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Microbial production of protease by *Bacillus Cereus* using cassava waste water

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ABSTRACT

The extracellular microbial proteases have several applications in various industries which account for approximately 40% of the total enzyme in the work market. In the present study, protease production from Bacillus cereus was studied and different parameters were optimized such as incubation time, pH, carbon and nitrogen were optimized. Maximum protease production was observed at 48 hours with pH 7; increase in production was observed when glucose and peptone were used as carbon and nitrogen sources in the production medium respectively. Further, cassava waste water was also used as an alternative carbon source for enzyme production, showing a maximum of protease production when supplemented with 6 % in the production medium.

Keywords: *Bacillus cereus*, Cassava waste water and Protease

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INTRODUCTION

Enzymes are biocatalysts that are produced by living cells to bring about specific biochemical reactions involved in the metabolic processes of the cells. Since its discovery, around 3000 different enzymes have been classified and found to be useful in biotechnological applications [1]. Even small improvements have been significant for commercial success in biotechnological enzyme production processes [2]. Proteases are important industrial enzymes synthesized by different types of microbes like fungi, bacteria, yeasts and also by some plants and animal tissues. Because of their rapid growth, less space requirement for their cultivation, microbes serve as a preferred source of protease enzymes [3]. Microbial proteases can be produced in large quantities and represent one of the largest classes of industrial enzymes, accounting for 40% of the total worldwide sales of enzymes by value [4]. Proteases are one of the important groups of industrial enzymes which have application in food industry, detergents, pharmaceutical industry, leather industry and bioremediation processes [5].

Bacillus sp. are one among the best protease producers studied so far since they exhibit properties like broad substrate specificity, significant activity, stability, simple downstream purification, short period of fermentation and low cost [6]. These properties make the bacterial proteases most suitable for a wider application in industries. Several *Bacillus* species involved in protease production are *B. sterothermophilus*, *B. cereus*, *B. megaterium*, *B. mojavensis*, and *B. subtilis* [3]. In order to sustain with the economic production of enzyme with industrial process, it is necessary to utilize alternative cheap substrates. Cassava waste water has a combination of high molecular

weight sugars which can be utilized for the enzyme production which gained much attention in recent years which can reduce the manufacturing costs with large scale production.

The aim of the present research study was to optimize the culture conditions of *Bacillus cereus* for maximal protease production and also to utilize cassava waste water as a cheap carbon source for enzyme production.

MATERIALS AND METHODS

Strain Selection:
Bacillus cereus MTCC 8301 was obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India and stored at 4°C. The culture was sub-cultured further in Nutrient agar (NA) for the present study.

Screening for Protease Production
Protease production by the bacterial strain was screened on agar plates supplemented with skim milk agar containing (1% skim milk, 1% peptone, 0.5% sodium chloride, 2 % agar). Screening medium was prepared, adjusted to pH-7.2, inoculated with *B. cereus* MTCC 8301 and were incubated at 37°C for 24-48 hours. Production of protease by *Bacillus cereus* MTCC 8301 was confirmed based on the zone of clearance formed after incubation.

Protease Enzyme Production
Protease production medium was adopted [7] with (g/L): glucose-0.1, peptone-1, yeast extract-0.02, K_2HPO_4-0.05, $CaCl_2$-0.01, $MgSO_4$-0.01 (pH 7.0). 100 ml of sterile medium was taken in 250 ml shake flask and inoculated with 5 ml of overnight *B. cereus* culture, and incubated at 37°C in a shaking incubator (150 rpm) for 24 hours.

Assay of Proteolytic Activity
After incubation, the culture was centrifuged at 10,000 rpm for 15 minutes at 4 °C. The cell free supernatant was used for protease assay [8]. The reaction mixture containing 1ml of enzyme was added to 1 ml of casein (1% w/v in 50 mM potassium phosphate buffer, pH 7.5) and the preparation was incubated for 10 minutes at 37 °C. The reaction was terminated by adding 2 ml of 10% trichloroacetic (TCA) acid reagent kept for 30 minutes incubation at room temperature and then centrifuged for 15 minutes at 10,000 rpm. Then 2 ml of filtrate was mixed with 3 ml of 500 mM sodium carbonate solution and optical density was observed at 280 nm. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µmol of tyrosine per minute under the defined assay conditions. The concentration with range 50–250 µg of tyrosine was used as standard. The prospective protease producer was utilized for further optimization studies to enhance the protease production.

Determination of Total Protein
The total protein content from the crude extract was estimated using Bradford method with bovine serum albumin as the standard protein [9].

Optimization of Physical Parameters on protease production
The effect of various physical parameters on protease production was assessed by growing bacterial culture in the production medium described earlier. Effect of varying incubation time was analyzed from 12 to 72 hours; while samples were withdrawn aseptically every 6 hours interval and protease activity was determined. Similarly, for the investigation of optimal pH, the medium was prepared by varying the pH from 4.5 to 8.5 and for the effect of agitation the bacterial culture was incubated at a range of 50–300 rpm at optimal incubation time for protease production.

Optimization of Chemical Parameters on Protease Production
The effect of various chemical parameters on protease production was studied by varying the carbon sources (glucose, fructose, sucrose, lactose, mannose and galactose at 0.1% w/v) and nitrogen sources (potassium nitrate, urea, ammonium nitrate, ammonium chloride, sodium nitrate, and peptone at 0.05% w/v). After 24 hours of incubation, the cell free supernatants were estimated for protease production.

Cassava waste water
From the local cassava flour factory, cassava waste water was obtained, boiled and cooled till it reached 5°C. The preparation was then centrifuged at 3000 rpm for 20 minutes at 4°C in order to solubilize the starch from the suspended solids and to eliminate the hydrogen cyanide present. The treated cassava water was then utilized for

protease production by replacing the carbon source (1 to 10 %) in the production medium and the protease activity was determined as described earlier.

RESULTS AND DISCUSSION

Screening for Protease Production:
The test strain *Bacillus cereus* MTCC 8301 was characterized for protease production by using skim milk agar, showing clear zone confirming the proteolytic activity.

Optimization of Physical Parameters
Incubation time plays a major role in the maximum production of enzyme during the fermentation process. In the present study, the protease production was gradually increased from 12 hours and reaches the maximum of 103 U/ml at 48 hours (Figure-1). Further incubation results in the decline of enzyme production due to the late exponential phase where the multiplication of bacteria started decreasing. These results are in accordance with observations made by Durhams [10], Gessesse [11] and Qadar and their co workers [7].Similar reports were also reported that maximum protease production was observed between 48 hours to 96 hours [12,13].

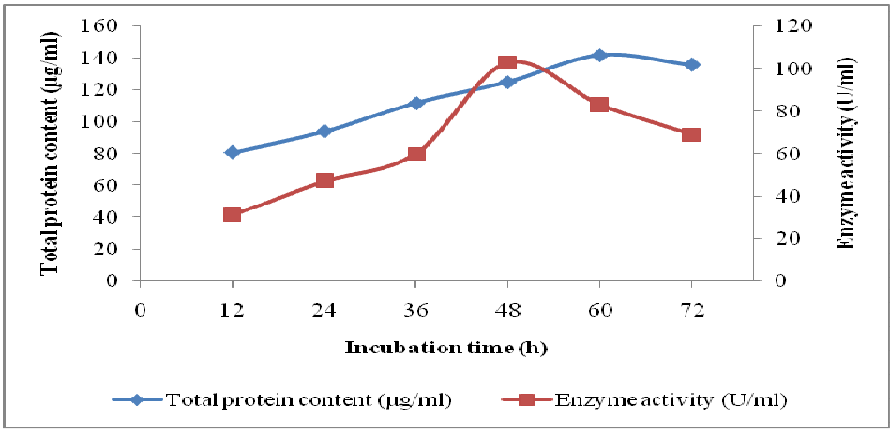

Figure 1: Protease production at various incubation time periods (hours)

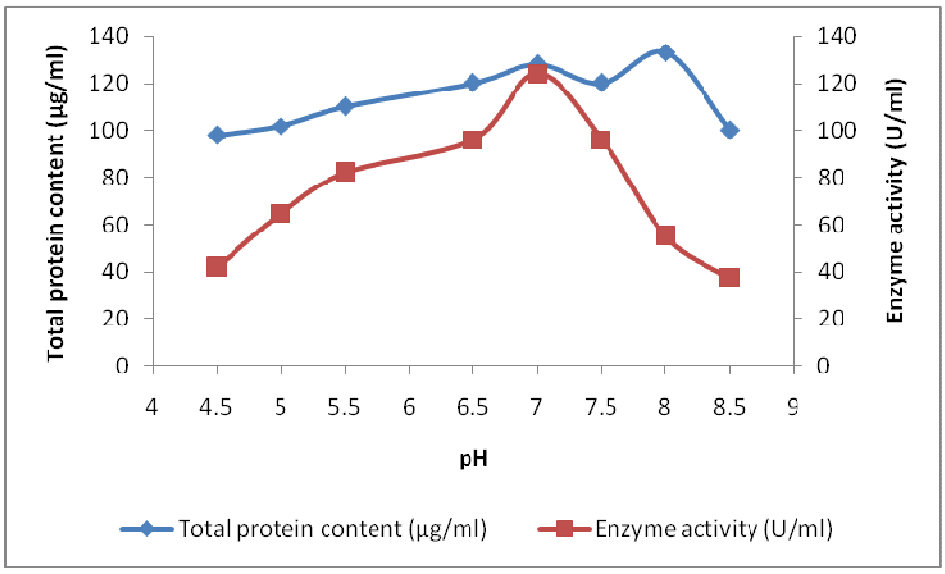

Figure 2: Protease production at various pH

The effect of pH and temperature was also studied for the protease production. Among the tested pH from 4.5 to 8.5, maximum production of protease activity of 124 U/ml was observed when the pH was maintained at 7. Similar studies were reported by Sarkar *et al.* [14] and Rahman *et al.* [15], where maximum enzyme production was observed at 6.0 and pH 6.5 pH respectively (Figure – 2). The results also show that there is a decrease in the enzyme production when it is below the optimal pH which may be due to the metabolites accumulation leading to inactivation of enzyme.

The effect of various agitation rates were studied for protease production and a maximum protease activity production of 130 U/ml (Figure-3) was observed at 150 rpm. *Bacillus subtilis* ATCC 14416 (Chu *et al.* 1992) and *B. licheniformis* (Fikret Uyar *et al.* 2011) showed optimum yields of alkaline protease production at 200 rpm which is similar to our studies.

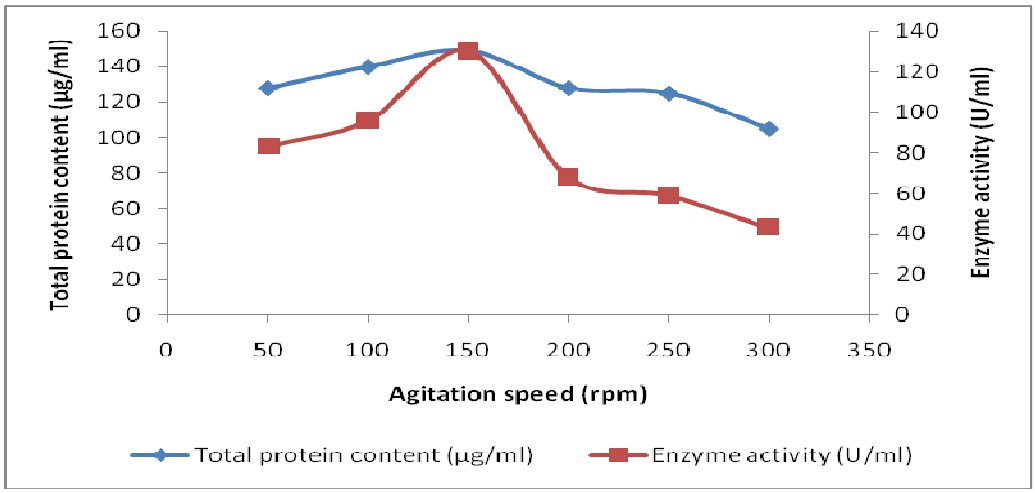

Figure 3: Protease production at various agitation speeds (rpm)

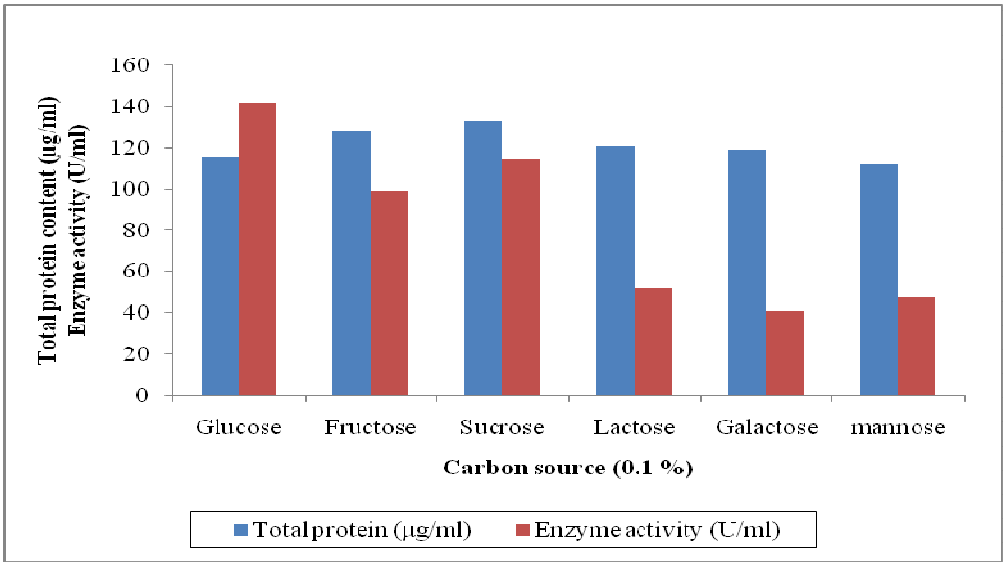

Figure 4: Effect of different carbon sources on Protease production

Optimization of Chemical Parameters

Among the different carbon sources utilized for protease production, maximum protease activity of 142 U/ ml was found when glucose was supplemented into the medium followed by sucrose and fructose (Figure-4). Similar findings were reported by Gomma *et al.* [16] and Sen and Satyanarayana, [17] where the maximum protease

production was observed when the carbon source was maintained at glucose and starch respectively. Recent results are in good accordance with previous studies showing maximum protease production observed when glucose was used as carbon source in the culture broth [12].

The effect of nitrogen source (peptone) on protease enzyme in the production medium was investigated and it was observed that maximum production of 152 U/ml when peptone was supplemented into the medium followed by urea and ammonium chloride (Figure-5). Similar results were observed by Nilegaonkar *et al.* when they used tryptone, peptone, casein and yeast extract as substrates for alkaline protease production [18]. Atalo and Gashe in their study found that yeast extract and peptone can induce protease production when supplemented in glucose medium [19]. Verma et al. also reported the production of alkaline protease was maximum when gelatin was maintained as a nitrogen sources followed by ammonium nitrate [20].

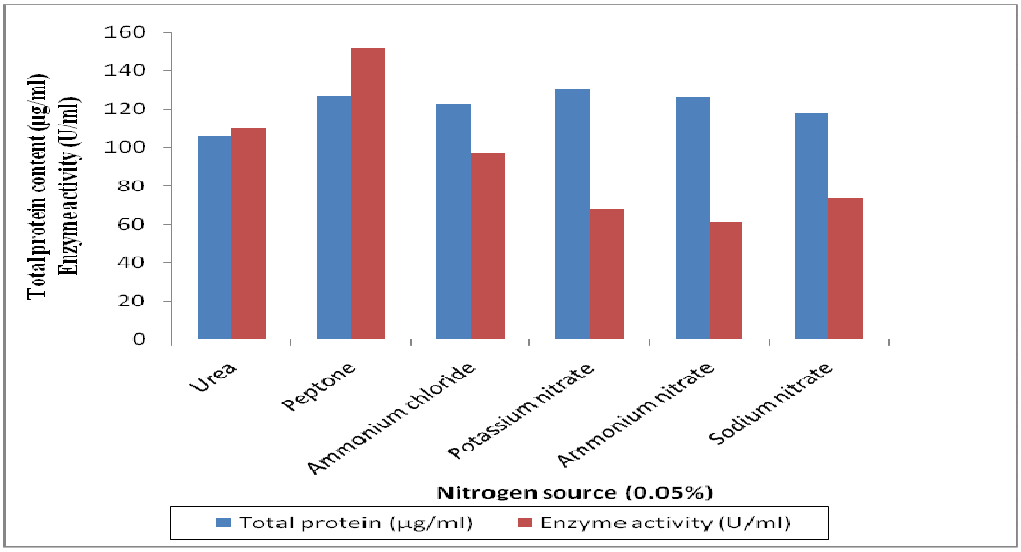

Figure 5: Effect of different nitrogen sources on Protease production

The liquid squeezed out during the cassava pulp fermentation was utilized as a source of industrial enzymes and various metabolites [21].The protease activity was maximum (114 U/ml) when the cassava waste water was supplemented at a concentration of 6 % in the production medium by replacing the carbon substrate. Increase in the concentration of cassava waste water more that 6 % shows a decline in the protease production. Similar studies were also reported by Uzochukwu *et al.* [22] where cassava waste water was utilized for the growth of different bacteria and fungi for metabolite production.

CONCLUSION

In conclusion, *Bacillus cereus* has been successfully exploited for the production of proteases. Various parameters were studied and optimized for maximum production of protease. Additionally, application of cassava waste water in the production medium was studied and a further investigation is needed for enhanced protease production for commercial scale.

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