Screening, Production and Partial Purification of Protease from *Pseudomonas Spp.* and their Potential Applications

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Abstract— The present study was carried out for the isolation of proteolytic bacteria, production, purification and designing the process parameters for optimum proteolytic enzyme activity as well as its potential application. Different soil samples taken from fish market, slaughter house, and poultry waste soil were processed according to the microbiological criteria for the isolation of proteolytic bacteria. All the isolates were screened for the protease ability and the isolate showing highest proteolysis was selected for further studies. The production of protease was carried out by fermentation by shake flask method at 37˚C. The supernatant of culture media were obtained after centrifugation, and was assayed for protease activity as well as total protein content. Partial purification of enzyme was done by ammonium sulphate precipitation followed by dialysis. Then the application of the enzyme was done by investigating the ability of the enzyme to solubilize blood clots, and de-staining blood stained white cotton cloth. Based on the biochemical studies the isolated organism was found to be *Pseudomonas spp*. The highest protease production was found after 24 hours of fermentation, while the optimum pH was 8.0, the optimum temperature of enzyme activity was 35˚C. The partially purified enzyme showed the ability to remove blood stain in the white cotton cloth, and the enzyme was also able to dissolve blood clots. Based upon the results of the study, this protease might find broad applications in detergents and pharmaceutical industries.

Index Terms— *Pseudomonas spp*., protease, partial purification, potential application.

1 Introduction

PROTEASES or proteolytic enzymes are degradative enzyme which catalyze the cleavage of peptide bonds in other proteins [18]. Chemically, proteases act through catalysis of proteins hydrolysis. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carboxyl group [15]. Advance in analytical techniques have demonstrated that protease conduct highly specific and selective modification of protein such as activation of zymogens form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, as well as processing and transport of secretory protein across the membrane [18].

Protease Enzymes can be found in animals, plants, bacteria, archaea and viruses. However, Protease from microbial sources are preferred over the enzymes from plant or animal sources for not only since they possess all most of the characteristics desired for biotechnological applications[14], but also Microorganisms excrete a wide variety of proteolytic enzymes, which are also found in mammalian systems[13]. Additionally, the microorganisms represent an alternative source of enzymes because they can be cultured in large quantities in a short time by fermentation and owing to their biochemical diversity and susceptibility to gene manipulation. Industries are looking for new microbial strains in order to produce different enzymes to fulfil the current enzyme requirements [13]. Microbial proteolytic enzymes from different fungi and bacteria are available. Most fungal proteases will tolerate and act effectively over a wide pH range (about 4 to 8), while with a few exceptions, bacterial proteases generally work best over a narrow range of about pH 7 to 8 [24].

Commercially proteases have been employed in a wide range of applications for many years with satisfactory results. Proteases represent one of the third largest groups of industrial enzymes and find applications in detergents, leather industry, and pharmaceutical industry and bioremediation processes [2], [9]. The vast variety, in contrast to the specificity of their action, has attracted to exploit their physiological and biotechnological applications [18].

The production of any biotechnological product begins with the screening of right microorganisms. The isolation and identification of promising bacterial strains, characterization of enzymes and optimization of products plays an integral part in this process. Protease producing bacteria were widely isolated from soil with high protein content [16]. Wastes from dairy, meat and poultry processing industries act as sources for large amount of protein rich material that can be biochemically transformed into recoverable products. Due to their high content of protein material, they may serve as excellent source for isolating proteolytic microorganisms [23].

Originally, commercial fungal and bacterial enzymes were produced by surface culture methods. Within the past few years, however, submerged culture methods have come into extensive use. Submerged liquid fermentation utilizes free

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flowing liquid substrates, and the bioactive compounds are secreted into the fermentation broth form which it will be purified. It was preferred due to the fact that purification of products is easier. The submerged method was originally developed and first extensively employed for production of penicillin and other antibiotics. In the laboratory, submerged cultures are grown in shake flasks or in aerated tubes or flasks. Commercially, deep tanks are employed which have provision for introduction of sterile air and for vigorous agitation. Descriptions of processing methods for preparing industrial microbial enzymes have been published [21], [10], [7], [17], [20].

Currently, a large proportion of commercially available proteases are derived from microorganisms notably bacteria and fungi. Therefore the selection of the right organisms plays a key role in obtaining high yield of desirable enzymes [1]. Fungal protease has been used for centuries in the Orient for the production of soy sauce, tamari sauce, and miso, a breakfast food [10]. At the present time, the majority of the proteases commercially available are derived from *Bacillus* species [19], even though fungi are being increasingly employed [5].

Proteases produced from *Bacillus subtilis* are present in large variety of commercially available enzymes differing in biological sources, activity, purity, physical form and characteristics such as pH and temperaturaure optima [4]. Based on the fact that the members of the genus *Pseudomonas* also demonstrate a great deal of metabolic diversity, and consequently are able to colonize a wide range of niches [11]. we set the principal objectives of this study as to screen other proteolytic microbial strain than *Bacillus*(mostly used) where emphasis was on *Pseudomonas spp.* as alternative strain, and to produce, partially purify the protease enzymes and to study their potential applications mostly as detergent and pharmaceutical applications such as anti-coagulant agent.

2. MATERIALS AND METHODS

2.1 Screening of Soil Sample for Protease Producing Organisms

Soil sample were aseptically collected from different protein rich areas, due to the waste debris they contain. Soil sample were collected from fish market, slaughter house, and poultry waste soil. Bacteria were isolated from 1g of each soil sample by serial dilution and agar plate method using nutrient agar medium.

The isolated bacteria were screened for proteolytic activity by streaking them on the skim milk agar medium and casein nutrient agar. Comparison between proteolytic potential of the isolates was made based on the zone of casein hydrolysis and individual colony diameter. The zone of hydrolysis was measured in mm. The isolate demonstrating the highest zone of casein hydrolysis was maintained on nutrient agar slants at 4˚C until use.

2.2 Identification of the isolate

The identification of the isolate was made based on both macroscopic and microscopic morphological and pigmentation characteristics of the colony. This involved culturing the isolate on nutrient agar plates for studying the appearance of colonies following which gram's staining and motility test were performed.

2.3 Protease Production by Shake Flask Method

A loopful culture was inoculated into the 20 ml nutrient broth and incubated at 38˚C in orbital shaker incubator at 140 rpm for 18 hours. Aseptically the 16 ml of the inoculum were introduced into 400ml of production medium (table 5) and incubated for 48-72 hours at 38˚C and shaking at 140 rpm.

TABLE 1
COMPOSITION OF PRODUCTION MEDIUM

COMPOSITION	QUANTITY(g/ml)
Casein	0.5
Starch	1
KH_2PO_4	0.42
K_2HPO_4	0.375
$(NH_4)_2SO_4$	0.244
NaCl	0.2
$MgSO_4.7H_2O$	0.005
$FeSO_4.7H_2O$	0.001

2.4 Protease Activity Assay

Protease activity was assayed by the modified method of Anson using casein as substrate the reaction mixture containing 0.5 ml of 1% (w/v) casein in 1.0 M phosphate buffer pH 8.0, and 0.1 ml of suitably diluted enzyme, was incubated at 37˚C reaction was terminated by the addition of 0.5 ml 10%(w/v) trichloroacetic acid and centrifuged at 10000rpm. To 1ml of supernatant, 2.5 ml of 0.5M Na_2CO_3 solution and 0.25ml of 3-fold-diluted Folin Ciocalteau reagent were added and mixed thoroughly. The colour developed after 30 min of Incubation (at Room Temperature in Dark) were measured at 660 nm.

One unit of protease activity was defined as the amount of enzyme required to liberate 1µg of tyrosine at 37˚C. The specific activity is expressed in units of enzyme of enzyme activity/mg of protein.

Enzyme activity = µg tyrosine/30 (1)

2.5. Optimization of the Enzyme Activity

Effect of incubation period

To investigate the optimum period for maximum enzyme yield, the fermentation experiments were carried up to 72 hours in triplicate. The samples were collected after 12, 24, 48, and 72 hours of fermentation, and assayed for enzyme activity.

Effect of temperature on Enzyme activity

5 test tubes containing the reaction mixture containing 0.5 ml of 1% (w/v) casein in 1.0 M phosphate buffer pH 8.0, and 0.1 ml of suitably diluted enzyme, were incubated at different temperatures i.e. 25, 30, 35, 40, 45˚C reaction was terminated by the addition of 0.5 ml 10%(w/v) trichloroacetic acid and centrifuged at 10000rpm. To 1ml of supernatant, 2.5 ml of 0.5M Na_2CO_3 solution and 0.25ml of 3-fold-diluted Folin Ciocalteau reagent were added and mixed thoroughly. The colour developed after 30 min of Incubation (at Room Temperature In Dark) were measured at 660 nm.

Effect of pH on the enzyme activity

A number of assay tubes were taken and the only varying parameter is the pH of the buffers added to each tube. The activity was measured under different pH values and draw a graph of pH versus activity optimum pH can be found in this way.

1 ml of 1 M phosphate buffer of varying pH that is 5.0, 6.0, 7.0, 8.0 and 9.0 was added to all test tubes. 0.5 ml of 1% (w/v) casein solution, and 0.1 ml of enzyme sample were added to all test tubes. All the reaction mixtures were incubated at 37˚C reaction was terminated by the addition of 0.5 ml 10% (w/v) trichloroacetic acid in each test tube and centrifuged at 10000rpm. To 1ml of supernatant, 2.5 ml of 0.5M Na_2CO_3 solution and 0.25ml of 3-fold-diluted Folin Ciocalteau reagent were added and mixed thoroughly. The colour developed after 30 min of Incubation (at Room Temperature in Dark) were measured at 660 nm

2.6. Purification and Application of the Protease Enzyme

Partial purification of the enzyme was carried out by ammonium sulphate precipitation of the filtrate of fermentation broth. This was followed by dialysis to remove ammonium sulphate.

De-clotting of blood

2ml of blood was taken in test tubes and allowed to clot. 1ml of enzyme was then added to the clot and incubated the reaction mixture at 37˚C. The effect of enzyme concentration on declotting of blood was investigated by adding 1.5 ml of blood in 5 Eppendorf and allowed to clot. Thereafter enzyme in varying amounts i.e. 0.25, 0.5, 1.0, and 1.5 ml was added into four Eppendorf tubes where one was kept as control. A buffer solution was added to make the final volume of 1.5ml. Thereafter, the reaction mixtures was incubated at 37˚C. A stop watch was used to monitor and record the time taken for blood de-clotting.

De-staining of blood

A white cloth (2.5cm X 2.5cm) was stained with blood. 1ml of enzyme solution was added and incubated. The de-staining activity of the enzyme was monitored in different time intervals with a stop watch.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Bacteria

From the processed soil samples 3 morphologically different bacteria species were identified. By gram staining and other biochemical tests conducted, they were identified to be *Pseudomonas sp.* These bacteria were sub-cultured to skim milk medium for screening protease production.

TABLE 1
BIOCHEMICAL TEST RESULTS DONE TO IDENTIFY THE BACTERIAL SPECIES ISOLATE

S.No	Tests	Result
1	Gram staining	Negative
2	Motility test	Motile
3	Pigment production	-ve
4	Indole test	-ve
5	Citrate utilization test	+ve
6	Catalase test	+ve
7	Urease test	+ve
8	Gelatin hydrolysis	-ve
9	Starch hydrolysis	-ve
10	Glucose & lactose fermentation on TSI	-ve
11	H_2S production	-ve

3.2 Screening of Bacteria Isolates for Protease Production

The isolate was sub cultured on skim milk agar plates, bacteria were incubated for 24 hours at 38˚C. They were observed for zone of clearance. The selection of bacteria was based on the zone of clearance. The organism showed high zone of clearance diameter was selected.

3.3 Enzyme Activity Assay

The protease activity of the collected supernatant after 18, 24, 48, and 72 hours of fermentation was determined and the result are depicted in figure 1. The results reveals that the production is faster at beginning but the longer fermentation take the production slows

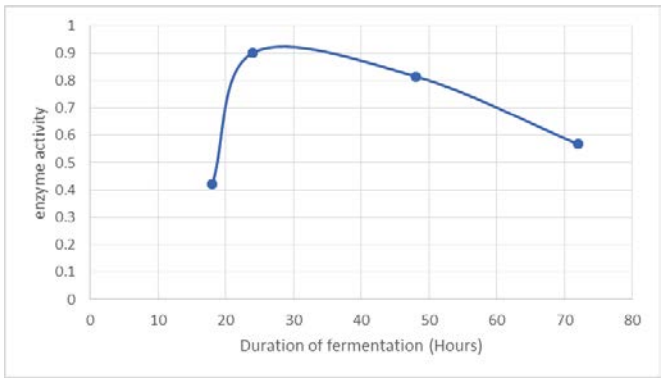

Fig. 1. Effect of duration of fermentation on Enzyme activity
The highest production of the enzyme was observed after 24 hours.

3.3. Optimization of the Enzyme Activity

Effect of pH on enzyme activity

The figure 2 shows the enzyme activity determined at different pH value. At lower pH values the enzyme activity is low. It increases with the increase of pH. The optimum pH was found to be 8

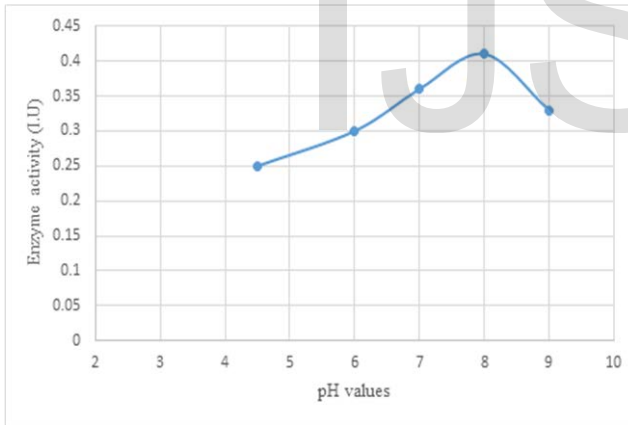

Fig. 2. Effect of pH on enzyme activity

3.4 Effect of temperature on enzyme activity

The table 9 and figure 3,shows the results of the assay on the effect of temperature on protease enzyme activity. The enzyme activity increases with the increase of temperature and decrease at temperature above the optimum temperature of 35˚C.

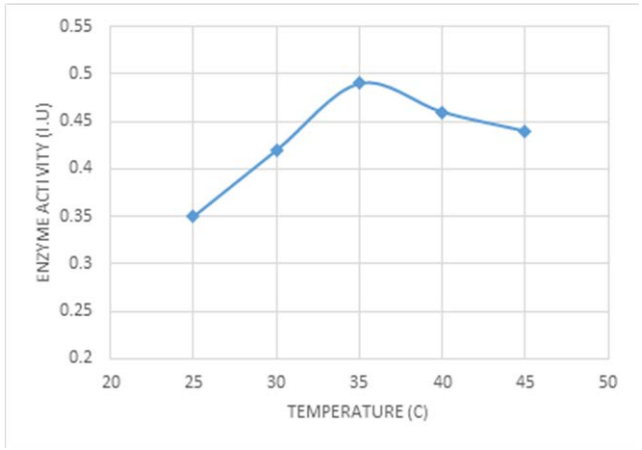

Fig. 3. Effect of temperature on Enzyme activity.

The optimum temperature was found to 35°C

3.5 Enzyme Purification

At each step of purification the total protein content and enzyme activity were determined. The table 10 depicts the results of these assays.

S.No	Purification step	Total activity (IU)	Protein Contents (µg/ml)
1	Culture supernatant	0.26	55
2	Ammonium Sulphate precipitation	0.83	74
3	Dialysis	1.26	82

3.6 Application of the Enzyme

Blood Declooting

As shown in figure 4(A and B) the insoluble form of blood clot was converted into soluble form. The blood clot was removed. The figure 5 shows results of the effect of enzyme concentration on declotting activity

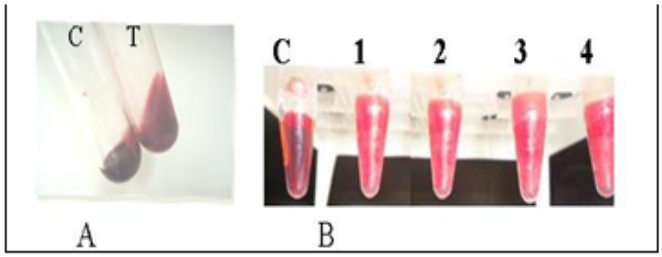

Figure 4: Blood de-clotting by Protease.

(A= Test reaction, B= assay of effect of enzyme concentration on declotting activity where C= control, T= Test)

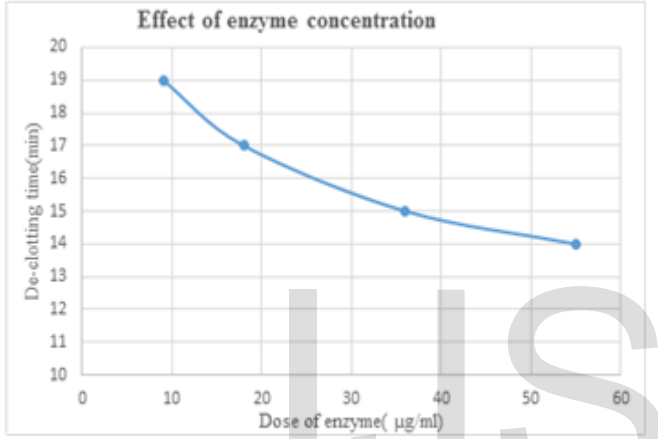

Figure 5: Effect of enzyme concentration on declotting activity

De-staining of blood

In the case of removing blood stain, as shown in Figure 6 the protease was able to remove blood stain very easily without addition of any detergent.

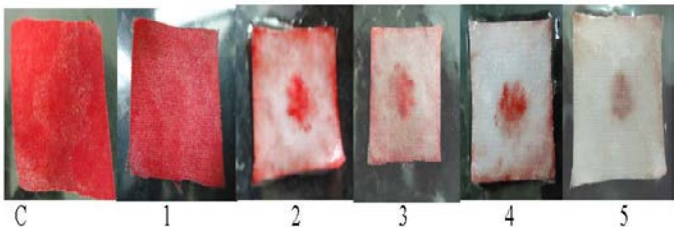

Figure 6: Removal of blood stain from white cotton cloth

The protease was added to the blood stained cloth and was incubated at 37C and monitored for different time. C= Control, 1=after 5min, 2=10 min, 3=15 min, 4=20min, 5=30 min

4. DISCUSSION

Our objective was to produce protease enzyme from *Pseudomonas spp*. Therefore we needed the organism, which produces more protease. To fulfill this we collected samples from fish market, slaughter house, and poultry waste soil, hoping that the possibility of getting protease producing bacteria enzyme will be more. As it is believed that due to their high content of protein material, they may serve as excellent source for isolating proteolytic microorganisms [23].

For isolation of the microorganisms serial dilution and pour plate method are best suited, because by using these techniques we can get minimum number of microorganisms population after each dilution. After isolation of *Pseudomonas spp*. it was sub cultured on skim milk agar to identify whether it was protease producing or not. A clear zone around the bacterial colonies indicated that, it produced protease and hydrolyzed the casein. The isolated bacteria was used in production of protease under fermentation.

Effect of fermentation period plays an important role in optimum production of any biotechnological product fermentation. Consequently, fermentation experiments were carried out up to 72 hours, and samples taken at different time interval were analyzed for enzyme activity. The maximum production (0.901 I.U) were observed after 24 hours of incubation, the same optimum period was observed during the study of alkaline protease production from *Bacillus spp*., under solid state fermentation and reported the optimum period of 24 hours [22].

Effect of pH and Temperature on enzyme activity towards casein were examined at various pH values at 37˚C. The enzyme were highly active between pH 7.0 and 8.0. The relative activities were 0.36 I.U and 0.41I.U respectively. And it is believed that, the high optimum pH is common characteristic of alkaline protease [8]. Therefore we believe that this enzyme is an alkaline protease. The highest protease activity 0.49 I.U was recorded at temperature of 35˚C. While it has reported that the protease had high fibrinolytic activity at temperatures 30C and 40°C with optimum temperature of 40˚C, but it could maintain its initial activity at less than 30°C [24].

For blood de-clotting, the enzyme showed the ability to remove blood clots. And hence it is able to digest natural proteins. The same protease was found to be able to digest not only blood clots but also coagulated egg white as well as cow skin when incubated for long time [12]. During fibrinolytic activity of the enzyme on human blood serum, it was presumed that the antithrombotic effect may due to proteolysis of any one of the blood clotting factors, leading to abolition of both thrombin generation and platelet aggregation [24]. The capability of the protease to digest natural substrate with base of fibrin, albumin and collagen suggest the usefulness of this enzyme for different applications such as extraction of collagen from skin for collagen therapy, waste treatment and related applications [12].

From our research this enzyme showed capability for removing blood stain in white cotton cloth without addition of any detergent powder or solution. Therefore its ability to act in the presence of solvents can be exploited for this purpose. The usefulness of protease from *Spilosoma obliqua* for removal of blood stains from cloth in the presence and absence of detergent was also reported[2]. Therefore we are hoping that this enzyme also can be more effective.

5. CONCLUSION

This study had the objective of isolation and screening of the proteolytic exhibiting bacterial strain, production, partial purification, optimization of the enzyme activity and potential applications of the enzyme. By using isolation and screening techniques, soil samples were serially diluted and poured on the nutrient agar, then biochemical characterization of the protease producing bacteria isolated was found to be *Pseudomonas spp*. This later was screened for protease production. The isolate showed high clear zone was subculture and stored for protease production. Optimization of incubation period for protease production was carried out, where samples were taken at time intervals such as 18, 24, 48, 72 hours of fermentation. The optimum incubation period was found to be 24 hours Optimization of pH and temperature of the enzyme activity was carried out. The optimum pH was found to be 8.0 while the optimum temperature was 35˚C. This shows that the enzymes works better in alkaline conditions. Partial purification of the enzyme was carried out by ammonium sulphate precipitation of the filtrate of fermentation broth. This was followed by dialysis to remove ammonium sulphate. The partially purified enzyme was then evaluated for the ability to dissolve blood clots and remove blood stains in clothes. It was shown by this study that the enzyme is able to de-clot blood and de-stain blood. Therefore its potential application may be in pharmaceutical or detergent industry.

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