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Isolation, Screening and Production of Bacterial Protease Enzyme

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ABSTRACT

The objective of present study was to isolate and screen proteolytic bacteria from poultry waste site at Rasipuram, Namakkal district, Tamilnadu, India. Soil samples were serially diluted and 0.1ml of sample was spreaded on nutrient agar plate at 37o C for 48 hrs. Four bacterial colonies isolated were screened for proteolytic activity by skim milk agar plate assay. The isolate RPB 2 showed maximum zone of hydrolysis (13mm) was selected and identified as Staphylococcus sp by morphological and biochemical test. Various factors influencing the protease production such as incubation time, initial pH, incubation temperature, inoculum level and casein concentration were studied. The results showed that maximum protease enzyme production occurred at incubation time 48hr (34 U/ml), pH 7.0 (42U/ml), 2% inoculum concentration (21.5U/ml), temperature 37o C (51U /ml) and 2% casein concentration (72U/ml).

Keywords : Protease, Poultry Waste Soil, Skim Milk Agar, Zone Of Hydrolysis, Fermentation, Staphylococcus.

I. INTRODUCTION

Proteases play pivotal regulatory roles in conception, birth, digestion, growth, maturation, ageing, and death of all living cells. Proteases regulate most physiological processes by controlling the activation, synthesis and turnover of proteins [1]. Proteases are also essential in viruses, bacteria and parasites for their replication and the spread of infectious diseases, in all insects, living cells and animals for effective transmission of disease, and in human and animal hosts for the mediation and sustenance of diseases. Proteases are also essential in viruses, bacteria and parasites for their replication and the spread of infectious diseases, in all insects, living cells and animals for effective transmission of disease, and in human and animal hosts for the mediation and sustenance of diseases.

Proteases, one among the three largest groups of industrial enzymes, account for about 60% of the total worldwide enzyme production and sales [2]. Proteases which include proteinases, peptidases or proteolytic enzymes break peptide bonds between amino acids of proteins. Microorganisms secrete a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products [3]**.** Extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes.

Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales. Within the bacteria, protease production in various species have been investigated *viz. Bacillus* spp. [4]**,** *Vibrio* sp. [5]**,***Pseudomonas* sp. [6]**,** *Lactococcus* sp. [7] and Staphylococcus [8]. Proteases perform a large variety of functions and have important applications in biotechnology based industries such as food, leather, detergent, pharmaceutical industries and bioremediation process [9].

In general, microbial proteases are directly secreted into the production medium because they are extracellular in nature, thus simplifying purification of the enzyme as compared to proteases obtained from plants and animals [10]. Hence, the aim of present investigation was to isolate the protease producing bacteria from soil and to optimize the enzyme production by submerged fermentation.

II. MATERIALS AND METHODS

Isolation of Proteolytic bacteria

The soil samples were collected from poultry waste site at Rasipuram, Namakkal district, Tamilnadu, India in a sterile container (about 100 grams). After collection of soil samples it was taken to the laboratory and these samples were stored at 4ºC till further use. Isolation of bacteria from soil sample was done by serial dilution agar plate technique. One gram of soil sample was transferred to aliquots of 9 ml of sterile distilled water and serial dilution was performed upto 10^{-6} dilution [11]. From each dilution 0.1 ml was spreaded on nutrient agar medium plates and incubated at 37°C for 48 hours. After incubation predominant bacterial colonies were isolated. After incubation predominant bacterial colonies were isolated and maintained on nutrient agar slants.

Screening of proteolytic activity by plate assay

Proteolytic activity of isolated bacteria was screened by plating them on skim milk agar medium. These plates were incubated at 37 °C for 48 hrs. Enzyme activity was indicated by the formation of a clear zone around colonies. Bacterial isolate which showed maximum zone of hydrolysis was selected for further studies.

Characterization of the bacterial isolate

The isolate which exhibited maximum proteolytic activity in skim milk agar plate assay was selected and identified by phenotypic characterization based on morphological, biochemical and physiological characters [12].

Production of protease by submerged fermentation

Production of protease enzyme by submerged fermentation was carried out using a basal medium. The enzyme preparation was obtained by inoculating bacterial isolate into 250 ml Erhlenmeyer flasks containing 100 ml of sterile fermentation medium. The basal fermentation medium with a composition (% w/v): glucose 0.5, Peptone0.75, MgSO4 0.02, KH2PO4 0.1, pH 7.0 [13]. Fermentation was carried out in an orbital Shaker at 160 rpm for 48 hrs at 37°C.

Enzyme extraction

At the end of incubation the medium was centrifuged at 10,000 rpm for 10 minutes. Pellet was discarded after centrifugation and clear supernatant was used as source of protease enzyme. The supernatant of crude enzyme was further used for subsequent studies [4].

Measurement of enzyme activity

Extracellular proteolytic activity was determined according to the modified method using casein as the substrate [14]. The reaction mixture contained 1 ml of 1.5% (w/v) casein in 0.1 M citrate phosphate buffer (pH 6.0) and 1 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The enzyme reaction was terminated by addition of 6 ml of 5% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 min and filtered through Whatman No. 1 filter paper. To 1 ml of filtrate, 3 ml of 0.5 M Sodium carbonate solution and 1 ml of 3-fold diluted Folin-Ciocalteau reagent were added and mixed thoroughly. It was incubated at room temperature in dark for 30mins for the development of colour and absorbance was read at 660 nm against reagent blank. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine from casein per minute under standard assay conditions described previously. All assays were carried out in triplicate.

Optimization of culture conditions for protease enzyme production

The various factors influencing the protease production were investigated, examining one factor at a time, keeping all other variables constant except one. They include incubation time, initial pH, incubation temperature, inoculum level and casein concentration.

Effect of incubation Time

The time course for enzyme production by the bacteria under submerged fermentation at room temperature (37±2°C) was studied by inoculating the fermentation medium with 1% (v/v) inoculum and incubating for upto 84 hrs. At regular time interval samples were withdrawn and centrifuged at 10,000 rpm for 10 minutes. Pellet was discarded after centrifugation and clear supernatant was determined for protease production.

Effect of different inoculum concentration

The effect of different inoculum concentration was investigated for the production of protease by the isolated bacteria. Optimal inoculum concentration which supports maximal protease enzyme production was evaluated using different concentrations of inoculums 1%, 2%, 3%, 4 and 5%.

Effect of pH and temperature on Protease enzyme production

To determine optimal pH, bacterial cultures were cultivated in a 150 mL flask containing 50 mL fermentation medium with different pH ranges from 4.0 to 9.0. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were incubated at 37°C for 48 hrs. In order to determine the effective

temperature for protease production by the bacterial isolate, fermentation was carried out at in the range of 20, 30, 37, 40 and 50°C.

Effect of substrate concentration of enzyme production

To study the effect of casein concentration on protease enzyme production by submerged fermentation was performed by inoculating media containing different casein concentrations (0.5-3.5%, w/v) and incubating at 37°C for 48 hrs. The protease activity was then determined as described earlier.

III. RESULTS AND DISCUSSION

Soil samples from poultry waste site of Rasipuram, Namakkal district was collected, and used for isolation of bacteria producing protease enzyme. After serial dilution of soil sample four morphologically distinct bacterial colonies were isolated by spread plate method sub-cultured on nutrient agar slants. Four bacterial isolate were given the code RPB1, RPB2, RPB3 and RPB4 were screened for the proteolytic activity by plate assay method on skim milk agar medium by inoculating each isolate separately and incubated at 37 ℃ for two days. The bacterial isolate RPB2 and RPB4 showed clear zone around colonies 13mm and 2mm diameter while the other two bacterial isolate RPB1 and RPB3 didn't showed clear zone of hydrolysis **(Table 1).**

Table 1: Screening of bacterial isolate for proteolytic activity by plate assay

Isolate	**Zone of Hydrolysis (mm)**
RPB 1	-
RPB2	13
RPB3	-
RPB4	2

Identification of the proteolytic bacterial isolate

The bacterial isolate produced maximum protease activity on skim milk agar plate assay was selected were preliminarily examined for morphological characteristics by gram staining, shape and motility. The isolate was further identified by biochemical characteristics. The results of the morphological and biochemical characteristics of the isolate were represented in **Table 2.** From the results it is inferred that the selected isolate was confirmed to be *Staphylococcus* sp.

Optimization of protease enzyme production

Maximum enzyme production could be obtained only after a certain incubation time which allows the

Table 2 Morphological and Biochemical characterization of bacterial isolate.

Test	Isolate RPB2
Grams reaction	Gram Positive rod
Shape and arrangement	Cocci, arranged in groups
MSA	Yellow colony
Catalase	Positive
Coagulase	Positive
Indole	Positive
MR Test	Positive
VP Test	Negative
Citrate test	Negative
Carbohydrate Fermentation	
Glucose	Positive
Lactose	Positive
Sucrose	Positive
Mannitol	Positive

Culture to grow at a study state .Enzyme production of each strain is based on the specific growth rate of the strain. Growth rate and enzyme synthesis of the culture are the two main characteristics which are mainly influenced by incubation time [15]. In tests to optimize the time, protease production increased gradually from 0 to 48 hrs, at which it was maximum, at 34 U/ml (Table 3). Further incubation resulted in a gradual decline in the enzyme production. This decline might be due to cessation of enzyme synthesis together with auto proteolysis (figure 1).Similar findings was also reported by previous worker [16] in which maximum protease

enzyme production by bacteria *Bacillus* sp. Was observed at 48 hours of incubation period. On contrary some authors reported that the protease enzyme production was maximum at 72 hrs of incubation period [17].

Inoculum volume determines the level of biomass and product formation and plays vital role in microbial fermentation. It is obvious that the inoculum size shows diverse impact on biomass formation and enzyme synthesis depending on the characteristics of microbial strains [18]. Varying concentrations of Inoculum levels were studied in this experiment and the various range of inoculum selected in this experiment ranged from 1% to 5% Enzyme production varied with percentage of inoculum and the maximum enzyme production was 30 U/ml with 3% inoculum concentration**.** Increase of inoculum level from 3% to 5% showed a marginal decrease in protease production (Fig 2). The optimum inoculum size for maximum protease production by *S.aureus* and *P.putida* was reported as 3% [17]. Similar result was observed by Niyonzima and More [19] in which they reported that maximum protease enzyme production (9.021 U/ml) was observed with 2.0% inoculum size and further increases resulted in decreases in alkaline protease production**.** The decline in protease level with larger inoculum size could be due to the reduced dissolved oxygen, shortage of nutrients available for the larger biomass and faster growth.

pH of the culture medium is important for cell growth [20,21], perhaps relating to its influences on nutrient solubility and uptake, enzymatic activity, cell membrane morphology, byproduct formation and redox reactions [22]. The bacterial isolate was allowed to grow in media of different pH ranging from 4.0 to 9.0. Maximum protease enzyme production by *Staphylococcus* sp. observed in medium of pH 7.0 (42 U/ml) (figure 3). The result of this findings is supported by earlier study of [23], and reported that the pH of the medium must be maintained above 7.0 throughout the fermentation period. It has been reported widely that protease production from microbial source can be acidic or alkaline proteases as reported by many researchers depending on the organisms and source of the isolation.
The optimal temperature for maximum protease production was studied by incubation of the inoculated media at different temperature ranging from 20 - 50°C and enzyme production was found to be highest at 37°C (51 U/ ml) (Figure 4) and it decreases at higher temperature Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield [24]. Abusham et al**.** [25] reported that in shaken cultures, 37°C was found to the optimum temperature for both protease production and bacterial growth.

Influence of different concentration of substrate on the production of protease by *Staphylococcus* sp. was evaluated. In this study, protease production was significantly enhanced when 2.% of casein was used as substrate in the medium with 72 U/mL of protease activity (figure 5) Generally, casein is a protein rich substrate, which supports high protease production. The concentration of casein at various levels (5 to 30 g/L) was evaluated by [26] **.** Casein at a concentration of 15 g/L significantly ($p<0.05$) yielded the maximum amount of protease (125.40 U/mL). Similarly Patil et al. [27] studied the optimum concentration of casein for maximum alkaline production was obtained to be 3%, where the protease activity of crude extract was 3PU/ml.

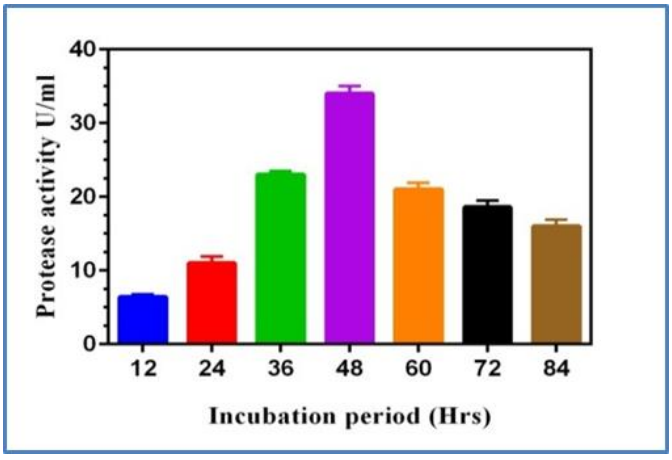

Figure 1. Optimization of Incubation period on protease production by *Staphylococcus* sp.

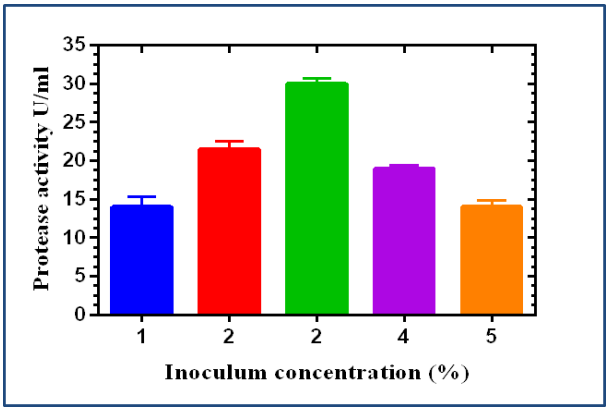

Figure 2. Effect of Inoculum concentration on protease production by *Staphylococcus* sp.

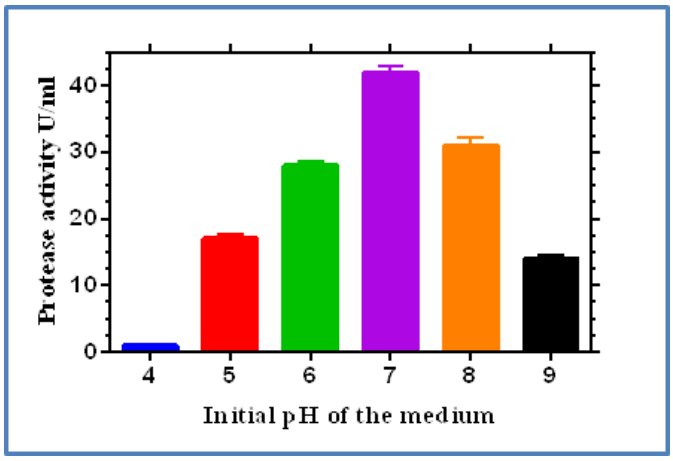

Figure 3. Effect of Initial pH of the medium on protease production by *Staphylococcus* sp.

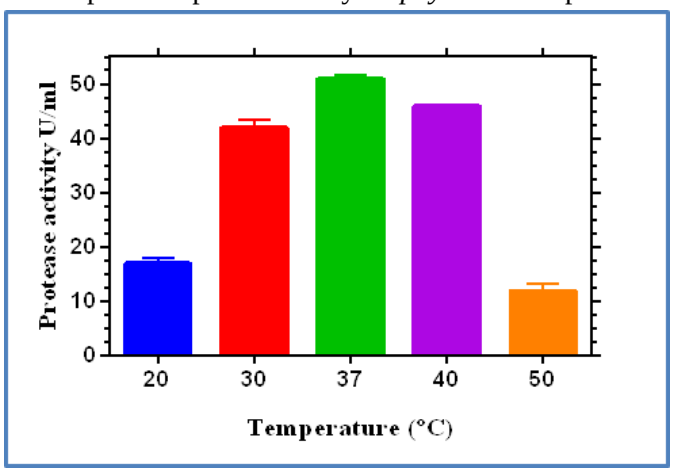

Figure 4. Effect of Incubation temperature on protease production by *Staphylococcus* sp.

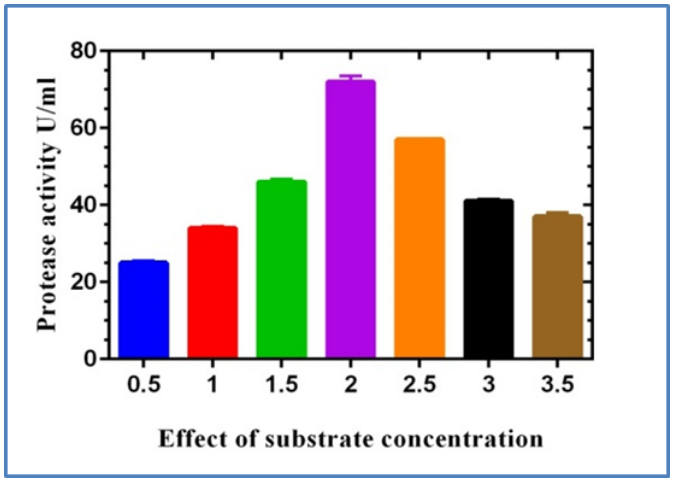

Figure 5. Effect of substrate on protease production by *Staphylococcus* sp.

IV. CONCLUSION

In the present study soil from poultry waste sit of Rasipuram shows the presence of protease producers. Bacterial isolates exhibited proteolytic activity by skim milk agar plate assay was selected and identified as *Staphylococcus* sp. Bioprocess variables like incubation time, inoculum concentration, pH, temperature and substrate concentration were optimzed for protease enzyme production. Keeping in view about use of protease enzyme it can be harnessed for biotechnological processes.

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