IJESRT

INTERNATIONAL JOURNAL OF ENGINEERING SCIENCES & RESEARCH TECHNOLOGY

Isolation And Characterization Of Proteolytic Bacteria And Its Protease

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Abstract

Soil samples from different habitats like river, garden, garbage dumping areas were collected. These samples were screened for the presence of proteolytic bacteria. The isolates were selected on the skim milk agar plates. The isolates forming larger zones were selected because of casein hydrolysis. The isolates were further screened for pure colonies. The bacteria found was rod shaped, gram positive, catalase positive, acid producing. The enzyme gave positive results for gelatin hydrolysis. The enzyme was active at 40°C to 50°C temperature and showed optimum activity at pH 5.

Source of Sample: Jalandhar, Punjab

Introduction

A protease is an enzyme that conducts proteolysis through the hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming protein. Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins.[1] 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*. Protease is responsible for digesting proteins in our food, which is probably one of the most difficult substances to metabolize. Because of this, protease is considered as one of the most important enzymes that we have.[2] If the digestive process is incomplete, undigested protein can wind up in our circulatory system, as well as in other parts of our body. Proteases belong to the class of enzymes known as hydrolases, which catalyse the reaction of hydrolysis of various bonds with the participation of a water molecule. 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; the others attack internal peptide bonds of a protein. Proteases, also known as proteinases or proteolytic enzymes, are a large group of enzymes. Proteases are divided into four major groups according to the character of their catalytic active site and conditions of action: serine proteinases, cysteine (thiol) proteinases, aspartic proteinases. Attachment of a protease to a certain group depends on the structure of catalytic site and the amino acid essential for its activity.[1]

Proteases occur naturally in all organisms. These enzymes are involved in many of the physiological reactions from simple digestion of food proteins to highly regulated cascades. Proteases can either break specific peptide bonds, depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids . The activity can be a destructive change, abolishing a protein's function or digesting it to its principal components; it can be an activation of a function, or it can be a signal in a signaling pathway. [3]

Proteolytic enzymes are very important in digestion as they breakdown the peptide bonds in the protein foods to liberate the amino acids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is notable based on several clinical studies indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation. Protease is 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 mechanism .[4]

Material and Methods

Isolation of the microorganism from the soil sample:

The soil from different habitats like river, garden and garbage dumping area was taken and serial dilution of the soil samples with 1 g soil sample and 9 ml water were made up to 5 dilutions. Then the dilutions were spread on the autoclaved skim milk agar plates

(CDH, 23 g/ l). The plates were incubated for 24 hours at 37º C for the growth. [5]

Isolation of Pure Colony:

The isolates forming the clear zones on the skim milk agar plates due to hydrolysis of the casein, were selected and transferred to the casein broth. This broth was put in the incubator shaker for 20 minutes at 37 degree Celsius. Then dilutions were made with 100µl broth and 1 ml water and 3 dilutions were made. These dilutions were spread on the autoclaved skim milk agar plates (CDH, 23 g/ l). These plates were incubated at 37º C for 24 hours for the growth. [5]

Further screening of the pure colony:

The isolates forming the clear zones were selected and were again streaked on the autoclaved skim milk agar plates (CDH, 23 g/ l). These plates were again incubated at 37 º C for 24 hours for the growth. [5]

Preservation of the Colony:

Nutrient Agar (25 g/l) slants of the pure colony were made for its preservation.

Physical Characterization:

Gram staining, Negative staining, Spore Staining, Acid fast staining, Catalase Activity, Motility test, Starch Hydrolysis Test, Carbohydrate Fermentation Test, Litmus Test, Citrate Hydrolysis Test were done. [8][9]

Enzyme Production:

The pure colony inoculum was transferred to the casein broth (10 g /l) and were put in the incubator shaker for 24 hours at 37º C. Then, centrifugation was done on 10,000 rpm for 10 minutes. [6]

Protein Estimation:

Lowry method was done for the protein estimation.

Enzyme Assay:

Agar well plate diffusion method :

The autoclaved skim milk (0.5 g/50ml skim milk powder, agar) well diffused agar plates were taken and the supernatant of different concentrations as 5µl, 10 µl, 15 µl, 20 µl were diffused in the wells. The plates were incubated at 37º C for 24 hours. [6]

Enzyme Activity:

Five test tubes were taken and the extract was added to each test tube making volume upto 3 ml. Then, the skim milk powder (0.5g/ml) was added to each test tube and put in incubator shaker for 20 minutes. Then, after 20 minutes, the TCA (trischloroacetic acid) was added to each test tube and put at 4º C for 60 minutes. Then, the solution was filtered and readings were taken by the Lowry method. [6]

Characterization of Enzyme:

- **Gelatin Hydrolysis Test:**

The well diffused gelatin agar plates were prepared and the supernatant was added to the wells and the plates were incubated at 37º C for 24 hours.

- **Temperature optimization:**

The enzyme was incubated at different temperatures as 20 º C, 30 º C, 40 º C, 50 º C, 60 º C for 30 minutes. Then, 3 test tubes with 0 µl, 200 µl and 400 µl enzyme, each for the different temperatures, were taken and the casein buffer (1ml) was added to each of them (0.5 g casein in 50 ml phosphate buffer, pH 7). Then, the test tubes were incubated for 20 minutes at the respective different temperatures. Then, 10% TCA (5ml) was added to each test tube and incubated at 4°C for 40 minutes. After that, the precipitates were filtered and the filtered 400 µl filterate was taken in each respective test tubes. Then, the Lowry method was done on the filtered enzyme with 4 ml alkaline $CuSO_4$ and 400 µl Folin-Ciocalteau reagent. The absorbance was taken at 660 nm. [7]

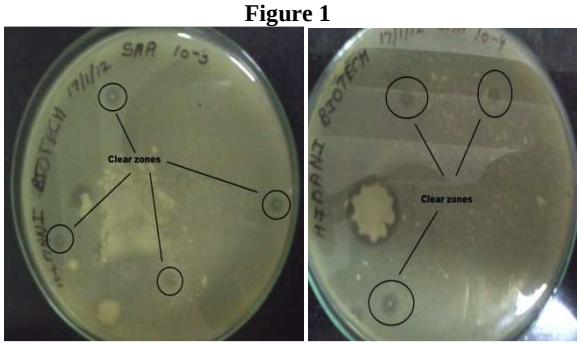
- **pH optimization**

The enzyme was added to the casein buffer (0.5 g casein in 50 ml buffer) made with different pH: Acetate buffer: pH 5, Phosphate buffer: pH 8, Glycine – NaOH buffer : pH 10.Then, the enzyme with buffer was taken in 3 different test tubes,0 µl,200 µl, 400 µl for each pH value and incubatedat 40° C temperature for 20 minutes. After 20 minutes, 10% TCA (5ml) was added to each test tube and incubated for 40 minutes at 4° C temperature. Then, the precipitates were filtered out and 0 µl, 400 µl an 400 µl filterate was taken in test tubes for each respective pH. Then, the lowry method was done on the filtered enzyme with 4 ml alkaline $CuSO_4$ and 400 µl Folin- Ciocalteau reagent. The absorbance was taken at 660 nm. [7]

Result

Isolation of the microorganism from the soil sample:

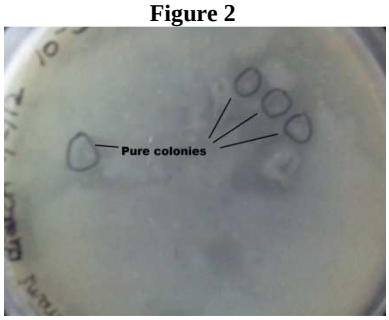
Figure 1



Microorganism presence was observed by the presence of clear zones of casein hydrolysis.

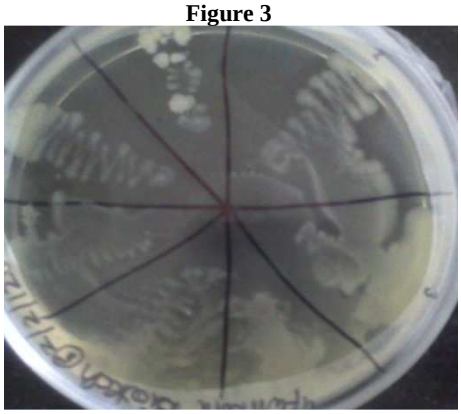
Isolation of Pure Colony:

Figure 2



Further screening of the pure colony:

Figure 3

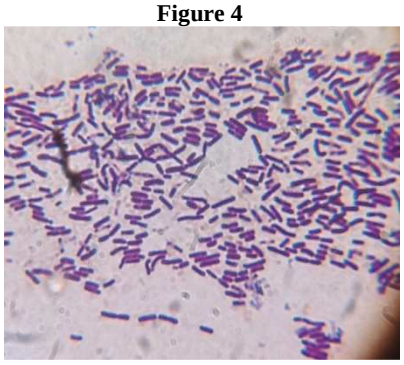


The pure colony was observed showing hydrolysis of casein through clear zones.

Physical Characterization:

- **Gram staining:**

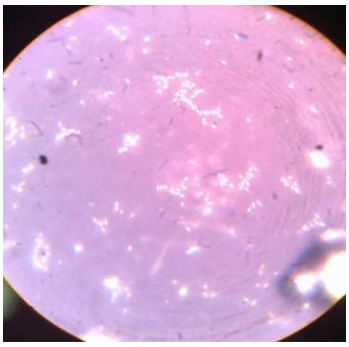
Figure 4



The bacteria having rod shape was observed and some were also present in the form of chains. The test showed bacteria to be gram positive.

- **Negative staining:**

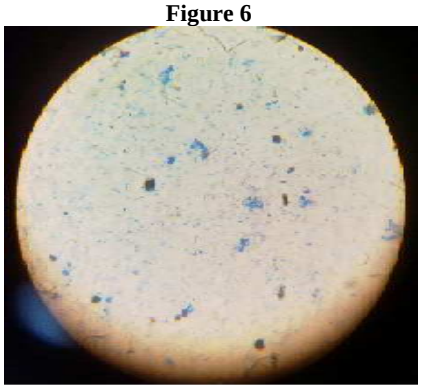
Figure 5



The bacteria with rod shape was visible on the black background.

- **Acid fast staining:**

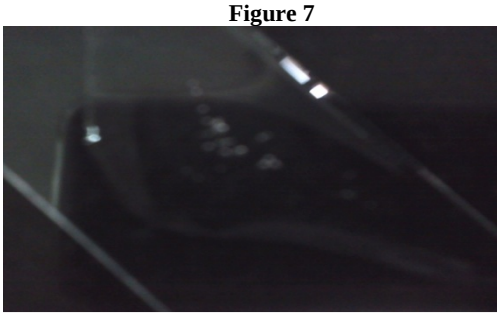
Figure 6



The blue stained *Bacilli* were observed, showing that the bacteria is acid fast negative.

- **Catalase Activity:**

Figure 7



The bubble formation indicated that the bacteria is catalase positive.

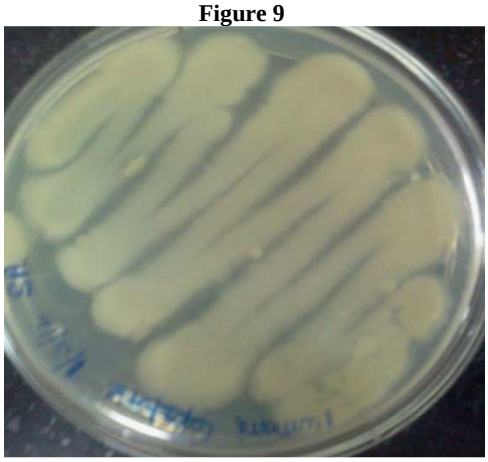
- **Motility test:**

Figure 8

Bacillus gave negative results.

- **Starch Hydrolysis Test:**

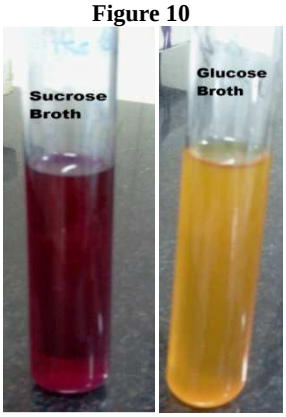
Figure 9



There were no clear zones indicating no starch hydrolysis.

- **Carbohydrate Fermentation Test:**

Figure 10



In glucose broth, there was color change from red to yellow and no bubble formation was observed, indicating that no carbon dioxide formation and acid formation.

In sucrose broth, there was no color change observed.

- **Litmus Test:**

Figure 11

There was discolouration of the litmus at the bottom of the tube, indicating reduction of litmus. Rennet curd was formed as soft, insoluble, semi solid clot was formed that flowed slowly when tube was tilted.Pink colour of the solution was formed indicating the acidic reaction by the bacteria.

- **Citrate Test:**

There was no change in the color indicating no citrate hydrolysis.

Enzyme Production:

The supernatant obtained was taken as the crude enzyme.

Enzyme Estimation:

- **For BSA Sample:**

Table 1

Sno.	BSA samples (ml)	Water (ml)	Absorbance (660 nm)
1.	0.1	0.9	0.116
2.	0.2	0.8	0.263
3.	0.4	0.6	0.478
4.	0.6	0.4	0.653
5.	0.8	0.2	0.841
6.	1	0	0.927

- **For Enzyme Sample:**

Table 2

Sno	. Sample (ml)	Water (ml)	Absorbance (660 nm)
1.	0.1	0.9	0.710
2.	0.2	0.8	1.158
3.	0.4	0.6	1.838
4.	0.6	0.4	2.234
5.	0.8	0.2	2.568
6.	1	0	2.882

- **These are the result readings when 3 dilutions of 0.1 ml and 0.2 ml sample were taken.**

Table 3

S no.	Sample (ml)	Water (ml)	Absorbance (660 nm)
1.	0.1	0.9	0.760
2.	0.1	0.9	0.76
3.	0.1	0.9	0.587
4.	0.2	0.8	1.005
5.	0.2	0.8	1.070
6.	0.2	0.8	1.070

Enzyme Assay:

The clear zone of different diameters were observed indicating the enzyme action on casein, provided as the substrate.

Table 4

S no.	Sample (µl)	Diameter (cm)
1.	5	1.6
2.	10	1.8
3.	15	2
4.	20	2.3

Enzyme Activity:

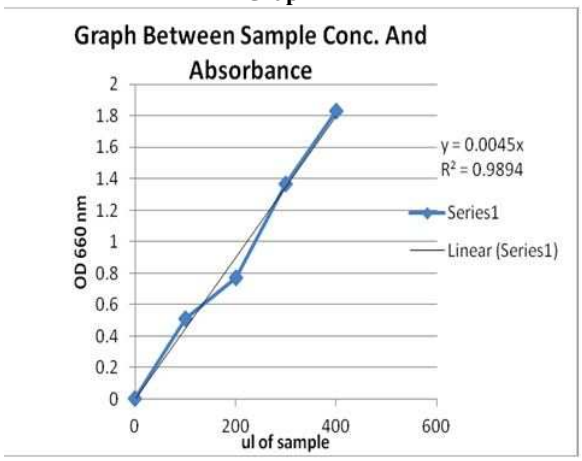
The readings were taken by the Lowry method on 660 nm .

Table 5

Extract (µl)	Absorbance (660nm)
0	0
100	0.51
200	0.77
300	1.37
400	1.83

Graph:

Graph 1

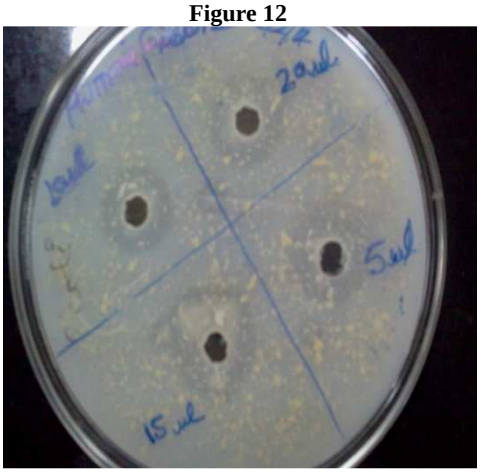


Enzyme activity is the µl of supernatant required to gain O. D. of 0.1 in 60 minutes with Lowry method. According to the graph, y = 0.004 x. So, for 0.1 y , x would be 25. 25 µl of the enzyme extract is needed for 20 minutes time interval.So, for 60 minutes, the extract needed would be its one- third, i.e. 8.33 µl.

Characterization of Enzyme:

- **Gelatin Hydrolysis Test**:

Figure 12



The clear zones were observed indicating the enzyme action on the gelatin.

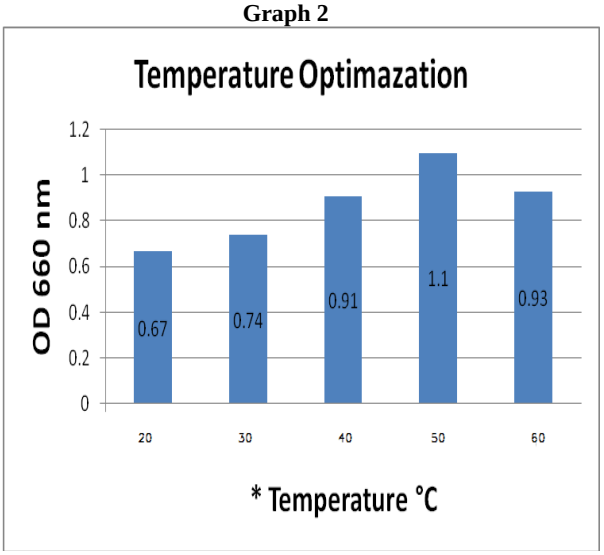
Temperature optimization:

Table 6

S.No.	Temperature (°C)	Sample (µl)	Absorbance (660 nm)
	20		
1.		200	0.31
2.		400	0.67
	30		
1.		200	0.55
2.		400	0.74
	40		

1.		200	0.72
2.		400	0.91
	50		
1.		200	0.93
2.		400	1.10
	60		
1.		200	0.73
2.		400	0.93

Graph 2

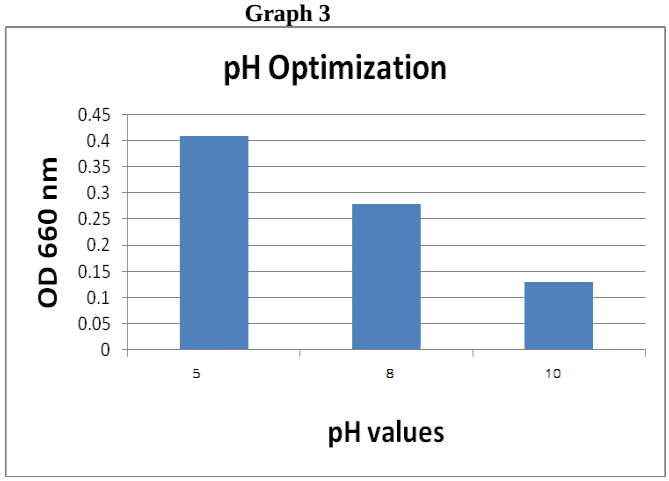


pH Optimization:

Table 7

S.NO.	pH	Sample (µl)	Absorbance (660 nm)
	5		
1.		200	0.23
2.		400	0.41
	8		
1.		200	0.12
2.		400	0.28
	10		
1.		200	0.01
2.		400	0.13

Graph 3



Conclusion

The bacteria isolated from the soil was *Bacillus* analyzed by the gram staining test. The bacteria gave positive test for spore staining, carbohydrate fermentation with glucose, gelatin hydrolysis, catalase activity test. The bacteria gave negative tests for motility, sucrose fermentation, starch hydrolysis, citrate hydrolysis. The crude enzyme was obtained from the supernatant and the enzyme presence was indicated by the casein hydrolysis on skim milk agar plates. The enzyme was optimized for different temperatures and pH, giving the results as the enzyme is active at temperature 40° C to 50°C and most active at 50°C temperature and at 5 pH range. The enzyme also gave positive results for the gelatin hydrolysis. The best substrate for the enzyme was casein. This enzyme can be exploited commercially.

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