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Isolation and Characterization of Extracellular proteases from *Pseudomonas aeruginosa* and *Bacillus subtilis* strains

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ABSTRACT

Protease, a digestive enzyme found in various plant and animal sources has wide array of industrial application for its ability to hydrolyze complex protein molecules. Microorganisms also secrete such protease enzymes which are also used in industrial sector considering their economical value. Here we evaluated the ability of bacteria such as Bacillus subtilis and Pseudomonas aeruginosa in producing protease and identified that both the bacterial isolates exhibited significant protease activity. Hence, considering their free living nature in the environment and ease of maintenance, these organisms can serve as an effective substitute for the conventional protease producing microorganisms for their use in industrial applications.

Keywords: Protease, *Bacillus subtilis* and *Pseudomonas aeruginosa*

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INTRODUCTION

Protease is a digestive enzyme that causes the breakdown of protein. Proteolytic enzymes have widespread applications in various industries like detergent, textile, leather, dairy products and pharmaceuticals [1]. Although proteolytic enzymes can be obtained from animal and plants, microorganisms are the major preferable source in industrial applications due to economical advantage. In the current scenario, proteases dominate worldwide enzyme market representing one of the most important groups of industrial enzymes and accounts 60-65% of the global market [2].

Proteases stand among one of the enzymes having high value commercial value [3]. Proteolytic enzymes in microorganisms are located within the cell, cell wall or excreted into the media [4]. Protease remains active in pH ranging from 3.5 to 9.5 and exhibits maximum proteolytic activity at pH 4.0 to 7.8. It is inhibited by diisopropyl flourophosphate but not by EDTA.

The proteolytic activity of microorganisms varies according to the bacterial species and environmental conditions [5]. Environmental conditions play a vital role in the production of extracellular proteolytic enzymes and could play an important role in the induction or repression of enzymes by specific compounds ([6].

Proteases of several catalytic types usually found in prokaryotic cells, including serine proteases, cysteine proteases and metalloproteases [7]. Bacterial proteases are mainly involved in the breakdown of peptide bonds which eases the need of consuming nutrients by microorganisms.

Proteases are involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino acid residues. Some of them can detach the terminal amino acids from the protein chain (exopeptidases, such as amino peptidases, carboxypeptidase A) and others attack internal peptide bonds of proteins (endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, elastase). Peptidases can either break specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (unlimited proteolysis).

Proteases are degradative enzymes and catalyze total hydrolysis of proteins [8]. The ability to synthesize serine and metalloproteases seems to be widespread among bacteria of the genus *Bacillus*.

Proteases are required for nutritional purposes and utilize degraded protein in plant cell wall [9]. Proteases able to hydrolyze almost all proteins as long as they are not components of living cells. Normal living cells are protected against lysis by the inhibitor mechanisms.

Proteases are highly necessary for living organisms and available in diverse sources such as plants, animals and microorganisms. Plant proteases are papain, bromelain and keratinases. Animal proteases are trypsin, chymotrypsin, pepsin and rennin. Microbial proteases produced by various bacteria and fungi are preferred than from plants and animal sources. Wide variety of fungi and bacteria produce proteases and in this study, we selected *Bacillus subtilis* and *Pseudomonas aeruginosa* for screening protease production.

Bacillus subtilis is a Gram-positive, catalase-positive bacterium commonly found in soil. A member of the genus Bacillus, *Bacillus subtilis* has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. *Bacillus subtilis* secrete major types of proteases, a subtilisin or alkaline protease and metalloprotease or neutral protease.

Pseudomonas aeruginosa is an opportunistic pathogen and can cause fatal infections in compromised hosts. The virulence is related to the secretion of several extracellular proteins. *Pseudomonas aeruginosa* secretes two proteases such as alkaline protease and elastase. *Pseudomonas aeruginosa* is a free-living bacterium, commonly found in soil and water. Hence the objective of this study is to isolate and detect the extracellular protease from *Bacillus subtilis* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Collection of samples

Samples were collected from various sources such as contaminated drinking water, spoiled vegetables, spoiled foods, soil and clinical samples such as pus, wound swab and urine sample.

Processing of sample

All the clinical samples were processed immediately. 1.0g of the environmental samples were weighed and homogenized with 9 mL of sterile distilled water to make a dilution of 10^{-1}. Serial dilutions were performed up to 10^{-8} 1mL of the serially diluted liquid sample was subjected to pour plate technique in appropriate media. It is incubated at 37˚C for 18-24 hours. The isolated colonies were picked up and sub-cultured on nutrient agar plates for isolation of pure cultures.

Isolation of micro-organisms from samples

The processed samples were inoculated in appropriate media and were incubated for 18-24 hours at 37˚C. After incubation, colonies were subjected to staining and other preliminary tests for identification.

Identification of isolates

After incubation, colonies from appropriate media were selected and subjected to Gram staining, motility, oxidase, catalase and other conventional bio-chemical tests. The results were compared with standard strains of *Bacillus subtilis,* and *Pseudomonas aeruginosa.*

Protease production

Identified isolates were then screened for the production of protease using skim milk agar, skim milk agar with tannic acid, gelatin agar, and in BHI agar with amidoblack.

RESULTS

Isolation and Characterization of Bacterial Isolates

Two different isolates were obtained from the samples processed. The results of various staining, motility, biochemical and physiological tests carried out on the known standards.

Reproducibility

Triplicates were prepared for each dilution and the mean counts of 3 plates were determined. The standard error of the mean was <10%. Each experiment was performed on 3 separate occasions.

Protease activity by Gelatin tube test

Gelatin tubes were inoculated and incubated at 25°C for a couple days, up to a week. The change of the medium form semisolid to liquid state was considered as positive for gelatinase activity.

Proteases activity by Gelatin agar

Gelatin agar plates were observed for clear zones and by using saturated ammonium sulphate solution or with mercuric chloride solution.

Proteases activity by Skim Milk agar

Skim Milk agar plates were observed for clear zones around the colonies and the isolates were selected for characterization and expressed as diameter of clear zone in mm.

Detection of microbial proteases by skim milk agar with tannic acid

After incubation the plates were observed for clear zones around the colonies. 10% tannic acid was flooded on the milk agar plate. TCA is sharp, distinct, clear and prominent zone of enzyme hydrolysis. Tannic acid sharply increases the colour contrast of the plate because tannins are polyphenolic compounds which forms insoluble complexes with proteins and a very powerful protein binding agent. The use of tannic acid is increases the clarity of the zone of hydrolysis.

Detection of extracellular proteases by brain heart infusion agar using amidoblack

Bacillus subtilis and *Pseudomonas aeruginosa* were screened for extracellular protease production by inoculation onto brain heart infusion yeast supplemented with gelatin. Protease activity was detected within 24 hours. Clear halos increased until the fifth day and remained constant upto ten days. All microorganisms hydrolyzed gelatin preferentially incorporated brain heart infusion agar.

Gelatin, extracellular protease detection was done after staining with 0.1% amidoblack in methanol-acetic acid-water 30:10:60 (v/v/v) for 1 hour at 28 ºC.

Enzyme activity was detected as clear areas, indicating the hydrolysis of the substrates. *Bacillus subtilis* zone of proteolysis was more than 2 mm from the margin of the colony. *Pseudomonas aeruginosa*, zone of proteolysis was more than 4 mm from the margin of the colony. Tables 1, 2 and 3 shows the extent of hydrolysis of the bacterial isolates on various media.

Table 1: Protease activity by Skim milk agar

S. No.	Organisms	Zones measured in diameter (mm)
1.	*Bacillus subtilis*	2 mm
2.	*Pseudomonas aeruginosa*	3 mm

Table 2: Influence of protein substrates (gelatin) and brain heart infusion agar in the production of extracellular protease

S. No.	Substrates – Media	Organisms	Zone measured in diameter (mm)
1.	Gelatin - BHI	*Bacillus subtilis*	2 mm
2.	Gelatin - BHI	*Pseudomonas aeruginosa*	4 mm

Table 3: Zones of proteolysis in brain heart infusion agar using amidoblack

S. No.	Substrates – Media	Organisms	Zone of Proteolysis
1.	Gelatin - BHI	*Bacillus subtilis*	+
2.	Gelatin - BHI	*Pseudomonas aeruginosa*	++

DISCUSSION

The number of enzymes secreted by various strains of *Bacillus subtilis* and *Pseudomonas aeruginosa* includes amylase, several proteases, and alkaline phosphates. Detection of extracellular proteases indicated that the optimum incubation period for protease production was within 24 hours.

Amidoblack staining technique is of high value for detecting extracellular proteases directly in the culture medium. The most predominant proteolytic activity was found in *Bacillus subtilis* and *Pseudomonas aeruginosa* hydrolyzed gelatin. *Bacillus subtilis* secretes metalloproteaes and subtilisin whereas *Pseudomonas aeruginosa* secretes two metallopeptidases and elastase and alkaline protease. Tannic acid method sharply increases the colour contrast of the plate because tannin are polyphenolic compounds which forms insoluble complexes with proteins and very powerful protein binding agent. The use of tannic acid increases the clarity of the zone of proteolysis activity.

CONCLUSION

Proteases are highly necessary for living organisms and the diversity of sources such as plants, animals and microorganisms. Proteases are degradative enzymes and catalyze the total hydrolysis of proteins. Proteases of several catalytic types usually found in prokaryotic cells, including serine proteases, cysteine proteases and metalloproteases.

The major uses of free protease occur in dry cleaning, detergents, meat process in, cheese making, silver recovery from photographic film, and in medical treatments. Stable proteases should be produced in high yield at a low cost. Cost of the growth medium accounts for about 30%-40% of the production cost of the industrial enzymes.

Tannic acid method is increases the clarity of the zone of hydrolysis thus making the process, easy, rapid and effective for the screening of large number of microorganism producing proteases. The use of gelatin in the culture medium provided us a qualitative assay which is simple, inexpensive technique for assessing the presence of proteolytic activity of the given colony. It cannot be used to quantitative degradation.

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