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Optimization of Culture Conditions for the Production of Protease from *Bacillus megaterium*

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Keywords

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Abstract

In this Study, *Bacillus megaterium* was used for protease production to examine the optimal condition for its maximum accumulation. Hence the production of enzyme was investigated at various pH, temperature, NaCl concentrations and different carbon and nitrogen sources. The medium amended with 3.0% NaCl, 1.0% gelatin, 0. 4% lactose, 0.5% peptone and pH 9, incubation at 40°C for 42 h were supported maximum protease production of 78.5 U/ml.

1. Introduction

Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. Microbial proteases are among the most important hydrolytic enzymes and have been considered extensively since the advent of enzymology (Gupta *et al*., 2002). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play a critical role in cellular metabolic processes but have also gained considerable attention in the industrial community. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. They are obtained in large quantities in a relatively short time by established microbial fermentation techniques. These are a single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Recently, the use of alkaline proteases has increased significantly in various industrial processes such as detergent and feed additives, dehairing, decomposition of gelatin on X-ray films and peptide synthesis. However, there is a considerable gap between the demand and supply of these enzymes. Proteases represent one of the three largest groups of enzymes and account for 60% of total worldwide sales of enzymes. Among the various proteases, bacterial proteases exhibit most significant applications in industries when compared with plant and animal proteases. Therefore there is an increased interest in microbial proteases.

Microorganisms represent an excellent source of enzymes owing to their broad chemical diversity and susceptibility to genetic manipulation. At present the overall cost of enzyme production is high due to expensive media used and therefore development of novel processes that minimize the cost of production and increase the yield of protease with respect to their industrial requirements is highly appreciable from the commercial point of view (Ashis *et al*., 2008).

Generally proteases of micro organisms are constitutive and are partially inducible in nature as well as under culture conditions. Although a number of microorganisms produced proteases, *Bacillus* strains are recognized as important sources of commercial alkaline proteases because of their ability to secrete large amounts enzymes with high activity (Christiansen and Nielsen, 2002; Joo *et al*., 2002; Beg and Gupta, 2003; Joo *et al*., 2003). It is well established that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, dissolved oxygen and incubation time (Kaur *et al*., 2001; Oberoi *et al*., 2001). *Bacillus* species produce extracellular proteases during post exponential and stationary phases.

In the present paper, we attempted to optimize the culture conditions of *Bacillus megaterium* for maximal protease productivity by using different physico-chemical parameters.

2. Materials and Methods

Organism

Bacillus megaterium, isolated from the red seaweed, *Kappaphycus alvarezii*, available as stock culture at the Centre for Advanced Studies in Botany, University of Madras, India, was used in the present study. The stock culture was maintained on nutrient agar at 4°C.

Extracellular protease activity

The presence of protease activity on plates was routinely determined by Ammar *et al.,* (1998)

method using Nutrient Agar medium supplemented with 2% gelatin. After incubation at 37°C for 48 h the plates was flooded with mercuric chloride solution ($HgCl_2$-15g and 20 ml of 6.0 N HCl made up to 100 ml with distilled water) and a complete degradation of the gelatin was observed as a clearance of the zone.

Media composition

The organism was grown aerobically in a 250 ml flask containing 50 ml basal medium contained: Glucose – 1%, Peptone – 0.5%, Yeast extract – 0.5%, KH_2PO_4 – 0.1, $MgSO_4.7H_2O$ - 0. 02%, Na_2CO_3 -1%, pH - 9.0, Seawater – 100 ml. Na_2CO_3 was sterilized separately and then added to medium.

Effect of pH and temperature on protease production

Bacillus megaterium was inoculated in the medium at different pH *viz*., 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 and incubated at different temperatures *viz*., 20, 30, 40, 50, 60, 70 and 80°C at pH 9.0. Incubation at specific temperatures was carried out for a period of 54 h. At every 6 h interval, the culture filtrates were collected and after centrifugation used for the estimation of protease activity.

Effect of different concentrations of sodium chloride on protease production

The medium (pH 9.0) was amended with different concentrations of sodium chloride *viz*., 1, 2, 3, 4 and 5%, then culture was inoculated and incubated at 37°C for 54 h. The protease activity of the culture was estimated at every 6 h interval.

Effect of different carbon sources on protease production

Different carbon sources namely glucose, sucrose, fructose, maltose, lactose, mannose, starch, cellulose, xylose and glycerol were used individually at a concentration of 0.2% in the medium amended with 3% NaCl at pH 9.0. The experimental flasks were inoculated with *B. megaterium* and incubated at 37°C for 54 h. Culture filtrates were collected by centrifugation at every 6 h interval and the supernatant was assayed for protease activity. In another experiment, different concentrations of lactose were tried at different concentrations *viz*., 0.2, 0.4, 0.6, 0.8 and 1.0% to study their effect of protease production.

Effect of different nitrogen sources on protease production

Different organic and inorganic nitrogen sources namely Peptone, $NaNO_3$, Yeast extract, Urea, NH_4Cl, KNO_3, NH_4NO_3, beef extract and ammonium sulphate were used at a concentration of 0.2% separately in the medium amended with 3% NaCl and lactose 0.4% at pH 9.0. *Bacillus megaterium* was inoculated and incubated at 37°C for 54 h. The culture filtrate was collected by centrifugation at every 6 h interval and the supernatant was used for the estimation of protease activity. The above medium amended with peptone at different concentrations *viz*., 0.5, 0.75, 1.0, 1.25 and 1.5% was also studied similar to the above procedure for its effect on protease production by *B. megaterium*.

Effect of different substrates on protease production

Six different substrates namely skim milk powder, gelatin, soybean, egg albumin, peptone and casein were used separately at a concentration of 1.0% in the medium amended with 3% NaCl, lactose 0.4% and peptone 0.5% at pH 9.0 to study the effect of these substrates on protease production by *B. megaterium*. Then the experimental flasks were inoculated with *B. megaterium* and incubated at 37°C for 54 h. The culture filtrate was collected by centrifugation at every 6 h interval and the supernatant was used for the estimation of protease activity. In addition, culture filtrates of the *B. megaterium* obtained by growing in the medium (pH 9.0) amended with gelatin alone at different concentrations *viz*., 0.5, 0.75, 1.0, 1.25 and 1.5% was also tested for protease production.

Protease assay

The protease activity was determined by caseinolytic assay method (Kanekar *et al*., 2002). The cell free supernatant (1.0 ml) was mixed with 4.0 ml of casein (0.625% w/v) and incubated at 60°C for 30 min. The reaction was stopped by adding 5.0 ml of 5% trichloroacetic acid. Enzymatically hydrolyzed casein was measured by the Modified Folin-Ciocalteu method against casein treated with inactive enzyme as blank. A standard graph was prepared by using tyrosine (10–100 μg/ml). One unit of protease activity was defined as the amount of enzyme which liberated 1μg tyrosine per min at 37°C.

3. Results

Bacillus megaterium showed hydrolysis of gelatin by culture grown for 48 h on nutrient agar plate supplemented with 2% gelatin. The cell free extract also showed clear zones in the gelatin cup assay (Fig. 1).

Fig. 1: Proteolytic activity of *Bacillus megaterium* on gelatin agar medium

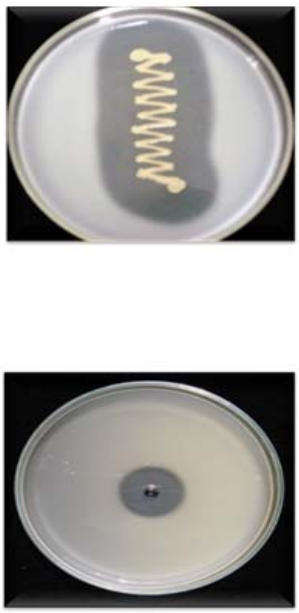


Fig. 2: Optimization of different pH on extracellular protease production by *Bacillus megaterium*

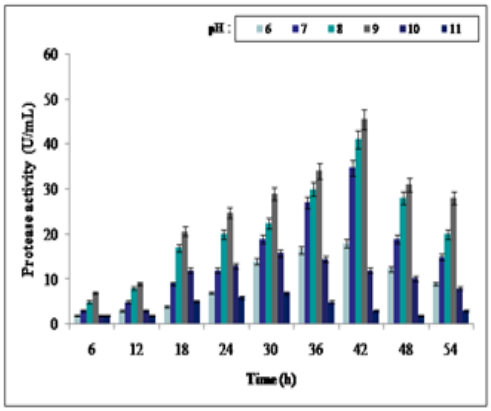


Fig. 3: Optimization of different temperatures for extracellular protease production by *Bacillus megaterium*

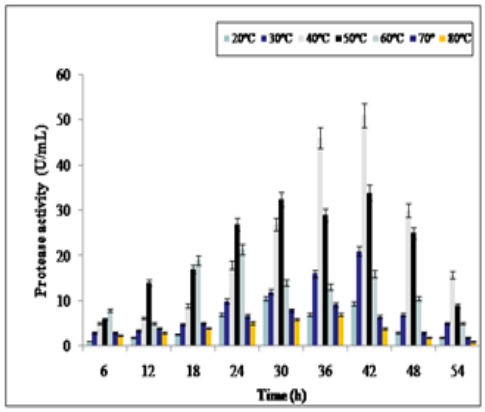


Fig. 4: Effect of different concentrations of sodium chloride on extracellular protease production by *Bacillus megaterium*

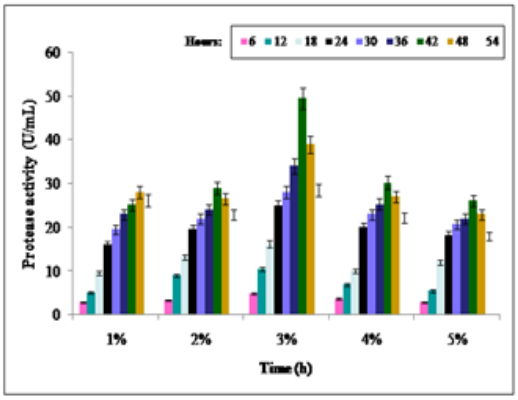
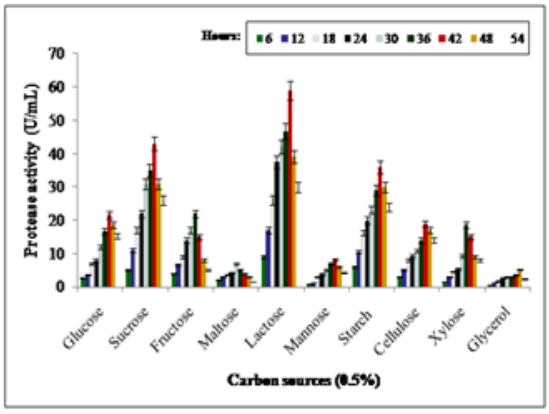


Fig. 5: Effect of different carbon sources on extracellular protease production by *Bacillus megaterium*



The initial pH of the medium greatly influenced the extracellular protease activity of *B. megaterium*. The bacterium was able to release a maximum of 45.5 U/ml protease at pH 9.0 after 36 h (Fig. 2). The data presented in Fig. 3 showed that *B. megaterium* produced maximum of 51 U/ml protease after 42 h at 40°C. Maximum extracellular protease by *B. megaterium* was observed in the medium containing 3% NaCl. Extracellular protease level in these cultures amounted to 49.5 U/ml at 42 h (Fig. 4).

Ten different carbon sources such as glucose, sucrose, fructose, maltose, lactose, mannose, starch, cellulose, xylose and glycerol were tested at 0.2% to assess their effect on extracellular protease production by *B. megaterium*. Among the different carbon sources tried, lactose supported a maximum protease production of 59 U/ml at 42 h followed by 43 U/ml by sucrose and 36 U/ml by starch. Glycerol and fructose as carbon source completely suppressed extracellular protease production in *B. megaterium* (Fig. 5). Among the different concentrations of lactose tested, the medium

amended with 0.4% lactose increased the extracellular protease production in *B. megaterium* to 55.6 U/ml at 42 h (Fig. 6).

Nine different nitrogen sources such as beef extract, sodium nitrate, yeast extract, urea, peptone, ammonium chloride, potassium nitrate, ammonium nitrate and ammonium sulphate were amended in the medium at 0.2% concentration to choose the best nitrogen source for high extracellular protease production in *B. megaterium*. A maximum protease production of 41 U/ml at 42 h was recorded in medium amended with peptone. Protease activity was initiated after 18 h of inoculation, attained peak at 42 h and declined thereafter (Fig. 7).

Peptone at 0.5% concentration made *B. megaterium* to secrete maximum quantities of extracellular protease i.e. 68 U/ml at 42 h (Fig. 8). Hence, peptone at 0.5% concentration was chosen for protease production in test organism for further study.

Six different substrates namely skim milk powder, gelatin, soybean, egg albumin, peptone and casein were tested as substrates of protease in the medium at 1.0% and assessed for their relative ability to induce the synthesis of extracellular protease by *B. megaterium*. Of the six different substrates, gelatin (1.0%) supported a maximum extracellular protease production to give an yield of 78.5 U/ml at 42 h. Protease in the extracellular fraction was observed after 18 h and the levels of the enzyme gradually increased to reach a maximum at 42 h (Figs. 9 and 10).

Fig. 6: Effect of different concentrations of lactose on extracellular protease production by *Bacillus megaterium*

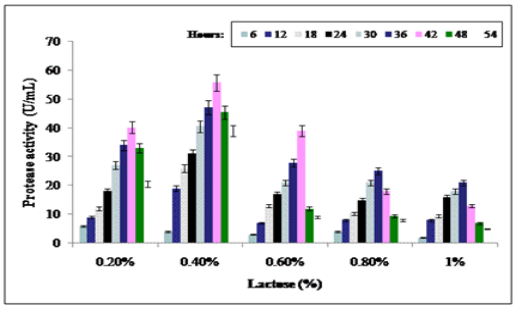


Fig. 7: Effect of different nitrogen sources on extracellular protease production by *Bacillus megaterium*

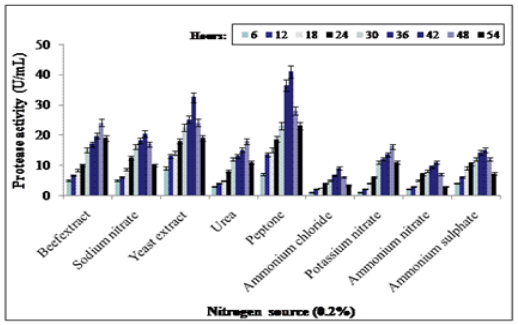


Fig. 8: Effect of different concentrations of peptone on extracellular protease production by *Bacillus megaterium*

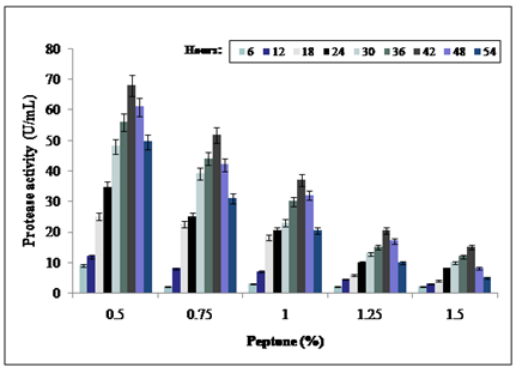


Fig. 9: Effect of different substrates on extracellular protease production by *Bacillus megaterium*

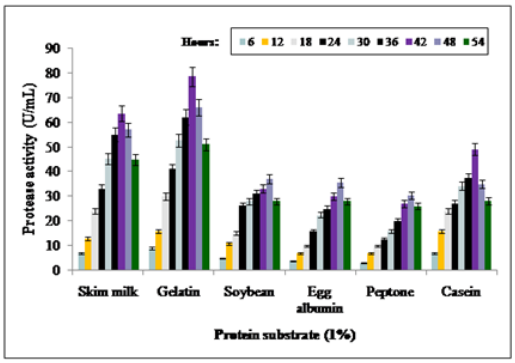
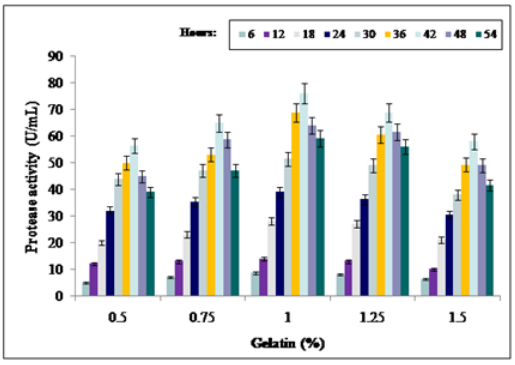


Fig. 10: Effect of different concentrations of gelatin on extracellular protease production by *Bacillus megaterium*



4. Discussion

Microbial enzymes possess almost all characteristics decided for biotechnological application. Thus, the microbes serve as preferred source of enzymes because of their rapid growth, limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various applications. Industries involved in detergent production and leather processing prefer enzymes with wide range tolerance to elevated temperature, alkaline pH and salt concentrations. By virtue of growing in extreme habitats, the microorganisms of these habitats can

be expected to be the best source of industrially useful enzymes with desired qualities.

Microbial proteases being extracellular in nature, they are directly secreted into the fermentation broth by the organism simplifying down-stream processing of the enzyme when compared to the proteases obtained from plants and animals. Despite, the long list of protease producing microorganisms, only a few are considered as appropriate producers for commercial exploitation, being Generally Regarded As Safe (GRAS), non toxic and non pathogenic (Gupta *et al*., 2002). Of these, strains of *Bacillus* spp. dominate the industrial sector (Gupta *et al*., 2002). Alkaline proteases have been given considerable attraction due to their potential application in leather processing and detergent industries. The demand for proteases has increased ever since their potential has been recognized. The production of proteases from natural sources is unable to meet the current industrial demands for the enzyme.

Culture conditions such pH, temperature, source of carbon, nitrogen, protein and metal ions are known to influence the synthesis and secretion of extracellular enzymes by microorganisms. Optimization of the culture conditions is hence, necessary in the selection of the bacterial source for industrial exploitation of their extracellular enzymes. Extracellular protease production in microorganisms is strongly influenced by medium components, e.g. variation in C/N ratio, presence of easily metabolizable sugars, such as glucose (Beg *et al*., 2002) and metal ions (varela *et al*., 1996). Besides these, factors such as aeration, inoculum density, pH, temperature and incubation period also influence the protease production by microorganisms in culture (Puri *et al*., 2002). The optimum period of growth in the medium for maximum protease production by the test bacterium *B. megaterium* was 42 h. This period of incubation or growth in the medium for optimum protease production differ with the bacterial source, *Bacillus laterosporus* AK1 was reported to require 38 h for maximum protease production (Arulmani *et al*., 2007). Abdul-Raouf (1990) reported that both *Bacillus anthracis*, S-44 and *Bacillus cereus* var. *mycoides*, S-98 synthesized maximum quantities of proteases at 24 h and the productivity reached up to 126.09 U/ml^{-1} for *B. anthracis* S-44 and 240.45 U/ml for *B. cereus* var. *mycoides* S-98. Johnvesly *et al.* (2002) observed a high level of extracellular thermostable protease activity in *Bacillus* sp. JB-99 was observed after 24 h of incubation in the selective medium.

The pH of culture medium plays a vital role in microbial growth and protease production. The observations made in the present study indicated that the organism *B. megaterium* preferred the pH 9.0 for maximum protease production. A similar observation was reported by Johnvesly and Naik (2001) and Han *et al.* (2002) for different strains of *Bacillus*. The pH required for maximum thermostable protease production by a thermo-alkaliphilic *Bacillus* sp. JB-99 was reported to be 11.0 (Johnvesly *et al*., 2002). Similarly, both the maximum production and activities of the protease from *Bacillus sphaericus* strain C3-41 were found to depend on a pH of 11.0 and the enzyme was stable in a wide range of pH from 5.0 to 12.0 (Sun *et al*., 1997).

Temperature optima for protease production varied from 34 to 60°C depending on the bacterial strain used. It has been reported that optimum temperature for maximum protease production in *Bacillus alcalophilus* (Kanekar *et al*., 2002), *Bacillus horikoshii* (Joo *et al*., 2002), *Bacillus* sp. (Mehrota *et al*., 1999), *B. clausii* (Ganesh *et al*., 2004) and *Bacillus* sp. JB-99 (Johnvesly and Naik 2001) was 30°C, 34°C, 40°C, 42°C, 50°C, and 55°C, respectively. Secades *et al.* (2001) reported that *Flavobacterium psychrophilum* produced maximum extracellular protease at temperatures between 25 and 40°C. Jobin and Grenier (2003) also observed that the optimum temperature for protease production by *Streptococcus suis* serotype 2 ranged from 25 to 42°C. In the present study, *B. megaterium* was found to require an optimum temperature of 40°C for maximum protease production.

Ten different carbon sources were investigated for inducing maximum protease production by *B. megaterium* in culture. The bacterium preferred lactose as the best carbon sources for maximum extracellular production of protease and the yield was 55.6 U/ml. The sugar lactose at a concentration 0.4% supported maximum extracellular enzyme production whereas the concentrations above 0.4% appeared to repress the synthesis of the enzyme. A similar observation was reported by Mabrouk *et al.* (1999) for *B. licheniformis* ATCC 21415. Increased yields of alkaline proteases with different sugars such as lactose, maltose, sucrose and fructose were reported by several workers (Malathi and Chakraborthy, 1991; Tsuchiya *et al*., 1991; Phadatare *et al*., 1993). Andrade *et al.* (2002) observed that the protease production reached a maximum in a medium amended with 0.4% glucose. In contrast, Madzak *et al.* (2002) recorded that sucrose was found a suitable substrate for extracellular protease production.

Different nitrogen sources such as Peptone, $NaNO_3$, Yeast extract, KNO_3, Urea, NH_4Cl, NH_4NO_3, beef extract and ammonium sulphate were also tested for maximum protease production by *B. megaterium*. It has been reported that the amendment of organic nitrogen enhanced the production of enzyme in microbes (Gajju *et al*.,

1996). *Bacillus megaterium* also preferred organic nitrogen than inorganic nitrogen for the production of the enzyme at optimum levels. Peptone amended medium in the present study enhanced protease production by *B. megaterium*. Peptone offers a balanced source of protein in terms of composition and accessibility to induce protease synthesis. Peptone was able to enhance extracellular protease secretion to the level of 68 U/L. The next best nitrogen source for protease synthesis was yeast extract. Synthesis and secretion of protease by *B. megaterium* appeared to be good when inorganic nitrogen compounds were used as the nitrogen source while reduced nitrogenous compounds (ammonium compounds) reduced the secretion of the enzyme into the medium. Johnvesly and Naik (2001) observed substantial increase in protease production by *Bacillus* sp. JB-99 in the presence of beef extract in their studies on the effect of organic N-sources as a function of graded concentrations.

Among the different substrates tested, gelatin at 1.0% (w/v) was able to increase the extracellular protease concentration to appreciable levels i.e. 78.5 U/ml at 42h. Similarly, *B. anthracis* also has been reported to synthesize maximum extracellular protease in medium amended with 1.0% gelatin as carbon source (Abdul-Raouf, 1990). In contrast, Drucker (1972) reported the preference of casein and skim milk powder as substrates for enhanced protease synthesis by *B. licheniformis* MIR 29.

As a result of adaption to their environment, many microorganisms have evolved unique properties of considerable biotechnological and commercial significance. The *Bacillus megaterium* is a good source of protease and may possess commercial importance.

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