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Isolation and Characterization of Protease Producing Organism Bacillus Species from Slaughter House Waste

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Abstract

The purpose of this investigation was to isolate protease producing bacteria from slaughter house waste collected in local area of Kaliakkavilai. The Bacillus sp. was identified by several biochemical and physiological tests. The effect of different production parameters such as pH, temperature, metal ions concentration and substrate concentration for protease production by the isolated bacterial strain were studied. The enzyme production was assayed in submerged fermentation with optimized parameters to produce maximum protease activity and specific activity was observed as 1.067 and 0.011 µmol/ml/min. The partial purification of protease was done by column chromatography.

Keywords: *Slaughter house, protease enzyme, column chromatography, Bacillus sp.*

INTRODUCTION

Protease is essential constituent of all forms of life on earth including prokaryotes, fungi, plants, and animals. Protease is one of the industrially important enzymes. Protease is highly exploited enzymes in food, leather, detergents, pharmaceutical, diagnostic, waste constituents and silver industry. The protease constituents two thirds of total enzymes used in various industries and its dominance in industrial market is expected to increase by the year 2005 [1]. In recent years, protease has been considered industrially significant and robust enzyme and alkaline protease account for approximately 60% of the world enzyme market [2]. Enzymes are proteins with catalytic properties due to its power to specific activation [3]. High production of alkaline protease is done by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium. These enzymes accounts for 30% of the total world enzyme production [4]. Among bacteria, *Bacillus Sp*., are specific producers of extra cellular alkaline

proteases [5]. Microbial proteases play important role in biotechnological process with worldwide sales representing about 60% of the total enzyme marked. Bacterial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymologist. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the industrial community [1]. Bacterial cells produce a large array of proteases which are intracellular or extra cellular. Microorganisms account for a two-third share of commercial protease production in the world [6]. Proteases are difficult to characterize because of their diversity of action and structure. Originally proteases were characterized based on molecular size, charge or substrate specificity. However, with the advent of molecular biology, proteases are now grouped into familiar based on the chemical nature of the catalytic or active site; mechanisms of action and the evolutionary relationship of their 3D structure [7,8]. The effectiveness of detergents protease depends upon pH and ionic strength of the detergents solution, the washing temperature and pH, mechanical handling, level of soiling, such as surfactants, builders bleaching agents, bleach activators, fillers, fabric softness and various other formulation aids.

MATERIALS AND METHODS

Isolation and Screening

The protease enzyme microbes were isolated by slaughter house soil sample. The isolatedmicrobes were screened by casein agar and skim agar medium. The casein agar medium was used for the enumeration of total bacterial population of soil sample.

Identification of Selected Isolate

The selected isolate were examined by different morphological, microscopical, biochemical and physiological tests. The bacterial identification studies were compared with Bergey's Manual of determinative bacteriology.

Measurement of Enzyme Activity

Enzyme assay was determined by the modified method of [9] as followed by [10]. Three milliliter of culture filtrates, 3 ml phosphate buffer and 3 ml 1% casein was taken in a 25 ml test tube. Then the test tube was placed in a water bath at 35^0 C for 1 h. After reaction, 5 ml 20% Trichloro acetic acid (TCA) was added with the solution for stopping the reaction, after one hour, the solution was filtered by whatman no. 540. Form the filtrate solution 1 ml enzyme substrate mixture was taken into a test tube and 2 ml, 20% Na_2Co_3 was added to it.

To this mixture 1 ml of Folinciocalteu reagent was added and immediately the contents of the tube were mixed well. After 30 min, 6 ml distilled water was added to it and the absorbance of the solution was measured at 650 nm in a spectrophotometer and calculated the amounts of amino acids released from a standard curve plotted from known concentration of tyrosine. The enzyme activity was expressed in unit. One unit of enzyme was defined as the amount of enzyme that releases 1 µg of tyrosine ml^{-1} of crude extract h^{-1}.

Protease Production

The culture medium used in this work to protease production contained different sources. In this medium will maintained at 37°C for 24 h in a shaking cum incubator. At the end of each fermentation period, the whole fermentation broth was centrifuged at 10000 rpm at 4°Cfor 15 min and clear supernatant was used as crude enzyme preparation.

Total Activity of Protease

0.2–0.1 ml of standard tyrosine solution was pipette out in different tubes. Add the volume was made up to the 5 ml with distilled water. To all the tubes 1 ml of Dinitro salicylic acid was added, mix well then all tubes are placed into the boiling water bath for 15 min and cooled. Then orange color was formed. Read the color development at 540 nm.

Preparation of Crude Enzyme

A loopfull of screened and characterized bacterial isolate was inoculated in 100 ml of nutrient broth aseptically and incubated in shaker cum incubator at 37°Cfor 24 h. 10 ml of culture was taken and centrifuged at 5000 rpm for 15 to 20 min. The supernatant and the pellet were collected aseptically. The polluted cells were then re-suspended in 1 ml of Tris Hcl(0.1 M) pH 8.0 and 10 µl of lysozyme was added and incubated at 37°Cfor 1 h in an incubation bath. Then it was centrifuged at 5000 rpm for extra 10 min. The intra cellular (cell lysate) and extra cellular (culture Supernatant) was made and used as a crude enzyme. Protease assay was made at different pH, temperature and varying concentration of substrate (casein) and metal ions (calcium chloride and mercuric Chloride).

Protease Activity inDifferent pH, Temperature

Protease assay was carried out using the method described by [11]. The casein (Substrate was prepared and tested for different temperature (10–80°C) The OD was measured at 650 nm.

ProteaseActivity of Metal Ions ($CaCl_2$ and $MgCl_2$) Concentration

The substrate of Calcium chloride and Mercuric chloride was prepared in different concentration (0.1–1) and add 0.1 M trisHcl buffer (pH8.0). To this1 ml of the enzyme sample was added. The mixture was incubated at 37°Cfor 1 h. Then one ml cold 10% TCA was added. It was then filtered by using whatman No.1 filter paper;color developed was accomplished with the addition of 500 µl of Folin phenol reagent. The OD was measured at 650 nm.

Purification of Protease

Purification of protease was done in coloum chromatography. ColoumChromatography apparatus was cleaned and add the crystal form of Silica gel in ¾ part of the coloum. The silica gel was washed with distilled after, then again washed with the solvent (acetone, benzene). After the washing the silica gel are poured on the coloum. Phosphate buffer was added above the silica gel. Then add the culture, the purified form of culture was obtained.

RESULTS AND DISCUSSION

Isolation of Protease from Slaughter House Waste

In order to screen the protease producing organisms, in this study totally five different type soil samples from different places of slaughter house were collected. Then this organism were characterized by (biochemical characterization) enumerated and found out the activity of protease producing organisms. In a skim milk agar medium the zone formation was observed.

Identification of *Bacillus Species*

The morphological identification of each bacterial pathogen was carried out by gram staining method. The biochemical characterization of *Bacillus species* was carried out by 12 different tests (Table1).

Total Activity of Protease

In this study, the total protease activity and a specific activity were evaluated using calorimetric methods. According to [9] mass cultivated protease was measured (Table 2).

Table 1: *Biochemical Characteristics of Bacillus Species.*

S.No	Characteristics	Results
1	Oxidase	Positive
2	Catalase	Negative
3	Hydrogen Sulphide Test	Negative
4	Carbohydrate Fermentation	Positive
5	VogesProskaur	Negative
6	Citrate utilization	Negative
7	Casein Hydrolysis	Positive
8	Gelatin Hydrolysis	Negative
9	Urea Hydrolysis	Negative
10	Starch Hydrolysis	Positive
11	Indole test	Negative
12	Methyl Red	Positive

Table 2: *Measurement and Total Activity of Protease.*

S.No	Activity of protease	µmol/ml/minute
1	Total Activity of protease	1.067 ±0.04
2	Specific Activity of Protease	0.011±0.01

Effect of pH on Protease Production

The effect of various pH (1–12) on protease activity was determined as per the standard procedure. The enzyme was observed to give an optimal activity pH of 8,according to the findings of [11]. Observed protease produced at a pH of 8. Generally are found to be active at alkaline pH (Figure 1). Optimum pH of protease activity was determined by using 50 mM of phosphate buffer pH (6–8) and Glycine buffer in the assay system. Maximum enzyme activity was observed at pH8. The enzyme activity was reduced to pH 3. This enzyme was active in alkaline conditions.

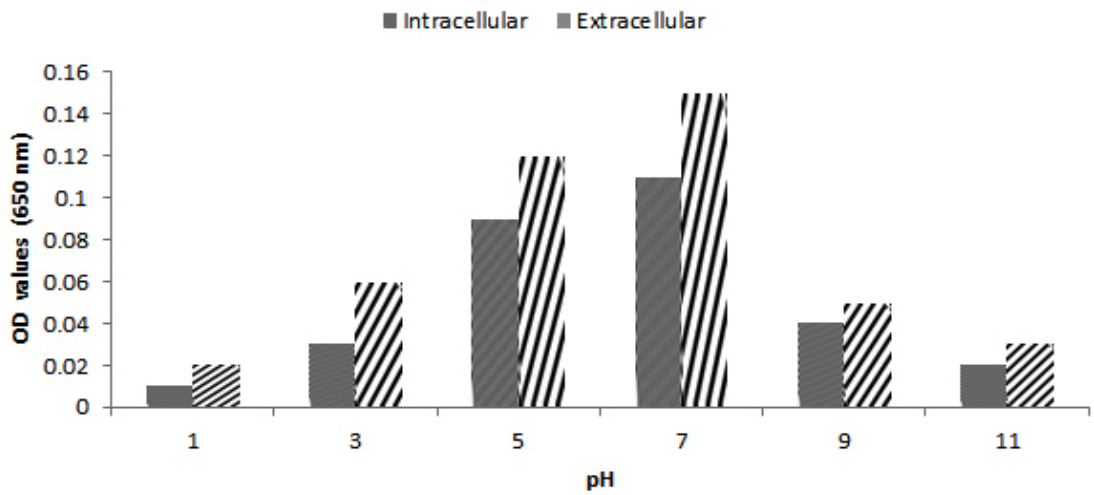

Fig. 1: *Effect of pH in Protease Production by Slaughter House Waste Soil.*

Effect of Protease Production in Temperature

The effect of various temperatures (10–80°C) on protease activity was determined as per the standard procedure. The enzyme was observed to give an optimal temperature of 40°Cas perthefindings of [11]. The temperature was recorded and is shown in Figure 2. The growth and enzyme activity of microorganisms is greatly influenced by different incubation temperature. The growth of microorganisms can be inhibited at one temperature but it can be activated at another temperature. So, it is essential to incubate microorganisms at their optimum temperature for their successful growth. The incubation temperature is usually determined by considering the sources from which the organism has been isolated. For this reason, to detect the optimum incubation temperature, the selected isolates were incubated at different incubation temperature. The bacterial isolates prefer 37°Cfor maximum production of protease which are in concurrence with the report of [12,13].

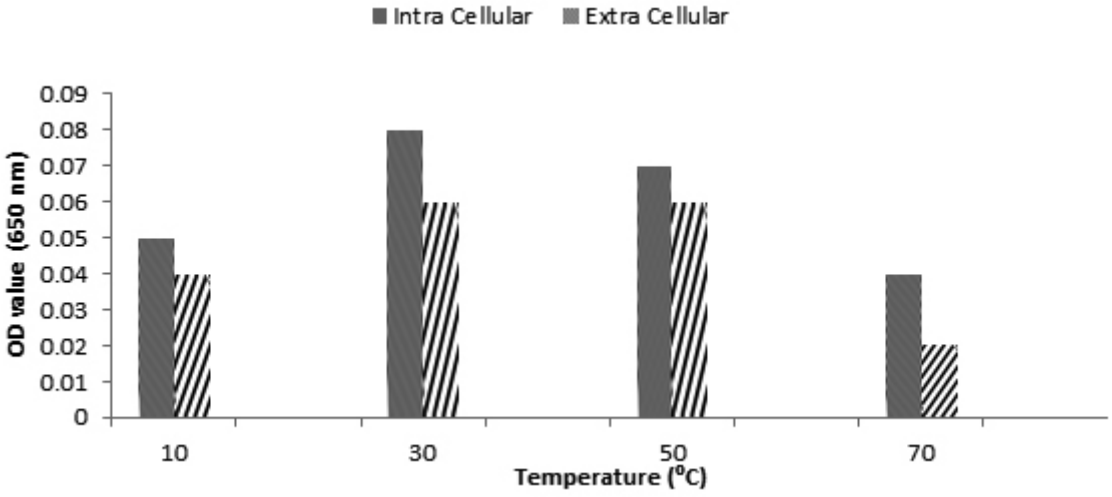

Fig. 2: *Effect of Temperature in Protease Production by Slaughter House Waste Soil.*

Effect of Protease Production in Substrate Concentration and Metal Ion Concentration

The effect of various Substrate concentrations (0.1 to5%) on protease activity was determined as per the standard procedure. The highest activity of protease was observed at 5% of casein (Figure 3). The metal ion concentration obtained in 1% of metal ionsconcentration (Mercuric chloride and Calcium chloride). The values were shown in Figures 4 and 5. The isolated crude enzyme when incubated with casein as substrate, then degradation of casein into amino acids was observed ina *Bacillus sp.* casein as a substrate. When compared with standard tyrosine it showed activity of 42 microgram/ml. Also, its activity was observed to be the maximum at pH 5.5 and temperature 27°C. *Bacillus species* was showed the maximum activity in pH 8 and Temperature is 40°C. Substrate concentration and metal ion concentration of protease obtained in 5% casein and 1% of metal ions, respectively.

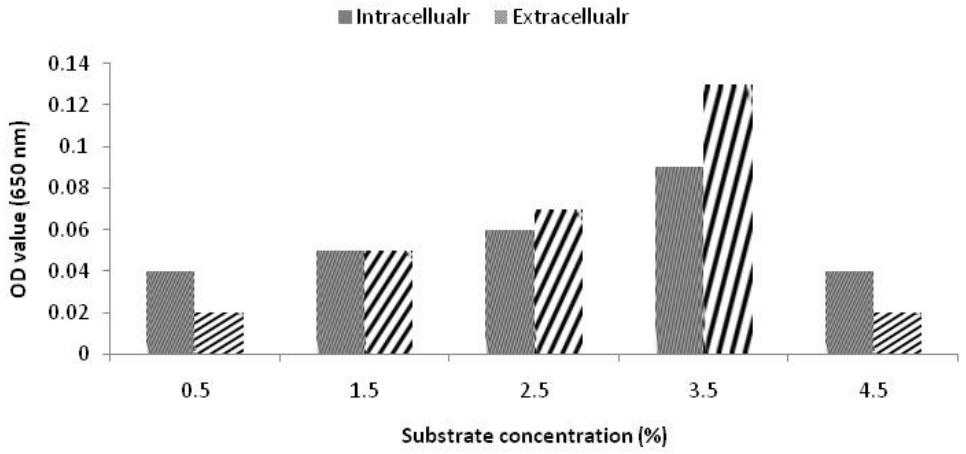

Fig. 3: *Effect of Substrate Concentration in Protease Production.*

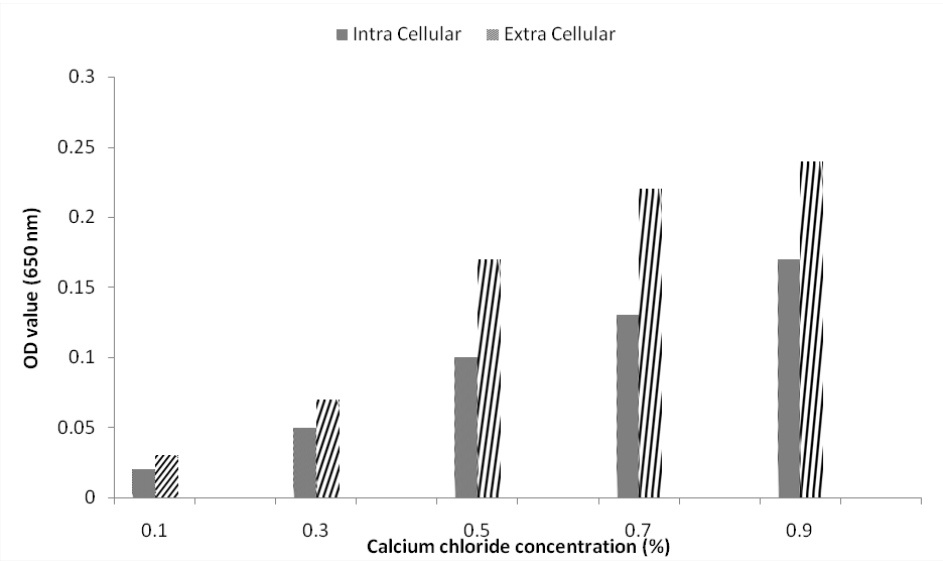

Fig. 4: *Effect of Calcium Chloride Concentration in Protease Production.*

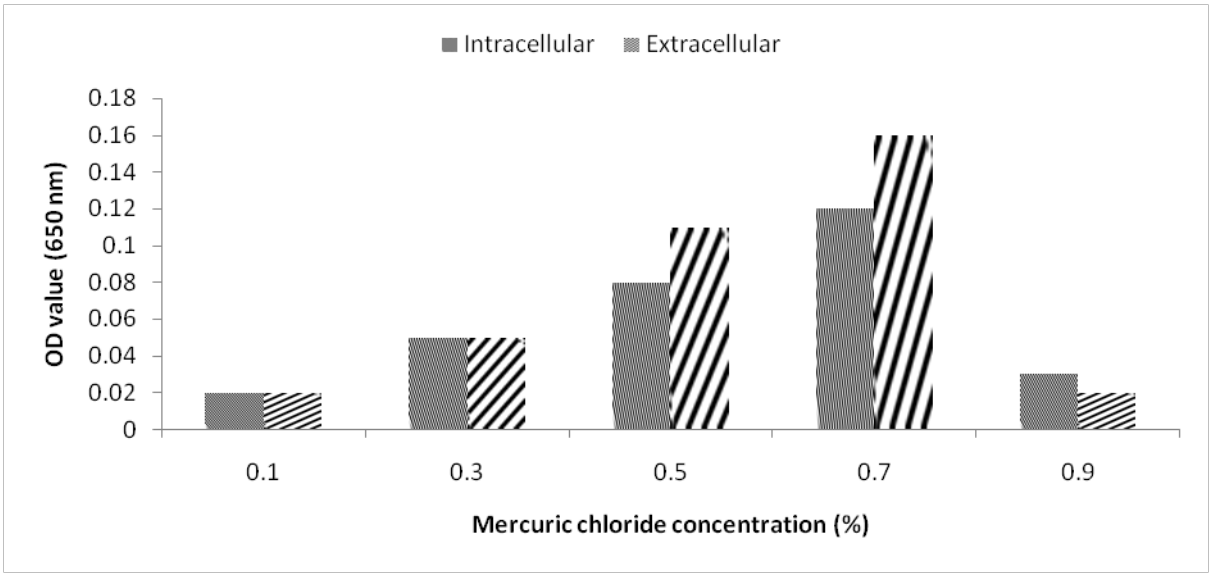

Fig. 5: *Effect of Mercuric Chloride Concentration in Protease Production.*

Purification

The protease enzyme was purified by coloum chromatography. The crude liquid was used in further studies.

CONCLUSION

Proteases have found a wide range of applications in various industries such as food, pharmaceutical, cosmetic etc. and have been widely commercialized by various companies throughout the world. Though the production of these enzymes have been improved significantly by the utilization of hyper-producing strains of fungi and bacteria and genetically modified microbes as well, efforts are still being done to find newer sources of enzymes, better production techniques and novel applications of these enzymes in unexplores fields.

The industrial production and use had assumed much significance and the process is important when compared to the application of this enzyme. There is no doubt that the use of this enzyme will be in favor of new context with new process evolved, and could model ideal catalyst with least problems of pollution and energy.

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