Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3

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A newly isolated strain of *Bacillus* sp. PCSIR EA-3 showed maximum protease production in 48 h. Cell free filtrate (CFF) from the strain was investigated for its proteolytic activity. The isolated strain exhibited highest proteolytic activity when grown on skim milk agar; average area of zone of inhibition was 480 mm\(^2\). Enzyme showed maximum activity at 35°C and pH 7.0 with glucose as an important medium component. It was strongly activated by metal ions, such as, Ca\(^{2+}\) ion. Protease from PCSIR EA-3 can easily be used in food industry, especially in cheese manufacturing due to its thermostability at neutral pH.

**Keywords:** *Bacillus* sp., calcium ion, culture optimization, protease

**Introduction**

Proteases constitute one of the most important groups of enzymes and have applications in different industries including detergent, food, pharmaceutical, silk and leather, and also for recovery of silver from used X-ray films\(^1-3\). They are also used for meat tenderization and in some medical applications\(^4,5\). Proteases account for 30% of the total worldwide enzyme production\(^6\). They are hydrolytic enzymes, which act upon native proteins to breakdown into small peptides and amino acids. Proteases do not refer to a single enzyme but a mixture of enzymes including proteinases, peptidases and amidases\(^7,8\). Among the various proteases, proteases from *Bacillus* sp. are the most significant, compared with animal and fungal proteases\(^9,10\).

*Bacillus* spp. mostly produce two groups of proteases, alkaline and neutral\(^11\). Bacterial neutral proteases are active in narrow pH range (pH 5.0-8.0) and have relatively low thermostolerance\(^12\). This property is advantageous for controlling their activity during the production of food hydrolysates with a low degree of hydrolysis. Bacterial neutral proteases generate less bitterness in hydrolyzed food proteins than animal proteases and, hence, are valuable for use in the food industry\(^11\). It has been reported that some neutral proteases belong to the metalloprotease (E.C. 3.4.24.4) type and require divalent metal ions for their activity\(^13\). On the other hand, some serine proteases are not affected by chelating ions\(^11,14\). In order to assess the utility of the *Bacillus* proteases for industrial use, it is desirable to search for new proteases with novel properties from as many different sources as possible. Production of enzyme for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process\(^15\). The aim of present study was to isolate the *Bacillus* sp. from the environment and optimize the conditions for maximum production of extracellular protease.

**Materials and Methods**

**Isolation of Strain**

Microorganisms isolated from air in the laboratory were plated on skim milk agar plates containing peptone (0.1%), NaCl (0.5 %), agar (2%) and skim milk (10%), and incubated at 37°C for 48 h. A clear zone of skim milk hydrolysis appeared after 48 h incubation and depending upon the zone of clearance, strain PCSIR EA-3 was selected for further experimental studies. It was maintained on nutrient agar slants at 4°C and was subcultured at 1 wk intervals. The isolated proteolytic strain was a spore forming, Gram positive and rod shaped bacterium, identified as *Bacillus* sp. PCSIR EA-3\(^16,17\).
Culture Conditions

Production of protease from Bacillus sp. PCSIR EA-3 was carried out in a medium containing the following (g/L): glucose 1.0, peptone 10.0, yeast extracts 0.2, CaCl\(_2\) 0.1, K\(_2\)HPO\(_4\) 0.5 and MgSO\(_4\) 0.1, and maintained at 37°C for 48 h. The pH of the medium was adjusted at 7.0 before sterilization.

Production of Protease

Sterile broth medium (900 mL) was inoculated with 100 mL inoculum and incubated at 35°C for 48 h, then centrifuged at 15000 rpm for 10 min at 0°C and the clear crude enzyme supernatant was stored at −20°C for further studies.

Enzyme Assay

Protease activity was determined by a modified procedure based on the method of Anson using casein as the substrate\(^\text{18}\). One unit is defined as the amount of enzyme that hydrolyzes casein to produce colour equivalent to 1.0 µmol of tyrosine per min at standard conditions.

Protein Assay

Total protein of the cell-free filtrate was determined by the method of Lowry et al\(^\text{19}\). Bovine serum albumin (250 µg/mL) was used as a standard.

Time Course of Protease Production

For protease production, culture medium was incubated at different time intervals (h) 6, 12, 18, 24, 48, 72, 96 and 120. Enzyme activity, total protein, final pH of culture media and wet cell mass were determined.

Effect of Substrate Concentration, Temperature and pH on Protease Production

Glucose concentration for the optimum enzyme production was varied from 0.05-1.0% in the culture medium having pH 7.0 at 35°C for 48 h. Optimum temperature for protease production was achieved by incubation of culture broth at 25°-50°C by the increment of 5°C for 48 h.

For protease production in cultivation medium, pH of culture broth was adjusted before autoclaving from 6.0-8.0 by the increment of 0.5 at 35°C for 48 h.

Effect of Calcium Ions and Peptones on Protease Production

Calcium ions were incorporated into the medium in the form of calcium chloride salt ranging from 0.001 to 0.025%, while peptone concentration for the optimum protease production was varied from 0.5-3% in the culture medium.

Results and Discussion

In the present study, environmentally isolated bacterium was identified as Bacillus sp. by using morphological and biochemical characteristics\(^\text{20}\). The Bacillus isolates were then characterized for protease production by using skim milk agar. The proteolytic activity was detected by the presence of clear zone. It was found that Bacillus sp. PCSIR EA-3 yielded the highest protease activity with a clear zone of hydrolysis.

Incubation time plays a substantial role in the maximum enzyme production. It has been reported that B. subtilis PE-11 showed maximum protease production in 48 h\(^\text{21}\); whereas B. subtilis 3411 gave maximum production in 72 h and Bacillus sp. K-30 in 96 h\(^\text{22,23}\). Results of the present study indicated that the production of protease was dependent on the bacterial cell growth (Fig. 1). The cells of Bacillus sp. PCSIR EA-3 started multiplication within 6 h of incubation and reached to the maximum growth in 48 h. The cells growth remained high upto 48 h and thereafter started to decline. It is clear from Fig. 1 that the maximum enzyme production was obtained during continuous growth of the culture at the late exponential phase and early stationary phase of the growth and thereafter number of viable organism decreased due to the depletion of readily available carbon source and other nutrients.

Various microorganisms and cultivation media have been studied for protease production\(^\text{24}\). Carbon sources greatly influenced the enzyme production and the most commonly used substrate was reported to be casein\(^\text{21}\). Reported studies showed that glucose was a promising alternative source for protease production for Mucor circinelloides. It has been noticed that lack
of glucose in a media resulted in dramatic decrease in the enzyme production\textsuperscript{4,25}. On the other hand, glucose has also been reported to suppress protease production\textsuperscript{26,27}. Present results are in good accordance with previous studies showing maximum enzyme production when 0.1% glucose was used as substrate instead of casein in the culture broth.

The effect of nitrogen source (peptone) on protease production by \textit{Bacillus} sp. PCSIR EA-3 was investigated. It was observed that among the different nitrogen sources, peptone was found to be best, followed by meat and yeast extract. The results of present study showed that 1% peptone concentration was optimum for maximum protease production (Fig. 2). The addition of peptone to the fermentation media shortened the lag period and increased the exponential period that resulted in enhanced enzyme production.

The effect of some physical factors, such as, pH and temperature, on the production of crude enzyme was investigated. Sarkar \textit{et al}\textsuperscript{27} have reported that maximum enzyme production was obtained when pH of the culture media was maintained at 6.0; while in case of \textit{B. subtilis} 38, the maximum protease production was reported at pH 6.5\textsuperscript{29}. The present results showed maximum protease production at the optimum pH of 7.0; the enzyme activity dropped gradually at pH above 7.5 and below 6.5 (Fig. 3). It was observed that the pH of fermentation broth reached a high value 7.5-8.0, probably due to the accumulation of metabolites that resulted in inactivation of enzyme.

It was reported that the maximum protease production was achieved at 47°C\textsuperscript{29}, while 60°C was the best temperature for protease production in case of \textit{B. subtilis} PE-11\textsuperscript{21}. Subsequently, 37°C was reported to be the best temperature for protease production for certain \textit{Bacillus} sp.\textsuperscript{8}. The present results showed maximum protease production at 35°C in cultivation medium (Fig. 4). However, \textit{Bacillus} sp. PCSIR EA-3 was not capable of producing the enzyme at temperature below 25°C. On the other hand, a progressive decline in enzyme production was observed at 40°C and no enzyme production was observed at 50°C.

Some of the metal ions, such as, Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and Mn\textsuperscript{2+}, have been reported to increase and stabilized the protease production by activation. These ions also have been reported to increase the thermal stability of other proteases\textsuperscript{30,31}. Thus, concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperature\textsuperscript{32,33}. Calcium chloride has been used by several workers as a source of calcium ions in protease producing media. The present results
In conclusion, the protease produced from Bacillus sp. EA-3 indicated that incorporation of 0.01% calcium chloride in the fermentation medium produced maximum protease as compared to the medium having no calcium ions (Fig. 5).

In conclusion, the protease produced from Bacillus sp. PCSIR EA-3 is a neutral protease having high thermostability. The neutral protease produced during log phase may play an important role in food industry for making cheese. Further studies are in progress in the application of protease in different commercial fields.

References


