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Studies on the effects of pH and incubation period on protease production by *Bacillus* spp. using groundnut cake and wheat bran

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Abstract: The production of alkaline protease by *Bacillus licheniformis* (MTCC 1483) and *Bacillus subtilis* of indigenous isolates using cheapest sources- groundnut cake (N_2 Source) and wheat bran (C source) was studied. The proteolytic activity was found maximum at 72 hr of the culture for both the bacterial types but the pH of the medium for maximal enzyme production varied as 9 for *Bacillus licheniformis* and *as* 8 for *Bacillus subtilis*. Thus investigations of this study reveal the possibility of employing cheapest nutrient source for microbial proteases that can be used on a commercial scale.

Keywords: pH, protease, *Bacillus licheniformis*, *B. subtilis*, groundnut cake, wheat bran.

Introduction

Proteases are found in several microorganisms such as viruses, protozoa, bacteria, yeast and fungi. They help in degrading proteins (Han-Seung Joo *et al*., 2004) so that the degradation products can become nutrients for microbial growth (Dixit *et al*., 2000). The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases which account for ~60% of the total worldwide enzymes sale (Beg *et al*., 2003, Ellaiah *et al*., 2003, Nascimento *et al*., 2004). In addition, proteases from microbial sources are preferred to the enzymes from plant and animal sources, since they possess almost all characteristics desired for their biotechnological applications (Gouda *et al*., 2006).

Proteolytic enzymes or proteases catalyze the cleavage of peptide bonds in proteins and can be broadly classified as exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. The protein degradation is initiated by endopeptidases secreted by microorganisms and further hydrolysed by exopeptidases at the extra- or intracellular site. Among the bacteria, *Bacillus* species are specific producers of extracellular proteases.

In this study, *Bacillus licheniformis* (MTCC 1483) and *B. subtilis* isolated from the soil (Aslim *et al*., 2002) nearby Bharath University, maintained on nutrient agar slants at 4 °C, was used for determining proteolytic activity. The identification of clear colonies in skimmed milk agar, gram stain, cellular morphology and motility confirmed presence of *B. subtilis* in the soil. Dynamics of protease production (Wellingta *et al*., 2004, Olajuyigbe *et al*., 2005, Wellingta *et al*., 2006) was examined at different pH and time period. The present study was conducted using cheapest medium sources, which includes groundnut cake as nitrogen source (Emmanuel *et al*., 2004) and wheat bran as carbon source (Krishna *et al*., 2005) for the production of alkaline proteases. The protein content (35%) of the groundnut cake was determined by Lowry's method (Lowry *et al*., 1951).

Materials and methods

Protease production

In the present study, glucose and wheat bran as sole carbon source, groundnut cake and ammonium sulfate as sole nitrogen source were used in 2 different Ehrlenmeyer flasks. Growth of the two species of *Bacillus* was carried out using submerged culture technique. Inoculums were prepared by transferring 1% suspension from a 24h old slant culture, into Ehrlenmeyer flasks of different pH (8, 9, 10). The culture medium used for protease production contained 1% (w/v) C source, 2% (w/v) N_2 source, 0.5%(w/v) $MgSO_4.7H_20$, 0.5%(w/v) KH_2PO_4 and 0.01%(w/v) $FeSO_4.7H_20$ maintained at 37 ºC in an orbital shaker (120 rpm) for 24h to 96h. At the end of incubation, the whole fermentation was centrifuged at 10,000 rpm at 4 ºC for 10 min and the clear supernatant was used as crude enzyme preparation.

Preparation of tyrosine standard

0.05, 0.1, 0.15 and 0.2 ml of 1.1mM L-tyrosine was taken in 4 separate test tubes. 5 ml of 500 mM Na_2CO_3 and 1 ml of diluted

Table 1. Activity of protease at pH 8.0 on various growth intervals of Bacillus spp.

Growth of the culture (hrs)	Protease Activity (Units/ml Enzyme) {Mean values and Standard Error (±)}			
	Groundnut cake (N_2 Source)		Wheat bran (C Source)	
	B. subtilis	*B. licheniformis*	*B. subtilis*	*B. licheniformis*
24	720±20	320±10	680±10	190±15
48	900±20	480±15	900±15	300±20
72	940±15	570±20	1160±20	465±10
96	440±20	330±10	220±10	285±15

Table 2. Influence of pH 9.0 on the protease activity at various growth intervals of Bacillus spp.

Growth of the culture (hrs)	Protease Activity (Units/ml Enzyme) {Mean values and Standard Error (±)}			
	Groundnut cake (N_2 Source)		Wheat bran (C Source)	
	B. subtilis	*B. licheniformis*	*B. subtilis*	*B. licheniformis*
24	580±10	430±15	620±10	380±10
48	740±15	570±20	840±15	510±20
72	920±20	615±10	1080±20	600±15
96	120±20	525±10	180±15	435±20

Table 3. Effect of pH 10.0 on the activity of protease at various growth intervals of Bacillus spp.

Culture growth (hrs)	Protease Activity (Units/ml Enzyme) {Mean values and Standard Error (±)}			
	Groundnut cake (N_2 Source)		Wheat bran (C source)	
	B. subtilis	*B. licheniformis*	*B. subtilis*	*B.licheniformis*
24	470±15	420±20	290±20	290±20
48	620±20	540±15	420±15	405±15
72	760±20	585±20	920±20	570±15
96	360±15	465±10	120±20	360±20

Folin-Ciocalteau's Phenol reagent was added in the test tubes. The total volume was made up to 8 ml using distilled water. Finally, the absorbance was measured colorimetrically at 620nm by keeping L-tyrosine as standard.

Protease assay

The enzyme was assayed by casein substrate method using L-tyrosine as a standard at different pH and sampled up to 96h with 24h interval. To the 1 ml of crude enzyme sample, 5 ml of 2% casein was added in the tube labeled 'test', after mixing by swirling, the tube was incubated for 10 minutes. Whereas, only 5ml of 2% casein was added in the tube labeled 'blank'. Later, 5 ml of 110 mM TCA was added in both the tubes. Then 1 ml of enzyme was added to the tube labeled 'blank' only and was incubated at 37 ºC for 30 minutes. The labeled 'test' tube was left without enzyme and incubation. Whatman's filter paper #150 was used for filtering both the 'test' and 'blank' tubes solution separately and the obtained filtrate was used for color development. 2ml of 'test' and 'blank' filtrate was then taken in separate tubes. Later 5ml of Na_2CO_3 and 1ml of Folin-Ciocalteu's reagent were added in both the tubes. The tubes were kept at room temperature for 30 minutes. Finally the absorbance was measured at 620nm in colorimeter using standard graph.

Determination of protease activity

Proteolytic activity is represented in terms of Units/ml enzyme, and is derived by:

$$\frac{\text{µmole Tyrosine equivalents released x Total volume (in ml) of assay}}{\text{Volume of enzyme (ml) x Time of assay (min) x Volume used in colorimeter (ml)}}$$

Figure 1. Groundnut cake source at pH 8.0

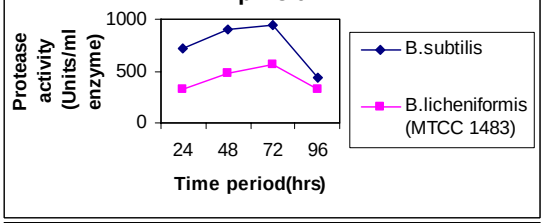


Figure 2. Wheat bran source at pH 8.0

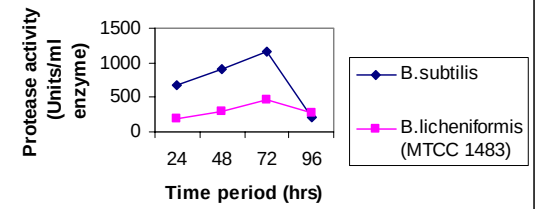


Figure 3. Groundnut cake source at pH 9.0

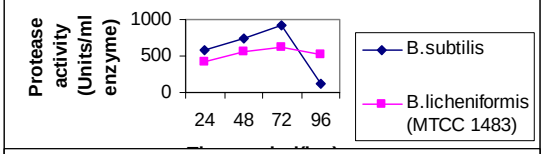


Figure 4. Wheat bran source at pH 9.0

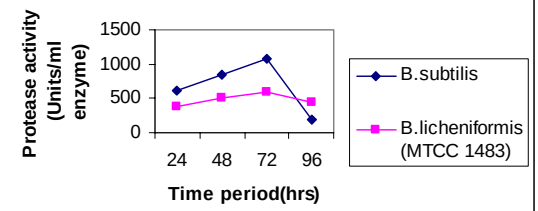


Figure 5. Groundnut cake source at pH 10.0

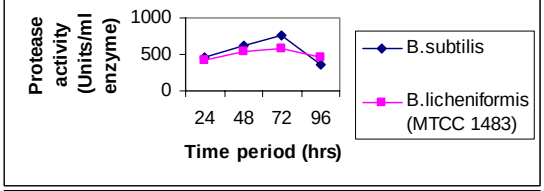
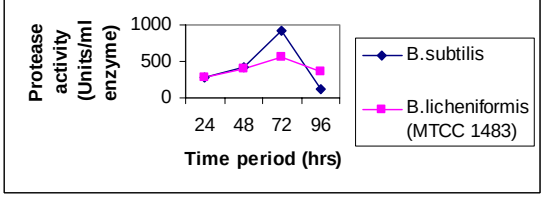


Figure 6. Wheat bran source at pH 10.0



One unit (Anson *et.al*., 1938) of enzyme will hydrolyze casein to produce color equivalent (Folin *et.al*., 1929) to 1.0 µmole (181 µg) of tyrosine per minute at pH 8.0 at 37 °C (color by Folin & Ciocalteau's reagent). The µmoles of tyrosine equivalents liberated were calibrated by using the standard curve. After evaluation, the protease activity was determined by the above formulae mentioned.

Results and discussion

Bacillus subtilis and *B. licheniformis* produce extracellular alkaline proteases (Pastor *et al*., 2001). The observations in the present study confirmed the wheat bran as the main carbon source yields the maximum protease activity of 1160 units/ml enzyme at pH 8.0 for *Bacillus subtilis,* which corresponds to the findings of Fikret *et al*., 2004 and 600 units/ml enzyme at pH 9.0 for *B. licheniformis* that relates to the study of Mabrouk *et al*., 1999. The present study confirms groundnut cake as the main nitrogen source yields the maximum protease activity of 940 units/ml of enzyme at pH 8.0 for *B. subtilis* and 615 units/ml of enzyme at pH 9.0 for *B. licheniformis*. The optimum enzyme activity was found at 72 hr and activity declined at 96 hr for both the species of *Bacillus* selected for the study. The present study also determines the *B. subtilis* isolated from the soil nearby Bharath University having a high potential with optimum enzyme activity compared to *B. licheniformis* (MTCC 1483). Bacterial growth analysis showed that slight statistically significant difference was observed between the two species of *Bacillus* using groundnut cake and wheat bran in the growth media ($P<0.05$, two tailored student's t-test).

pH plays an important role in activation and inactivation of enzymes. Each enzyme has an optimum pH for maximum enzyme activity. Optimum pH for *B. subtilis* was found to be 8.0 (Olajuyigbe *et al*., 2005) and for *B. licheniformis* was found to be 9.0 (Abu Sayem *et al*., 2006). The present study recorded the enzyme activity of *Bacillus* species at pH 8.0 in Table 1; Fig.1 & 2, pH 9.0 in Table 2; Fig. 3 & 4 and pH 10.0 in Table 3; Fig. 5 & 6 over different growth intervals in batch culture.

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