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ISOLATION OF BIOTECHNOLOGICALLY IMPORTANT ENZYME PRODUCING RHIZOBACTERIA FROM SEASONAL FLOWER BEDS

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Abstract

This study aimed to isolate biotechnologically important enzyme like amylase and protease producing rhizobacteria from rhizospheric soil. Soil samples were collected from the rhizospherc of five different flowering plants from Curzon hall, University of Dhaka. A good number of bacteria were isolated. Among them 12 bacterial isolates were selected for detailed study based on their amylolytic and proteolytic activity. Starch hydrolysis ratio (SHR) of the studied isolates ranged in between 2.06 and 4.67, casein hydrolysis ratio (CHR) ranged in between 2.27 and 3.92. All the selected isolates were Gram positive and rod shaped and provisionally identified as the member of the genus *Bacillus* with five different species viz. *Bacillus alcalophilus*, *B. subtilis*, *B. pumilus*, *B. firmus* and *B. lentus*. Amylase and protease production of the studied bacteria were estimated and it was ranged in between 29.11±9.57 to 35.78 ± 9.05 and 230.27±37.08 to 276.47 ± 55.01 U/ml. The highest amylase and protease producer were *Bacillus alcalophilus* (Ce104/S9/L) which could produce 35.78 ± 9.05 U/ml and *Bacillus subtilis* (Ca71/S5/L) could produce 276.47 ± 55.01 U/ml of protease, respectively. Maximum production of both amylase and protease was achieved in 24 h of incubation period at 37°C and pH 7.0.

Introduction

Amylase, any member of a class of enzymes that catalyze the hydrolysis of starch into smaller carbohydrate molecules such as maltose (a molecule composed of two glucose molecules). Although amylases are produced from different sources (microorganisms, plants and animals), microbes are most suitable for industrial production due to their short growth period, low cost effective production, eco-friendly behavior, less handling issues for workers, productivity[1] and their easy manipulation[2]. Extensive application of amylase in food, starch liquefaction, saccharification, brewing, detergent, paper, textile and distilling industries, has brought about a greater attention for the increase in the indigenous production of α-amylase by indigenous bacteria. However, different strains have different optimal growth conditions and enzymatic production profile. Now a days *Bac illus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are industrially used as the source of amylase.

Proteases are group of enzymes which catalyze hydrolysis of peptide bonds in proteins. They are also called as peptidases or proteinases or proteolytic enzymes. Proteases can

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be obtained from various living organisms i.e. plants, animals, bacteria and archaea. For industrial production of the proteases, microorganisms are preferred so that large scale production of enzymes can be achieved. *Bacillus, Aspergillus, Pseudomonas* etc. are the organisms that produce protease. Bacteria are most important alkaline protease producers with the genus *Bacillus* being the most prominent source, because of their ability to produce large amount of protease having significant proteolytic activity and stability at high pH and temperature[3]. Proteases are largely used in biotechnology based industries such as food, leather, detergent, pharmaceutical industries and bioremediation process.

Materials and Methods

The soil samples were collected from rhizospheric region of five separate bed of seasonal flowering plant beds from Botanical garden, Curzon Hall, University of Dhaka[4]. Starch hydrolysis test[5] on Starch Nutrient Agar (SNA) was used to screen out the bacteria having the potentiality to produce amylase enzyme. Casein (Milk protein) hydrolysis test[6] on Skim Milk Agar (SMA) was performed to identify proteolytic activity. For this test, SNA and SMA plates were point inoculated with test organisms and the plates were incubated at 37°C for 24 h. After incubation, the surface of the SNA plates was flooded with iodine solution. Starch Hydrolysis Ratio (SHR) and Casein Hydrolysis Ratio (CHR) were calculated using following formula to quantify amylolytic and proteolytic activity of the isolates.

$$\text{SHR or CHR} = \frac{\text{Colony Diameter}}{\text{Zone Diameter}}$$

The isolates selected based on SHR and CHR were purified through repeated plating (by streak plate method). Isolates were provisionally identified according to Bergey's Manual of Systematic Bacteriology Vol. II[6]. Amylase producing broth medium was used for evaluation of amylase activity consisted of 1% soluble starch, 0.6% bacteriological peptone. 0.05% $MgSO_4.7H_20$, 0.05% KCl and pH of the medium was adjusted at 7.0. Bacterial isolates were cultured in 50 mL of broth medium and incubated at 37ºC on rotary shaker (DAIHAN-LABTECH) at 100 rpm for 24 h. Optical Density (OD) of each inoculum was fixed to 1.00 at 600 nm using UV-spectrophotometer (UV-1800 Shimadzu, Japan). Forty nine mL production medium was inoculated with 1mL of inoculum of and incubated at 37°C on rotary shaker at 120 rpm for 24 h. The experiments were performed in triplicates. The culture fluid was withdrawn and centrifuged (Thermo Scientific Sorvall ST 8R, Germany) at 9,000 rpm for 15 min. The cell free supernatant was used for crude enzyme assay. The DNS method[7] was used to determine the amylase activity. Enzyme activity (U/ml) was calculated using the following formula.

$$\text{Enzyme Activity (U/ml)} = \frac{\mu\text{g of glucose}}{\text{ml of enzyme} \times \text{incubation time}}$$

Protease producing broth medium was used for protease production. The medium consisted of (% w/v): CaCl2-0.01, K2HPO4,-0.05, yeast extract-0.02, peptone-1, MgSO4-0.01, glucose-0.1, pH 7.0. One ml of inoculum was transferred in 100 mL of production medium flasks were placed in a shaker incubator at 37ºC, 120 rpm for 24, 48 and 72 h. Two ml of fermentation broth was centrifuged at 8000 rpm, 4ºC for 15 minutes (Thermo Scientific Sorvall ST 8R, Germany). Bacterial cell pellet was removed and cell free supernatant was used as a source of crude enzyme for protease assay and protein estimation.

Protease activity in the crude enzyme extract was determined[8] by using casein as substrate. Two test tubes were taken and labeled as test (T) and blank (B). Five mL of 0.65% casein solution (prepared in 50 mM potassium phosphate buffer, pH 7.5) was added in both the test tubes. One mL of crude enzyme extract was added in T-test tube. Enzyme was not added in blank test tube. Both test tubes were placed at 37ºC for 30 minutes for enzymatic reaction to occur. Five ml of Trichloroacetic acid solution (110 mM) was added in both test tubes to stop the enzymatic reaction. One ml of crude enzyme extract was added to blank test tube to bring the total volume to 11 ml. Solution from both test tubes was filtered using filter paper (Whatmann No 1). Two ml of each filtrate (test and blank) was taken in two new test tubes. Five ml of sodium carbonate (500 mM) was added in both test tubes followed by addition of 1 ml of 2 fold diluted Follin Ciocalteus phenol reagent. The resulting solutions in both test tubes were placed in dark at room temperature for 30 minutes for the development of blue color. The unknown concentration of tyrosine liberated after enzymatic reaction in T-test tube was measured at 660 nm against a reagent blank using tyrosine standard.The efficiency of phosphate solubilization and IAA production of the selected isolates were studied at incubation temperature 25°C, 30°C, 37°C and 40°C and media was adjusted at different pH values 5, 6, 7, 8 and 9 with incubation period of 24h, 48h, 72h and 96h.

Results and Discussion

The pH of the collected soil samples were found to be almost neutral (data not shown). A good number of bacteria were isolated from the collected soil samples. Based on their amylolytic and proteolytic activity, 4 isolates were selected for detailed study. The major biochemical characteristics were shown in the Table 1. All the bacterial strains were rod shaped and Gram positive. (Table 2). Considering biochemical characteristics and microscopic observations two isolated organisms were presumptively identified as *Bacillus alcalophilus* and two isolates were *Bacillus subtilis* (Table 2). All the 12 isolates were protease producer and 8 of them were amylase producer. Based on their ability to produce amylase and protease 4 isolates were selected for enzyme estimation (Table 3).

Table 1. Biochemical characteristics of the selected isolates.

Isolates No.	V.P. test	M.R. test	Deep glucose agar	Utilization of		Lecithinase production	Nitrate reduction	Lipase
				Citrate	Propionate			
Pg10/S2/N	+	+	FA	-	-	-	+	+
Cr71/S5/L	+	+	FA	-	+	+	+	+
Ca104/S9/L	+	+	FA	-	+	-	-	+
Gg126/S10/L	+	+	FA	-	-	+	-	+

FA=Facultative Anaerobic

Among the 12 protease producing isolates 3 best producer were selected for the evaluation of both amylase and protease production (Fig. 1). During 48 h of incubation, the optimum condition for maximum production of amylase was 35.78±9.05 U/mL by *Bacillus alcalophilus* (Ce/S9/L) at pH 7 and temperature 37°C. Basma *et al.*[9] reported maximum amylase production (72.5 U/ml) by the *Bacillus amyloliquefaciens* and Jogezai *et al.*[10] reported 79.0 U/ml by *B. subtilis*. Different incubation periods have been reported by other studies for maximum amylase production: 24h in *B. cereus*[11] and 48h in *B. subtilis*[12]. Optimum enzyme activity at the incubation period of 48h was reported by Pokhrel *et al.*[13]. The optimum temperature for the maximum amylase production was 35°C reported by Pokhrel *et al.*[13] which was closer to the present study (optimum production at 37°C). Shruti and Banik[14] reported the maximum amylase activity at pH 7 which is similar to this study.

Table 2. Microscopic observations and provisional identification of the selected isolates.

Isolate No.	Vegetative cell characteristics	Gram reaction	Spore	Presumptively identified bacteria
Pg10/S2/L	Rod shaped, occur both as single cells and in chains	+	+	*Bacillus subtilis*
Cr71/S5/L	Long rods, occur both as single cells and in chains	+	+	*B. subtilis*
Ca104/S9/L	Rod shaped, occur both as single cells and in chains	+	+	*B. alcalophilus*
Gg126/S10/L	Rod shaped, occur in chains	+	+	*B. alcalophilus*

Table 3. Estimation of amylase and protease activity.

Isolate No.	Bacteria	Amylase activity (U/ml)	Protease activity (U/ml)
Po10/S2/L	*Bacillus subtilis*	29.11±9.57	247.50±33.46
Cr71/S5/L	*B. subtilis*	29.33±10.41	276.47±55.00
Ca104/S9/L	*B. alcalophilus*	35.78±9.05	242.00±1.10
Gg126/S10/L	*B. alcalophilus*	29.11±9.57	230.27±37.08

The maximum protease production was observed as 276.47±55 U/ml by the isolate *B. subtilis* (Ca/S5/L). Sharma *et al.*[15] worked on soil bacteria and reported maximum protease production as 52.29 U/ml by the isolate AKS-4. In this study, the optimum incubation period for the maximum production of protease was 24 hour for all the isolates. Sinha *et al.*[16] reported maximum prodution (124 U/ml) at 24h of incubation period. In the present study, optimum pH for maximum protease production was found to be 7.0. Josephine *et al.*[17] reported maximum protease production (87 U/ml) by the *Bacillus* sp. SNRO1 strain at pH 7. Kumar *et al.*[18] stated pH 9.0 as the optimum pH for protease production. In our study, optimum temperature for the protease production was 37°C. Agarwal *et al.*[19] reported the optimum temperature for maximum protease activity at 40°C by the *Bacillus* sp.

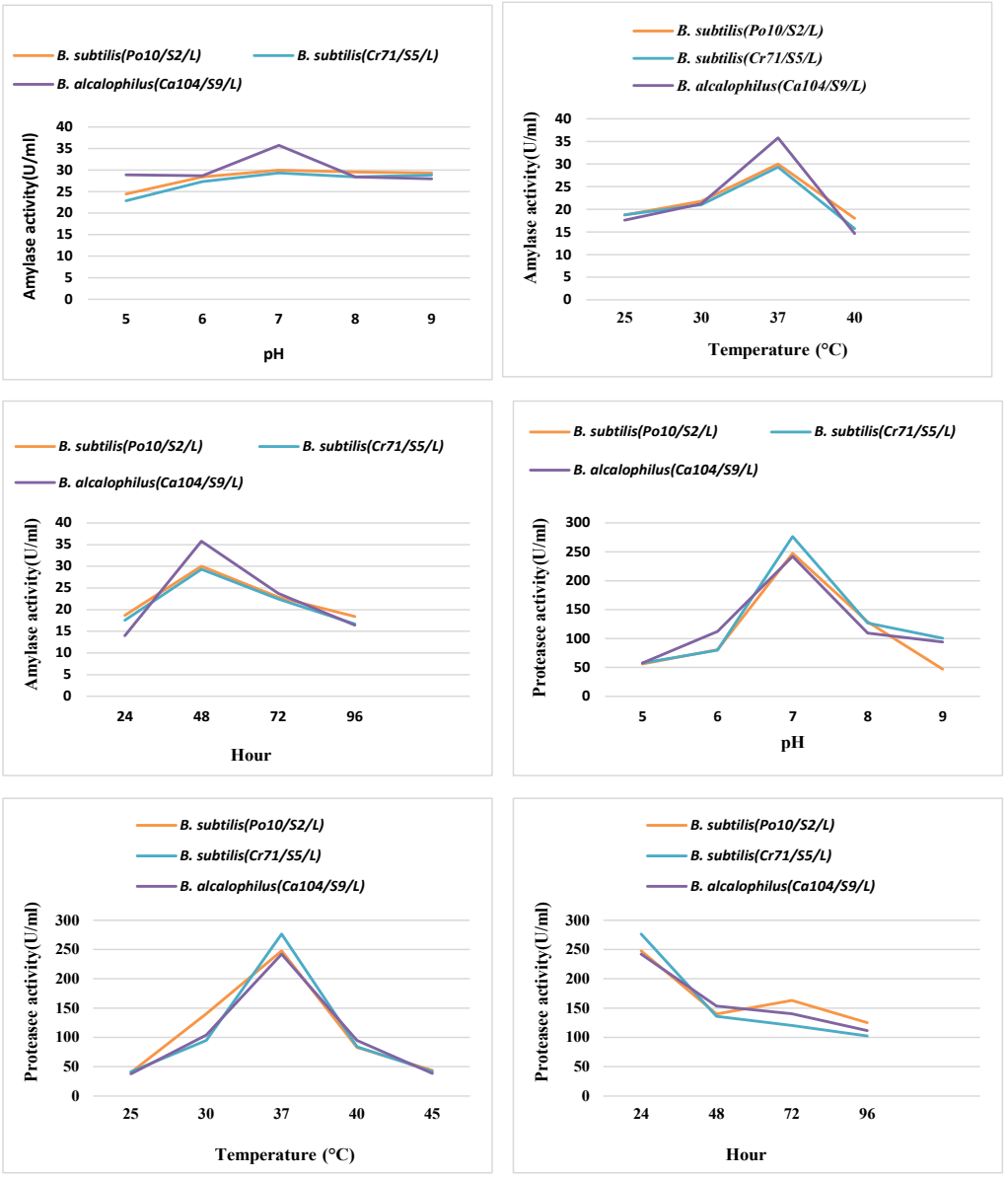

Fig. 1. Optimization of amylase and protease activity by three bacteria under different pH, temperature and incubation period.

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