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Screening and Optimization of Extracellular Alkaline Protease Production from *Bacillus Spp*

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Abstract

Protease enzyme catalyzes the hydrolysis of protein. Among the various proteases, bacterial proteases are most significant when compared with animal and fungal proteases. In the present study a protease producing bacteria were isolated from soil collected from Govt. Holkar Science College, Indore campus and identified as Bacillus spp. They were grown within a temperature range between 25°C & 45 °C and pH range of 6.0 to 11.0. The optimum condition for protease production obtained was 35 °C at pH 9. The best carbon and organic nitrogen sources for this bacterial strain were fructose and yeast extract, respectively, while the most effective inorganic nitrogen sources was urea. It is envisaged that the isolate can be a potential source of alkaline protease for use as additive in industrial applications like detergent industry.

Keywords*: Alkaline protease, Bacillus sp, Screening*

Introduction

Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications (Mohen *et al*., 2005). They constitute two thirds of the total enzymes used in various industries and it account for at least a quarter of the total global enzyme production (Kumar *et al.,* 2002). These enzymes are used primarily in detergent additives, hence holding more than 50% of total enzyme market. Among bacteria, *Bacillus* spp. are specific producers of extracellular alkaline proteases (Godfray *et al.,* 1985). These enzymes occupy a pivotal position due to their wide application in food processing (Pastor *et al.,* 2001), pharmaceutical industries (Anwar and Saleemuddin, 1998; Gupta *et al.,* 2002), peptide synthesis (Kumar and Hiroshi, 1999), leather processing (George *et al.,* 1995) and in weaving processing (Helmann, 1995). Alkaline proteases occur widely in plants, animals and microorganisms.

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The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Kumar *et al.,* 2008). 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 , 2006). Alkaline proteases are produced by a wide range of microorganisms including bacteria, moulds and yeasts. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B. licheniformis, B. horikoshii, B. sphaericus, Bacillus furmis, Bacillus alcalophilus, Bacillus subtilis* (Ellaiah *et al.,* 2002).It is well established that extracellular protease production in microorganisms is greatly influenced by media components. Therefore, the effect of various carbon and nitrogen substrates, divalent metal ions, environmental and fermentation parameters were evaluated (Adinarayana and Ellaiah , 2002). The present investigation is aimed at optimization of growth conditions and other parameters which have been predicted to play a significant role in enhancing the production of alkaline proteases. For this, various parameters of nutritional and

environmental factors were tested and growth and protease activity were measured.

Material and Methods

Isolation: Soil samples were collected from college garden, Indore. One gram of soil was dissolved in 100 ml of distilled water. One ml of thoroughly mixed sample was used for serial dilution. The serially diluted samples were plated on basal medium of pH 8.5-11. The bacterial isolates were inoculated onto nutrient milk agar plates and incubated at 37^{o}C for 24 h. Proteolytic bacterial isolates showing zone of clearance was picked up, purified by repeated streaking on the same medium and finally transferred to nutrient milk agar slants and maintained at 4 °C.

Production media and culture conditions: The culture was grown in 250 ml of erlenmeyer flasks containing 100 ml medium consisting of glucose 1.5 g %, Urea 2.0 g %, KH_2PO_4 0.2 g %, K_2HPO_4 0.2 g %, $MgSO_4.7H_2O$ 0.1 g %, $CaCl_2$ 0.1g %, milk (Sanchi Shakti) 7.5% (v/v) for 24 h and inculated with loop full of 24 h old culture prepared in basal media. The pH of the media was adjusted to 9.0 and the supernatant were collected after centrifugation at 12,000 rpm for 10 minute as the crude enzyme source.

Protease assay: Proteolytic activity in the culture supernatant was determined by using the spectrophotometric method [Hagihara *et al.*,1958] with slight modification. 0.5ml of enzyme solution was incubated with 0.5 ml of 1% casein in glycine-NaOH buffer (0.2 M, pH 9) at 35 ^{o}C for 15 min and the reaction was terminated by the addition of 3 ml trichloroacetic acid (5%). The reaction mixture was allowed to stand for 15 minute. Tyrosine released was estimated using Folin Ciocalteau's Reagent. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of tyrosine/min/ml under standard assay conditions.

Optimization of temperature and pH:

The effect of temperature was determined by growing the isolate in production media at varied temperatures (25-45^{o}C). The effect of pH on protease production of the isolate P-2b was determined by growing the isolate in production media of different pH in the range of 6-11 using appropriate buffers, Tris-HCL buffer (pH 6.0–8.0), glycine-NaOH buffer (pH 9.0– 11). All the flasks were incubated at 35^{o}C for 24 hours. The resulting culture was subjected to centrifugation at 10,000 rpm, 4^{o}C for 15 minutes. Finally the protease activity was assayed.

Optimization of various carbon and Nitrogen sources

Effect of Carbon Sources: The sterilized production broth was prepared with Glycine-NaOH buffer (pH-9) with the various carbon sources like fructose, sucrose, lactose and maltose. These carbon sources were used to replace the carbon source available in the media. The isolate P-2b was inoculated into different carbon sources flasks and the flasks were incubated at 35^{o}C for 24 hours. The resulting culture was subjected to centrifugation at 10,000 rpm, 4^{o}C for 15 minutes. Finally the protease activity was assayed.

Effect of Nitrogen Sources: The sterilized production broth was prepared with Glycine-NaOH buffer (pH-9) with the various nitrogen sources like Peptone, Yeast extract, Ammonium Sulphate and Potassium nitrate. These nitrogen sources were used to replace the nitrogen source available in the media. The isolate P-2b was inoculated into different nitrogen sources flasks and the flasks were incubated at 35^{o}C for 24 hours. The resulting culture was subjected to centrifugation at 10,000 rpm, 4^{o}C for 15 minutes. Finally the protease activity was assayed.

Results and Discussion

The extracellular protease enzyme was synthesized by *Bacillus* sp. isolated from soil of Govt. Holkar Science College garden, Indore. The results obtained in the present study revealed the ability of collected *Bacillus* sp. to produce extracellular protease. *Bacillus* spp. usually produces extra cellular protease during late exponential phase (Ward, 1985). Different culture conditions were used to obtain the maximum levels of protease production by *Bacillus* spp. (Plate 1).

Effect of temperature and pH on growth and protease production: The effects of different incubation temperatures on protease production were evaluated. It is known that temperature is one of the most critical parameters that have to be controlled in bioprocess (Chi and Zhao, 2003). It is obvious from the results (Fig. 1) that 35 ^{o}C was generally more favourable for protease production.

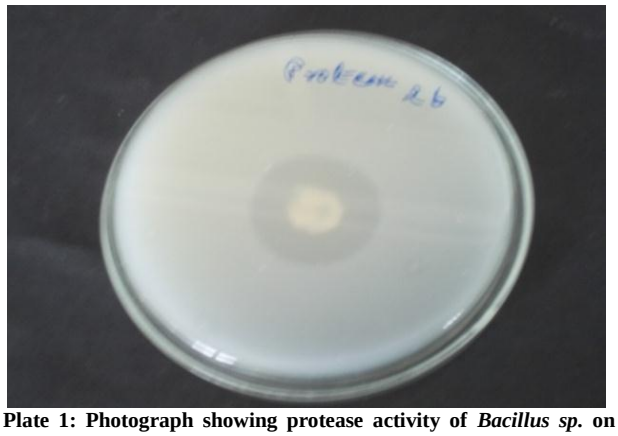
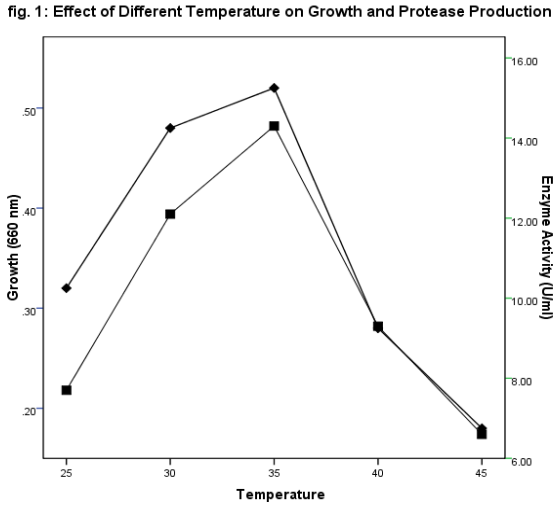
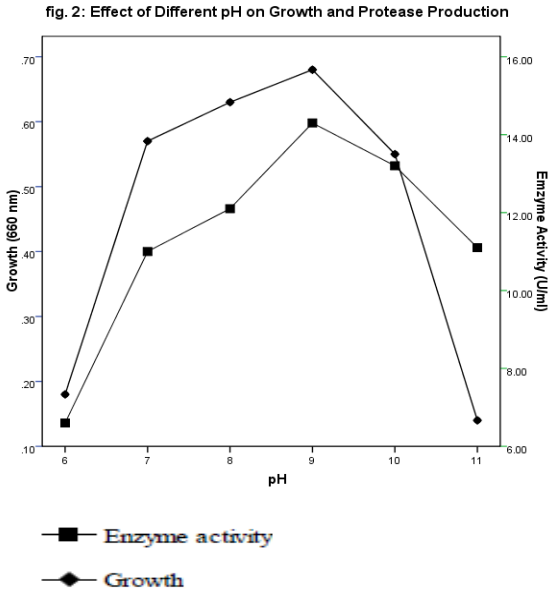


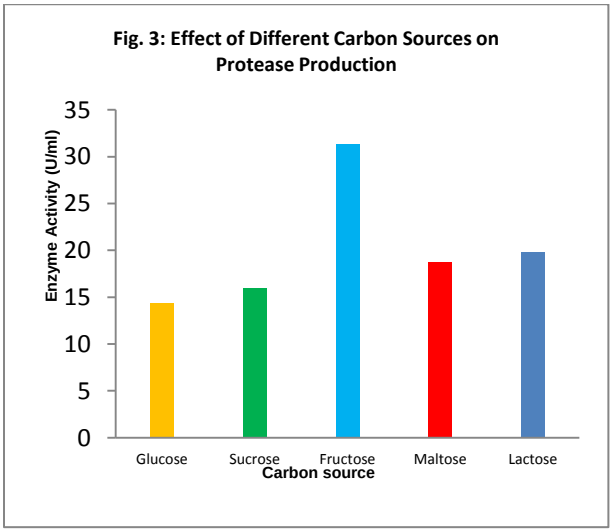
Plate 1: Photograph showing protease activity of *Bacillus sp.* on nutrient milk agar media



However, the temperature below or above 35 ºC resulted a sharp decrease in protease yield as compared to the optimal temperature. It has been noted that the important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production (Kumar and Tagaki, 1999). The production medium was adjusted at different pH values of different buffers. The results of pH studies showed (Fig. 2) that the best buffer was Glycine-NaOH buffer with pH 9.0 for protease production. A notable decline in the enzyme productivity occurred at both higher and lower pH values.

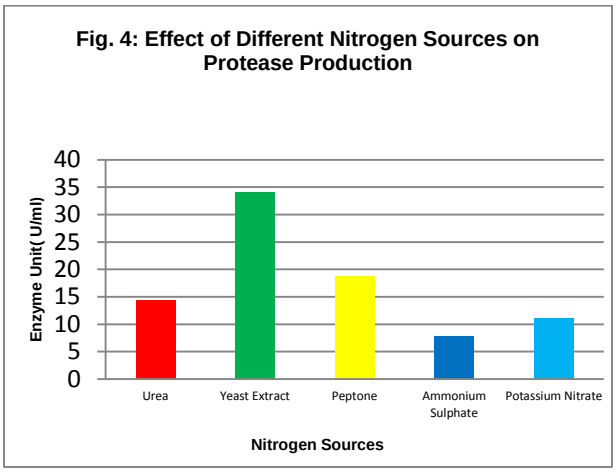
Effect of Carbon Source: Various sources of carbon such as fructose, lactose, maltose and sucrose were used to replace glucose which was original carbon source in production media. Results obtained showed that, fructose brought the highest protease production as compared to other carbon sources at 24 hrs of incubation (Fig 3). Hence, fructose was the best carbon source for protease production. Fructose has been reported as the best carbon source for alkaline protease production by *Bacillus licheniformis* S40 (Sen and Satyanarayana, 1993).





Effect of Nitrogen Source: Production of extracellular protease has been shown to be sensitive to repression by different carbon and nitrogen sources (Haulon *et al.*, 1982). The effect of nitrogen sources was studied in the production

medium, where urea was replaced with peptone, yeast extract, ammonium sulphate and potassium nitrate. Among various nitrogen sources tested, yeast extract found to be best nitrogen source for protease production (fig 4).



Conclusion

The media optimization is an important aspect to be considered in the development of fermentation technology to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. However, particularly the *Bacillus* spp. are known for their ability to produce proteolytic enzymes with potential use in industries. In the present investigation we have determined the optimum parameter for maximum production of alkaline protease. The best carbon source for protease production was fructose while organic nitrogen sources were better for growth and enzyme production compared to inorganic ones.

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