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PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYME FROM NOMURAEA RILEYI

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ABSTRACT: *Nomuraea rileyi* (Farlow) Samson is an entomopathogenic fungus capable of producing a variety of enzymes including proteases and chitinases. Protease derived from microorganisms such as fungi, bacteria, and yeast has established wide spread applications in fields such as in the food, detergent and other industries. This study investigates the strategy for partial purification and characterization of protease enzyme from *Nomuraea rileyi*. The fungus was primarily screened on skim milk agar plate and a clear zone of skim milk hydrolysis confirmed the protease production. The protease activity of 89 U/100 ml was observed in crude sample and then subjected to partial purification by ammonium sulphate precipitation followed by dialysis. The protease enzyme was partially purified to 3.64 fold with a yield of 3.5%. The partially purified protease exhibited optimum activity at pH 8.0 and temperature at 40ºC. Proteases can be used for various commercial purposes such as detergent, waste treatment, leather industry for dehairing the animal skins and degradation of natural proteins. In the present study protease were tested upon blood stain and dye and was found to exhibit good detergent activity after 15 mins.

INTRODUCTION: The entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson is capable of producing a variety of enzymes including proteases and chitinases. Proteases are enzymes that are often considered vital in facilitating the host infection process [1]. According to [2], fungal proteases are believed to play an important role in cuticle penetration of insects. [3] reported that fungi have a wide variety of enzymes than bacteria and proteases are among the most important enzymes produced by fungi. The protease from the entomopathogenic fungi is believed to be an important pathogenic factor for the fungal attachment on cuticle of insects [4].

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Proteases are one of the most important classes of enzymes which are expressed all the way through the animal kingdom, plant and as well as microbes [5]. The immense diversity of proteases, in spite of their mode of action and specificity, has attracted worldwide consideration to exploit their physiological and biotechnological application. Alkaline protease of microbial origin hold considerable industrial potential due to their biochemical diversity and wide applications in silver recovery, tannery and food industries, medicinal formulations, detergents and processes like waste treatment [6, 7, 8].

Proteases are one of the most leading industrial enzymes, accounting for nearly 65% of total worldwide sales, employed primarily as cleansing additives. Protease derived from microorganisms such as bacteria, fungi, and yeast has found wide spread applications in many fields such as in the food, detergent and other industries [9, 10].

To make clear the precise role of the enzymes in the insect infection process, it is crucial to characterize and purify the enzymes. Clarification of the extracellular enzymes to the fungal infection process is held up mainly because of unavailability of pure enzyme preparations. The ideal purification strives to acquire the maximum recovery of the desired enzyme, with minimal loss of activity, combined with the maximum removal of other contaminating enzymes [11].

The aim of this work is to explain the processes which can be used to extract, purify and characterize protease enzyme related to *Nomuraea rileyi*. Such studies surely increase the knowledge on enzyme production in entomopathogenic fungi which opens new avenues for the utilisation of protease.

MATERIALS AND METHODS:

Microorganism and culture condition: Pure culture of *Nomuraea rileyi* (MTCC 4171) was obtained from Institute of Microbial Technology, Chandigarh, India and used for the present study. The strain was revived using potato dextrose agar media and preserved at 4ºC for further use.

Primary screening: The fungal strain *Nomuraea rileyi* was primarily screened for protease enzyme production using skim milk agar test. The fungus was cultured on skim milk agar plate and incubated at 37 ºC for 24 hours. A clear zone of skim milk hydrolysis indicates the production of protease enzyme.

Enzyme production: The fungus was grown in Czapekdox medium containing (g/l): Casein, 20; Sucrose, 30; KCl, 0.5; $FeSO_4$, 0.01; $MgSO_4$, 0.5; K_2HPO_4, 1.0; $NaNO_3$, 2.0 (pH 8.0). After three days, the culture was taken and filtered through Whatman No. 1 filter paper to remove the unwanted mycelia and cell debris. The culture filtrate was centrifuged at 10000 rpm for 10 mins and the supernatant was taken as crude enzyme source.

Protease assay [12]: The protease activity was assayed using casein as a substrate. 0.5 ml of 0.5% casein solution was taken in two different test tubes and 0.2ml of enzyme was added in one test tube and the other was added with water and served as blank. After 10 minutes of incubation, 2 ml of Trichloroacetic acid was added and incubated at 37ºC for 30 minutes. After the incubation time, the mixture was filtered through whatman filter paper and the filtrate was used for further colour development reaction. 5ml of 500 mM sodium carbonate was added to both the test and blank filtrate.

To that, 1:4 ratio of Folin Ciocalteu's Phenol reagent was added and incubated at 37ºC for 30 minutes. Absorbance was read at 660 nm using Hitachi U 2900 UV-Spectrophotometer and the enzyme activity were calculated using tyrosine standard. One unit activity is the amount of enzyme hydrolyzes casein to produce colour equivalent to 1µmole of tyrosine per minute.

Purification of Enzymes:

- **Ammonium sulfate precipitation:** The crude enzyme extracted from *Nomuraea rileyi* was subjected to ammonium sulphate precipitation. The filtrate is taken and 70% fraction of ammonium sulphate was added slowly to the supernatant. While adding ammonium sulphate the cultures were kept in ice cubes. Then the mixture was incubated for overnight in refrigerator at 4ºC. Next day the mixture was centrifuged at 12000 rpm for 10 mins, the pellet was collected and dissolved in 1 M Tris HCl.

Dialysis:

- **Preparation of dialysis bag:** Dialysis was carried out following the method of [13]. 8-10 cm of dialysis membrane was taken and boiled in 100 ml of distilled water for 10 minutes with slow stirring. The membrane was decanted from boiled water and placed in 100 ml of 2% sodium carbonate solution in boiling conditions. After ten minutes, the sodium carbonate was discarded and fresh distilled water was added, kept boiled for 10 mins and allowed to cool. The membrane was taken and tied with rubber tightly on one side of the membrane then the pellet which was dissolved in 1 M Tris HCl was poured and tightly packed. Then the dialysis tube was kept in magnetic stirrer for 8 hours in 0.1 M phosphate buffer.

For every one hour the buffer was changed and finally the dialysis tube was kept overnight with distilled water. After dialysis, protein solution was centrifuged at 6000 rpm to remove large molecules.

Characterization of Enzymes:

1. **Effect of pH:** The effect of pH on protease enzyme activity was carried out at normal room temperature. The pH ranging from 4 to 10 was taken for the study. The different pH buffers were prepared by using 0.2M phosphate buffer and the enzyme assays were carried out with the same procedure as mentioned previously.

2. **Effect of Temperature:** The effect of temperature on enzyme activity was conducted at varying temperatures ranging from 20-80ºC under controlled conditions. The substrate was preincubated at desired temperature before the addition of enzyme. Enzyme assay was measured as described earlier.

3. **Effect of substrate concentrations:** The effect of substrate concentration on the rate of enzyme activity was tested. The substrate concentration of 100 µg to 500 µg was taken for this experiment. The kinetic constants Km and V_{max} were estimated by double reciprocal plots of the data according to standard method [14].

RESULTS AND DISCUSSION:

Primary screening: The fungal strain *Nomuraea rileyi* was cultured on skim milk agar plate around which a clear zone was observed (**Figure 1**). This indicated that the strain has capability to produce protease enzyme.

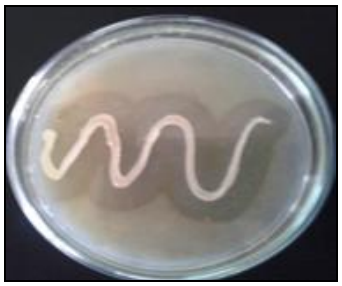

FIGURE 1: ZONE OF HYDROLYSIS OF *NOMURAEA RILEYI* ON SKIM MILK AGAR

Partial purification of protease enzyme from *Nomuraea rileyi*: The supernatant with protease activity of 89 U/100ml and specific activity of 0.51U/mg was used as a crude enzyme solution and subjected to partial purification by ammonium sulphate precipitation and the results were presented in Table 1. The crude enzyme was precipitated at 70 % saturation with specific activity of 1.12 U/mg and 2.24 fold purification. The ammonium sulphate treated solution was subjected to dialysis against low salt buffer for several hours. The enzyme was partially purified to 3.64 fold with a yield of 3.5 % and specific activity of 1.82 U/mg. A similar trend was observed by [13].

TABLE 1: PURIFICATION TABLE FOR PROTEASE ENZYME OF *NOMURAEA RILEYI*

S. No.	Purification steps	Total volume (ml)	Total enzyme activity (Units)	Total protein content (mg)	Specific activity U/mg	Purification fold	% Yield
1	Crude	100	89	174	0.51	1	100
2	Ammonium sulphate	20	20.2	18	1.12	2.24	10.16
3	Dialysis	10	11.5	6.3	1.825	3.64	3.5

Characterization of Protease Enzyme:

Effect of pH on protease enzyme: In the present investigation, enzyme activities at different pH had a considerable effect upon enzyme production and hence enzyme activity of the fungus. The experiment was carried out to investigate the effect of different pH values on the partially purified protease enzyme. The partially purified protease enzyme was incubated at different pH values ranging from 4-10 using 0.2M phosphate buffer.

The enzyme assay was carried out as described earlier using casein as a substrate. The results (**Figure 2**) showed that the partially purified protease exhibit maximum activity at pH 8.0 whereas any further pH gave decreased activity. A similar trend was observed by [15].

The maximum activity at pH 8.0 showed that it may be alkaline protease.

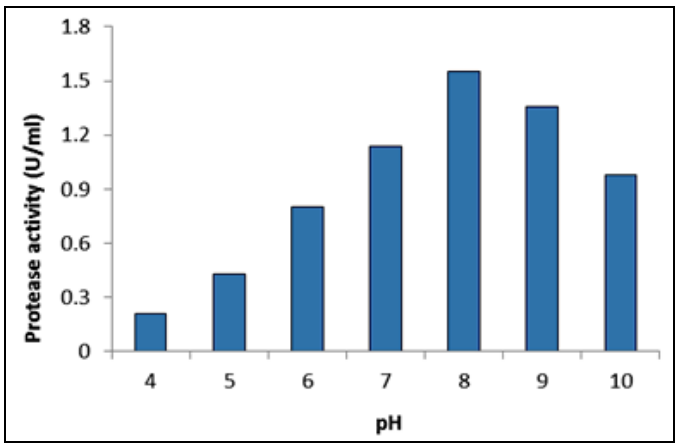

FIGURE 2: EFFECT OF VARYING pH ON PARTIALLY PURIFIED PROTEASE ENZYME

Effect of temperature on protease enzyme: In the present study, the effect of varying temperature on partially purified protease was tested. The partially purified protease was incubated at different temperatures ranging from 20-80ºC and the results are presented in **Figure 3**. The enzyme showed maximum activity of 10.36 U/ml at 40ºC. At temperatures higher than 40ºC enzyme started to loss its activity. So the optimum temperature of partially purified protease from *Nomuraea rileyi* was found to be 40ºC. Similar results were observed by [13] who recorded 40ºC to be optimum for protease enzyme from *Bacillus* sp. From the statistical analysis there is no significant difference between the various temperatures on enzyme activity.

Effect of substrate concentration on protease enzyme: Effect of casein concentration on protease activity was determined by incubating the enzyme with 100 to 500 µg of casein at 40°C, pH 8 (**Figure 4**). The protease activity was increased with increase in substrate concentration until certain saturation level occurs. The maximum protease activity of 0.85U/ml was observed at 300 µg casein concentration. The concentration beyond 300 µg of substrate showed slight inhibition in the enzyme activities.

The kinetic parameter of enzyme was estimated at various concentrations of casein substrates (**Figure 5**). Kinetic parameter V_{max} and Km values for the hydrolysis of casein by the protease were determined by plotting the reciprocal of substrate concentration versus reciprocal of enzyme activity. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate.

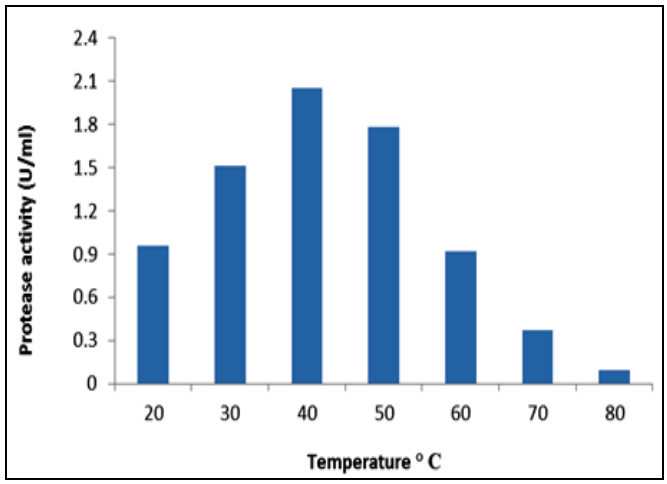

FIGURE 3: EFFECT OF VARYING TEMPERATURE ON PARTIALLY PURIFIED PROTEASE ENZYME

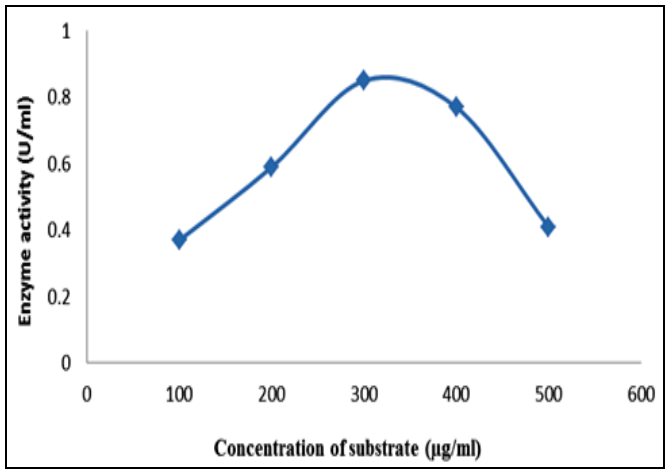

FIGURE 4: EFFECT OF SUBSTRATE CONCENTRATION ON PARTIALLY PURIFIED PROTEASE ENZYME

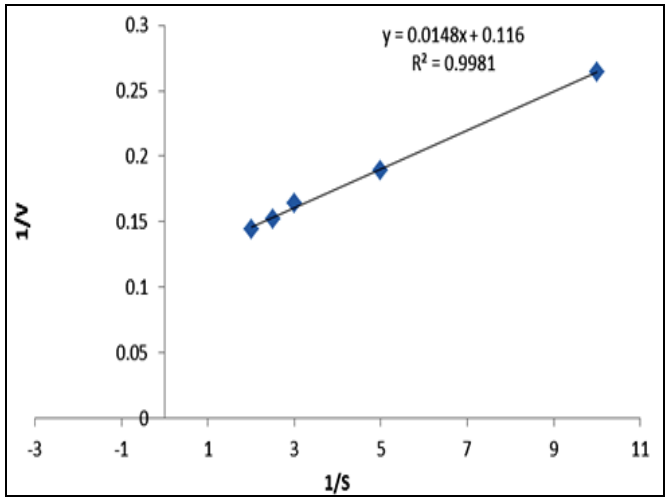

FIGURE 5: DOUBLE RECIPROCAL LINEWEAVER BURK PLOT OF PARTIALLY PURIFIED PROTEASE ENZYME

The Km and Vmax of partially purified protease were calculated from double reciprocal Lineweaver-Burk plot and found to be 130 µg and 0.87 U/ml respectively (Figure 5). The present study differs from the work of [16] who obtained a higher value of 148U/ml with V_{max} its corresponding Km value of 58µM.

Application of Protease Enzyme:

Destaining activity of protease on blood: When protease was applied on a white cotton cloth, the blood stain was removed by incubating the cloth with partially purified protease for different time intervals (**Figure 6**). After 15 minutes of incubation the blood was completely destained by protease enzyme. It was observed that the partially purified protease from *Nomuraea rileyi* had high capability of removing the blood stain, which indicated that the protease enzyme has potential application in detergent industries [17] reported the protease from *Bacillus clausii* has the ability to remove the blood stain very effectively.

Destaining activity of protease on dye: The methylene blue dye stained cloth was incubated with partially purified protease enzyme of *Nomuraea rileyi* at different time intervals to check the detergent activity of protease enzyme and the results are presented in **Figure 7**. After 15 minutes of incubation the dye was properly destained by protease enzyme. It revealed that the partially purified protease from *Nomuraea rileyi* had high capability of removing the staining dye methylene blue, which revealed that the protease enzyme had good detergent activity.

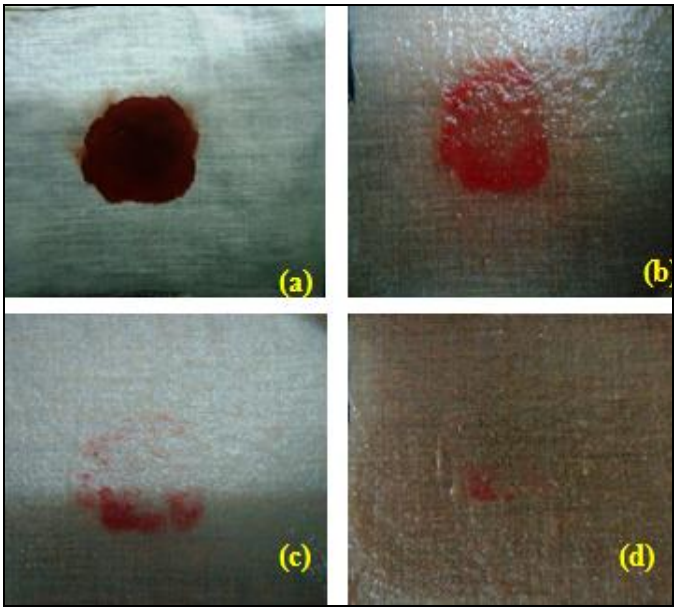

FIGURE 6: DESTAINING ACTIVITY OF PROTEASE ENZYME ON BLOOD. (a) Control (b) after 5 min (c) after 10 min (d) after 15 min

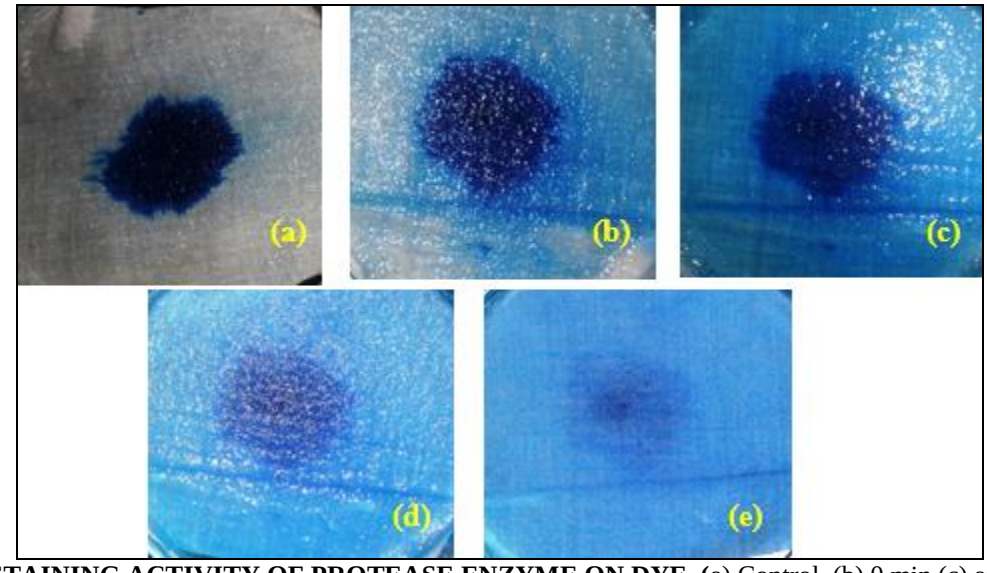

FIGURE 7: DESTAINING ACTIVITY OF PROTEASE ENZYME ON DYE. (a) Control (b) 0 min (c) after 5 min (d) after 10 min (e) after 15 min

CONCLUSION: From the present study it was revealed that *Nomuraea rileyi* was capable of producing a considerable quantity of the protease enzyme and it was subjected to ammonium sulphate precipitation followed by dialysis. The protease enzyme was partially purified to 3.64 fold with a yield of 3.5 % and specific activity of 1.82 U/mg. The partially purified protease has optimum pH at 8.0 and temperature of 40ºC which showed a positive result in the removal of blood stains and

dye from the cloths. Therefore this enzyme can be extensively used in the industrial applications in pilot scale.

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