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Isolation and Screening of An Extracellular Protease Producing Bacteria From Soil Sample

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Abstract

An attempt to isolate bacteria, from local environment, which can produce extracellular protease, was made. We isolated and cultured an isolate for which physical and biochemical characterizations were performed. Gram staining, negative staining, acid fast staining, citrate and starch hydrolysis test, sucrose and glucose fermentation test were performed for this. Enzymatic activity was measured for the isolate culture. Assessment of pH and temperature for obtaining best enzymatic activity were made. The bacteria were found to be gram positive, rod shape and acid fast negative. It was not able to ferment sucrose and glucose. It was not able to hydrolyze starch and citrate. It was producing extracellular protease and best temperature and pH for its production was found to be 40 C and 5 respectively.

Introduction:

Protease represents an important class of industrial enzymes which are utilized in pharmaceutical, food, and detergent industries [1]. The global market value is set to high for these enzymes [2]. These enzymes are industrially produced from fermentation systems which utilizes live microorganisms in submerged or solid media for production [3, 4]. Owing to their importance and growing market value, a substantial research plan is adopted in every industry which aims at developing new technologies and pathways which can deliver production in a sustainable and economical way [5]. One of the areas in which this research is invested is screening of novel microorganism which may produce better titer of enzymes or which may produce product using cheap substrates.

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1. Material and methods

2.1 Screening and isolation of bacteria: Soil sample from garbage dumps was taken for screening of protease producers. 1 gm of soil was mixed thoroughly in 10 ml of tap water by vortexing for 5 min. The soil was allowed to settle and the supernatant was then diluted by serial dilution up to 10 times in distilled water. 20 µl of the last three dilutions were spread on skimmed milk agar plates. The plates were incubated for 24 hrs at 37 C.

Single colony having clear zone of clearance around it were picked and pure cultured by streaking on skimmed milk agar plates.

2.2 Characterization of bacteria

2.2.1 Physical: The isolated bacteria were physically characterized by gram staining, negative staining and spore staining. For gram staining, culture was heat fixed and stained with grams iodine and subsequently washed with ethyl alcohol. Then it is counterstained with safranine.

For negative staining, Negrosin was used. A drop of negrosin was placed in glass slide and culture was mixed in it. Using a second slide a smear was prepared. This was air dried and observed under microscope.

For spore staining, heat fixed smear was flooded with malachite green and dried over water bath. It was rinsed with tap water, couterstained with safranine, and observed under microscope.

2.2.2 Biochemical: Catalase activity, starch hydrolysis test, carbohydrate fermentation test and citrate hydrolysis test were performed for the biochemical characterization of bacteria.

Catalase test was performed by adding small amount of active culture in few drops of hydrogen peroxide on slide. Development of bubbles of oxygen gives positive result for presence of catalase activity [6].

For starch hydrolysis, bacteria were streaked on starch agar plates which are incubated for 24 hrs [7].

Sucrose and glucose fermentation test was carried out by inoculating small amount of culture in separate test tubes containing sucrose and glucose respectively. Durahm's tubes were placed in test tubes to capture the bubbles of carbon dioxide evolved during fermentation [8]. The tubes were placed air tight in incubator at 37 C.

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Citrate hydrolysis test was performed by streaking isolated bacterial culture on slants of citrate agar. It was then incubated for 24 hrs at 37 C [9].

2.3 Enzyme production and assay

For production of enzyme, inoculums from pure culture were transferred to 100 ml casein broth (10g/L) in a shake flask which was incubated on a shaker incubator (Remi RS12) at 37 C, 200 RPM for 24 hrs. 10 ml of aliquot was then taken and centrifuged at 1000 RPM for 10 min in refrigerated centrifuge (Remi R-8C laboratory centrifuge). Supernatant was taken and was used as sample for subsequent assays.

Protease assay for activity determination was performed as per the standard protocol with slight modification [10]. 3 ml of solution containing different dilution (five in number) of the supernatant were taken and 1 ml of (0.5 g/ml) casein powder was added in each tube. These were incubated with shaking at 37 C for 20 min and after that 1 ml of 10% TCA was added in each tube to stop the reaction. The tubes were put at 4 C for 60 min. The solution was filtered and undigested protein was estimated by Lowry's method [11]. BSA (10 mg/50 ml) was used to produce standard curve to estimate amount of protein in digested product. One unit of enzyme activity is defined as amount of digestion production produced per unit volume of supernatant used at a given incubation period [12].

2.4 Optimization of enzyme activity

2.4.1 Temperature: One variable at one time approach was used to determine the temperature for incubation resulting in highest enzyme activity. Cultures of bacteria were grown at 20 C, 30 C, 40 C, 50 C, and 60 C and processed as explained earlier. Supernatant was used to determine the enzyme activity.

2.4.2 pH: The best temperature from the above values was used to determine the incubating pH which may yield best enzyme activity. Three different pH i.e, 5, 8, and 10 were used in study. Enzyme activity was evaluated as described earlier to ascertain best pH for enzyme production.

2. Results and discussions

3.1 Screening and isolation of bacteria: Small, circular single cell colonies having clear zone of casein hydrolysis were obtained as shown in Fig 1. These colonies were picked for further

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pure culture and characterization. One of them was used for characterization, enzyme production, and optimization.

3.2 Physical characterization: Gram staining reveals gram positive, rod shaped bacteria (Fig 2). Photograph of negative stained cells is shown in Fig 3. It also suggested that bacteria are rod shaped. Cells were found to be acid fast negative as suggested by acid fast staining.

3.3 Biochemical characterization: Cells were found to be catalase positive, non starch hydrolyzing, non sucrose and glucose fermenting. There was no change in the color in the citrate agar indicating no citrate hydrolysis reaction.

3.4 Enzyme activity and protein content of supernatant: BSA (Sigma Aldrich) was used to prepare standard curve used to estimate protein concentration in unknown samples by extrapolation (Fig 4). The supernatant sample was found to contain 0.566 mg/ml of total protein.

Table 1 consolidates the results of experiment done for enzymatic activity measurement. It can be seen from there that the average enzymatic activity was found to be 0.889±0.1 ml (Fig 5).

3.5 Optimization: Variation of enzyme activity with temperature and pH are shown in Fig 6 and Fig 7 respectively. 40 C was found to be best among the chosen values of temperature and pH value of 5 was found to be best as producing highest enzymatic activity.

3. Conclusion

A bacterial isolate able to produce extracellular protease was successfully isolated from soil sample taken from garbage dump. The bacteria was rod shaped, gram positive, acid fast negative, catalase positive, non starch hydrolyzing, glucose and sucrose non fermenting, non citrate hydrolyzing. It was found to produce extracellular protease as confirmed from protease activity assay. Incubating temperature and pH were optimized for protease activity. Further studies can be carried out for improvement in protease production of the isolate by mutagenesis and genetic engineering.

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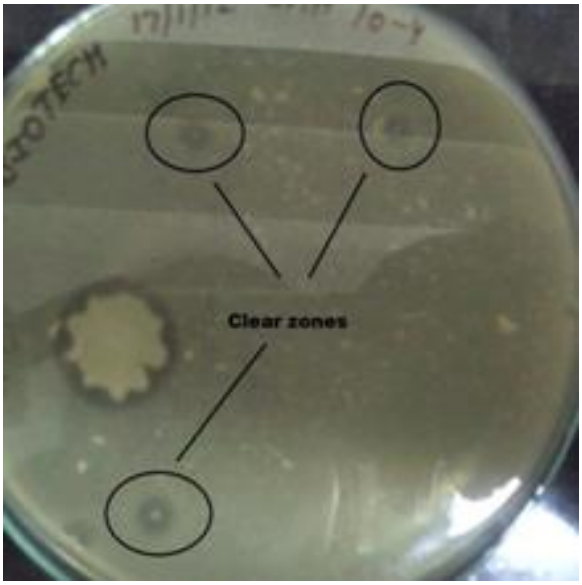

Fig 1. Skimmed milk agar plate showing clear zones of hydrolysis

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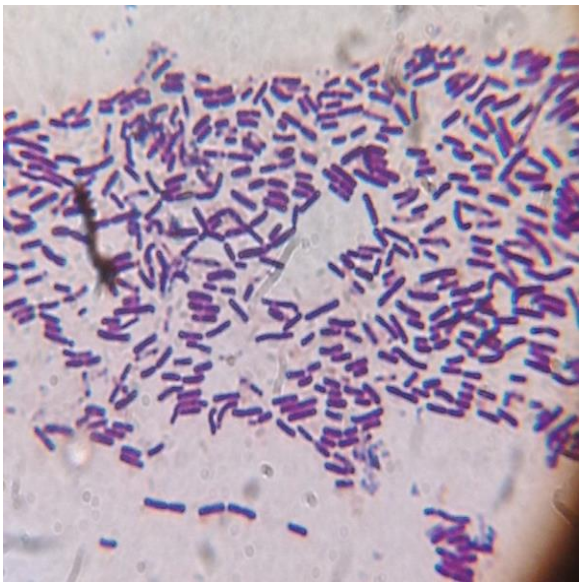

Fig 2: Photograph of Gram staining for isolated bacteria 10 40X zoom.

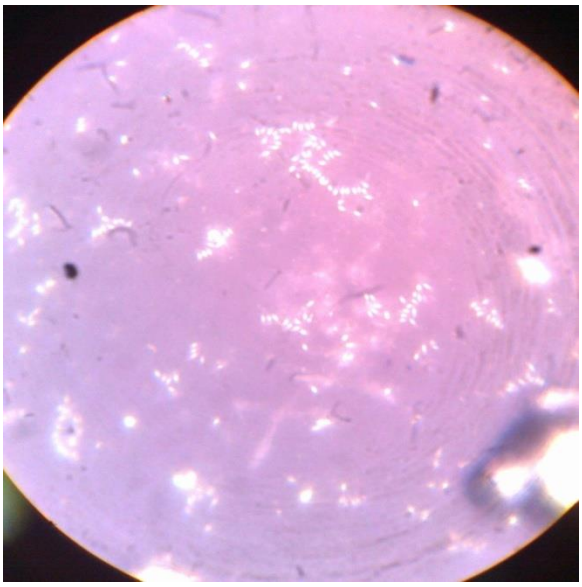

Fig 3: Photograph of negative staining of bacterial isolate.

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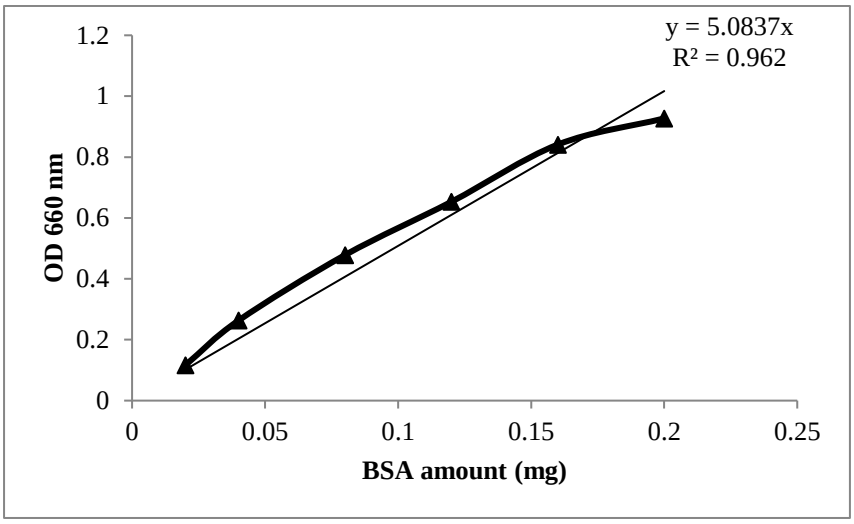

Fig 4: BSA calibration curve

Volume (ml) of supernatant added	Absorbance (660nm)	Amount (mg) of protein in supernatant	Amount (mg) of protein produced per ml of enzyme supernatant added	Volume (ml) required to produce 1 mg of protein
0.1	0.51	0.10033445	1.003344	0.996667
0.2	0.77	0.15148534	0.757427	1.32026
0.3	1.37	0.26952587	0.89842	1.113066
0.4	1.83	0.36002361	0.900059	1.111038

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Table 1: Consolidate table for calculation of enzymatic activity

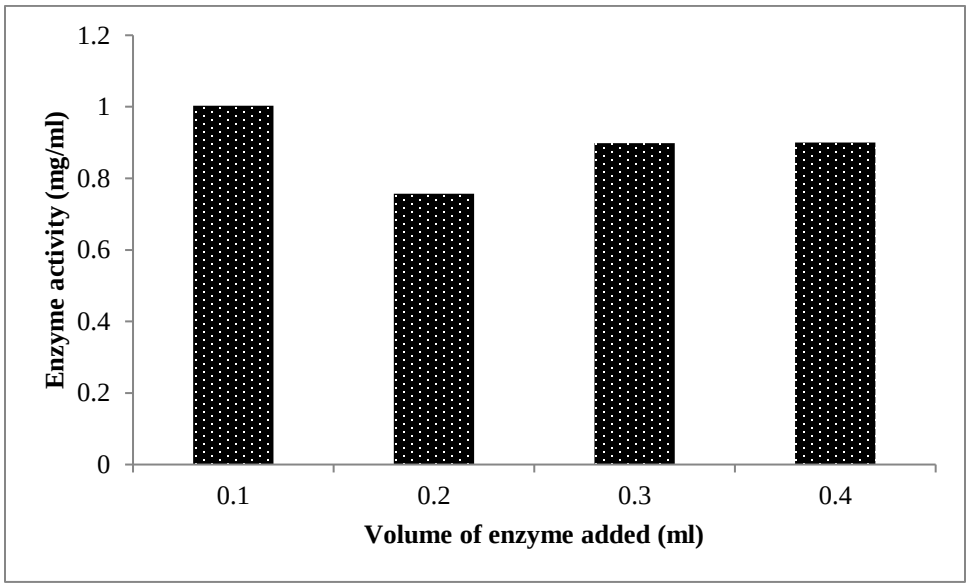

Fig 5: Bar graph of the enzyme activity

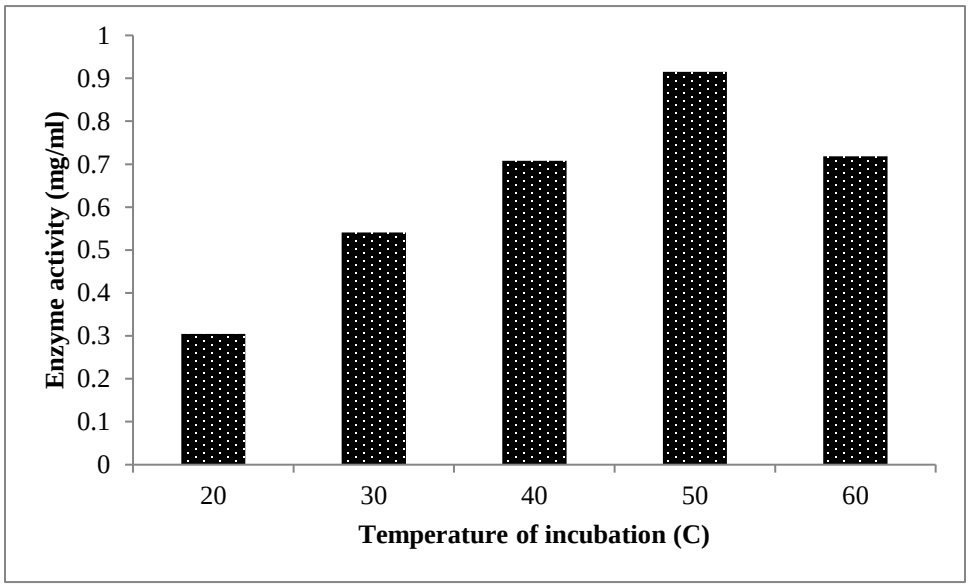

Fig 6: Bar graph of activity at different incubation temperatures

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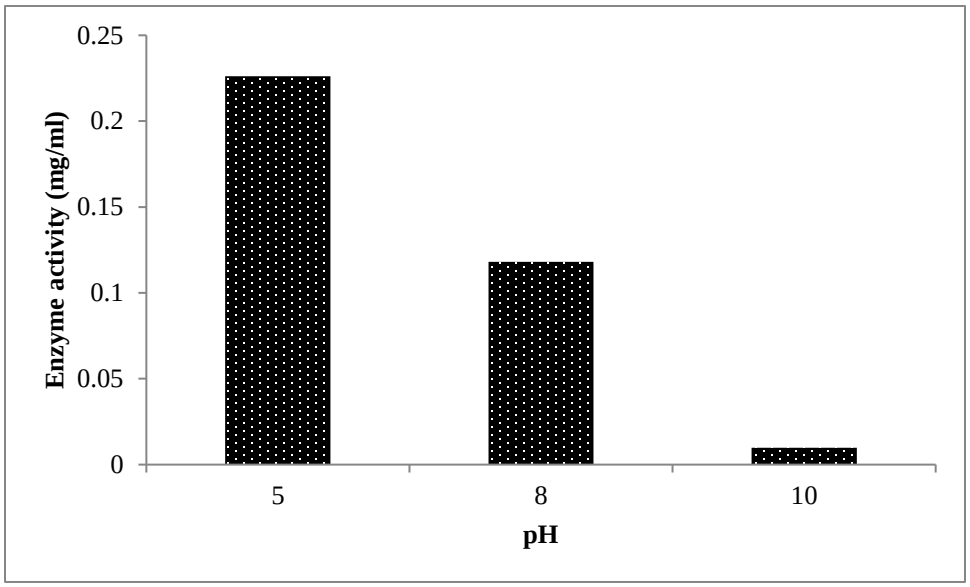

Fig 7: Bar graph of activity at different pH