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ISOLATION AND IDENTIFICATION OF PROTEASE PRODUCING BACTERIA FROM SOIL

Vishnu Priya V G (1), Preethi S (1), Karthikeyan S (1) and Ramesh Babu N G (1)

(1) Department of Biotechnology, Adhiyamaan College of Engineering (Autonomous), Hosur – 635 109, Tamil Nadu, India

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Abstract

Protease producing bacteria was isolated from marine soil sample from Marakkanam Saltern, Tamil Nadu. Based on their morphology, it was identified as Pseudonomonas fluorescens biovar. The bacteria were inoculated in gelatin agar for the production of protease enzyme. The crude enzyme extract was purified by ammonium sulphate precipitation method to obtain 85% saturation. The protease enzyme activity of P. fluorescens biovar was 7.5 U/ml after 24 hours of incubation. The maximum extracellular protease production was observed at 37 ^{0}C with a specific activity of 49.02 U/mg.

Key words: *Pseudomonas fluorescens biovar, Protease enzyme, protease activity, Gelatin, Bacteria, Marine Soil Sample.*

1. INTRODUCTION

Enzymes are the biocatalysts used for enhancing metabolic rate of reactions. A large number of enzymes are produced *in vivo* having great importance in industries. Protease is the most important enzyme produced industrially. Protease enzyme is naturally present in all organisms and it corresponds to 1-5% of total protein content [1].Protease is the third largest group of industrial enzymes and has a worldwide sale of 60% [2]. Proteases can hydrolyse peptide bonds in proteins and they are also called peptidase or proteinase or proteolytic enzymes [3].

Proteases are classified into three groups based on their acid base behavior that is, acid, neutral, and alkaline proteases [4]. Acid proteases have a pH range of about 2.0-5.0 and they are produced only by fungi. Neutral pH of protease ranges from 7.0-8.0 and they are mainly of plant origin and finally proteases with pH above 8 are said to be alkaline proteases [5].

Proteolytic enzymes are ubiquitous in nature and they are found in all living organisms such as plants, animals and microbes [6]. The microbial production of proteases is preferred more than others sources because microbes can be grown and cultivated in a very small space. They grow faster and can be genetically modified easily [3].

Protease enzymes can produce eco-friendly products and so they play a vital role in modern biotechnology industries [7]. The microbial protease was lacks pathogenicity. Therefore they can be grow easily in culture medium and they have wide industrial applications [8].

Protease enzymes are produced in the marine environment as a bioactive compound. Different parameters viz; temperature, pressure, salinity, density control the synthesis of these bioactive substances. They are capable of retaining activity in extreme conditions. [9]. Marine enzymes are used to monitor pollution [10].

Protease enzymes are majorly used in various industries such as in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestives and for certain medical treatments of inflammation and virulent wounds [11]. Bacterial protease is used for accessing genetic manipulations[12].The protease producing bacterial strains are as *Bacillus subtilis, Bacillus licheniformis, and Bacillus thuringiensi.*[3].The current study aims to isolate protease producing bacterial stains from the marine soil sample. This study analysis presents the production and purification of extracellular protease by *Pseudomonas fluorescens.*

2. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

The soil samples were collected from Marakkanam Saltern, Tamil Nadu. The marine soil samples were collected in a sterilized plastic container and aseptically transferred in to a sterile polythene bag. The samples were used for bacteriological analysis.

2.2 ISOLATION AND IDENTIFICATION OF MICROORGANISMS

One gram of marine soil sample was weighed aseptically and diluted with 9ml of distilled water. The solution was diluted serially to obtain 10^{-4}, 10^{-5}, 10^{-6} dilutions. 1 ml of each of these dilutions was spread on the nutrient agar plates and they were incubated at 37 ^{0}C for 24 hours. Gelatin agar was used for screening bacterial strains having proteolytic activity. The isolates were streaked on the gelatin agar and incubated at 37 ^{0}C for 24 hours. The bacterial colonies with high zone of clearance around their colonies when flooded with saturated ammonium sulphate were selected and maintained on nutrient agar.

2.3 IDENTIFICATION OF MICROORGANISM

The bacterial strain was identified by microscopic examination (Gram Staining and Motility test) and Biochemical tests such as Citrate utilization test, Oxidase test, Methyl red test, Indole test, Voges Proskauer test, Urease test, Nitrate reduction test and

Catalyses test (according to Bergey's Manual of Systemic Bacteriology).

2.4 PRODUCTION OF ENZYME

The bacterial colonies were inoculated in the Gelatin agar plates and flasks were incubated at 37 ^{0}C for 24 hours. After growth for 24 hours, the culture was centrifuged at 10,000 rpm for 10 minutes. The crude enzyme obtained from the culture medium was used for further analysis.

2.5 ESTIMATION OF ENZYME ACTIVITY

The enzyme activity was estimated according to the method of Carrie Cupp Enyard by using gelatin as the substrate [3]. In brief, Gelatin solution containing 0.1g of gelatin in 50 mM potassium phosphate buffer, pH 7.5 was prepared. The crude enzyme was added in the test sample alone and incubated at 37 ^{0}C for 10mins. Trichloroacetic acid (TCA) was added to the assay mixture for stopping the enzymatic reaction. Then 500 mM sodium carbonate and 0.5 M Follin Ciocalteus phenol reagent was added to the assay mixture and incubated at room temperature for 30 min. Finally, the absorbance was recorded at 660 nm by using spectrophotometer (systronic). Tyrosine was as a standard for estimation of enzyme assay.

2.6 PROTEASE UNIT

One protease unit was defined as the amount of enzyme released by 1µM of tyrosine at 37 ^{0}C for one minute, pH 7.5. The enzyme activity (U/mL) was calculated by the following formula (Arun Kumar Sharma *et al.*, 2015)

Enzyme Activity (U/mL) =

$$\frac{\mu\, mol\ tyrosine\ equivalent\ releases * Total\ volume\ of\ assay}{Volume\ of\ enzyme\ taken * incubation\ time}$$

2.7 ESTIMATION OF TOTAL PROTEIN CONTENT

The amount of protein produced was estimated by using Coomassie Brilliant Blue (CBB) reagent as suggested by Bradford [13]. 50 µl of crude enzyme was diluted with 950 µl of double distilled water in 3ml of CBB reagent. The sample was incubated at room temperature for 5mins. The vial contained 500 µl of double distilled water in 1.5 ml of CBB reagent kept as blank. Finally, absorbance was observed at 595 nm by using a spectrophotometer. Bovine serum albumin (BSA) was used as a standard for protein estimation.

2.8 SPECIFIC ACTIVITY

Specific activity is the activity of an enzyme per milligram of total protein (expressed in µmol/min/mg). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins. [3]

The specific enzyme activity (U/mg) was calculated by the following formula

$$\text{Specific enzyme activity} = \frac{Enzyme\ Activity}{Total\ Protein\ Content}$$

3. RESULTS AND DISCUSSION

Isolation and identification of bacterial strains producing protease enzyme from the marine soil sample was collected from Marakkanam Saltern, Tamil Nadu. The isolation and screening was done on nutrient agar medium. Protease producing bacterial isolates was screened by the growth on gelatin agar. A group of 6 proteases producing bacteria were identified and among these, 3 strains showed highest zones of clearance. These three isolates were maintained for further studies and were named as S_A, S_B, and S_C. The screened isolates were preserved on nutrient agar plates [6].

Out of the 3 screened isolates, S_B showed maximum growth in gelatin medium and turned the gelatin broth turbid after growth for 24 hours at 37°C [3]. The isolate, S_B was identified as *Pseudomonas fluorescens* based on their microscopic morphology as gram negative and biochemical tests. The results of biochemical tests are shown in Table 1.

Table 1: Results of biochemical tests of S_B bacterial Strain

Biochemical Test	S_B Bacterial Strain
Indole production	Negative
Methyl red	Negative
Voges Prokaurer	Positive
Citrate utilization	Negative
Urea hydrolysis	Negative
Catalyses	Positive
Oxidase	Positive
Starch hydrolysis	Negative
Nitrate reduction	Negative

The bacterial strains were unable to convert tryptophan into indole and produce stable acids by the mechanism of mixed acid fermentations of glucose. The bacteria showed positive result for Voges Prokaurer test, oxidase test (Figure 1.) and catalase test. They neither hydrolyse urease and starch nor reduce citrate and nitrate. All these characteristics suggested that the bacteria was *Pseudomonas florescence sp*.

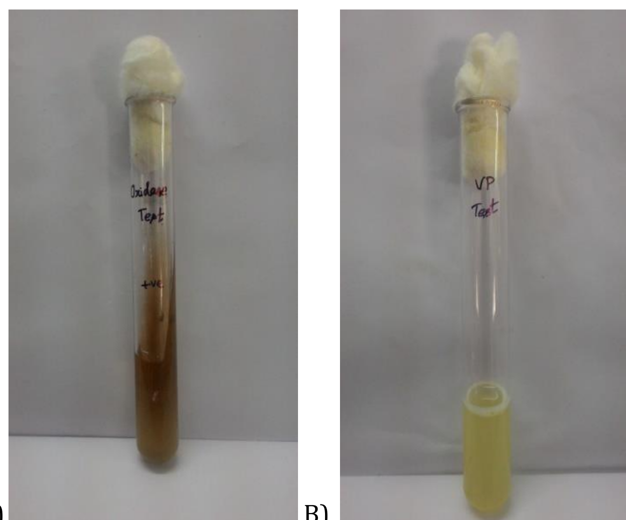

Figure 1. Results of Oxidase test (A) and Voges Prokaurer test (B)

Among the isolates, *Pseudomonas fluorescens* (S_B) was a potent producer of protease enzyme (Figure 2).The maximum amount of protease production was found after growth for 24 hours at 37 ˚C. The activity of the protease produced was determined by using potassium phosphate buffer according to Arun Kumar Sharma *et al.*, (2015)(14). The results of enzyme activity of isolated strains were shown in Table 2. These results showed that *Pseudomonas fluorescence sp* (S_B) showed maximum proteases activity (7.5 U/ml). The protease activity was minimum as reported by Arun Kumar Sharma *et al.*, (2015) who reported the enzyme activity of 243 U/mL using casein as a substrate.

Table 2: Protease activity of isolated strain

Bacterial Strain	Specific Activity (U/mg)
S_A	37.07
S_B	49.02
S_C	28.85

The total protein was estimated using protein dye binding assay as suggested by Bradford (1976). The total quantity of protein produced after 24 hours of incubation was found to be 153 µg/ml. This result was in partial in agreement with Arun Kumar Sharma *et al.*, (2015) who observed highest protease activity from *Bacillus Sp*.

The purification of the crude enzyme extract was carried out by using ammonium sulphate precipitation to obtain a saturation of 85%. After centrifugation, the precipitate was dissolved in PBS and then purified by dialysis against double distilled water over night. This sample showed higher alkaline protease activity, with a specific activity of 49.02 U/mg.

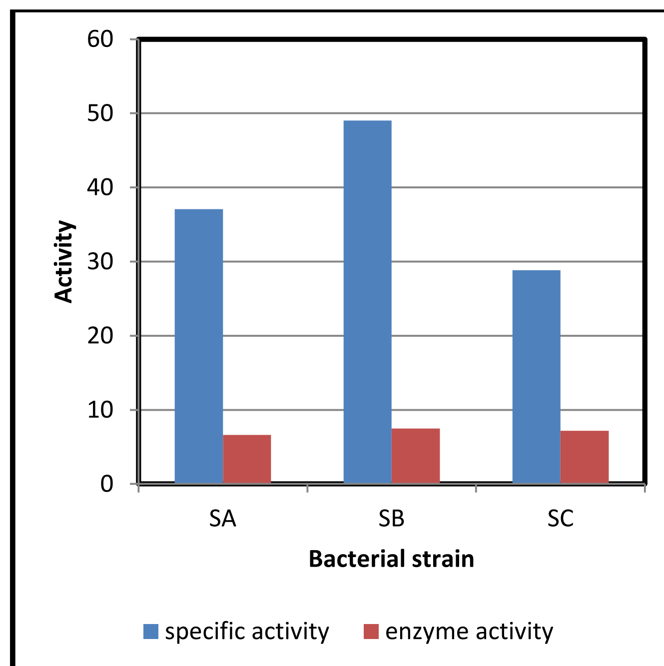

Chart - 1: Protease activity and specific activity.

4. CONCLUSION

The protease enzyme producing bacteria is still explored to achieve high quality industrial grade enzyme to produce goods. Commercial enzymes from bacteria still have demerits and to overcome with these demerits, newer organisms from newer environment may be helpful in finding a bacteria.

The present study of isolated bacterial strains showed that protease activity was maximum when using gelatin as a substrate and grew most 37 ^{0}C for 24 hrs. Further characterization is needed to explore these selected isolates as industrial protease producing strain.

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