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Research Article

Production of Protease through SSF by *Bacillus Subtilis* NCIM 2724

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ABSTRACT

Production of protease employing the laboratory isolates of Bacillus sps. under solid state fermentation. The present study was designed for study of increasing the production of protease feasible at commercial level and an attempt has been made to optimize the different physico chemical parameters required for obtaining the maximum production of protease using Bacillus subtilis and Bacillus lincheniformis. Bacillus subtilis gives the maximum enzyme production by using papaya peel as the substrate with the optimized conditions of incubation time 24hr, temperature 300C, moisture content 40%w/v, and inoculum level of 0.8%w/v and with substrate concentration of 10g and pH 8.0, glucose concentration 2.0%w/v. The maximum production of protease enzyme considering all optimum conditions of various parameters was found to be 0.69 mg/ml.

Keywords: Bacillus subtilis, B. licheniformis, Incubation time, Optimisation, Moisture content, Papaya peel, Solid state fermentation.

INTRODUCTION

Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds[1]. Each type of protease has a specific kind of peptide bonds it breaks. Examples of proteases include: fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin[2]. Their use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation.Contrary to old beliefs

several studies have shown that orally ingested enzymes can bypass the conditions of the GI tract and be absorbed into the blood stream while still maintaining their enzymatic activity. Commercially, proteases are produced in highly controlled aseptic conditions for food supplementation and systemic enzyme therapy[3,4]. The organisms most often used are *Aspergillus niger* and *A. oryzae*. Proteases are important tools in studying the structure of protiens, peptides and the estimation of kinetic parameters[5]. Heavy metals, such as lead (Pb) and mercury (Hg), exert their poisoning effect by binding to ionizable or sulfhydryl groups of proteins, including vital enzymes. Once they bind to an essential functional protein, such as an enzyme, they denature and/or inhibit it. This interaction of heavy metals to proteins can lead to degenerating diseases, nerve damage or even death[6].The protease enzymes are applied as to improve blood circulation, to prevent abnormal blood clotting, to reduce pain and inflammation associated with Phlebitis, to alleviate the pain, inflammation, and discomfort of varicose veins; to minimize muscle pain that occurs after exercise, to minimize the inflammation and pain associated with Osteoarthritis and Rheumatoid Arthritis, to alleviate the symptoms of Sinusitis and to alleviate[7] (reverse) Edema.

MATERIALS AND METHODS

Micro Organism: *Bacillus subtilis* and *Bacillus licheniformis*[8]. The selected species were obtained from NCIM, Pune and are maintained on nutrient agar medium at 30^0c for 24hrs and preserved by sub culturing every 4 weeks.

Substrate: Papaya peel[9].

Inoculum preparation: The production of protease requires the preparation of inoculum culture was scraped and washed from the slant culture with 10 ml sterile water and 2 ml of this inoculum was added to each 250 ml flasks containing production medium.

Solid State Fermentation: 10g of substrate was taken in 250 ml Erlenmeyer conical flasks and to this 4ml of water was added. The contents were mixed thoroughly and autoclaved at 121^0c for 15 min. After cooling the flasks to room temperature, the flasks were inoculated with 2ml of 24 hr grown culture strain under sterile conditions. The inoculum was prepared by adding sterile distilled water to a 24 hr old slant; the contents were mixed thoroughly and incubated in a slanting position to provide maximum surface area at 30^0C temperature[10].

Enzyme Extraction: After incubation period, the enzyme was extracted according to the method take 50 ml of 0.2M glycine –NaOH buffer pH 10 was added and was kept for shaking in an orbital shaker for 30 min. Then the mixture was filtered using a whatman No.1 filter paper. The extracts were collected and then centrifuged. The supernatant was as enzyme source for protease.

Enzyme Assay: Alkaline protease activity was estimated by the modified Auson-Hagihara method. 1ml of the enzyme solution was added to 6 ml of casein (0.6 gm of casein mixed in 100 ml of 0.2 M NaOH glycine buffer) and the mixture was incubated at 37^0C for 10 min. Then 6.0 ml of TCA was added and then incubated for 30 min at room temperature. The mixture was centrifuged for 10 min and the 2 ml of Folin-Ciocalteau reagent was added to the 1 ml of the supernatant taken and then incubated for 30 min at room temperature. The absorbance was read at 660 nm.

RESULTS AND DISCUSSIONS

Effect of physico chemical parameters: Protease enzyme was produced using the papaya peel by *Bacillus subtilis.* The enzyme physic chemical parameters like incubation time, temperature, pH, inoculums level, moisture content, substrate concentration and nutritional supplementation studies were carried out and optimized. The effect of kinetic parameters on the production of protease was also studied and the results are shown in the form of tubular forms and also studied and the results are shown in the form of tabular forms

and also shown in the graphs plotted against the obtained values. The results are discussed based on the available literature.

Effect of incubation of time: To determine the effect of incubation time on protease production, the inoculated flask was incubated for 12, 24, 48, 96 hours duration. After incubation period the enzyme was extracted and assayed. Results indicate that there was a steep increase in the protease production with the increase in time. The maximum protease production was found at 24 hours. The maximum protease concentration was found to be 0.543mg/ml at 24 hours. After 24 hours of incubation the enzyme production started to decline as the organism has reached the stationary phase and so the production remain constant with the increase in the biomass and finally the enzyme production decreases due to self digestion.

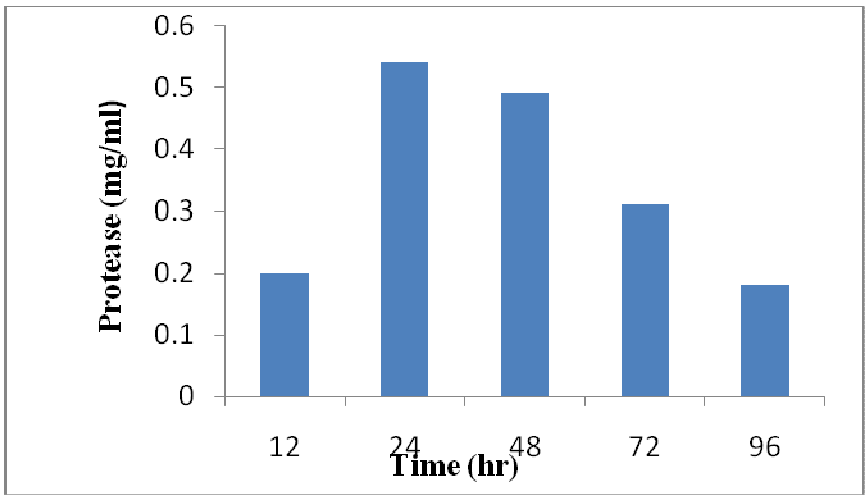

Fig.1: Effect of incubation of time

Effect of incubation temperature: Temperature is one of the important parameter influencing the growth of the bacterial species. Due to the high fermentation temperature, it is relatively easy to collect the product and there by avoid the decrease of yield. And it is associated with product increase. The production medium was prepared in 250 ml flask and each flask was inoculated and incubated with different temperatures like 24^0C, 27^0C, 30^0C, 33^0C and 36^0C for *Bacillus subtilis*. The maximum enzyme production was found to be 0.64mg/ml at 27^0C. After 27^0C there has been a decrease in the production of protease enzyme. Further increase in the temperature decrease the enzyme production. This is probably due the link between the enzyme synthesis and energy metabolism in *Bacilli*.

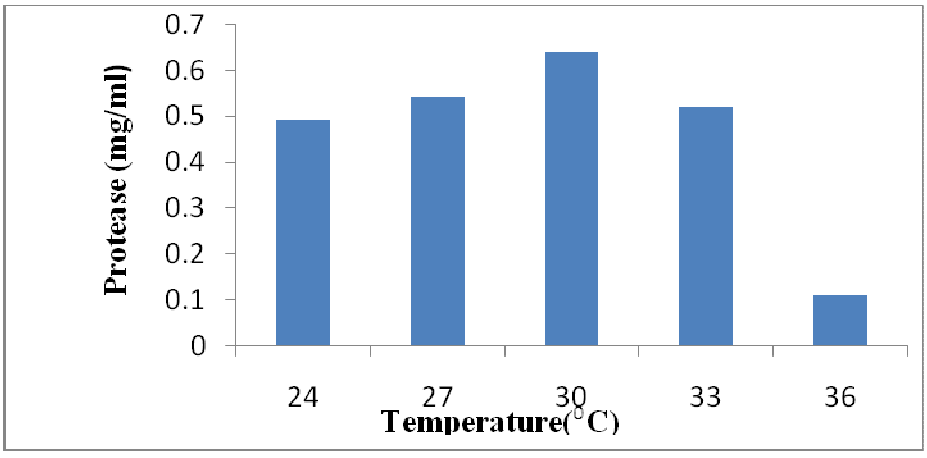

Fig.2: Effect of incubation temperature

Effect of pH: pH strongly influences many enzymatic process and also transport of various components across the cell membrane, which in turn supports the cell growth and product production. To determine the effect of pH, different values are considered such as 7, 8, 9 and 10. Flasks were incubated for 24 hours. The highest enzyme production was recorded at pH 8. The micro organism plays a significant role in maintain pH at a relatively constant value. When the pH differs from the optimal values then there will be an increase in maintenance energy requirement. The optimal pH of the medium often effects the growth and product formation by influencing the uptake of nutrient, metabolic pathway and in other physiological activities.

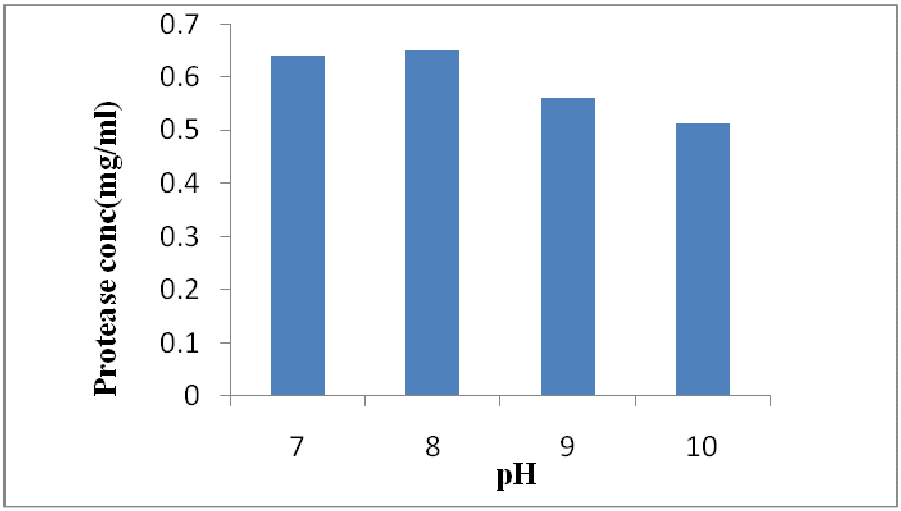

Fig.3: Effect of pH

Effect of Inoculam level: The nature of inoculum as well as its size may affect the microbial process. To evaluate the affect of inoculum level on the protease production varying cell concentration were added to different flask. Different inoculum levels of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 % w/w were added to the production medium and kept in incubator for 24 hours at temperature 30^0C. The maximum production was obtained at 0.8% w/w for *Bacillus subtilis* (0.66mg/ml). With the increase in inoculum level, the production of enzyme declined due to the exhaustion of nutrient in the production medium. Every excess liquid present in an un-absorbed form gives rise to a diffusional barrier imposed by the solid nature of the substrate. This leads to a decrease in enzyme production and growth.

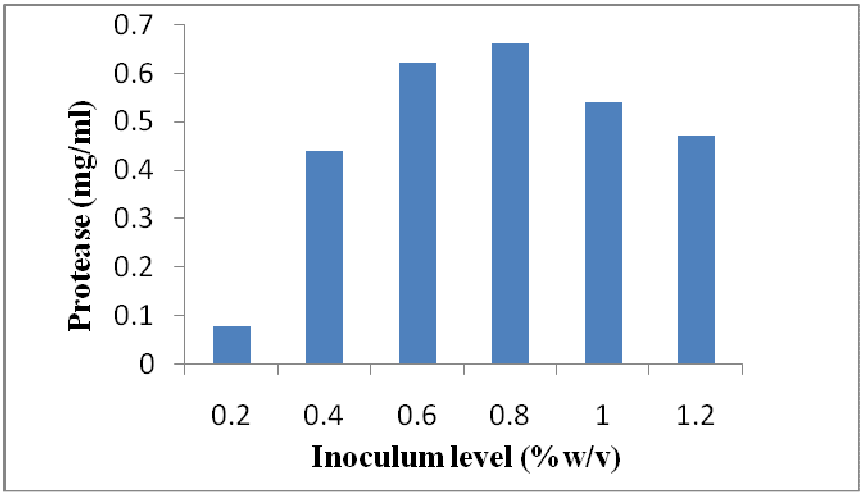

Fig.4: Effect of Inoculam level

Effect of Moisture Content: Moisture content on water activity is one of the most important factors in SSF. In solid state fermentation microbial growth and product formation occurs at or near the surface of the solid substrate having low moisture content. To determine the moisture content effect on the production media, the different moisture contents are been used i.e., 20, 30, 40, 50 and 60. The optimum moisture content was recorded at 40% for *Bacillus subtilis*. The maximum production was 0.54mg/ml.

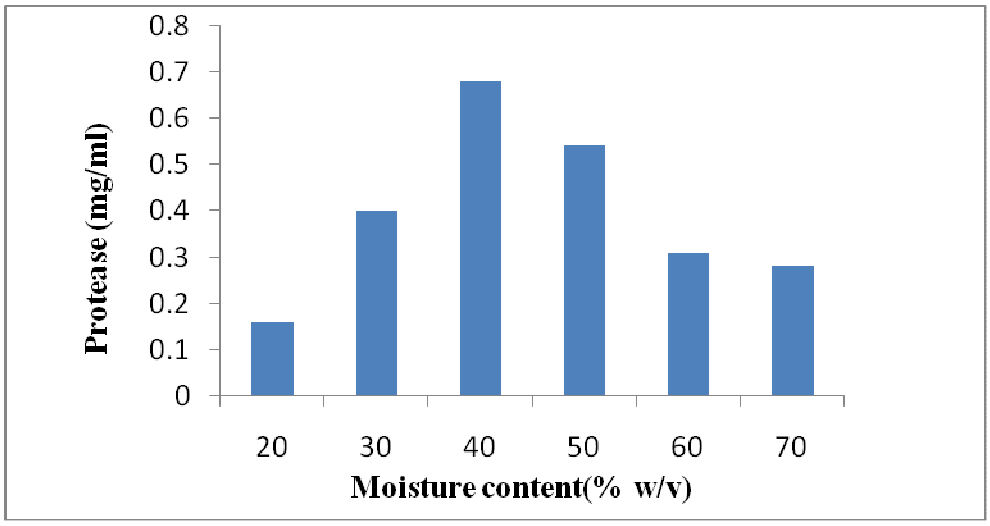

Fig.5: Effect of Moisture Content

Effect of *S*ubstrate concentration:To determine the effect of substrate concentration on protease production, the production medium of different concentration i.e. 7, 8, 9, 10, 11 and 12 grams was prepared in 250 ml flasks, was inoculated and incubated at 30^0C in an incubator for 24 hours. The results indicated that the concentration from 7 to 10 grams showed that the enzyme production increased for *Bacillus subtilis*. After 10 grams the enzyme production decreased with the increase in substrate concentration.

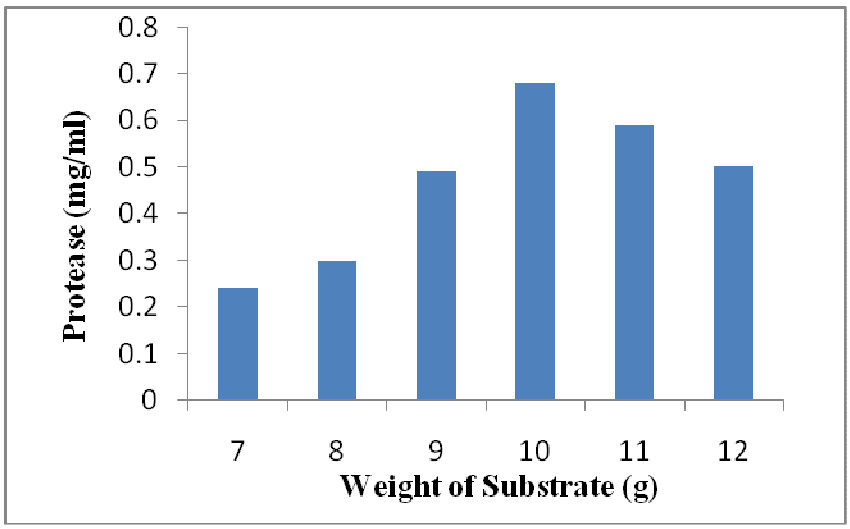

Fig.6: Effect of *S*ubstrate concentration

Effect of carbon supplements: To determine the effect of different carbon sources used which includes glucose, fructose, lactose and mannose. Each of these at a concentration of 1% w/v was added to the

production medium. The flasks was inoculated and incubated at 30^{0}C in an incubator for 24 hours. Out of the four samples study glucose showed maximum production of protease reported as 0.59 mg/ml.

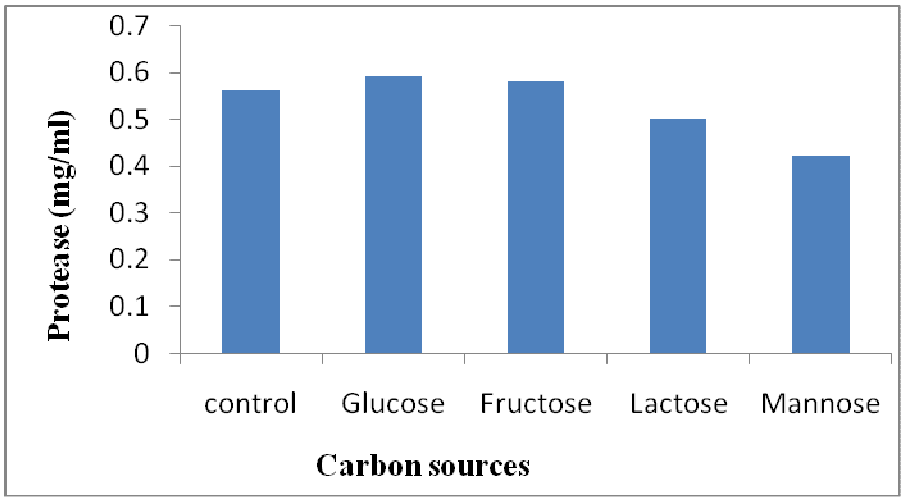

Fig.7: Effect of carbon supplements

Effect of different glucose concentrations: To determine the effect of carbon supplement concentration on protease production medium was prepared using 0.5, 1, 1.5, 2.0, 2.5 and 3.0 % w/w of glucose were added to the substrate. The flasks were incubated at 30^{0}C in an incubator for 24 hours. Enzyme is assayed. The result indicate that the maximum enzyme was produced was 0.68 mg/ml using *Bacillus subtilis* at 2.0% w/w.

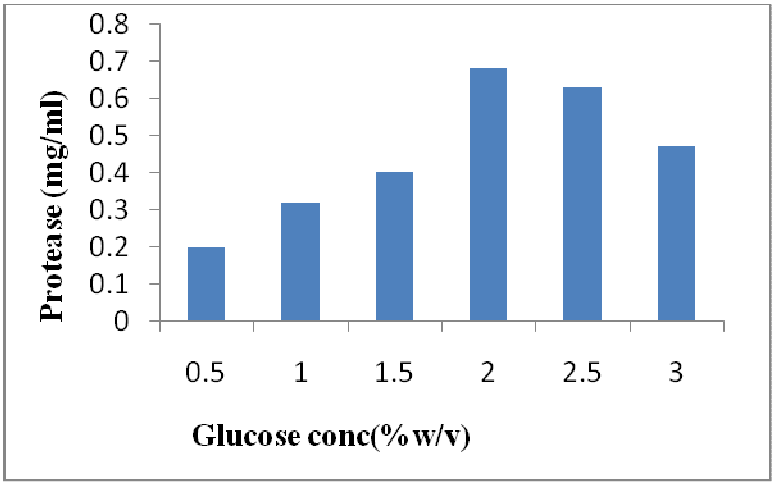

Fig.8: Effect of different glucose concentrations

Effect of substrate on protease production taking on all optimum conditions: The maximum production of protease enzyme taking all optimum conditions of various parameters was found to be 0.69 mg/ml.

CONCLUSION

Proteases are the single class of enzymes which occupy pivotal portion with to their applications in both physiological and commercial fields. The present study was intended to produce protease from papaya peel employing *Bacillus subtilis* (NCIM 2724) and its various physic chemical parameters have been studied. The microorganisms were screened for protease production and *Bacillus subtilis* was fond to be a good producer for the protease with the above conformation an attempt has been made to study the physic chemical

parameters as they greatly influence the production was observed at incubation time 24hrs, temperature 30^0C, pH 8, inoculum level 2.0, moisture content 40%, and substrate concentration 10gms. Even the chemical parameters which include different carbon supplements have also been studied. Out of all carbon supplements which include glucose, fructose, lactose, and mannose. Glucose has been found to be the best carbon supplement which produces maximum protease (2% w/w).

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