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Optimization studies of Protease enzyme in in-vitro conditions from *Bacillus licheniformis*

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Abstract

Protease enzymes are of immense commercial value and find its applications in various industrial sectors. Microbes serve as a major source for proteases enzyme especially Bacillus strains. In this study protease producing bacteria was isolated from the soil sample. Screening was done on skim milk casein agar media. The colony with the highest protease production was used as the test organisms and maintained on the nutrient agar slants. The isolated protease producing microorganism was characterized morphologically and biochemically by Bergey's Manual of Determinative Bacteriology and identified to be as Bacillus licheniformis. Optimization studies were taken up with two physical and two chemical parameters. The organism showed maximum enzyme production at pH of 8.0, temperature 37°C, with 1.5% carbon source and 1.0% nitrogen source. Mass production was carried out for the protease enzyme with optimized media parameters and purified by centrifugation, ammonium sulphate precipitation (salting out), dialysis and Sephadex G-100 gel chromatography. The enzyme activity of the purified protease was assayed to be 6.66 U/ml. The 12% SDS-PAGE has revealed a unique linear band with a molecular weight of 52-53kDa. The results of this study revealed that the bacterial strain Bacillus licheniformis is a potent source for protease enzyme.

Keywords: Protease, Skim milk casein agar, *Bacillus licheniformis*, SDS-PAGE.

Introduction

The enzyme Proteases are capable of hydrolyzing proteins with the addition of water molecules to peptide bonds and catalyze peptide fragment synthesis in organic solvents and in the solvents with low water content[1]. Proteases are the class of enzymes, which holds an important place in both physiological and commercial fields[2]. Proteases are one of the largest and most diverse families which primarily known as enzymes of digestion. Proteases are classified under Group III Hydrolases by International Union of Biochemistry and Molecular Biology[3].

Proteases are obtained from plants, animal and microorganisms, with the greatest part acquired from bacteria and fungi[4]. Approximately microbial protease stands 40% of the total enzyme sales worldwide[2]. Proteases from microbial sources are preferred from plant and animal sources because of its desired characteristics for their biotechnological and industrial applications[1]. There are several reports on the proteases from microorganisms like *Bacillus sp*, *Microbacterium*[5,6] *Penicillium sp*, *Aspergillus sp*[7]. *Bacillus* is being the most prominent its serve as an ideal source for enzyme production because of their rapid growth[8]. Several species are involved in the protease production such as *Bacillus stearothermophilus*[9], *Thermus aquaticus*[10], *Bacillus licheniformis*[11], *Bacillus subtilis*[12], *and Bacillus licheniformis*[13].

Proteases account for nearly 60% of the total industrial market [8,14] and have wide applications in many industries viz., textiles, detergents[15,16], food processing especially for cheese ripening, meat tenderizing, animal nutrition, pharmaceuticals, paper industry and food industry[16,17].

In the present study, we report the isolation of protease enzyme producing *Bacillus sp* from soil sample, and then focus on optimizing the media parameters for increased production of extracellular protease.

Material and Methods

Sample collection: Two soil samples were collected using sterile plastic bag at a depth of around 15cm from dump yards in Domlur, Bangalore, India.

Isolation and Screening for Protease enzyme: The isolation was carried out for the two soil samples by standard isolation procedure and incubated at 37ºC for 24 hours. Individually isolated bacterial colonies were characterized morphologically, purified and subcultured on nutrient agar slants. Obtained bacterial isolates were screened for protease enzyme production by spot inoculating it on the skim milk agar (Skim milk powder 28g/l, Casein hydrolysate 5g/l, Yeast extract 2.5g/l, Dextrose 1g/l, Agar 15g/l, pH -7.0) plates and incubation at 37°C for 24-48 hours. Proteolytic activities of the isolated organisms were detected by clear zone of hydrolysis. Colonies which showed

maximum zone of clearance were selected for further experimental studies.

Biochemical Identification: Bacteria which showed maximum protease production was maintained as the test organisms and identified by various biochemical tests as per *Bergey's Manual of Determinative Bacteriology*[18].

Quantitative Determination of Protease Enzyme: Protease enzyme was assayed by the method described by Folin O. et al 1929 [19] with slight modification. 0.5 ml of the clear enzyme supernatant was added with 1.0 ml of substrate solution (1% casein in 20mM Tris-HCl buffer of pH 7.4) and incubated at 37°C for 30 minutes. To stop the reaction 1.0 ml of 10% Trichloroacetic acid was added with incubation at 37°C for 1 hour. To the above mixture 1.0 ml of 500mM Na_2CO_3 and 1.0 ml of Folin-Ciocalteu's solution (1:1 dilution) were added and incubated at 37°C for 30 minutes. The absorbance (O.D) was read at 660 nm against blank. 25-1000µg/ml Tyrosine was used to obtain standard curve. One unit (U) of protease was defined as the amount of enzyme that would be required to produce 1 µg of tyrosine in one minute under the defined assay conditions.

Media Optimization: Optimization was done for maximum protease production by varying two physical and chemical parameters of Production media (w/v: Peptone 0.5%, Glucose 0.75%, $MgSO_4$ 0.5%, KH_2PO_4 0.5%, $FeSO_4$ 0.01% Casein 0.5%) [20] Such as pH, Incubation Temperature, Concentration of Carbon source and Nitrogen source.

Effect of pH for enzyme production: The effect of pH on protease enzyme production was determined by preparing broth with varying the pH from 5.5 to 9. pH was adjusted with 1N NaCl or 1N HCl. Protease enzyme quantification was performed after 48 hours of inoculation with the identified *Bacillus licheniformis*.

Effect of Temperature for enzyme production: The effect of incubation temperature on protease enzyme production was determined by preparing broth with pH 8. The media was then inoculated with *Bacillus licheniformis* and incubated at different temperature such as 15°C, 20°C, 25°C, 30°C, 37°C and 55°C. Protease enzyme quantification was performed after 48 hours of incubation.

Effect of Carbon source concentration on enzyme production: Optimization of carbon source was done by preparing the production media with different concentration of glucose ranging from 0.1% to 2.5% keeping the other constituents constant. Protease enzyme quantification was performed after 48 hours of inoculation with the identified *Bacillus licheniformis*.

Effect of Nitrogen source concentration on enzyme production: Optimization of nitrogen source was done by preparing the production media with different concentration of peptone ranging from 0.1% to 2.5% keeping the other constituents constant. Protease enzyme quantification was performed after 48 hours of inoculation with the identified *Bacillus licheniformis*.

Mass production: Protease production was carried out in 1.5 liter Sartorius B-Lite fermentor for the *Bacillus licheniformis* using the optimized media (w/v: Peptone 1.0 %, Glucose 1.5%, $MgSO_4$ 0.5%, KH_2PO_4 0.5%, $FeSO_4$ 0.01% Casein 0.5%) with pH-8 and temperature 37°C for 48 hours. After completion of fermentation the product was drawn out in a sterile conical flask and subjected to centrifugation at 10,000 rpm for 10 minutes. The upper clear supernatant was used as crude enzyme.

Enzyme Purification: Enzyme precipitation was carried out overnight at 4°C with 75% ammonium sulphate saturation. Precipitated protein was dissolved in 50mM phosphate buffer (pH-7.2) and dialyzed against same buffer three times at 4ºC. Around 2ml of the partially purified enzyme was subjected to Sephadex G-100 (MP Biomedicals, Mumbai) gel chromatography for further purification. 3ml of sample fractions was collected in test tubes at the flow rate of 20ml/hour with the same phosphate buffer as elution buffer and absorbance was read at A_{280nm}. Fractions with maximum absorbance were assayed for the protease activity. The molecular weight of column chromatography purified protease was determined by 12% sodium do-decyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) [21].

Results and Discussion

Isolation of microorganisms: Two soil samples were used for isolation. The 10^{-5} dilution of the soil sample 1 has showed 102 CFU/ plate and soil sample 2 has showed 49 CFU/ plate.

Morphological characteristics: Isolates from two different soil samples were categorized morphologically into 9 types on the basis of its colony morphology and Gram staining.

Screening and identification: Morphologically categorized bacterial isolates were screened for protease enzyme producing ability on skim milk agar. The clear zone of hydrolysis was observed for the samples S-4 and S-8. The sample S-4 was found to have maximum enzyme production with 16mm diameter clear zone compared to the sample S-8 with 9 mm. The test organism S-4 was identified based on Bergey's manual determinative bacteriology as *Bacillus licheniformis*.

Media optimization: Optimization studies were carried out for pH, Temperature, Carbon source and Nitrogen source. The enzyme quantification was done by Folin-Ciocalteu's method. The pH and temperature studies of *Bacillus licheniformis* have showed the maximum protease production at pH -8 (figure-1a) and Temperature 37 ºC (figure-1b). Upon optimization of carbon and nitrogen source with different percentage, 1.5% Glucose (figure-1c).and 1.0% Peptone (figure-1d) has given maximum production.

Mass production and Purification: Production was carried out for 48 hours and the enzyme product was purified partially by ammonium sulphate precipitation and Dialysis. The dialyzed proteins then subjected to Sephadex G-100 column. The fractions given maximum absorbance at 280nm (figure-2) were assayed for the proteolytic activity. The fraction 8 showed maximum enzyme activity of 6.66 U/ml with tyrosine standard curve (figure-3). Protease in the active fractions are analyzed by 12% SDS-PAGE (figure-4) and showed presence of a single band near to 52-53 kDa marker protein.

Discussion: Proteases are the important biological enzymes involved in cell division, protein synthesis, protein degradation, protein transportation across membranes and gene expression. In the present study, Screening of micro- organisms isolated from two soil samples has showed two positive organisms for protease enzyme by using the casein agar plates. Maximum protease producing bacteria was identified as *Bacillus licheniformis* by using morphological and biochemical characteristics.

The optimization studies for enzyme production increased proteolytic activity of the micro-organisms[22,23]. The optimization studies on *Bacillus licheniformis* have showed maximum enzyme production at pH 8, confirming with the previous studies obtained by Folasade M[24] and Udandi[25].

As earlier studies reported[26,27,20] the maximum enzyme production was obtained at the temperature 37°C for *Bacillus licheniformis*. For carbon source, 1.5% glucose concentration has showed highest and for nitrogen source 1.0% peptone was found maximum with enzyme production.

75% ammonium sulphate saturation has showed precipitation of crude protease enzyme as given by the earlier studies[27]. Further purification by dialysis and Sephadex gel chromatography enzyme has showed a unique band on 12 % SDS-PAGE. The approximate molecular weight of the purified enzyme was found to be 52-53 kDa[28] using standard protein markers.

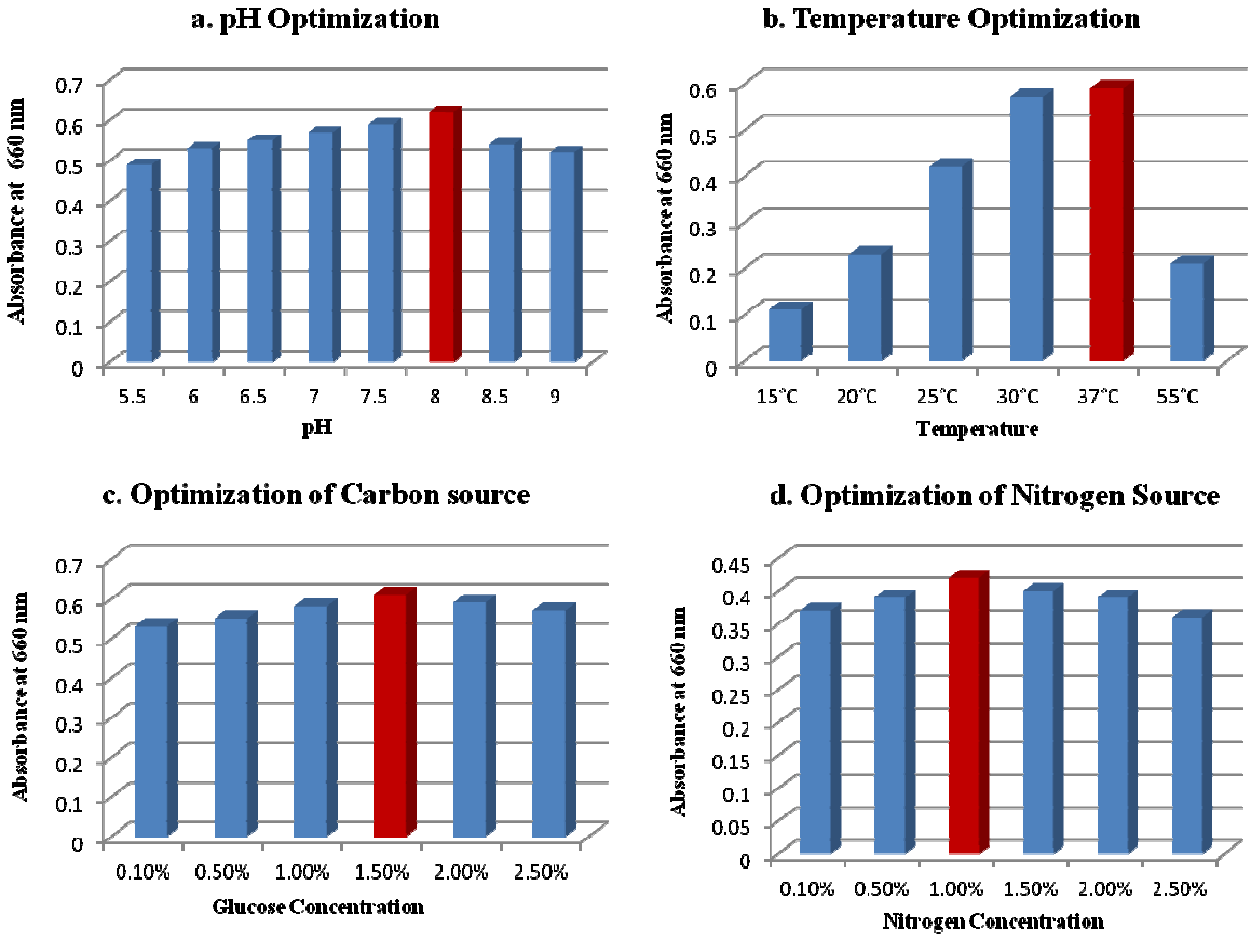


Figure-1
Effect of pH, Temperature, Carbon source and Nitrogen source on Enyme production

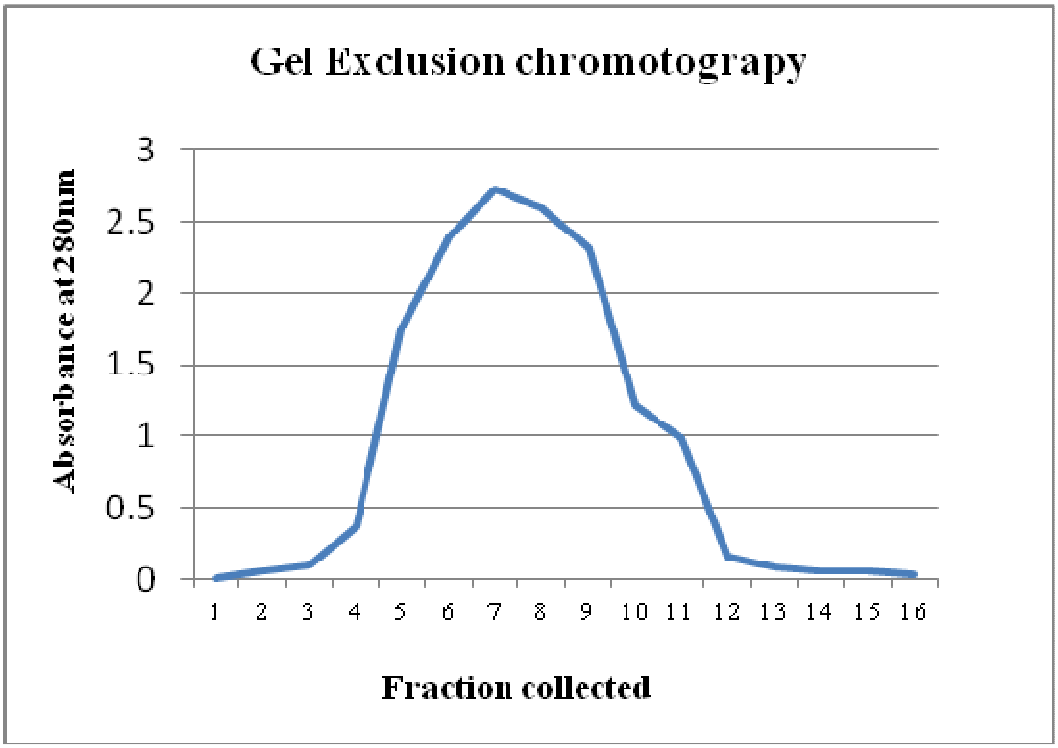


Figure-2
Purification by Sephadex G-100 Gel chromotography

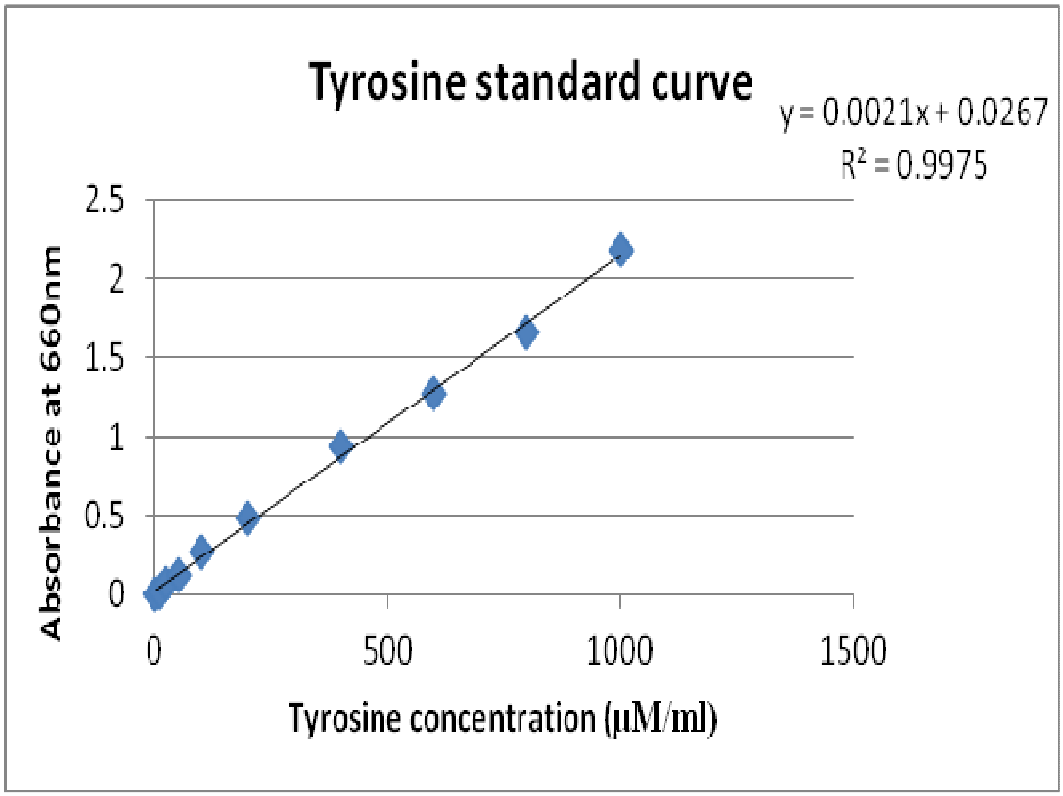
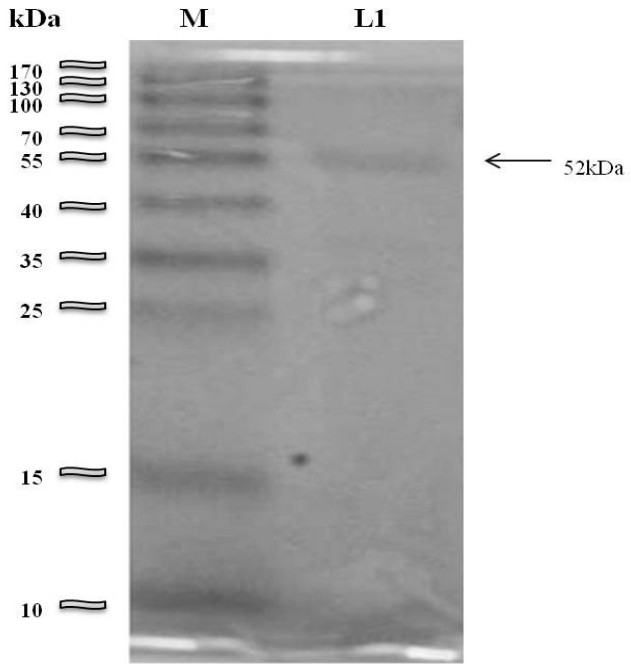


Figure-3
Tyrosine standard curve for quantification



M- Marker, L1- Purified enzyme
Figure-4
12% SDS-PAGE gel with Protein ladder

Conclusion

Bacillus licheniformis which has ability to grow at 55ºC can be a potent source of protease enzyme. Studies on *B. licheniformis* showed that chemical factors such as carbon, nitrogen and Physical factors such as pH, temperature can influence production of protease enzymes. Purified enzyme can be a used for many industrial purposes. A further research is needed to enhance the enzyme production to meet the commercial needs.

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