

Original Research Article

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## Optimization of Protease Production from *Bacillus cereus*

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### ABSTRACT

#### Keywords

Protease production, *Bacillus cereus*, culture conditions, enzyme activity.

#### Article Info

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A soil isolate of *Bacillus cereus* exhibited highest proteolytic activity when grown on casein agar plates with a zone of inhibition of 34mm. The production medium was optimized with respect to physical and chemical factors using submerged fermentation. The optimum pH, temperature and incubation period for protease production were pH 7 at 37°C for 72 hrs at 150rpm. Glucose was found to be an important media component in this. The protease production of this isolate was also studied with respect to the inoculum size, nitrogen sources and metal ions. Ca<sup>2+</sup> was found to be a potent enhancer. The extracted crude enzyme showed the ability to remove blood stains and so can be used in detergent industry.

## Introduction

Proteases are enzymes which are degradative and they catalyse the hydrolysis of proteins. They are one of the third largest groups of industrial enzymes and hold 60% of the total worldwide sale of enzymes (Chu *et al.*, 2007). This belongs to the class of enzymes which plays an important role in various commercial fields (Godfrey *et al.*, 1996). Among various proteases, microbial proteases play an important role in biotechnological process and industrial applications (Gupta *et al.*, 2002). Various microorganisms are used for protease production, among them *Bacillus* is the most predominant and an ideal source for enzyme production and that is because they grow

rapidly (Ibrahim *et al.*, 2013). Proteases produced by *Bacillus* sp find applications in detergent, leather, food and pharmaceutical industries (Banerjee *et al.*, 1999; Banik *et al.*, 2004). Hence it is always desirable to search for new proteases showing different properties and isolating them from various other sources.

## Materials and Methods

### Sample Collection and Screening

Soil samples from protein rich areas were collected from various regions of Hyderabad, Telangana and were screened

for protease producing bacteria. One gram of each soil sample was dispensed in 10ml of sterile distilled water and diluted serially. 0.1ml of dilutions  $10^{-3}$  was plated on casein agar plates and incubated at 37 °C and 45 °C respectively for 24 to 72 hrs. *Bacillus cereus* was the organism that was screened showing good enzymatic activity compared to other isolates. Further analysis was done by subjecting the isolate to submerged fermentation (SmF) and optimizing the conditions for its maximum enzyme production.

### **Production Medium**

Inoculum was prepared by inoculating a loopful of 24 hr culture into 100ml of sterile inoculum medium. The composition consisted of 0.2% glucose, 0.05% casein, 0.05% peptone, 0.05% Yeast Extract and salt solution 5ml (Salt solution containing 0.5%  $\text{KH}_2\text{PO}_4$ , 0.2%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ). The pH of the medium was adjusted with 1N NaOH or 1N HCl. This medium was autoclaved at 121°C for 15 minutes. The flasks were incubated at 37°C for 24 h and agitated at 150 rpm in a shaker. 98ml of the production medium was prepared in 250ml Erlenmeyer flask. The composition of production medium consisted of 0.5% glucose, 0.5% Peptone and salt solution 5ml (Salt solution containing 0.5%  $\text{KH}_2\text{PO}_4$ , 0.2%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ). The pH of the medium was adjusted with 1N NaOH or 1N HCl. This medium was autoclaved at 121°C for 15 minutes. 2% of overnight grown inoculum was transferred to 98 ml production medium. The flasks were incubated at 37 °C for 72 h and agitated at 150 rpm in a shaker. After 72 h of incubation, the broth culture was centrifuged in a cooling centrifuge at 10,000 rpm for 10min. The supernatant was collected in separate sterile tubes. This crude enzymatic suspension was used to determine the enzymatic activity.

### **Protease Assay**

Protease enzyme was assayed by Folin's method (Folin *et al.*, 1929). 1 ml of the clear enzyme supernatant was added with 5.0 ml of substrate solution (0.65% casein in 50 mM Potassium Phosphate buffer, pH 7.5) and incubated at 37°C for 10 minutes. To stop the reaction 5.0 ml of 110 mM Trichloroacetic Acid Reagent was added with incubation for 30 min. To the above mixture 5.0 ml of 500mM  $\text{Na}_2\text{CO}_3$  and 1.0 ml of Folin-Ciocalteu's solution (1:1 dilution) were added and incubated for 30 minutes.

The absorbance (O.D) was read at 660 nm against blank. 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  $\mu\text{mole}$  of tyrosine in one minute under the defined assay conditions.

### **Media Optimization**

Optimization of medium composition was done to maintain a balance between the various media components, thus minimizing the amount of unutilized components at the end of fermentation. In addition, no defined medium has been established for the optimum production of alkaline protease from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

### **Effect of Incubation Period**

The inoculum size of 2% was inoculated and incubated at varying periods viz., 24hr, 48hr, 72hr, 96 hr respectively in a shaker incubator and the enzyme activity was recorded.

### **Effect of Inoculum size**

The effect of inoculum size was determined by growing the isolate in fermentation media and inoculating in varied inoculum size viz., 1%, 2%, 5%, 10%, and 15% and the enzyme activity was noted.

### **Effect of pH**

The effect of pH on protease production was carried out. The pH was adjusted using 1N HCl and 1N NaOH and the assay was performed to know the enzyme activity.

### **Effect of Temperature**

The effect of temperature was studied by maintaining the medium at varying temperatures of 5°C, 20°C, 30°C, 37°C, 40°C, 50°C and the protease assay was performed.

### **Effect of Different Carbon Sources**

Optimization of carbon source was done by adding 1% of different sugars like glucose, fructose, starch, sucrose, maltose, lactose to the production, the protease enzyme quantification was done.

### **Effect of Nitrogen Sources**

Production media was optimized with 1% of various nitrogen sources like ammonium sulphate, ammonium chloride, sodium nitrate, ammonium nitrate, potassium nitrate, casein, tryptone, urea, yeast extract, beef extract and the enzyme activity was recorded.

### **Effect of Metal Ions on Protease Production**

The effect of metal ions on protease production medium was carried out using 10mM of different metal ions like

MnSO<sub>4</sub>.7H<sub>2</sub>O, CaCl<sub>2</sub>, MgSO<sub>4</sub>.7H<sub>2</sub>O, ZnSO<sub>4</sub>.7H<sub>2</sub>O, FeSO<sub>4</sub>. 7H<sub>2</sub>O. The protease assay was done and activity of enzyme recorded.

### **Application of Crude Protease**

The application of alkaline protease in removing the blood stains was observed according to the method of Najafi (Najafi *et al.*, 2005) with slight modification. On a clean piece of pure white cotton cloth a drop of blood was added and allowed to dry for 30min. The dried cloth was cut into equal sizes (4x4 mm<sup>2</sup>) and was treated with 1:1 dilution of crude enzyme of pH 8 at room temperature at different time periods (5, 10, 15, 20 and 25 min). After which the cloth was rinsed in tap water for 30sec without scrubbing and then air dried. A control was prepared following the same procedure without enzyme treatment.

### **Results and Discussion**

*Bacillus cereus* was gram positive and a spore former that showed good protease enzyme activity. Optimization studies were carried out using this isolate for increase protease production.

Incubation period plays an important role in maximum enzyme production. In earlier studies it was seen that *B. subtilis* PE-11 showed maximum protease production at an incubation time of 48 h (Pastor *et al.*, 2001), there were also reports on *B. subtilis* 3411 that gave maximum production at 72h while *Bacillus* sp K-30 showed maximum production at 96h (Naidu *et al.*, 2005 ; Gibb *et al.*, 1987).

The present study with *Bacillus cereus* shows maximum protease production at 72h as shown in (Fig. 1) which is similar to the study with *B. subtilis* 3411.

Inoculum size creates a balance with the available materials that enhance the protease production. With 5% inoculum size, maximum protease production was recorded in the study as shown in (Fig. 2). This result is in accordance with the result reported on 5% inoculums size for optimum protease production by *Bacillus subtilis* (Abusham *et al.*, 2009).

The physiological character of an organism is dependent on the pH of culture medium. Maximum protease production was observed at pH 7 followed by pH 8, and the lowest was seen at pH 5 as shown in (Fig 3). This work is in relevance to the previous study (Kanekar *et al.*, 2002; Horikoshi 1971).

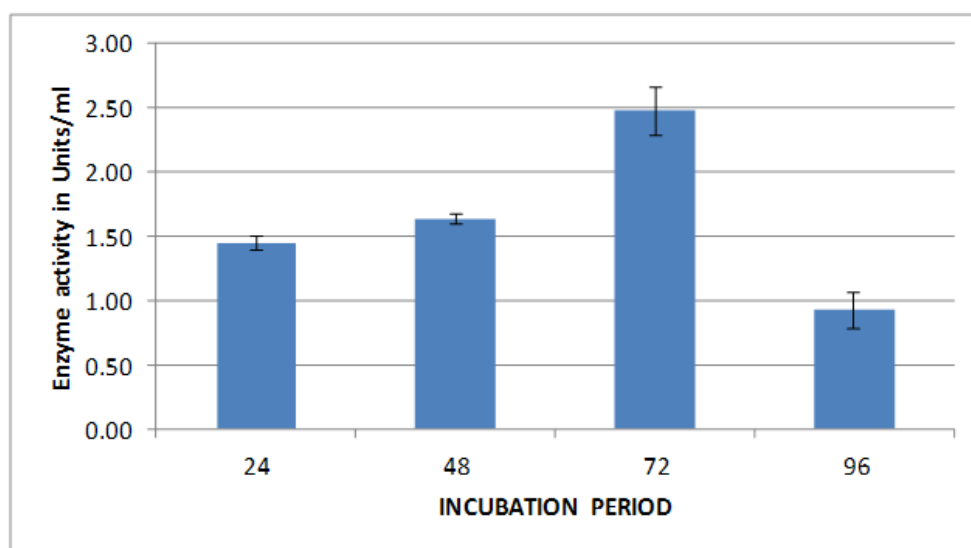
Previous study reports shows that maximum protease production was seen at 47 °C (Rahaman *et al.*, 1994) and the best protease production at that temperature was seen in case of *B. subtilis* PE-11 (Pastor *et al.*, 2001). There were also reports indicating that 37 °C was the best temperature for

protease production for certain *Bacillus* sp (Adinarayana *et al.*, 2002). The present study with *Bacillus cereus* showed maximum enzyme production at 37 °C as seen in (Fig. 4).

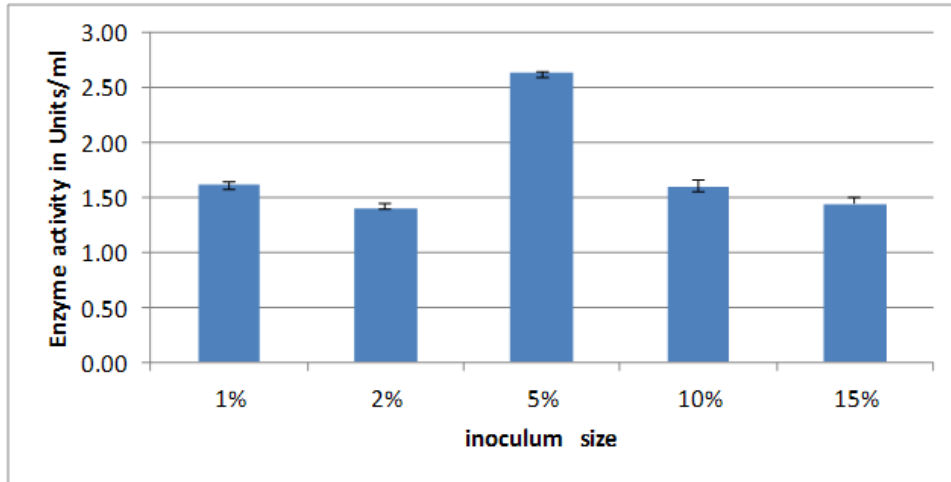
Different culture media are used by various microorganisms (Sen *et al.*, 1993). Carbon sources influence protease production. Lack of glucose in media resulted in dramatic decrease in enzyme production (Gajju *et al.*, 1996; Sonnleitner 1983). Among the various carbon sources, glucose showed maximum enzyme production as shown in (Fig. 5).

Among the various organic and inorganic nitrogen sources, tryptone was found to be the most suitable for protease production in the present work as seen in (Fig. 6) which is in relevance with the earlier investigation. It was reported that the enhancement of protease production in *Conidiobolus coronatus* by organic nitrogen sources like tryptone, peptone and yeast extract (Phadataré *et al.*, 1993).

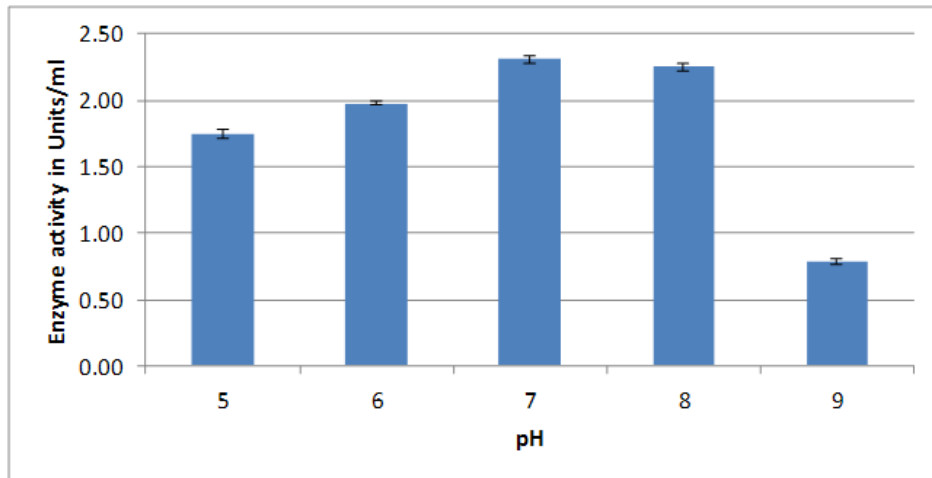
Fig.1 Effect of incubation period



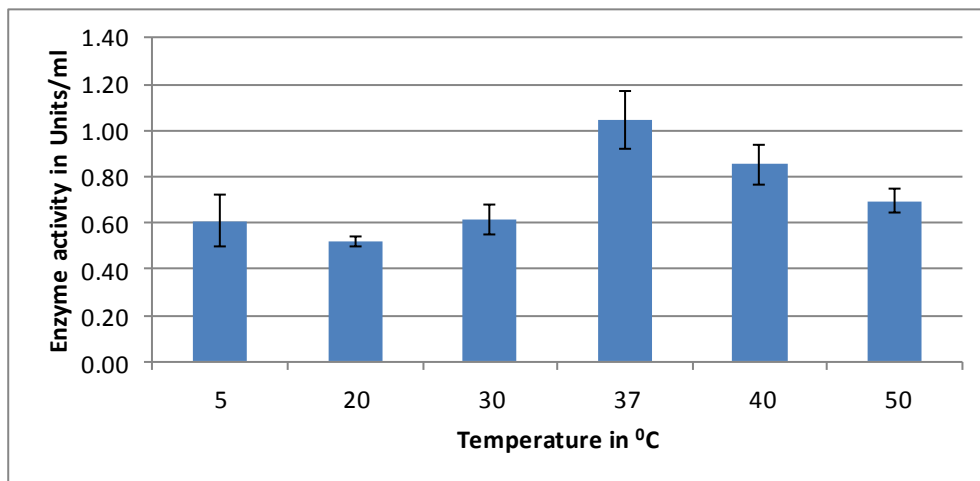
**Fig.2** Effect of inoculum size



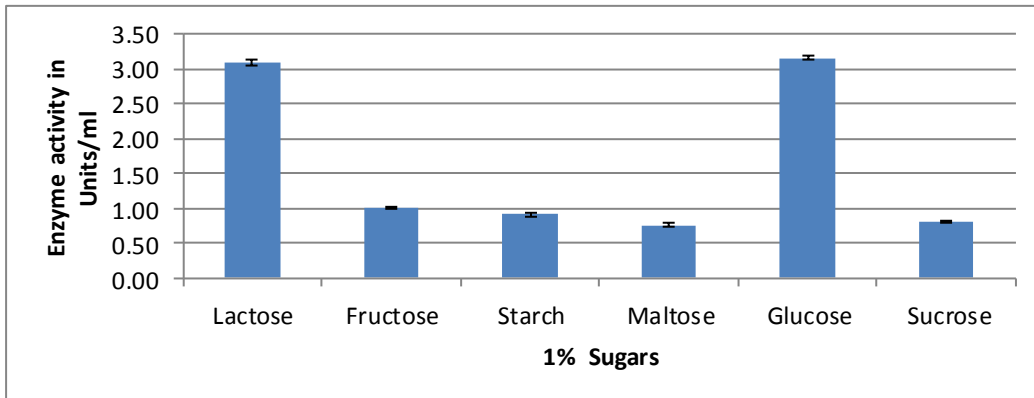
**Fig.3** Effect of pH



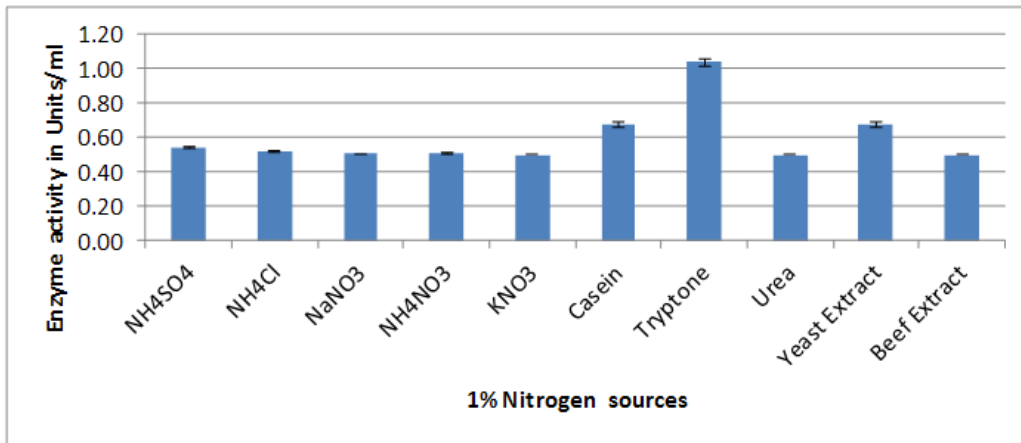
**Fig.4** Effect of temperature



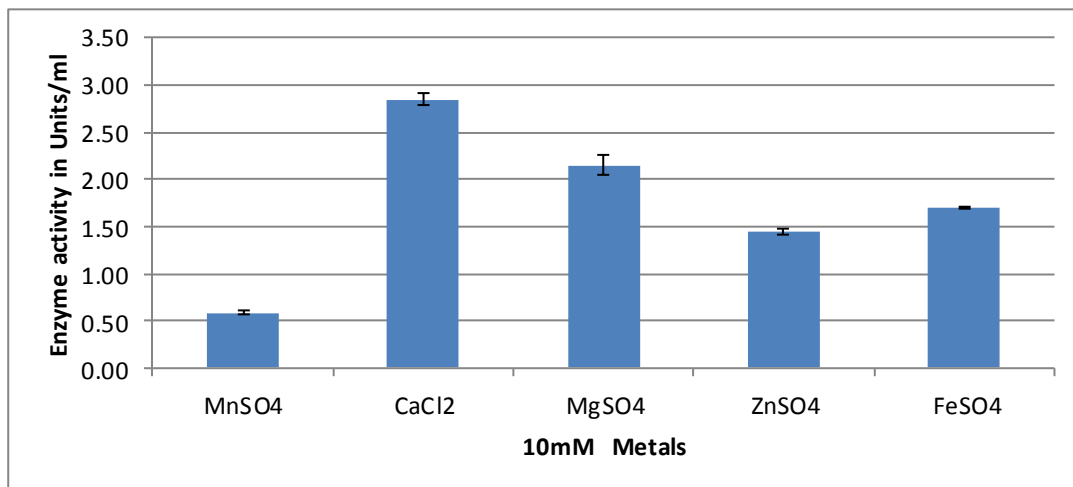
**Fig.5** Effect with different sugars



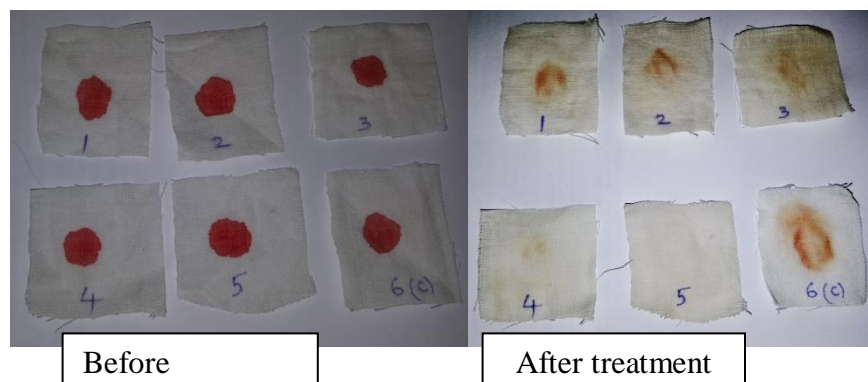
**Fig.6** Effect with various Nitrogen sources.



**Fig.7** Effect with metals.



**Fig.8** Removal of blood stain from cotton cloth pieces after exposure to 1:1 dilution of crude enzyme sample extracted from *Bacillus cereus* at different time intervals 1=5min, 2=10min, 3=15min, 4=20, 5=25min, 6(c) =control (no enzymatic treatment)



Inorganic nitrogen sources did not show any significant increase in the production. Many other investigations reported that organic nitrogen sources are better for enzyme production than inorganic nitrogen sources (Beg *et al.*, 2002; Feng *et al.*, 2001; Joo *et al.*, 2002).

Some metals like  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$  and  $\text{Mn}^{+2}$  have reported increase and stability of protease production. They are reported to increase the thermal stability of other proteases. The present studies showed that there was a significant increase in the production of protease on addition with  $\text{CaCl}_2$  as shown in (Fig.7).

### Application of Enzyme in Detergent Industry

*Bacillus cereus* has very high capability to remove blood stains in absence of detergents. The diluted crude enzyme has completely removed the blood stain at 25 min (Fig.8).

In conclusion, *Bacillus cereus* is a potent source of protease enzyme. Studies revealed that physical factors like temperature, pH, incubation period, inoculum size and the

chemical factors like carbon, nitrogen, metal ions can increase the production of protease. Since the enzyme produced can remove the blood stains, it can be used in the detergent industry.

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