PRODUCTION OF PROTEASE ENZYME FROM *BACILLUS SUBTILIS* USING SKIMMED MILK

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ABSTRACT: *Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management, silver recovery, healing of the wound, cosmetics, etc. Of all proteases, alkaline proteases produced by Bacillus species are of great importance in the detergent industry due to their high thermostability and pH stability. Submerged fermentation technique was employed for the production of Protease by Bacillus subtilis using cheese whey & skimmed milk broth followed by ammonium sulfate precipitation and molecular weight determination by SDS PAGE. Protease characterization showed that it has a maximum enzyme activity at pH 9, temperature 45°C, incubation period 20 minutes and gelatin substrate is suitable in enzyme assay.*

Key Words: Proteases, *Bacillus subtilis*, fermentation, skimmed milk

INTRODUCTION

Proteases enzymes are bio-catalytic alkaline having the ability to hydrolyze the peptide bond of proteins[1] are with its many roles in living organisms that include in all cell differentiation and growth and they are also known as industrially important as the previous study notified its industrial importance because of 60% dependency of total enzymes market. Proteases or biocatalytic enzymes present in plants, animals, and microorganisms. These are extracellular enzymes [2], these microorganisms produce enzymes faster than mammalians[3] and plants. *Bacillus subtilis* are a wide source of certain enzymes such as amylase and Protease [4]. It formed the heterologous proteins and 8 types of extracellular protease [5]. Protease enzymes from a microbial origin is in huge demand to be manipulated in industrial processes [6]. Past studies have observed that there are many organisms that have the ability to producing proteases such as *Serratia marcescens, Aspergillums oryzae and Bacillus subtilis* [1]. Among them, Bacillus species are preferred because there growth rate is rapid and the cultivation time period is low, needs less space and is easy to be genetic manipulated [7]. *Bacillus subtilis* is known as hay bacillus and grass bacillus [8], is a rod-shaped Gram-positive bacterium. Proteases do not refer to a single enzyme but a mixture of enzymes including proteinases, peptidases, and amidases. These proteolytic biological catalysts are important biochemicals that have magnificent importance and advantages into the renewable sources[9] & biodegradability[10] and these proteases have advantages in the chemical industry, pharmaceutical industry food processing leather beverages[11]. The thrombolytic agent is very expensive, in order to avoid the cost, bacillus species is used for the formation of protease which acts as a thrombolytic agent [12] Proteases from the bacillus can apply in food for obtaining the bioactive peptides. Proteases are used in the cure of certain diseases such as healing the wound, Osteoarthritis and removes dead tissues [13]. Protease is manipulated in bioremediation [4] as a biological control agent [14] and silver recovery [15]. It is stable at various temperatures, pH and formed stable products in a rigid environment [16]. The crude enzyme is can be utilized to avoid the purification cost [17].

The tremendous biodiversity of microorganisms that improves their biotechnological importance gives a reason to search more about proteases enzyme production. In this study, we produced protease enzyme from skimmed milk using *Bacillus subtilis* isolated at PCSIR Lahore and determine which temperature, pH, substrate and incubation period is good for protease production followed by partial enzyme characterization.

MATERIALS AND METHODS:

Source of Strain:

Bacillus subtilis already prepared slant culture of strains PCSIR-NL23, PCSIR-NL27, PCSIR-NL31 were obtained from the Molecular Biology laboratory at FBRC, Pakistan Council of Scientific and Industrial Research (PCSIR), Lahore, Pakistan. The culture was further sub-cultured onto Nutrient agar for the purpose of the present study.

Inoculum Preparation:

Skim milk broth (2% skim milk, 1% tryptone, 0.5% sodium chloride, 0.5% yeast extract) was inoculated with PCSIR-NL23, PCSIR-NL27, and PCSIR-NL31, and then incubated at room temperature in a shaker incubator for 48 hr. In the other hand control medium (50ml) was also prepared, autoclaved and incubated.

Selection of Best Strain:

Protease assay and Lowry Protein assay was performed after 48hours to determine the proteolytic enzyme activity and total protein in the broth of (NL-23, NL-27 and NL-31) to find out the best strain by the results of Optical Density (OD) at 660nm.

Protease Assay

One milliliter of inoculum solution from Skim milk media containing inoculum of *Bacillus sp* i.e. NL23, NL27, NL31 into 3 aliquots and one control was centrifuged at 10,000rpm for 5 minutes. The supernatant (500ul) was transferred to four clean test tubes. 500ul of 1% casein solution was added in each test tube containing the supernatant. These test tubes were placed in a water bath at 50°C for 15 minutes. Two milliliters of 5% TCA (Trichloroacetic acid) was added to the test tubes to stop the reaction. One ml from these test tubes was aliquoted and centrifuged at 10,000 rpm for 20 minutes. 1ml supernatant was transferred into a new test tube and 3ml of $Na_2Co_3$was added in a test tube followed by the addition of 200ul (1:1 diluted) folin reagent into a test tube. The reaction was incubated at room temperature for 15 minutes. Optical density at 660nm was measured. All the enzyme assay experiments were carried out in triplicate and the mean

values were recorded. The standard curve of tyrosine was plotted.

One Unit is defined as the amount of enzyme that is used to hydrolyze casein to produce color equivalent to 1 µM of tyrosine under standard assay conditions.

Enzyme Extraction and partial purification:

For enzyme extraction, one liter of skim milk broth was prepared, inoculated with PCSIR-NL23 and incubated at 37°C for 48 hours. After the incubation completed all the protease producer media was centrifuged at 10000rpm for 15 min at 4°C. Centrifugation recovered the clear supernatant and crude enzyme was processed for further procedure and purification by the salting-out method by using $(NH_4)_2SO_4$, ammonium sulfate.

Dialysis

Dialysis tubing was sterilized by indirect heating at temperature 95^{o}C for 15 minutes. After this, the knot was tied at one end. The precipitated crude enzyme was poured into dialysis tubing and knot on the other end was tied. The enzyme was dialyzed against 50mM glycine buffer in a clean beaker on a magnetic stirrer for 24 hours at 4^{o}C. Performed the dialysis for 2-3 times.

Molecular weight determination

Polyacrylamide gel electrophoresis was conducted to determine the purity and molecular weight of the proteolytic enzyme extracted. Native PAGE was run according to the method of Davis (1964).

Characterization of Protease enzyme

1-Temperature effect on the activity of Protease:

Incubation Temperature varies from 20-100°C was used to analyze the activity of protease with an interval of 5^{o}C for 15 minutes. The protease enzyme assay is performed at each temperature to evaluate the optimal temperature for the activity of the protease.

2-pH effect on the activity of protease:

pH effect was observed on protease activity at different pH ranging from 2.0 to 11.0 by incubating the enzyme-substrate using following buffers 0.5M citrate buffer (pH 5), 0.5 M phosphate buffer (pH6 & 7),0.5 M Tris HCl (pH: 8 & 9) and 0.5 M glycine NaOH buffer (pH: 10 & 11) at 50°C for 15 minutes from skim milk broth.

3-Time frame study

Protease's best activity time was studied by carrying out different assay incubation time ranging from 5 minutes to 60 minutes with an interval of 5 minutes.

4-Substrate concentration effect

The activity of protease was carried out by varying the concentration of casein as a substrate. The various concentration range of substrate was 0.1-1.5%.

Results and Discussion

Extracellular proteases are important for the hydrolysis of protein in cell-free environments and enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes. During the study reported here, the proteases enzyme was produced using skimmed milk as a substrate. Figure 1 showed the proteolytic activity of the strains on a casein agar plate in the form of clear zones.

Figure 1: Proteolytic Zone on Casein Agar Plate

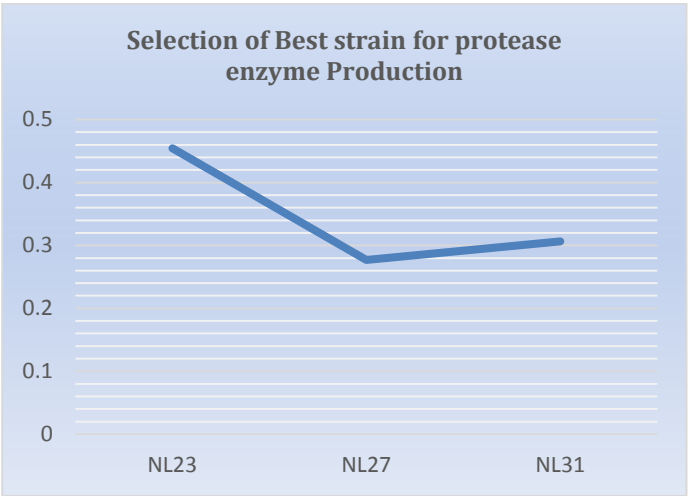

Figure 2: Strain Selection

Protease assay was performed in order to determine which strain possesses maximum protease production. The assay was performed for the extracellular proteases produced from three strains used in the study i.e. PCSIR-NL23, PCSIR-NL27, PCSIR-NL31. Among them, PCSIR-NL23 gave an exuberant protease activity while the rest of them falls down.

Assay for proteases activity was performed at various temperatures such as 30°C, 37°C, 45°C, 55°C, 60°C. Protease activity was highest at 45°C temperature (Figure 3).

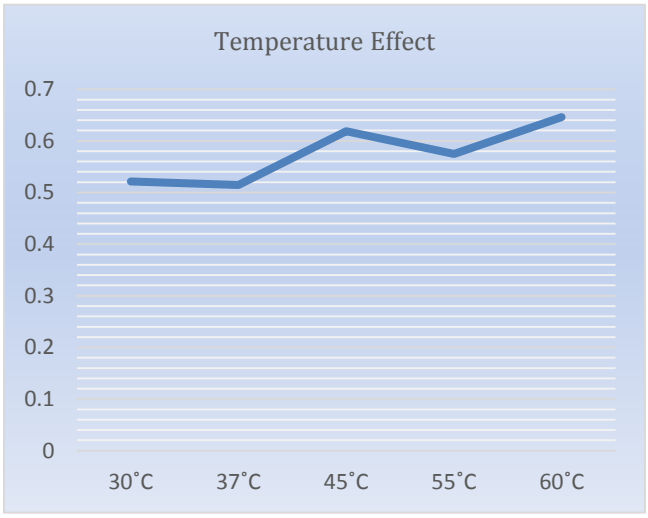

Figure 3: Effect of Temperature on Enzyme Assay

Protease assay was performed at different pH using buffers such as Citrate pH 5, Phosphate pH 7, Tris pH 9, and Glycine pH 10 in order to know which pH shows the maximum protease activity. Tris pH 9 showed the maximum protease activity (Figure 4). The protease was generally showed

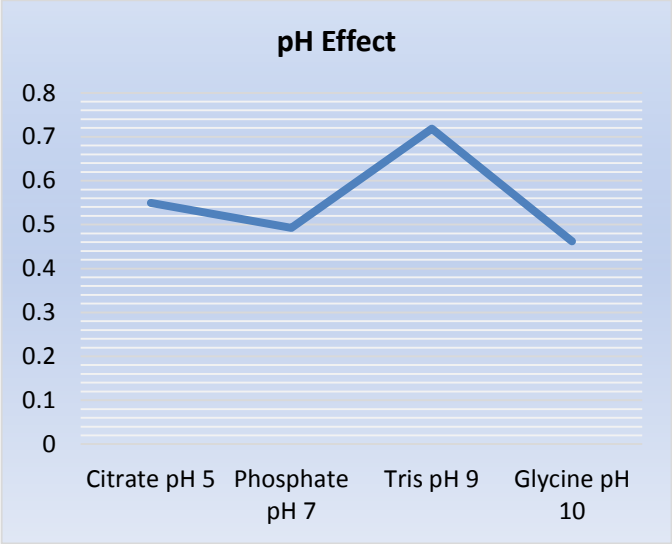

Figure 4: Effect of pH on Enzyme Activity

maximum activity at pH 7.5-8.0 [4], Noh, *et. al.*, reported protease that was stable at pH 8.0–10.0 and temperature 50–60°C [18] while our protease worked best at alkaline pH 9. Extracellular enzymes secreted by bacillus species can tolerate alkaline pH [19]

Protein was partially purified by 80% ammonium sulfate precipitation and dialyzed. The partially purified extracellular enzyme was run on SDS-PAGE for determination of molecular weight that was found ~40 kDa. Lane 1 was prestained protein marker 10-170kda (26616) ferments, lanes 6 & 7 were crude protease and lane 8 showed partially purified proteases.

The proteases enzyme assay was performed to compare three substrates (Figure 5), gelatin was found good during our study.

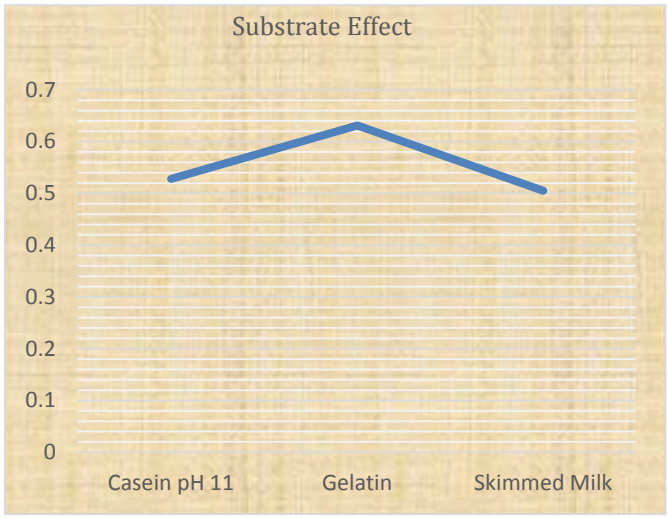

Figure 5: Substrate Effect in Proteases Assay

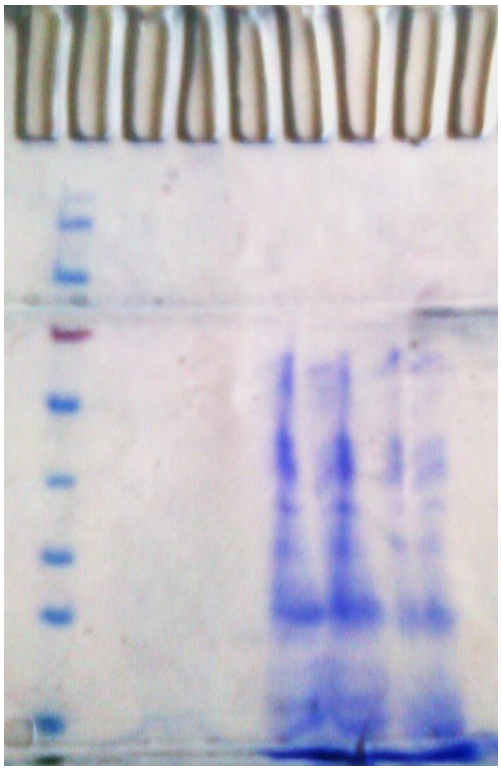

Figure 6: SDS PAGE analysis of partially purified Proteases enzyme

CONCLUSIONS

In this study protease production from dairy by-products is a cheaper, time saving and effective method. We conclude that Protease from *Bacillus subtilis* showed optimum production at 45˚C in media with pH 9 for 96 hours incubation. We explore1.5% gelatin in place of casein in the enzyme assay and found it best. The protease was used in the industrial and health sector and can play a vital role in the economic growth of the country.

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