over UV illuminator on 1% agarose gel after electrophoresis. It can be seen that the 16S rDNA bands of isolate 14 which migrated along the lanes of wells 3 and 4 are slightly lower than 1,650 bp when compared to the ladder which migrated along the lane of well 5. Lane 1 indicates the DNA band of positive control and lane 2 shows that no band can be seen for the negative control*

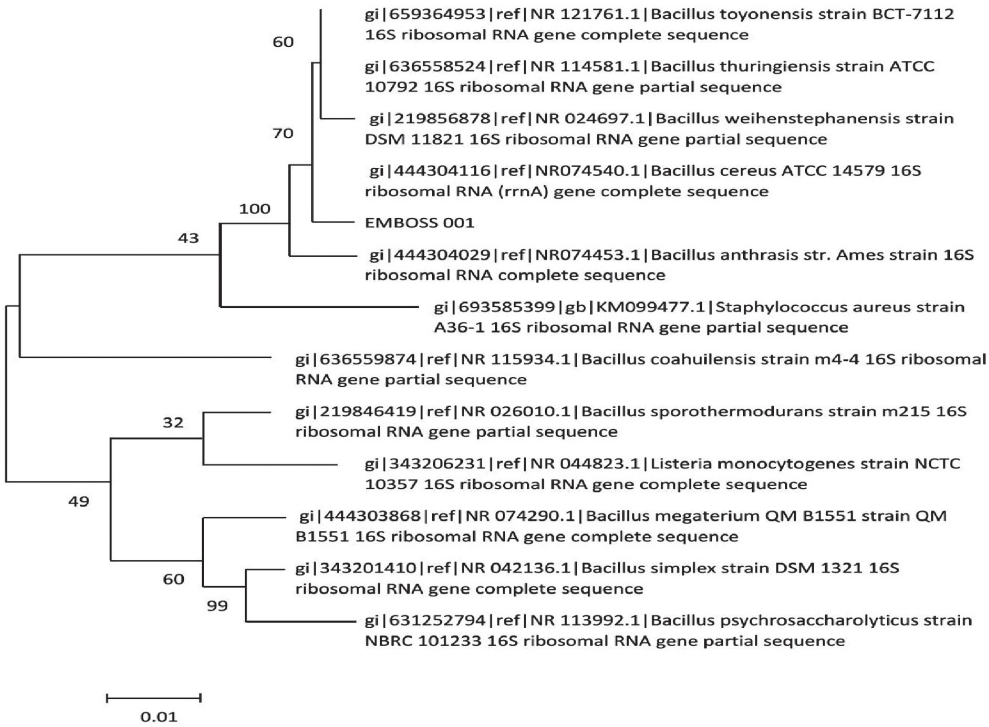

Figure 6. *Molecular Phylogenetic analysis by Maximum Likelihood method. The query sequence in this tree is subjected as 'EMBOSS 001'. It can be observed that the query sequence shares the same node with Bacillus cereus ATCC 14579 16S ribosomal RNA (rrna) gene complete sequence and the bootstrap value is 70. This confirms that isolate 14 is a strain of Bacillus cereus*

frequency as amylase producer. They have been isolated as the second most common amylase producers after *Bacillus subtilis* in a study conducted by Verma *et al*[16] who isolated the bacteria from soil collected from a potato waste dump site. However, this might not be the case every time because the abundance of the type of amylase producers depends on the type of soil they are isolated from.

The enzyme characterization revealed that the enzyme from this *Bacillus cereus* isolate had a low activity from 25 °C until 55 °C. At 65 °C, there was a boost in the activity and it increased even further at 75 °C (Figure 7). This was considered to be the optimum temperature for maximum enzyme activity. The activity was seen to decrease at 85 °C. The result is similar to those found in studies by Cordeiro *et al*[17] and Fattah *et al*[8]. The activity of this enzyme was seen to be 0.724 U/ml at 75°C. However, in

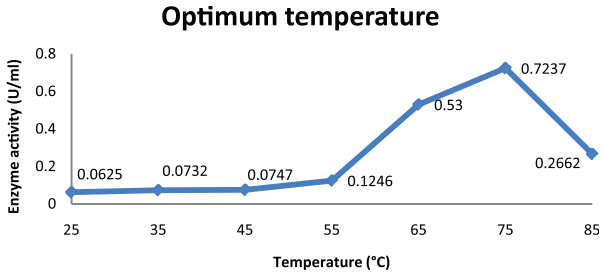

Figure 7. *Effect of temperature on the activity of crude amylase of isolate 14*

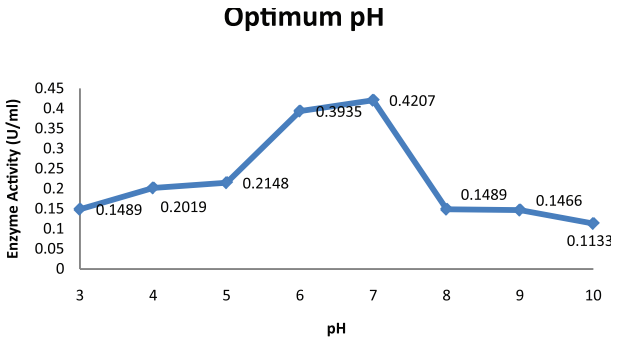

Figure 8. *Effect of pH on the activity of crude amylase of isolate 14*

a study by Asad *et al*[18], the activity of amylase enzyme was found to be around 1.25 U/ml. Hence, it can be postulated that this enzyme is suitable to be used in different processes that require high temperature, such as starch liquefaction which is generally carried out at temperatures of 70 –90 °C[19]. This temperature, on the other hand, is not too high that a large amount of energy will be needed to heat up the fermenters.

The activity of the enzyme was fairly moderate at pH 3. With an increase in pH, the activity of the enzyme was observed to increase as well. There was a boost in activity from pH 6 to pH 7 (Figure 8). However, the enzyme activity decreased from pH 8 to 10. The result correlates completely with those obtained in studies conducted by Vaidya and Rathore[20] and Vaseekaran *et al*[6]. The activity of this enzyme (0.421 U/ml) is similar to that obtained by Asad *et al*[18] where the activity was found to be around 0.5 U/ml at pH 7. However, in the research by Vaidya and Rathore[20] , the activity was 9.19 U/ml for the amylase at pH 7 which is a much greater find compared to the one found in this study. Since the optimum pH for this amylase is 7, a neutral condition is appropriate for the enzyme to work best which may prevent the need to make the fermentation condition too alkaline or acidic for the enzyme to work. However, many glucose fermentation processes release acetic acids, formic acids, ethanol and carbon dioxide and hence pH of the medium needs to be controlled in that case[21].

According to this research, the soil sample used is a moderate source of amylase producing bacteria. Its high activity at high temperature and neutral pH indicates its potentiality in various industries and biotechnological studies. *Bacillus* is a good choice of source for industrial amylase production because the enzyme from *Bacillus* is thermostable, has retention to extreme pH, osmolarity, and high pressure. Other advantages of this bacterial enzyme production include the use of short fermentation cycles, the capacity to produce extracellular enzymes, simple, cost effective production, eco-friendly behavior, less handling hazard for workers, mass production and easy manipulation of bacterial genes[2].

However, further research work is necessary in order to determine the enzyme's thermostability, pH stability, the effect of different metal ions and different substrates. The bacterial isolate can also be characterized to determine optimum fermentation conditions, the strain can be improved using genetic modification techniques, the crude amylase can be purified and the encoded sequence of amylase can be determined that would allow specific modifications. With all this information in hand, in the near future, it is hoped that the demand of commercial amylase in Bangladeshi industries will be fulfilled.

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