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Protease Producing Bacillus sps. from Soil: Implications in Anti-coagulation Activity

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Abstract

The use of isolates with enzymatic activities of potential advantage and biotechnological applications is considered important now that environmentalists are concerned with the safety which might be endangered by the release of genetically engineered microorganisms (GEMs) in the environment. Studies which target the diversity of microbial communities in contaminated sites or those which highlight their applications in bioremediation or any other biotechnological processes are of constant need due to the ever-increasing problems related to increase in pollution and depletion in natural resources. This work is complementary to a number of existing studies devoted to the use of this bacterial protease in treatment of congestive cardiac failure as an initial step for anti-clotting agent.

Introduction

Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks. Examples of proteases include: fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin. Proteolytic enzymes use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation. Proteases represent a class of enzymes with important roles in physiological process. Besides this, they have commercial applications, being one of the three largest group of industrial enzymes, accounting for about 60% of the total worldwide sale of enzymes. These enzymes are involved in

essential biological processes like blood clotting, controlled cell death, and tissue differentiation. They catalyse important proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms and viruses. This makes proteases a valuable target for new pharmaceuticals. They also participate in protein catabolism in degradative or biosynthetic pathways and in the release of hormones and pharmacologically active peptides from percursor proteins. They conduct highly specific and selective modifications of proteins such as activation of enzymes by limitated proteolysis and colaborate with the transport of secretory proteins across membranes. Besides this they have a variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes, and in pharmaceutical industry for preparation of medicines such oitments for debridement of wounds. Proteases assist the hydrolysis of large polypeptides in smaller peptides and aminoacids, thus facilitating their absorption by the cells. The extracellular enzymes play a major hole in nutrition due to their de-polymerizing activity. On the basis of their acid-base behavior, proteases have been classified in to three categories i.e., acid, neutral and alkaline proteases. The acid proteases are those which have pH optima in the range of 2.0-5.0 and are mainly fungal in origin. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. They are mainly of plant origin however; some bacteria and fungi also produce neutral proteases. While proteases that have pH optima in the range of 8.0-11.0 are grouped under the category of alkaline proteases. Some of the important alkaline proteases are solanain, hurain and proteolytic enzymes of *Bacillus* and *Streptomyces* species. Alkaline proteases are envisaged to have extensive applications in leather industry. In the presence study we isolated *Bacillus* species, which is more resembling *Bacillus subtilis.* This species was identified by using microbiological and biochemical tests. The species was characterized by using 16s rDNA ribotyping. Further studies on the species showed the expression of proteases, which play an important role in the inhibition of blood clotting.

Materials and Methods

Isolation of Bacteria

The diluted samples were plated onto isolation media (LB Agar) by pour plate method and incubated at 37°C for 24 hours. Subculturing was done by streak plate method taking the isolated colonies of bacterial cultures which were obtained from pour plate method and again incubated at 37°C for 24-48 hrs.

Identification of Bacteria

The isolated bacterial identification was done based on morphological characters, staining techniques and various biochemical tests.

Culture Conditions for Protease Production

Production of protease from Bacillus subtilis were carried out in a medium containing

the following (g/200mL): glucose 0.2, peptone 2, yeast extract 0.04, Calcium chloride 0.02, K_2HPO_4 0.1, and $MgSO_4$ 0.02 and maintained at 37°C for 48hrs. The pH of the medium was adjusted at 7.0 before sterilization.

Production of Protease

Sterile broth medium (450mL) was inoculated with 50mL inoculum and incubated at 35°C for 48 hrs, then centrifuged at 10000rpm for 15min at 0°C and the clear crude enzyme supernatant was stored at -20°C for further studies.

Protein Estimation – Lowry's Method

Total protein of the cell free filtrate was determined by the method of Lowry et al. Bovine serum albumin was used as standard. Different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ ml) and water in a test tube. The final volume in each test tube was 5 ml. The BSA range was found as 0.05 to 1 mg/ ml. From these different dilutions, 0.2 ml protein solution was taken in to different test tubes and added with 2 ml of alkaline copper sulphate reagent (analytical reagent). The solutions were then mixed well. This solution was incubated at room temperature for 10 mins. 0.2 ml of reagent Folin and Ciocalteau solution (reagent solutions) was then added to each tube and incubated for 30 min. Colorimeter was adjusted to zero with blank and the optical density was measured at 660nm. The same above procedure was followed for different volumes of test samples (0.2ml, 0.4 ml, 0.6ml, 0.8ml). A graph was plotted with absorbance on Y-axis and protein concentration on X-axis. The absorbance of unknown sample was measured and plotted to find the concentration of the unknown sample in the standard curve.

Enzyme Assay

Sigma's non-specific protease activity assay may be used as a standardized procedure to determine the activity of proteases. In this assay, casein acts as a substrate. When the protease we are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute. To begin the assay four clean test tubes were taken. 5mls of 0.65% casein solution was added to each of four test tubes, and it was equilibrated in a water bath at 37°C for about 5 minute. Different volumes (0.2, 0.4, 0.6, 0.8 mL) of enzyme test solution were added. The solutions were mixed well and incubated for 37°C for exactly ten minutes. After 10 minutes of incubation, 5 ml of the TCA reagent was added to each tube to stop the reaction. Then an appropriate volume of enzyme solution was added to each tube so that the final volume of enzyme solution in each tube was 1 ml. This was done to account for the absorbance value of the enzyme itself and ensure that the final volume in each tube was equal. The solutions were then incubated at 37°C for 30 minutes. Meanwhile, tyrosine standard dilutions were set up using 6 test tubes. 0.05, 0.10, 0.20, 0.40, 0.50 mls of 1.1 mM tyrosine standard stock

solutions were taken in the test tubes. Blank was prepared without adding tyrosine standard solution. An appropriate volume of purified water was added to make the final volume of 2 mls. After the 30 minute incubation, 5mls of sodium carbonate and 1 ml of Folin's reagent were added to all of the tubes. Sodium carbonate was added to regulate the pH drop created by the addition of the Folin's reagent. Similar volumes of Sodium carbonate were then added to the test samples. The solutions became cloudy after the addition of sodium carbonate. Then, the Folin's reagent was added, which react primarily with free tyrosine. The test tubes were then mixed by swirling and incubated at 37ºC for 30 minutes. An appreciable color change was observed after incubation of the test samples. Absorbance values were recorded using the spectrophotometer.

Anti-clotting activity

Anti-clotting activity test was performed using human blood sample and 0.05M sodium phosphate buffer. Two clean glass slides were taken. Two drops of human blood (200ul) were placed separately at two extreme ends of glass slide. To one of the blood sample, 200ul of sodium phosphate buffer was added and to the other drop of human blood sample, 200ul of bacterial supernant (M sample) was added. Then both the blood samples were observed for anticlotting activity. The same above procedure was repeated for bacterial supernant (S sample).

Results

The Bacillus speciesisolated from soil, were then used to produce Protease. The media selected for protease production consists of glucose, peptone, yeast extract, calcium chloride, K_2HPO_4, and $MgSO_4$ and temperature of 37°C for 48 hrs. Among various pH ranging from 6-9, the pH of 7.0 was considered to be the best. Among various temperatures like 25°C, 37°C and 40°C, the temperature of 37°C supported the growth of test organisms. After optimization the mass production was carried in 500 ml of media in orbital shaker at 37°C for 48 hrs. After 48 hrs of incubation the culture was centrifuged and crude extract of enzyme was obtained.(Figure:14,15)

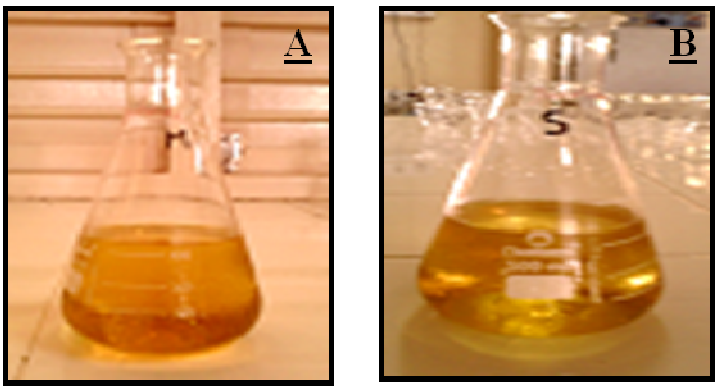


Figure 1A: represents supernatant of sample M and Figure 1B represents the supernatant of sample S.

Quantitative estimation of protease from soil Bacillus sps.

The concentration of so obtained protease extract was determined using Lowry et al method with varying volume of test samples. The average concentration of M sample was found to be 2.43mg/ml and the average concentration of S sample was found to be 0.89mg/ml.

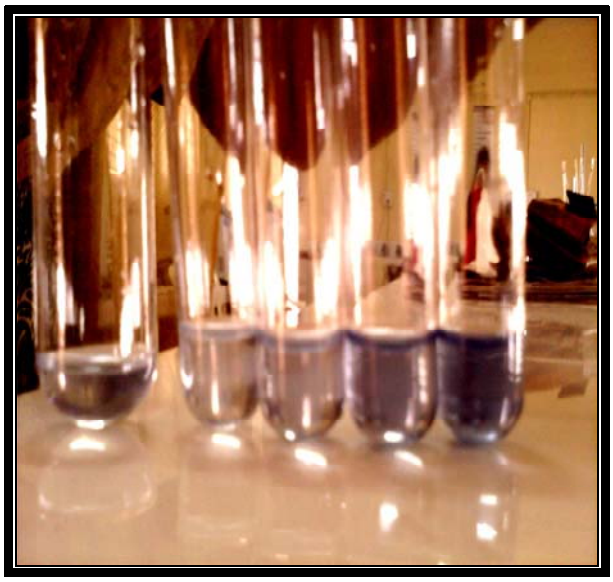


Figure 2: Lowry test for estimation of proteins.

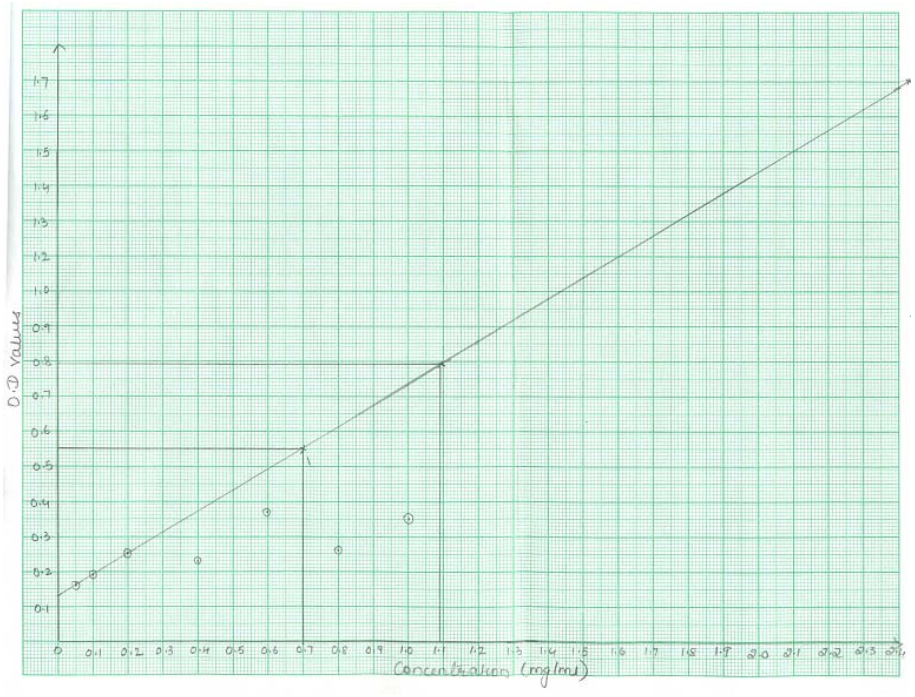
Table 1: Results of Protein Estimation

Standard samples

Sample conc(mg/ml)	O.D 600nm
0.05	0.16
0.1	0.19
0.2	0.25
0.4	0.23
0.6	0.37
0.8	0.26
1.0	0.35

Test samples

Sample conc(mg/ml)	O.D 600nm
0.2	0.55
0.2	0.79
0.6	1.70
0.8	2.43



Graph 1: represents the protein estimation of proteases using Lowry's method.

Catalase activity test

The enzyme activity of protease extract was determined by Universal method using casein as substrate with different volumes of test samples such as 0.2ml, 0.4ml, 0.6ml, 0.8 ml. The enzyme activity of M sample was found to be 0.865 units/ml and the enzyme activity of S sample was found to be 0.765 units/ml.

Figure 3: Catalase test.

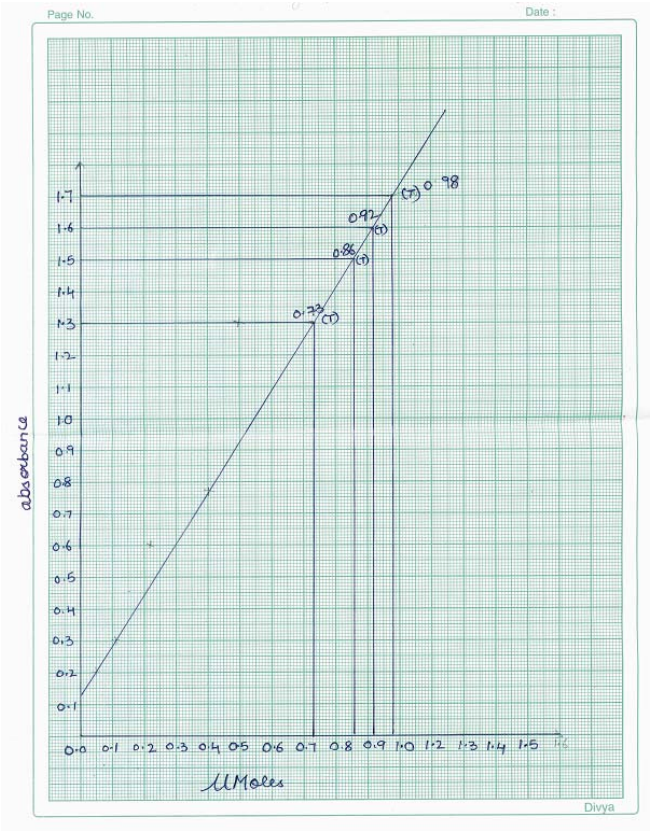
Table 2: Results of Protein Activity

Standard samples

uMoles	Absorbance values
0.055	0.22
0.111	0.3
0.221	0.62
0.442	0.77
0.553	1.3

Test samples

uMoles	Absorbance values
0.86	1.5
0.98	1.7
0.73	1.3
0.92	1.6



Graph 2: represents the protease activity using catalase assay.

Anti-coagulant activity of the culture supernatant

The anti-clotting activity test was performed using clotting time delay method of Baughman (1968). The clotting time was done by adding buffer as control (0.05M sodium phosphate buffer) and culture supernatant to 200µl of human blood. The clotting time for human blood mixed with 0.05M sodium phosphate buffer was found to be 90 sec. Surprisingly The clotting time for human blood mixed with same volume of culture supernatant was found to be 300 seconds. We perfumed the same with sample 2 and the clotting time was delayed.

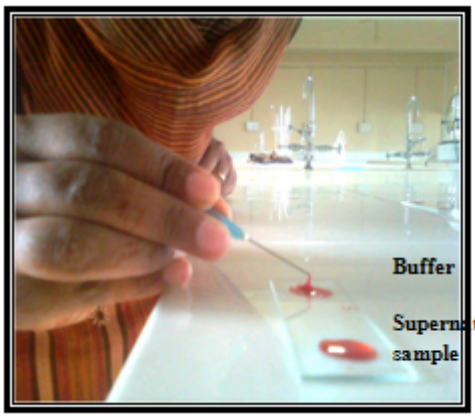


Figure 4: Represents the clotting assay and the formation of fibrin network structure when 200 µl of PBS was mixed with 200 µl of human blood.

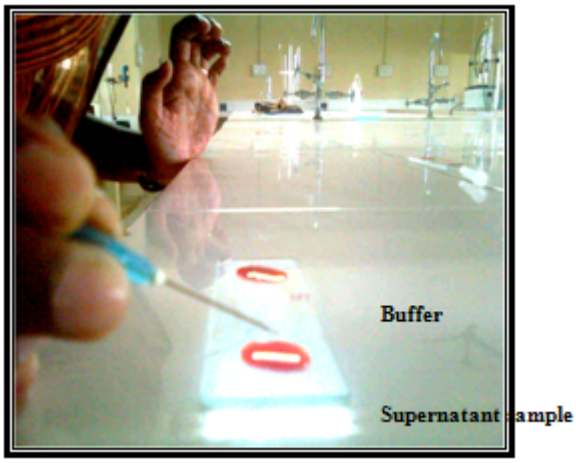


Figure 5: Represents the clotting assay and the no formation of fibrin network structure when 200 µl of culture supernatant was mixed with 200 µl of human blood.

Discussion

In biotechnological applications, the key is always the choice of the appropriate microorganism, it is important to select the suitable microorganism to carry out a desired biotechnological process. The isolation of microorganisms from extreme conditions or contaminated sites offers microorganisms with unusual properties and activities. Studies undertaken to examine the identification and characteristics of environmental samples revealed the true diversity of microorganisms and their unique functionality which arise from their biological system that produce enzymes to make them tolerate or adapt to their environments. The use of molecular techniques adds more precision and accuracy to the phylogenetic identification and also to the true reflection of microbial diversity. An isolate of *Bacillus spp.* obtained from textile waste water was studied for its potential in hydrogen peroxide tolerance in combining

Advanced Oxidation Processes (AOP) and biological processes in textile wastewater treatment. This isolate was further studied to determine its taxonomic position and characteristics; it was phenotypically identified as *Bacillus maroccanus* and was given the description of extremely halotolerant, facultative psychrophilic and facultative alkalophilic, it also exhibited tolerance to hydrogen peroxide up to 442 mM above which it showed no colony forming ability). The isolation of a spore forming probiotic *Bacilli sp.* was successful which had extraordinary abilities to produce anti-clotting protease. The biochemical tests were not decisive in defining the exact strain of microorganism as no previous reports of a spore forming *Bacilli sp.* with a anti-clotting ability was reported. The 16SrRNA sequencing revealed the strain to be *Bacillus subtlis* closely related to *Bacillus subtilis subsp.* Previously reported strain *Bacillus subtilis KL88* has the ability to ferment lactose at temperatures ranging between 0-10°C. We acclaim anti-clotting ability at room temperature by *B. subtilis* to its source of isolation from soils of Visakhapatnam. Over a period of time this *B. subtilis* strain would have evolved to reduce the viscosity of the blood. The sequencing data of this new strain will be submitted to National Centre for Biotechnology Information (NCBI), Europian Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ). This particular strain of *Bacillus subtilis* can be used as to isolate protease, which has anti-clotting agent in hyperlipidemia and hypertension patients.

Conclusion

The screening of environmental microflora should extend to their gene stability prior to their storage by classical techniques. This work is complementary to a number of existing studies devoted to the use of this bacterial protease in treatment of congestive cardiac failure as an initial step for anti-clotting agent. Further study of this protease in the bacteria and potentiality of over-expression of this protease is needed.

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