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Production of Protease Enzyme from *Bacillus Clausii* Sm3.

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Abstract: *Soil was collected and serially diluted using sterile distilled water. Totally 5 bacterial colonies are isolated, all the 5 strains showed clear zone in protease plate assay, particularly the strain SM3 exhibited clear zone around the colony on skim agar plates. On amylase assay the activity was found to be 0.99 U/min/ml. Considering the macroscopic, microscopic, biochemical and physiological characteristics the strain was identified as Bacillus clausiii SM3. It was seen that protease produced by Bacillus clausii SM3 had high capability of removing the blood stain, which indicates its potential in detergent industries. From this study it was came to be know that the study organism (Bacillus clausiii SM3) isolated from soil can be used as an effective source for the production of protease enzyme.*

Keywords: *Bacillus, subculturing, detergent, U/min/ml,*

I. Introduction

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of total worldwide sales, of these alkaline protease are employed primarily as cleansing additives. Among various proteases, bacterial proteases are most significant, compared with animal and fungal proteases. Protease derived from microorganisms such as bacteria, fungi, and yeast has found wide spread applications in many fields (Fujiwara *et al*., 1991). Proteases constitute one of the most important groups of industrial enzymes, being extensively used in the food, detergent and other industries. Ideally, proteases used in a detergent formulation should have high level of activity over a broad range of pH values and temperatures. Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives, holding more than 50% of total enzyme market. They play a specific catalytic role in the hydrolysis of proteins (Godfrey *et al*., 1985).

Many *Bacillus* species produce a variety of extracellular and intracellular proteases. This protease is the most important group of industrial enzymes and certainly forms a major portion of the world wide scales. Bacteria are the most important alkaline protease producers with the genus *Bacillus* being the most prominent source, because of their ability to produce large number of alkaline proteases having significant proteolytic activity and stability at high p^H and temperature (Venugopal and Saramma, A, 2007). The enzyme should also be stable in the presence of oxidizing agents, surfactants, bleaches and other additives that might be present in the formulation. The largest application of the proteases is in the laundry detergents, where they help in removing protein based stains from clothing during washing. Considering these facts, in the present study was attempted to screen some low cost and easily available medium yield of alkaline protease by locally isolated *Bacillus clausii*. It has also shown tremendous activity for the removal of blood stains from cotton cloths.

II. Materials and methods:

2.1 Isolation of bacteria

Soil was collected using sterile polythene bags and brought to the laboratory of Department of Microbiology, Malankara Catholic College, Mariagiri. The collected samples were serially diluted using sterilize distilled water. From 10^{-1} suspension, 1ml was transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2} - 10^{-7} dilution. From the required dilutions, 0.1ml suspension was drawn and spread over the surface of nutrient agar medium (Himedia). The isolated bacteria were sub-cultured in nutrient agar slants and incubated at room temperature for 48 h to achieve good growth and then preserved in refrigerator for further analysis.

2.2Screening for protease producers

The isolated colonies were screened for protease production using skim milk medium. All the isolates were streaked on to skim milk agar plates and the plates were incubated for 48 h at room temperature. The clear zones around the colonies were evaluated as protease producers.

2.3 Cultural characterization of protease producer

Microscopic, macroscopic, biochemical, and physiological characteristics of the potent protease

producer (SM3) were determined by using microbiological methods (Cappucino and Sherman, 2004).

2.4 Production of enzyme

The cultivation of isolate was carried out in 1L Erlenmeyer flasks containing 250 ml of nutrient broth as liquid medium. The isolates were inoculated and incubated for 48 h at 37°C with agitation of 250 rpm.

2.5 Protease assay

Protease activity was assayed by the modified method of Keay *et al*., 1970; 1ml of the diluted enzyme was mixed thoroughly with 1ml of 2% casein solution. The mixture was incubated at 37°C for 10 min. Then the reaction was terminated by the addition of 2ml of 0.4 M trichloro acetic acid and the mixture was again incubated for 20 min at 37°C .The incubated solution was filtered through Whatmann no:1 filter paper. Then 1 ml from the filterate with 5ml of 0.4M sodium carbonate and 1ml of 0.5 N folin phenol reagents was added and mixed thoroughly. Again the mixture was incubated at 37°C for 20 min and the final solution was measured at 660nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1µ of tyrosine in 20 min at 37°C).

2.6 Removal of the blood stains

It was performed according to the method of Najafi *et al*., 2005. A clean piece of pure white cotton cloth was soaked in animal blood for 15 min and then allowed to dry at 80°C for 5 min in hot air oven. The dried cloth was cut into equal sizes (4x4 cm^2) and incubated with crude enzyme at 40°C for different incubation periods (10, 20, 30, 40 and 50 min). After a given incubation, the cloth was rinsed with tap water for 2 min without scrubbing and then dried in open air. The same procedure was done with the control without the enzyme exposure.

III. Results

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers. Of these, strains of Bacillus sp. dominate the industrial sector (Gupta *et al*., 2002).

3.1 Isolation and screening of protease producers

Based on the colony morphology and stability in subculturing, 5 strains showed clear zones around the colonies on skim milk plates. Among these strain, SM3 exhibited large clearing zone around the colony on skim milk agar plates. Therefore this strain SM3 was selected for further production and purification of extra cellular protease. Coolbear *et al*., (1991) who established that, there is no necessarily good correlation between zones of clearing around colonies on milk-agar plates and levels of proteinase activity.

3.2 Protease assay

The 5 bacterial strains produced proteases at varying levels (Table 1). Among the cultures tested the strain SM3 obtained from soil gave the maximum yield of 0.99 U/min/ml. Microbial proteases are produced from high yielding strains including species of *Bacillus* sp., *Alcaligenes faecalis, Pseudomonas fluorescens* and *Aeromonas hydrophilia* grown under submerged culture conditions. Among these, *Bacillus* sp. is the most important group of bacteria that are involved in the enzyme industry and this bacterium is also known to produce proteolytic enzymes quite effectively (Boominadhan *et al*., 2009). Asokan and Jayanth, 2010, Das and Prasad, 2010, Kumar and Vats, 2010 and Ramakrishna *et al*., 2010 studied the production of alkaline protease from different *Bacillus* species.

Table 1 Assay for protease enzyme production

Sl no	Enzyme producers	Protease assay (U/min/ml)
1	SM1	0.65
2	SM2	0.70
3	SM3	0.99
4	SM4	0.56
5	SM5	0.56

3.3 Culture characterization of protease producer

The strain SM3 was identified as *Bacillus clausii* based on the Bergey's manual Classification of determinative Bacteriology (Table 2).

Table 2 Macroscopic, Microscopic, biochemical and physiological identification of amylase producer

S.NO	Macroscopic Analysis	
1.	Colony Morphology	Filamentous with filamentous margin
2.	Colour	White
Microscopic Analysis		
3	Gram stain	Gram +ve, Rods
4	Motility	Motile
5	Spore staining	Presence of spores
Biochemical Analysis		
6	Indole Production	-ve
7	Methyl – Red	-ve
8	Voges Proskuer	+ve
9	Citrate utilization	+ve
10	Triple sugar iron test	Alkaline slant / Acid Butt
11	Catalase	-ve
Physiological Analysis		
12	Starch hydrolysis	+ve
13	Gelatin hydrolysis	+ve
14	Casein hydrolysis	+ve
15	Urea hydrolysis	-ve

3.4 Removal of blood stains

The blood stain was removed from a white cotton cloth by incubating the cloth in purified protease for different time intervals. It was seen that protease produced by *Bacillus clausii* SM3 had high capability of removing the blood stain, which indicates its potential in detergent industries (Fig 1). Anwar and Saleemuddin, 1998 reported the effectiveness of protease on blood stain removal from cloth in the presence and absence of detergents. In the present study the removal of blood by Bacillus clausii producing protease enzyme is a promising additive for detergent industry and that well established by many authors. Nadeem *et al*. (2008) studied the high capacity of blood stain removal by *B. licheniformis* N-2. Vijayalakshmi *et al*., 2011 studied the removal of blood stain by *Bacillus* RV.B2.90. Elela *et al*., 2011 reported the removal of blood stain by alkaline protease production by alkaliphilic marine bacteria *Bacillus cereus.*

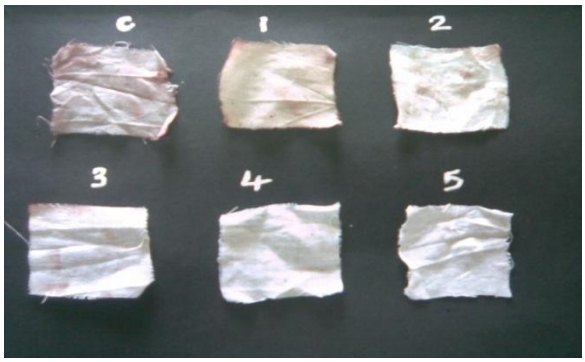

Fig 1 Removal of blood stains by protease enzyme produced by *Bacillus clausii* SM3 at various intervals (1-10 min, 2- 20 min, 3- 30 min, 4-40 min, 5- 50 min).

IV. Conclusion

From the present study it was revealed that the protease enzyme produced by the soil microorganism *Bacillus clausii* SM3 showed a positive result in the removal of blood stains from the cloths. Therefore this enzyme was used in the industrial applications in pilot scale.

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