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Production of Protease from *Bacillus Subtilis* by Submerged Fermentation Technology

Keshamma E*

Associate Professor, Department of Biochemistry, Maharani Science College for Women,Palace Road, Bengaluru, Karnataka, India

***Corresponding Author**
Dr. Keshamma E
Associate Professor
Department of Biochemistry,
Maharani Science College for Women,
Palace Road, Bengaluru-560 001,
Karnataka, India
Email: keshamma.blr76@gmail.com

Abstract

Industries of food, pharmaceutical, agricultural and medical have been taking advantage of using *Bacillus sp*. because of their wide range of physiological characteristics and ability to produce enzymes and other metabolites. *Bacillus sp.* are attractive industrial microorganisms recognized as generally recognized as safe, which have high growth rates leading to shorter fermentation times and possess the ability to secrete extracellular proteins. Production of protease was carried out generally using submerged fermentation. It is advantageous than other methods due to its consistent enzyme production with defined medium, better process conditions and improved downstream processing. Therefore, current study was conducted with the main objectives of isolation of bacterial strain producing protease enzyme and optimization of submerged fermentation parameters for enhanced production of protease enzyme. Slaughter house waste from local market of Chikkaballapura were collected and screened for protease producing bacterial strains on skimmed milk agar plates. Protease positive bacterial strains were identified and recorded based on the clear zone formation around the bacterial growth. Protease producing bacteria was grown on the optimized media in submerged fermentation method. Optimization of cultural conditions like incubation period, pH, temperature, carbon & nitrogen sources was carried out. Folin Ciocalteu method of enzyme assay was followed and activity was expressed in U/mL. Study findings delineated that newly isolated *Bacillus subtilis* produced extrasellar protease optimally when it was cultured at 50ºC, pH 8.0 for 36 hours under submerged fermentation. Furthermore, newly isolated *B. subtilis* utilized fructose and yeast extract as carbon and nitrogen sources

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respectively for optimal production. In conclusion, newly isolated *B. subtilis* in this study has potential industrial biotechnological applications. Therefore, large scale production studies utilizing newly isolated *B. subtilis*could be recommended to carry out in order to exploit for industrial applications.

Keywords:*Bacillus subtilis*, Submerged fermentation (SmF), Protease, Industrial applications

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Introduction

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are physiologically necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale.[1] They are generally used in detergents,[2] food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds.[3,4] They also have medical and pharmaceutical applications.

Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins.[5,6] The molecular weight of proteases ranges from 18 – 90 kDa.[7] These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium.

Industries of food, pharmaceutical, agricultural and medical have been taking advantage of using *Bacillus sp.* because of their wide range of physiological characteristics and ability to produce enzymes and other metabolites.[8-10]*Bacillus subtilis* and *Bacillus licheniformis* species are attractive industrial microorganisms recognized as generally recognized as safe, which have high growth rates leading to shorter fermentation times and possess the ability to secrete extracellular proteins.[11-13]Production of protease was carried out generally using submerged fermentation. It is advantageous than other methods due to its consistent enzyme production with defined medium, better process conditions and improved downstream processing.[14] Microbial proteases producing industries are always in search of new and cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme.[15] Hence, in the present study we aimed isolation of bacterial

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strain producing protease enzyme and optimization of submerged fermentation parameters for enhanced production of protease from the newly identified microorganism by using submerged fermentation technology.

Materials and Methods

Sample Collection and Screening

Slaughter house waste from local market of Chikkaballapura were collected in sterile container according to microbiological procedures and taken to the laboratory for further analysis. The collected samples were serially diluted and streaked on skimmed milk agar plates. The plates were incubated 37ºC for 24-36 h. Microbial strains capable of producing protease enzyme were selected by observation of zone of hydrolysis around the colonies as described by Genkal et al., (2006).[16]

Identification of Bacterial Strains

All the bacterial strains capable of producing protease enzyme were identified based on morphology, cultural and biochemical characteristics as described by Koneman et al., 1994).[17]

Assay of Protease Enzyme

Protease activity was determined according to the modified Anson's method. 1.0 ml of the culture broth was taken in a 100 ml flask and 1.0 ml of pH 7.0 phosphate buffer added to it. One ml of the substrate (2% Hammersten's casein pH 7.0) was added to the buffer enzyme solution and incubated at 37oC for 10 minutes in a water bath. At the end of 10 minutes, 10.0 ml of 5N TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was then filtered off and 5.0 ml of the filtrate were taken in a test tube. To this 10.0 ml of 0.5N NaOH solution and then 3.0 ml of the Folin Ciocalteu reagent (one ml diluted with 2 ml of distilled water) were added. Final readings were taken in a spectrophotometer at 750 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate.

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Production of Protease by SmF

Protease producing bacteria was grown on the optimized media of protease enzyme production (10 g fructose, 5 g KNO_3, 150 g NaCl, 5 g K_2HPO_4, 0.4 g $MgSO_4$, 0.2 g $CaCl_2$ and 10 g Tween-80) dissolved in one litre of distilled water, was prepared,[18]and incubated on a rotary shaker for 48 hours at 37°C. Enzyme was extracted by centrifuging the incubated broth at 5,000 rpm at 4°C for 5 minutes. Supernatant was used as crude enzyme source of protease.

The experiment was carried out in 250 mL plugged Erlenmeyer flasks, each containing 100 mL sterile optimized media of protease enzyme production and inoculated with 1% of standard inoculum (2.30×106 CFU ml^{-1}) for the tested bacterial isolate which was incubated at 50°C on rotary shaker at 150 rpm for 48 h. The fermented medium was centrifuged at 10,000 rpm for 10 min in order to determine periodically the cell dry weight and protease activity in the precipitate and supernatant respectively.[19]

Optimization of SmF Parameters

Optimization of cultural conditions for the optimum production of protease under SmF using newly isolated bacterial strain *Bacillus sp.* was conducted for the following parameters.

Incubation period

To study the effect of incubation period on protease production by newly isolated *Bacillus sp.* under SmF was carried out at different incubation periods *viz.* 24 h, 48 h, 72 h, 96 h and 120 h. The optimum incubation time achieved by this step was fixed for subsequent for experiments.

pH

In order to optimize the pH of the fermentation medium, the protease activity produced from newly isolated *Bacillus sp.* was measured at different pH of fermentation medium *viz.* 6.00, 7.00, 8.00, 9.00 and 10.0. The optimum pH achieved by this step was fixed for subsequent for experiments.

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Temperature

To study the effect of temperature on protease production using newly isolated *Bacillus sp.* in SmF was carried out at different temperatures *viz.* 30°C, 40°C, 50°C and 60°C. The optimum temperature achieved by this step was fixed for subsequent for experiments.

Carbon sources

The fermentation medium was prepared with different carbon sources such as arabinose, fructose, sucrose, and lactoseat 1.0% concentration, and assessed for protease production.

Nitrogen sources

Different organic nitrogen sources such as ammonium chloride, beef extract, peptone and yeast extract at concentration of 1.0% were incorporated in to the fermentation medium and assessed for their effect on protease enzyme production.

Results and Discussion

Out of six isolates, three bacteria showed the zone of clearance on skimmed milk agar plates and among three, one isolate was identified as *Bacillus subtilis.* showed the maximum zone of clearance on the skimmed milk agar plate (Figure 1), and hence, it was selected for the further study.

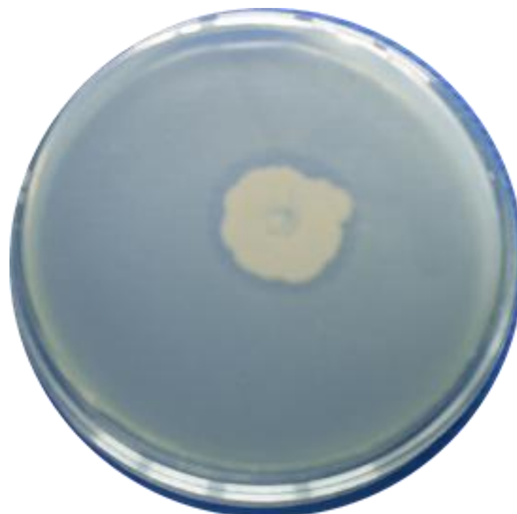

Figure 1: Zone of clearance on skimmed milk agar plate

Optimization of SmF Parameters

Incubation period

There was increase in activity of protease enzyme produced from newly isolated *B. subtilis* was observed between 24-36 h of incubation period. However, maximum activity of protease was observed at 36 h (Figure 2). Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased. This is because the cells would have reached decline phase with lowered enzyme synthesis. It might be also due to the depletion of the nutrients, death phase of organism or due to the production of amylase in the medium.[20]

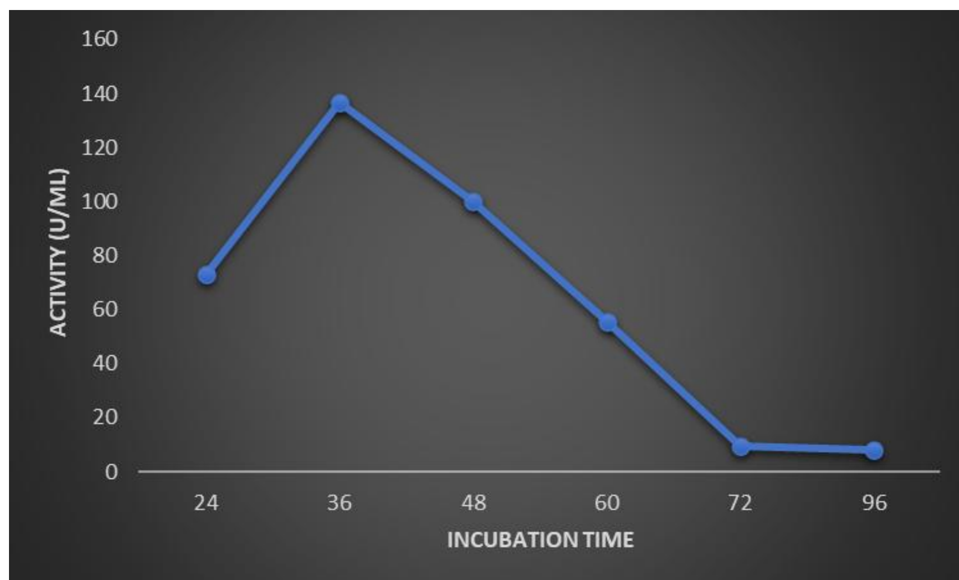

Figure 2: Effect of incubation period on protease production

pH

Protease production by microbial strains strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production.[21]In the present study, the peak protease production was achieved at pH 8.0 i.e., activity of proteaseproduced from *B. subtilis* was optimum at pH 8 (245 U/mL)(Figure 3).

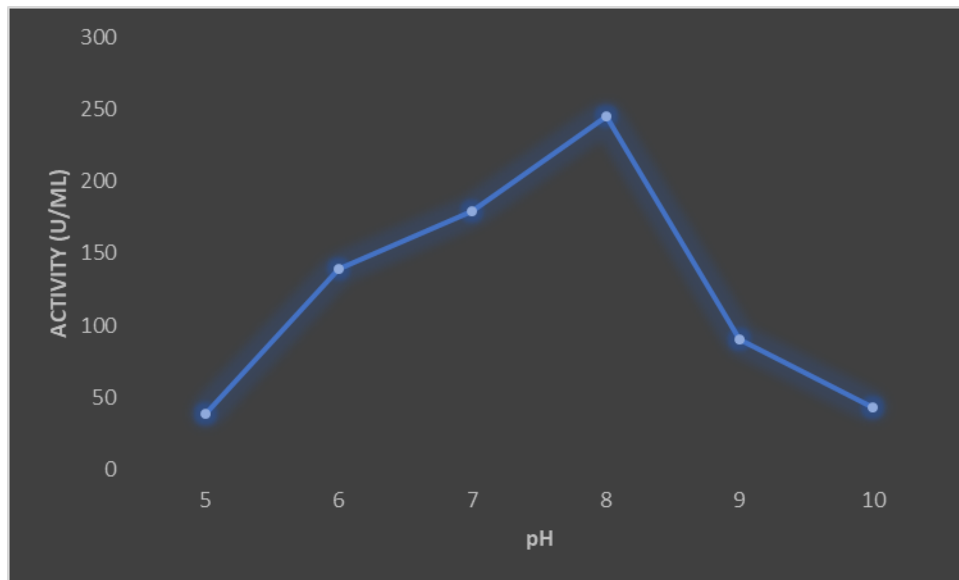

Figure 3: Effect of pH on protease production

Temperature

In our study at 50°C optimum production of protease was estimated as 204 U/mL (Figure 4). The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure.[22]

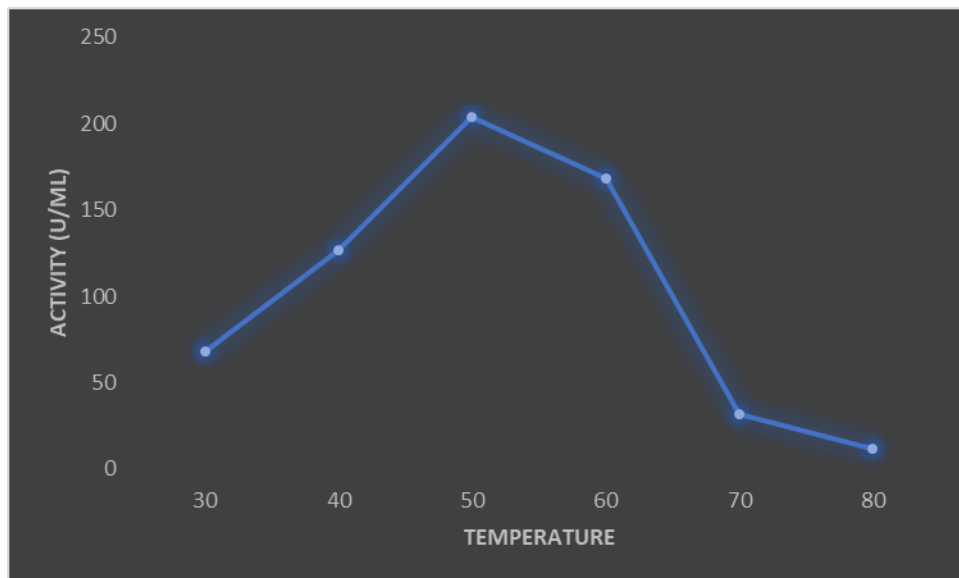

Figure 4: Effect of temperature on protease production

Carbon sources

In the current study maximum protease production from newly isolated *B. subtilis.* was measured when production medium was supplemented with fructose*i.e.,*145 U/mL (Figure 6). Literature findings evidenced that depending on the species and source of the organism

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carbon source requirement varies.[23] Atalo and Gashe (1993) showed that yeast extract and peptone can induce the alkaline protease production in glucosemedium. In our study newly isolated *B. subtilis*prefer to use fructose as a source of carbon for the optimum production of protease enzyme.[24]

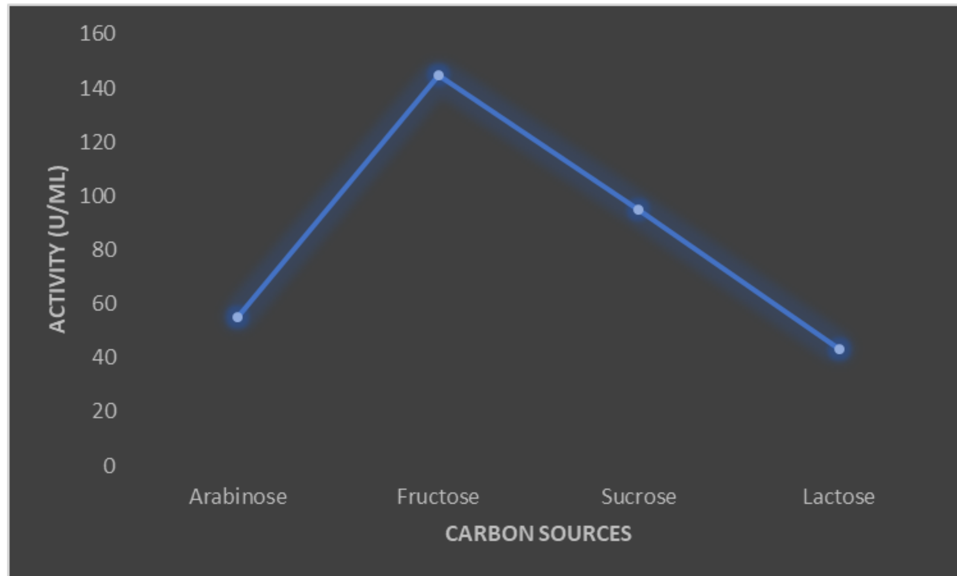

Figure 6: Effect of carbon sources on protease production

Nitrogen sources

Literature reports evidenced that high yield of enzyme production was obtained when organic nitrogen source compounds were used.[25] In our study the supplementation of nitrogen sources on protease production showed that yeast extract was found to be a better nitrogen source for the production of protease i.e., 163 U/mL (Figure 7).It was understood from the literature reports that certain nitrogenous salts tend to decrease the pH of the culturemedium and had the adverse effect on enzyme production although they supported the growth of the organism.[26]

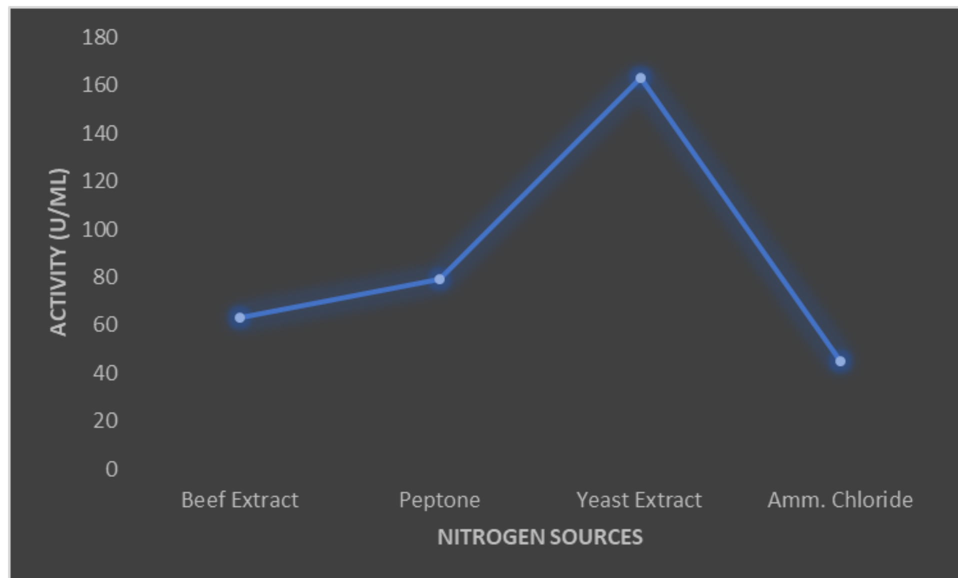

Figure 7: Effect of nitrogen sources on protease production

Conclusion

In conclusion, the newly isolated *B. subtilis*produced extrasellar protease optimally when it was cultured at 50ºC, pH 8.0 for 36 hours under submerged fermentation. Furthermore, newly isolated optimum utilized fructose and yeast extract as carbon and nitrogen sources respectively for optimum production. Newly isolated *B. subtilis* in this study has potential industrial biotechnological applications. Therefore, large scale production studies utilizing Newly isolated *B. subtilis* could be recommended to carry out in order to exploit for industrial applications

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