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Isolation, Production, Purification and Applications of Proteases from *Pseudomonas aeruginosa*

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ABSTRACT

Proteases are the enzymes which have a host of applications. They are used in the industrial, pharmaceutical and food industry etc. Proteases contribute to two third of the global requirements. The bacterial sources form an interesting sources for the isolation of the protease enzymes. The production of this crucial enzyme has hence gained importance. In the present experiment the isolation, purification, production, media optimization of the protease enzyme was performed from the *Pseudomonas aeruginosa*. The optimum activity was found to be at pH 8 and temperature 30 °C. The effective Nitrogen source and Carbon source are found to be Peptone and sucrose with the highest specific activity of 41 IU/ml and 13 IU/ml respectively.

Keywords: Protease, Industrial uses, *Pseudomonas aeruginosa*, Casein media, Soil bacteria

Introduction

Proteases refer to a group of enzymes which hydrolyze proteins and are hence called as proteases (or) proteolytic enzymes [1]. Due to their wide applications in the pharmaceutical, leather, food and agricultural industries, they are regarded as the most important groups of enzymes [2]. Among these host of applications the proteases are widely used in the process of bioremediation [3,4]. The proteases are seen in all the living organisms like animals, plants and microorganisms with majority being the fungi.

The bacteria *Pseudomonas aeruginosa* was the organism of interest for several reasons from treatment of infection to decomposition of natural materials to bioremediation [5-8]. *Pseudomonas* has an advantage over other organisms as it forms biofilms which helps the organism to survive under different conditions [9]. Quorum sensing is the regulatory mechanism by which the

organism forms biofilms and produces many exo products [10,11]. During the infections by *pseudomonas*, the proteases are the enzymes which are suspected to play a very important role [12,13].

The objective of the present experiment is to screen the microorganisms *Pseudomonas* locally and to extract the enzyme protease from it.

Materials and Methods

1. Isolation of the microorganism:

The organism for the present study was taken from the previously isolated technique [14]. To this IMVIC test was performed to doubly confirm organism. The organism was inoculated in the Casein nutrient media and the colonies were observed. Later the qualitative plate assay method was done for further confirmation.

A. Qualitative Plate Assay:

Casein nutrient medium and gelatin nutrient medium are prepared and are poured into separate petri plates and allowed

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to solidify and kept for contamination check for 24 hrs. The culture is streaked onto both the plates. The plates were then incubated for 24-48 hrs. After incubation, the plates were flooded with HgCl_2 solution. The plates were transferred to refrigerator for proper reaction of indicator with the medium. The results were observed in the form of clear precipitation zones.

2. Isolation of the enzyme proteases:

A. Preparation of crude enzyme

The isolated *Pseudomonas* species was inoculated from slant into 50ml of Casein-Nutrient medium with vigorous shaking (150 rpm) at 37 °C for 24hrs. The culture was centrifuged at 10,000 rpm for 5 mins at 4 °C. The supernatant was collected and used as the enzyme solution.

B. Estimation of tyrosine by Anson method [15]

Protease activity was assayed by the modified method of Anson using casein as substrate. Take 1 ml of dialyzed enzyme (cultured filtrate), incubate at 37 °C for 5min, then add 1 ml of 2 % casein solution. Incubate the reaction mixture at the same temperature for 10 mins. Terminate the reaction mixture by adding 2 ml of 0.4 M TCA and incubate for another 20 min. Filter the reaction mixture through Watman filter paper No.1 into a clean test tube. Take 1 ml of the above solution and 5 ml of Na_2CO_3 (20 %) and 1 ml of diluted Folin ciacoteau (FC) reagent and incubate at 37 °C for another 30 min. Measure the intensity of blue colour which is developed at 660 nm in spectrophotometer. Prepare the blank by adding TCA at zero time in casein and then incubate for 10 min. The amount of amino acid released can be read from a standard graph drawn using tyrosine.

C: Purification of the Crude Enzyme

The purification of the crude enzyme was done by the following method. The bacterial cells were allowed to centrifugation at 10,000 rpm for 20 min at 35 °C. The supernatant formed is used for the purification.

a Ammonium sulfate precipitation

The supernatant of the volume 75 ml was precipitated with 70 % ammonium sulfate. The resultant was allowed to centrifugation at 10,000 xg for 45 min at 4°C and the precipitate is dissolved in 20 mm of Tris-HCl buffer. The enzyme is collected and the specific activity is determined.

b. Sephadex G-50 chromatography

The resultant enzyme from the above step was loaded on the Sephadex column. After the purification process, the enzyme is checked for the specific activity.

c. Cm-Sephadex chromatography

The enzyme obtained from the previous step was allowed to pass through cm - Sephadex, which is pre equilibrated with 100 mM citrate buffer at pH 6. The enzyme was extracted and the specific activity was analyzed.

3. Standardization and media optimization:

A. Effect of pH on production of the protease

The pH effect on the production of the proteases was performed by taking different pH gradients i.e. 2, 4, 6, 8 and 10 at temp 30 °C. The optimum pH was determined by analyzing the specific activity after 36 hrs.

B. Effect of temperature

The temperature effect on the production of the enzyme was determined by taking the following temperature concentration 10, 20, 30, 40 and 50 at pH 7.4. The specific activity of the enzyme was studied after 36 hrs.

C. Effect of N_2 source

The effect of N_2 source was studied by taking yeast extract, peptone and BSA as the Nitrogen sources and were incubated at pH 7.4 and temperature 30 °C. The specific activity was determined after 36 hrs.

D. Effect of carbon source

The effect of carbon source on the protease production was done by taking glucose, starch, cellulose and sucrose as the carbon sources. The specific activity was determined after 36 hrs.

4. Applications:

A. Digestion of natural protein [16]

20ml of crude enzyme was taken and incubated with coagulated egg white along with 20 mM Tris-HCl and pH is set to 7.5 at 35 °C. The conditions of substrate were monitored at different time incubation times 12, 14, 24, 48 and 72 hrs.

B. Removal of blood stain [16]

A clean piece of cloth was soaked in blood and allowed to dry. Then the cloth was soaked in 2 % formaldehyde for 35 min and the excess of formaldehyde was removed by washing with water. The cloth was cut to equal sizes and they were incubated with a crude enzyme solution at 40-45 °C for different time intervals at 12, 14 and 24 hrs respectively.

C. Dehairing of skin [16]

Sheep skin was cut to 5 cm pieces and incubated with the crude protease solution in 50 mM Tris-HCl (pH 7.5) at 45 °C. The skin was checked for removal of hair at different incubation times 1, 2 and 3 hrs respectively.

Results and Discussions

1. Isolation of the organism

The organism isolated was then added to the casein media and the colonies were observed. The organism was subjected to IMVIC test (Table1 and Figure 1).

The casein media was used with the following composition Casein 1.0 g/L, Peptone 1.0 g/L, Beef extract-1.0 g/L, KCl- 1.0 g/L and NaCl 0.5 g/L at pH 7.7.

Based on the qualitative assay, *Pseudomonas* was found to be showing clear and distinct zone of clearance in casein medium when compared to gelatin medium. Further work was done with Casein medium. The production of protease by *Pseudomonas* species was further evaluated by Quantitative assay.

Table 1: IMViC Results

S.No	Name of the test	Result
1.	Indole	-ve
2.	Methyl Red	-ve
3.	Voges-Proskauer	-ve
4.	Citrate	+ ve

Figure 1: *Pseudomonas* Culture

2. Purification:

The crude enzyme was purified by the $(\text{NH}_4)_2$ precipitate, Sephadex G-50, chromatography, CM-sephadex. The purification fold and the yield of the protein is found to be as given below (Table 2).

Table 2: Purification

S.No	Step	Total activity	Specific activity IU/ml	Purification (fold)	Yield %
1	Crude supernatant enzyme	1953	5	1	100%
2	Ammonium sulfate	1782	20	2.8	69 %
3	Sephadex	983	60	10	50
4	CM – Sephadex	548	248	38	29

3. Standardization and media optimization:

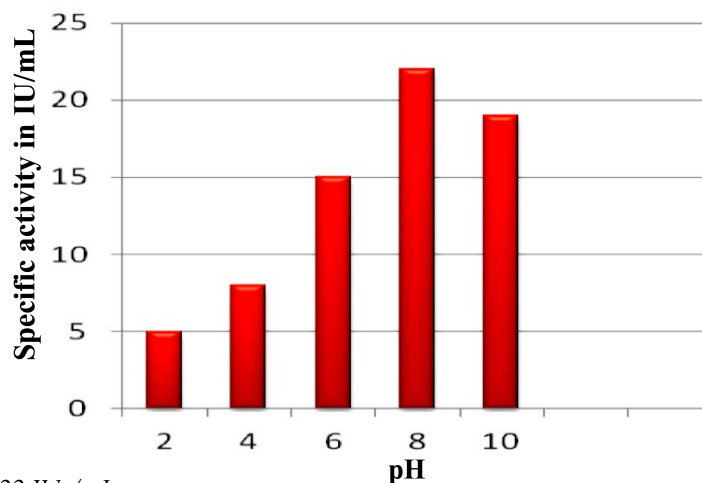
A. Effect of pH

The effect of pH on the specific activity was noted (Table 3 and Graph 1)

Table 3: Effect of pH

S.No	pH values	Specific activity IU/ml
1	2	5
2	4	8
3	6	15
4	8	22
5	10	19

Graph 1: Effect of pH



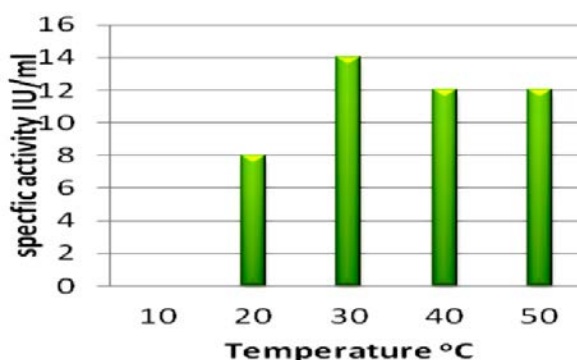
The optimum pH is found to be 8 with the specific activity of 22 IU /mL.

B. Effect of temperature

The temperature effect on the production of the enzyme was studied.

Table 4: Effect of Temperature

S.No	Temperature	Specific activity IU/mL
1	10	-
2	20	8
3	30	14
4	40	12
5	50	12

Graph 2: Effect of Temperature

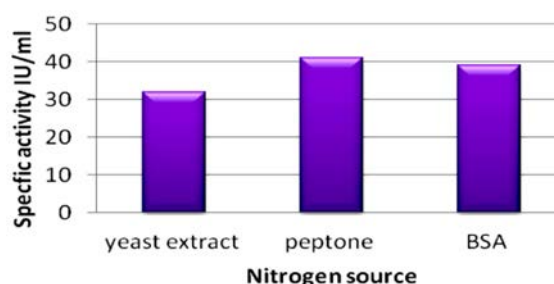
The optimum temperature is found to be 30 °C with the specific activity of 14 IU /mL

C. Effect of Nitrogen source:

The effect of nitrogen source on the production of the protease enzyme was studied.

Table 5: Effect of Nitrogen Source

S.No	N ₂ Source yeast extract	Specific activity IU /ml
1	Yeast extract	32
2	Peptone	41
3	BSA	39

Graph 3: Effect of Nitrogen Source

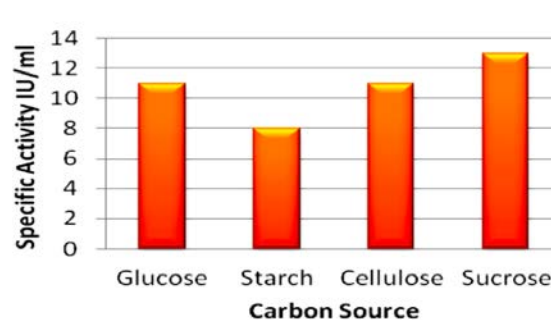
The nitrogen source which produced the highest specific activity of 41 IU /mL is Peptone.

D. Effect of Carbon source:

The effect of carbon source on the production of the protease enzyme was studied.

Table 6: Effect of Carbon Source

S.No.	Carbon source	Specific Activity IU/mL
1	Glucose	11
2	Starch	8
3	Cellulose	11
4	Sucrose	13

Graph 4: Effect of Carbon Source

The carbon source which produced the highest specific activity of 13 IU /mL is sucrose.

4 Applications:**Digestion of Natural Proteins:**

The protease enzyme was found to be successful in digestion of natural proteins and the digestion was found at 24 hours.

Destaining of the Blood:

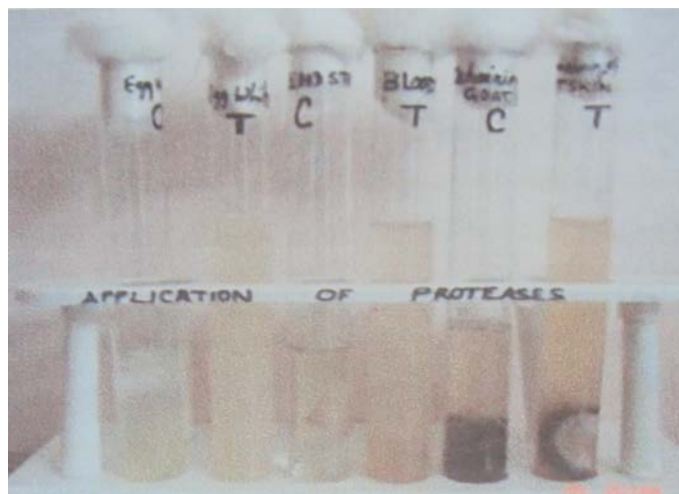
The protease showed efficiency in destaining the blood. The

destaining of the blood was seen at 12 hours.

Dehairing:

The Dehairing mechanism of the enzyme was found to be seen at 3 hours of time interval.

Figure 2: Application of Proteases



Conclusion

Proteases are the group of enzymes which are having a wide range of applications in the fields of pharmaceutical, food, leather and waste processing industries [17,18]. Thus, so important enzyme should be produced in large quantities. This was achieved with the bacterial organism, *Pseudomonas aeruginosa* and the media optimization was done.

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