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Partial Purification and Characterization of a Haloalkaline Protease from *Pseudomonas aeruginosa*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

A study was conducted to partially purify and characterize a haloalkaline protease isolated from *Pseudomonas aeruginosa.* The enzyme was purified in a two-step procedure involving acetone precipitation and chromatography. The enzyme was shown to have a relatively low molecular weight of 30 kDa. The haloalkaline protease enzyme was purified 2.2-fold with a relative activity of 67.25%. The maximum activity of the enzyme was noticed at 35℃ at pH 9, with casein as a substrate. The partially purified enzyme was almost 100% stable at 5% sodium chloride supplemented medium even after 1hr of incubation. The effect of sodium dodecyl sulphate on partially purified protease activity revealed that the maximum activity was found to be at 50 mM. The impact of ethylene diamine tetra acetic acid on the partially purified protease activity revealed that the maximum activity was found to be at 50 mM. The compatibility of the enzyme was studied with commercial and local detergents. The partially purified enzyme has improved the sanitization power of the detergents tested.

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1. INTRODUCTION

Halophilic microorganisms require very high salt (2 to 5 M NaCl) concentrations for growth and are found in salterns and hypersaline lakes. Many extreme and moderate halophiles have been isolated and investigated for possible biotechnological applications. Proteases encompass a group of industrial enzymes, which alone form about 60% of the total world-wide enzyme yield [1]. Among the variety of enzymes, haloalkaline proteases constitute an important group of enzymes that have high catalytic potential at high salinity. Microbial proteases are an important group of enzymes that can have application in various industries such as leather processing, food processing, pharmaceutical and bioremediation [2]. The applications of proteases include their protein hydrolysate use in cheese-making, cosmetic industries, beer clarification and pharmaceutical products [3]. These potential applications of the proteolytic enzymes in biotechnology are widely common. Hence the search for new haloalkaline proteolytic organisms and enzymes is still a point of interest. In contrast to other types of proteases, the activity of the aspartic proteases is dependent on pH conditions [4]. Proteases have also been extensively studied for their role in inflammatory diseases and blood clotting [5]. Looking into the depth of microbial diversity in India, the chance of haloalkaline protease producing bacterial strains and its exploitation due to their wide variety of industrial application, an attempt was undertaken to isolate microorganisms capable of producing haloalkaline proteases.

2. MATERIALS AND METHODS

The elephant dung was collected from the Shenbagathoppu hills, Srivilliputur, Tamilnadu, India by using sterile container and used. From this sample microbes were isolated and plated on nutrient agar initially and on skim milk agar selective medium which has the capacity to degrade protease.

2.1 Isolation and Screening of Protease Producing Bacteria

The isolated colonies were streaked on the skim milk agar plates and incubated at 37℃ for 48 hours. The zone around the bacterial growth indicated a positive reaction for protease activity [6].

2.2 Protease Enzyme Assay

The haloalkaline protease enzyme activity was studied by the modified method of Hagihara [7] using casein as the substrate. One unit of protease enzyme activity is defined as the enzyme that releases 1 µg/ml of tyrosine per min under standard assay conditions [8].

2.3 Partial Purification of Crude Protease and Characterization

The crude protease extract was partially purified by acetone (75% m/v) precipitation followed by dialysis. The precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4℃ and dissolved in 0.01 M phosphate buffer (pH 7.0). The solution was dialyzed against the same buffer at 4℃ for 8 h with 3 changes of the dialysis buffer, and protein concentration was measured as per the method of Lowry et al. [9] using Bovine Serum Albumin as standard protein.

2.4 Enzyme Purification and Molecular Mass Determination of Protease

Purification of enzyme was carried out at 4℃. Enzymes (supernatant of fermented broth obtained after centrifugation at 10,000 g for 10 min) were precipitated by adding two volumes of acetone and kept for 1 h at 0-4℃ to allow complete precipitation. The resulting precipitate was collected by centrifugation (10,000 rpm, for 30 min) and the pellet was air dried and resuspended in a minimal volume of 20 mM Tris HCl buffer, pH 7.0. After the removal of insoluble materials by centrifugation (10,000 rpm for 30 min), the supernatant was subjected to ion exchange chromatography. Ion exchange chromatography was performed on a column of DEAE- Sephadox (2.5 to 30 cm), which was equilibrated with 20 mM Tris HCl pH 7.2. After loading the sample, the column was washed with the same buffer until the optical density of the elution at 280 nm was zero. The bound proteins were then eluted with a linear gradient of sodium chloride in the range of 0.1-1 M as the equilibrating buffer. Fractions (4.0 ml each) were collected and the enzyme activity and protein content of each fraction were determined. The approximate molecular weight of the partially purified enzyme was determined by (Sodium

Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis) SDS-PAGE [10].

2.5 Effect of SDS on Enzyme Activity

The enzyme was pre-incubated with various concentrations of 10 mM, 25 mM, 50 mM, 100 mM and 150 mM Sodium Dodecyl Sulphate (SDS). The enzyme activity was then determined at 37℃ by using 0.5 ml of casein as the substrate. The protease activity was checked using carbonate buffer. The residual activities were quantified under standard assay conditions.

2.6 Effect of EDTA on Enzyme Activity

The enzyme was pre-incubated with various concentrations of 10 mM, 25 mM, 50 mM, 100 mM and 150 mM EDTA. The enzyme activity was then determined at 37℃ by using 0.5 ml of casein as the substrate. The protease activity was checked using carbonate buffer. The residual activities were quantified under standard assay conditions.

2.7 Effect of pH and Temperature on Partially Purified Enzyme Activity

Stability of the purified protease was studied and the pH of the reaction mixture was adjusted using one of the following buffers: Sodium acetate, sodium phosphate and Tris HCl. To ensure the pH stability and the enzyme reaction mixtures were incubated at 40℃ for 1 h and the relative activity was measured. The enzyme stability of the purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 25, 30, 35, 40, 45 and 50℃ for 1 h and then relative activities were assayed at standard assay conditions.

2.8 Compatibility of Protease Enzyme with Ariel and Power Commercial Detergents Activity

Ariel and Power detergent powder was used for protease compatibility studies. The enzymes were pre-incubated with various concentrations of 10 µl, 25 µl, 50 µl, 100 µl and 150 µl of Ariel and Power detergent. The enzyme activity was then determined at 37℃ by using 0.5 ml of casein as the substrate. The protease activity was checked using carbonate buffer. The residual activities were quantified under standard assay conditions.

3. RESULTS

3.1 Isolation and Screening of Protease Producing Bacterial Strains

A total of ten bacterial strains were isolated from the elephant dung sample of Shenbagathoppu hills, Srivilliputur, Tamilnadu, India and screened. Only one bacterial strain was selected on the basis of maximum protease production by hydrolysis of casein and used for successive studies.

3.2 Identification of Protease Positive Colony

Depending upon the morphological, physiological and biochemical tests the selected colony was identified as *Pseudomonas aeruginosa* following the standard keys of Bergey's Manual of Determinative Bacteriology (Table 1). Consequently, the strain was identified as a protease producer and was utilised for further experimental studies. Phylogenetic studies revealed that the 16S rRNA gene sequence of *Pseudomonas aeruginosa* has 97**%** similarity with the nearest match in the Gene bank.

3.3 Enzyme Purification

The protease activity of the crude enzyme extract was concentrated by acetone precipitation. Eighty nine percentage (89%) of the protease recovery was achieved with 1.6-fold purification (Table 2). Following acetone precipitation the resuspended solution was applied to a column of DEAE- Sephadex. Fig. 1 shows that the maximum protease activity was observed fraction in 30 kDa. This step resulted protease recovery (2.2 – Purification of fold) with a specific activity of 4.4 U/mg^{-1} of protein. The purified protease migrated as a single band of 30 kDa in the SDS-PAGE (Fig. 2) under reducing conditions, suggesting that the purified protein was homogenous. This report is similar for *Bacillus cerus* resulted in molecular weight of 34 to 45 kDa.

3.4 Effect of pH on Protease Stability

Table 3 shows the effect of pH on the protease activity and stability under various experimental pH. Results inferred that protease activity of *Pseudomonas* sp. was more active at pH 9.0 after 30 min of incubation. The minimum protease activity was recorded in pH 6.0.

3.5 Effect of Temperature on Protease Activity

Effect of different temperatures on protease activity was studied after 30 min of incubation. Among different temperatures tested, the maximum activity of *Pseudomonas* sp. was registered at 35℃, whereas the variation in temperature above and below 40℃ showed reduction in the protease activity (Table 4).

3.6 Effect of Surfactants (SDS) and EDTA on Enzyme Activity

Enzyme was tested at various concentrations of SDS at 1 h incubation. The maximum enzyme activity was recorded at 50 mM, while the least activity was noticed at 150 mM concentration (Table 5). Similarly, the effect of EDTA was tested against the purified enzyme. Table 6 shows maximum enzyme activity at 50 mM, while the least enzyme activity was noticed at 150 mM concentration.

3.7 Effect of Commercial Detergents on the Enzyme Activity

The enzyme was tested at various concentrations of detergents like Ariel and Power detergent after 10 min of incubation. Both detergents showed maximum enzyme activity at 50 µl and least activity at 150 µl (Table 7).

Table 1. Biochemical tests of the haloalkaline protease producing strain *Pseudomonas aeruginosa*

Tests	Results
Indole	Negative
Methyl red	Negative
Voges – proskauer	Negative
Citrate	Positive
Catalase	Positive
Oxidase	Positive
Urease	Positive

Table 2. Results of the purification of haloalkaline protease from the *Pseudomonas aeruginosa*

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Relative %
Culture supernatant	192	114	1.6	1	100.00
Acetone precipitation	171	65	2.6	1.6	89.06
DEAE-Sephadex	115	19	4.4	2.2	67.25

Table 3. Effect of pH on the enzyme activity of haloalkaline protease

S. no.	pH	U/ml/h^{-1}
1	6	41
2	7	64
3	8	65
4	9	75
5	10	66
6	11	66

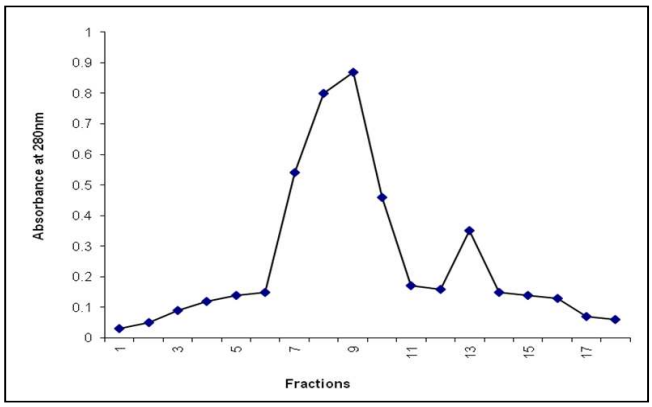

Fig. 1. Elution profile of haloalkaline protease from *Pseudomonas aeruginosa* using DEAE -Sephadex

Table 4. Effect of temperature on haloalkaline protease activity

S. no.	Temperature	U/ml/h^{-1}
1	25℃	39
2	30℃	58
3	35℃	74
4	40℃	63
5	45℃	47
6	50℃	39

Table 5. Effect of various concentrations of SDS on haloalkaline Protease activity

S. no.	Surfactants	U/ml/h^{-1}
1.	Control	81
2.	10 mM	73
3.	25 mM	81
4.	50 mM	92
5.	100 mM	70
6.	150 mM	64

Table 6. Effect of various concentrations of EDTA on haloalkaline protease activity from *Pseudomonas aeruginosa*

S. no.	EDTA	U/ml/h^{-1}
1.	Control	80
2.	10 mM	70
3.	25 mM	79
4.	50 mM	85
5.	100 mM	75
6.	150 mMl	49

Table 7. Effect of various concentrations of Ariel and Power detergent on haloalkaline protease activity

S. no.	Detergent	Ariel U/ml/h	Power U/ml/h
1.	Control	80	80
2.	10 µl	79	77
3.	25 µl	85	86
4.	50 µl	96	91
5.	100 µl	75	70
6.	150 µl	42	58

4. DISCUSSION

In the partial purification of the highly haloalkaliphilic protease produced by *Pseudomonas aeruginosa*, precipitation by acetone was important in the exclusion of contaminating exopolysaccharides and proteins [11]. The above mentioned method was previously employed for the purification of the halophilic serine protease from *Natronococcus occultus.* An advantage of the enzyme purification is an increase in its enzyme activity, making the after purification more specific for industrial applications. In order to elucidate the specific functions of the enzyme, there is a need to characterize an enzyme after purification.

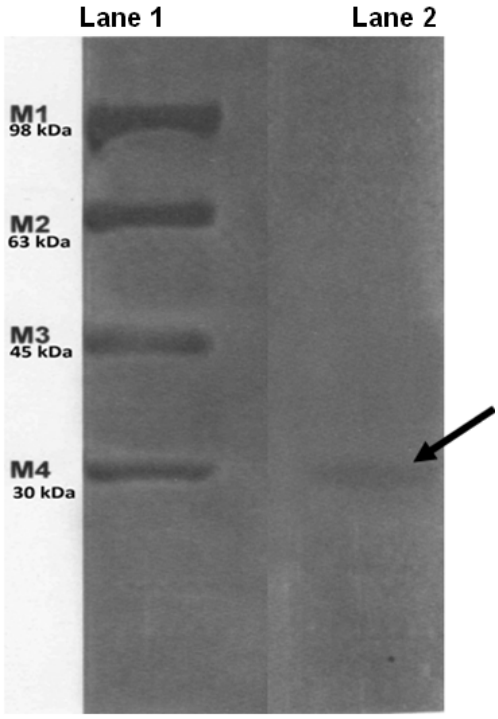

Fig. 2. SDS – PAGE for molecular weight detrermination of *Pseudomonas aeruginosa* protease

In the present investigation, the enzyme was purified in a 2- step procedure involving acetone precipitation and DEAE - Sephadex G-100 chromatography. The enzyme was shown to be homogenous and had a relatively low molecular weight of 30 kDa. From this study, it was observed that the partially purified protease of *Pseudomonas aeruginosa* was homogenous and recorded a single band with molecular weight of 30 kDa. This is similar to the protease produced by the *Bacillus pumilis* that was found to be 31 kDa [12].

In this study, the effect of temperature on the partially purified protease showed that the optimum protease activity was found to be at 35℃ and the optimum pH for the enzyme activity recorded was at 9.0 with casein as the substrate. The present study is in accordance with the

previous report of Jadwiga [13], who has reported a similar optimum temperature range from 40 to 50℃ for the purified protease, from *B. cereus* [14]. Some marine bacterial strains such as *Vibrio*, *Pseudomonas* and *Bacillus* sp. have produced noval thermal protease [15]. He also reported that the optimum pH of alkaline protease from *B. subtilis* CN2 and *B. subtilis* PE-11 was found to be at pH 10.

In the present study, the maximum protease activity was recorded at 50 mM EDTA added mixture. This result is identical to the earlier report of Hoshino et al. [16] who have also reported the maximum protease activity obtained in reaction mixture containing EDTA after 48 hours of incubation.

The partially purified protease showed admirable stability and compatibility in the presence of locally available detergents such as Power and Ariel. The present report is analogous to the previous report of Adinarayana et al. [17], who reported that the alkaline protease from *B. subtilis* PE-11 showed good stability and compatibility with a broad range of locally available detergents in the presence of $CaCl_2$ and glycine as stabilizers at 60℃. Bhosale et al. [18] also reported that the activity of alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20) showed good compatibility and stability with locally available commercial detergents.

5. CONCLUSION

The halophilic protease purified from *Pseudomonas aeruginosa* in this study exhibited stability towards ionic, non ionic surfactant and commercial detergents. Considering the promising properties of *Pseudomonas aeruginosa* enzymatic preparation, we suggest that the purified enzyme could be a better choice for biodetergent potentiality.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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