

Screening and Optimization of Protease Enzyme from *Bacillus* sp.

¹S. Kavi Karunya, ¹A. Santhoshvar, ¹K. Sathiyaseelan,
²P. Saranraj and ³Abdel Rahman Mohammad Al-Tawaha

¹Department of Microbiology, Shanmuga Industries Arts and Science College,
Tiruvannamalai, Tamil Nadu, India

²Department of Microbiology, Sacred Heart College (Autonomous), Tirupattur, Tamil Nadu, India

³Department of Biological Sciences, Al-Hussein Bin Talal University, Maan, Jordan

Abstract: Enzymes occurring in nature as inside or on the surface of living organisms such as plants, animals and microbes especially proteolytic enzymes (proteases) are ubiquitous being found in all living organisms and are essential for cell growth. The extracellular proteases are of commercial value and find multiple applications in various sectors. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases which account for the total worldwide enzymes sale for various industrial applications. The protease producing *Bacillus* sp. was isolated from soil sample, identified by various standard bacteriological methods. The *Bacillus* sp. showed highest protease enzyme producing using skim milk agar as a substrate. The enzyme activity was showed 0.62 U/ml of *Bacillus* sp. The production of *Bacillus* sp. was optimized under different pH, temperature, carbon source, nitrogen source and incubation periods. The maximum chitinase production was observed at pH 7.0, temperature 40°C, sucrose as carbon source and yeast as a nitrogen source at 24 hours of incubation. The enzyme was partially purified by Dialysis method. Protein concentration of 250µg/ml was estimated according to Lowry's method.

Key words: Protease • Protease Activity • *Bacillus* Sp. • Optimization and Partial Purification

INTRODUCTION

Enzyme technology is an interdisciplinary field and enzymes are used in many eco-friendly industrial sectors and advancement in biotechnology especially in the area of genetics, protein engineering [1]. Proteases are a group of enzymes that have been found in several microorganisms like bacteria and fungi which are involved in breakdown of complex protein molecules into simple polypeptide chains [2]. Extracellular protease high commercial value and multiple application in various industrial sectors, such as detergent, food, pharmaceutical, leather, diagnostic, waste management and silver recovery industries [3].

Microbial proteases, especially from *Bacillus* sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide with major application in detergent formulations [4]. Proteolytic enzymes can be produced by submerged and solid state

fermentation. For the growth of fungi, Solid state fermentation is most appropriate method because it resembles the natural habitat of the fungi [5]. For the prospective uses of proteases and their high demand, the need exists for the invention of new strains of marine bacteria that produce enzymes with novel properties and the development of low cost industrial media formulations [6].

Microbes which produces alkaline protease needs to be screened and should be optimized to produce substantial amount of protease by adapting favourable conditions like optimal pH, temperature and favourable media should be demonstrated to increase its yield. Alkaline protease from extreme organisms should be produced commercially in high yield at a low-cost method [7]. Alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from

food, textile, tannery and potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources [8].

MATERIALS AND METHODS

rSample Collection: Soil sample was collected from the Tiruvannamalai central Market in different area. The soil samples are taken from 2 to 3 cm depth with the help of sterile spatula and put in sterile plastic bags and it mouth was tied properly and brought to laboratory for further processing.

Isolation of *Bacillus* sp.: One gram of soil sample was serially diluted up to 10^{-9} dilution and 0.1 ml of serially diluted sample from 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} was taken and transfers to Nutrient agar medium by spread plate technique, the inoculated Plates were incubated at room temperature for 48 hours. After the incubation the colonies were observed. To reduce the number of colonies in the inoculum, quadrant streaking is carried out.

Colony Characterization: The *Bacillus* isolates were observed under macroscopically, the colony morphology was noted with respect to colour, size, shape and nature of colony.

Identification of *Bacillus* sp.: The identification of *Bacillus* sp. was done by standard bacteriological methods viz., Light microscopy, motility demonstration, spore staining, hydrolysis of starch, hydrolysis of gelatin, plating on selective media and performing biochemical tests.

Screening of Protease Activity: The skim milk agar medium was used for the protease screening. After inoculation of the *Bacillus* sp. isolates in Skim milk agar medium, the plates were incubated for 3 to 4 days at 50°C. Opaque halos around the colonies were taken as the indication of protease activity.

Protease Assay: The culture broth was incubated at 50°C in a rotary shaker operated at 200 rpm for 24 hrs. Afterwards the bacterial cell cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was collected and assayed for protease activity. The reaction mixtures containing 1 ml enzyme solution and 1.5 % casein were incubated in a water bath at 50°C for 10 min.

The supernatant was obtained by centrifugation at 10,000 rpm for 10 min. Next, 0.4 M Na_2CO_3 and Folin's reagent were added to terminate the reaction and the reaction mixture left to stand at room temperature for 10 min. Protease activity was determined spectrophotometrically at 660 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 U/ml of tyrosine per min at 660 nm under control conditions. Specific activity was expressed as units per mg of protein of the enzyme extract.

Optimization of Cultural Conditions

pH Optimization: The effect of pH values was carried out to determine the optimum pH value for protease productivities by *Bacillus* sp. The pH was adjusted at pH – 5, pH – 6, pH – 7 pH – 8 and pH – 9 for the production media using 1 N NaOH or 1 N HCl.

Temperature Optimization: The effect of temperature values of was carried out to determine the optimum temperature value for protease productivities by *Bacillus* sp. Protease production was studied by incubating the production medium at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C temperature.

Carbon Sources Optimization: The effect of different carbon sources was studied for protease production by *Bacillus* sp. Four different carbon sources viz., Glucose, Maltose, Galactose, Lactose, fructose and Sucrose were selected for this present study. (Add reference).

Nitrogen Sources Optimization: The effect of different nitrogen sources was studied for protease production by *Bacillus* sp. Four different nitrogen sources viz., Peptone, Ammonium chloride, tryptone, meat extract, Yeast extract and Ammonium citrate were selected for this present study. The protease production by replacing 1 % tri-sodium citrate in the production medium with 1 % and carbon source. The flasks were incubated at 55°C on shaker for 24 hrs.

Incubation Optimization: The optimization of incubation time required for protease productivities by *Bacillus* was studied at 12, 24, 48, 72 and 96 hrs.

Partial Purification by Dialysis: The culture supernatant fluid was subjected to precipitation with ammonium sulphate to 80 % saturation and stirred for 2 hours. Then the precipitate was allowed to stand for overnight

and then collect by centrifugation at 10000 rpm at 4°C for 30 min. The precipitated pellets were dissolved in phosphate buffer and centrifuged and dialyzed against the same buffer which was then partially purified.

Estimation of Protein Content: The protein concentration was estimated by the following the method of Lowry *et al.* [9] using Bovine serum albumin as the standard

RESULTS AND DISCUSSION

Proteolytic enzymes (proteases) are ubiquitous being found in all living organisms and are essential for cell growth. The extracellular proteases are of commercial value and find multiple applications in various sectors. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases which account for the total worldwide enzymes sale [10].

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids [11]. Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [12]. *Bacillus* produces a wide variety of extracellular enzymes, including proteases. Several *Bacillus* species involved in protease production are e.g. *Bacillus cereus*, *Bacillus sterothermophilus*, *Bacillus mojavensis*, *Bacillus megaterium* and *Bacillus subtilis* [13].

The proteolytic bacteria was isolated from Market soil of Tiruvannamalai district and further identified, characterized by their features as Gram positive, rod shaped motile organisms. Finally, the morphological and biochemical test tentatively indicated that the isolated organisms were *Bacillus* sp. Sara Seifzadeh *et al.* [14] grown the bacterial strains in Nutrient media containing salt at various concentrations (up to 15 % (w/v)). The strain was able to grow at temperatures as high as 50°C. The combination of morphological, physiological and biochemical data suggested that this strain belonged to the *Bacillus* species.

The isolated *Bacillus* sp. strains with proteolytic activity on Skim milk agar plates. In total 5 isolates were isolated from the Tiruvannamalai Market soil samples.

Among them, two isolates *Bacillus* sp. (B2) (16 mm) and *Bacillus* sp. (B5) (15 mm). Among these two isolates *Bacillus* sp. (B2) was selected for further studies (Fig. 1). Protease activity was determined at 50°C by using casein as a substrate. The enzyme activity was showed 0.62 U/ml of *Bacillus* sp. (B2).

The effect of isolated *Bacillus* sp. isolate (B2) for the protease enzyme production was studied at different pH viz., pH – 6, pH – 7, pH – 8 and pH – 9. The maximum protease activity of *Bacillus* sp. (B2) was noticed at the pH – 8 (Figure 2). The protease enzyme productions were determined at differ rent temperatures viz., 30°C, 40°C, 50°C, 60°C, 70°C and 80°C (Figure 3). The maximum protease activity of *Bacillus* sp. (B2) was observed at the temperature 40°C. Sepahy and Jabalameli. Sepahy and Jabalameli [15] reported the *Bacillus* sp. in the production of protease at the pH 7 and pH 8 was relatively comparable. Proteases from *Pseudomonas aeruginosa* MN1 and some other *Bacillus* species have been described with optimum temperature of 60°C [16].

Optimization of *Bacillus* sp. by different carbon sources viz., Glucose, Maltose, Lactose, Galactose, Fructose and Sucrose. The maximum protease activity was recorded in the presence of Sucrose (Figure 4). The isolated *Bacillus* sp. isolate (B2) were estimated for its protease enzyme production against the different nitrogen sources viz., Peptone, Ammonium chloride, Meat extract, Yeast extract, tryptone and Ammonium citrate. Maximum protease production was exhibited in the presence of Yeast extract as nitrogen source (Figure 5).

Kumara *et al.* [17] have evaluated various carbohydrates such as glucose, maltose, lactose, sucrose, mannitol, sorbitol, raffinose, xylose, fructose and starch for their effect on protease production. Carbon sources greatly influenced the enzymes production and the most commonly used substrate was reported to be glucose. Joo *et al.* [18] and Patel *et al.* [19] found that different nitrogen sources such as soybean meal, glucose, casamino acids and peptone were effective medium ingredients for the protease production by *Bacillus* sp. Vonothini *et al.* [20] and Manivasagan *et al.* [21] also observed maximum enzyme activity in the beef extract as nitrogen source in action bacteria.

The effect of isolated *Bacillus* sp. isolate (B2) for the protease enzyme production were studied at different incubation hours viz., 12, 24, 48, 72 and 96 (Figure 6). The maximum protease activity was exhibited at 24hrs of incubation. Shumi *et al.* [22] reported maximum production of proteases with 48 h of incubation. Ammar *et al.* [23]

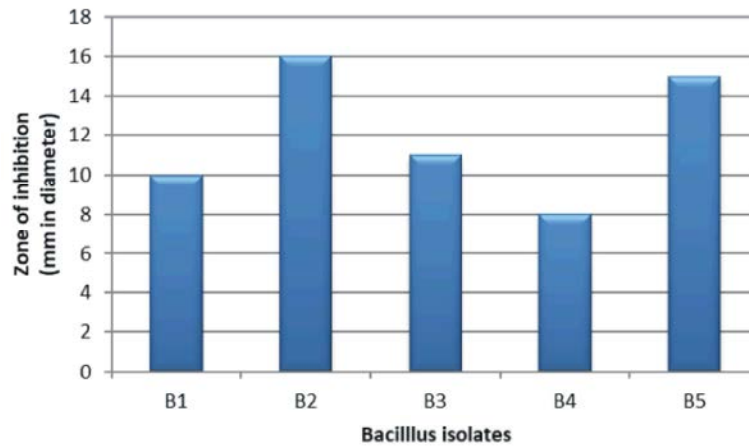


Fig. 1: Screening of *Bacillus* sp. for its protease production

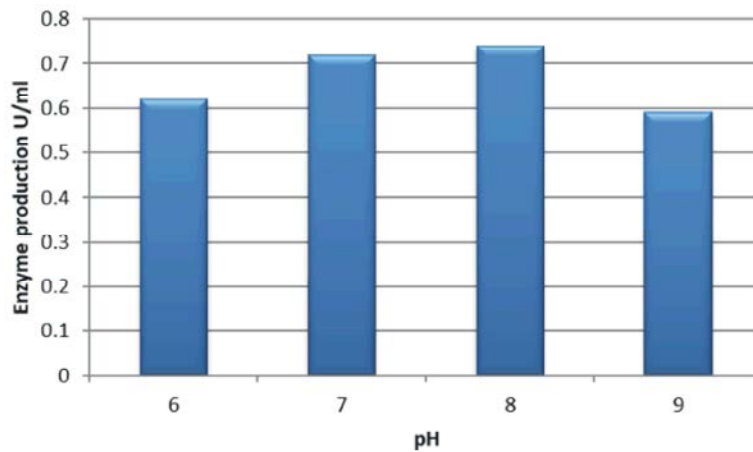


Fig. 2: Different pH on protease production by *Bacillus* sp.

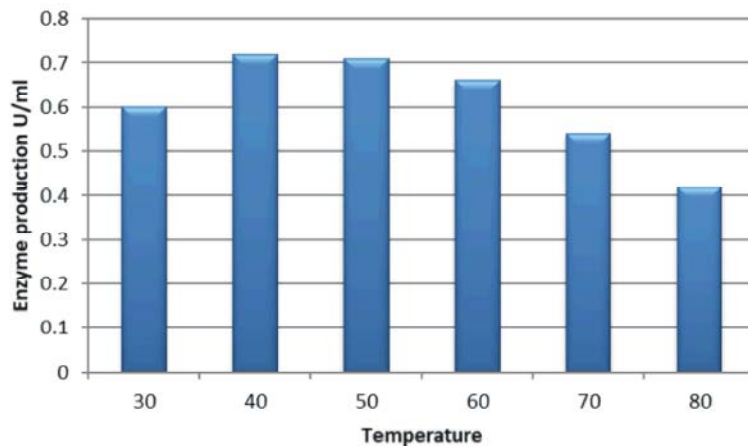


Fig. 3: Different temperatures on Protease production by *Bacillus* sp.

also reported that the optimum incubation period for thermo stable purified protease enzyme as ranging from 60 to 72 hrs. The amount of protein present in the culture precipitate was estimated and the culture was partially

purified by Dialysis method. The concentration of protein was estimated by Lowry's method using bovine serum albumin as substrate and the protein concentration was 250 µg/ml.

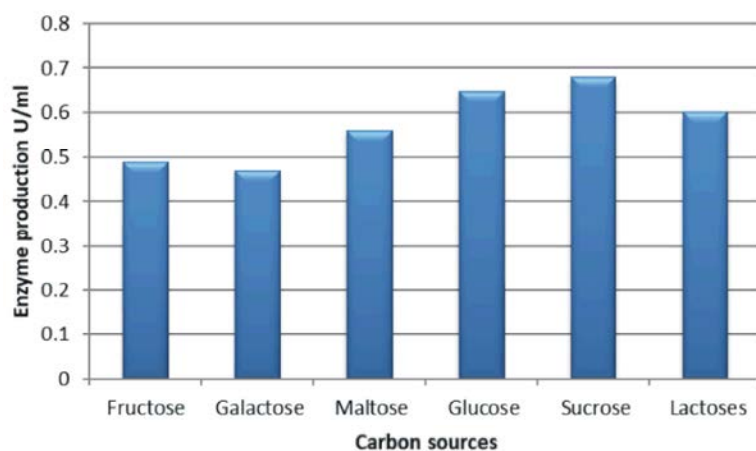


Fig. 4: Different carbon sources on Protease production by *Bacillus* sp.

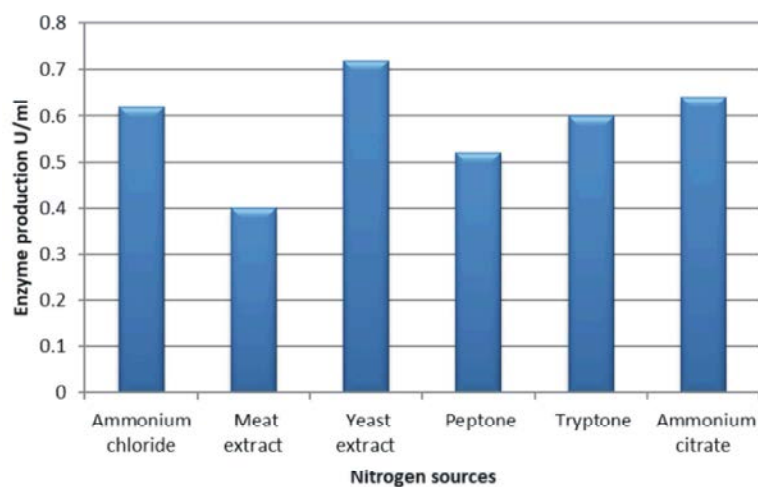


Fig. 5: Different nitrogen sources on protease production by *Bacillus* sp.

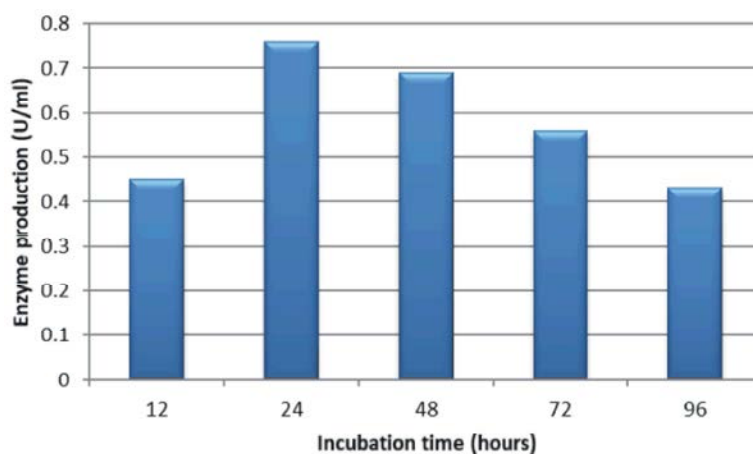


Fig. 6: Different incubation on protease production by *Bacillus* sp.

CONCLUSION

Protease constitutes one of the most important groups of commercial enzymes accounting for

approximately 60 – 65 % of the global enzyme market. Proteases can be produced from a wide range of organisms such as Bacteria, yeast, molds, plants and animals. However, bacterial proteases represent an

excellent source of protease enzymes in comparison with others. Protease enzymes are of particular interest for Bioengineering and Biotechnological applications. Proteolytic bacteria isolated from the soil samples, these isolates were studied in respect of enzyme activity, purification. The best results are obtained with casein as substrate in the pH-8, temperature 40°C, lactose as sucrose source, Ammonium citrate as the nitrogen source. The present investigation produces more protease enzymes and it to be applied for various applications.

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