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PRODUCTION AND OPTIMIZATION OF EXTRA CELLULAR PROTEASE FROM *BACILLUS* SP. ISOLATED FROM SOIL

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ABSTRACT

A thermophilic *Bacillus* sp. was isolated from the soil and characterized for the production of protease enzyme. The bacterium was stable at high temperature (65^0C). Maximum enzyme activity was observed when culture was grown at 55^0C (0.61 U/ml). At 48h of incubation period, the highest enzyme activity of 0.58 U/ml was observed. At alkaline pH, enzyme activity was optimal and the highest was observed at pH 9. Among the various carbon and nitrogen sources studied, maximum activity was recorded when glucose (0.60 U/ml) and peptone (0.57 U/ml) were used in the medium.

Keywords: Alkaline protease, casein hydrolysis, enzyme activity, *Bacillus* sp.

[I] INTRODUCTION

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in detergents, pharmaceuticals, brewing, leather, food industry and waste treatments [1]. Proteolytic enzymes are the most important industrial enzymes, representing worldwide sale about 60% of total enzyme market [2]. Proteases are hydrolytic enzymes, which act upon native proteins to breakdown into small peptides and amino acids. Proteases are obtained from plant, animal and microbial sources. Microorganisms are the preferred source for obtaining proteases because of their fast growth rate, easy to manipulate for getting highly stable enzymes through genetic engineering and requires shorter time for production and purification steps [3, 4]. From plants papain and ficin are important proteases, trypsin and chemotrypsin from animals and alkaline proteases from microorganisms like *Bacillus sp*, Microbacterium [5, 6], *Penicillium sp, Aspergillus sp* etc. [7, 8].

Among the various proteases, proteases from *Bacillus* sp. are the most significant, compared

with animal and fungal proteases [9]. *Bacillus* sp. produces alkaline and neutral protease. Bacterial neutral proteases are active in narrow pH range (pH 5.0- 8.0) and have relatively low thermostability [10]. The alkaline proteases are active in pH range (pH 9-11) hydrolyze extended spectrum of peptide bands [11]. There are several reports on the thermostable proteases produced by many thermophiles such as *Bacillus stearothermophilus* [12], *Thermus aquaticus* [13], *Bacillus lichiniformis* [14], *Bacillus subtilis* 3411 [15], *Bacillus licheniformis* [16]. Biochemical properties of the enzymes produced from these thermophiles have also been well investigated.

With increasing industrial demands for biocatalysts that can cope with the industrial processes at harsh conditions, the isolation and characterization of new promising strains are possible ways to increase the yield of such enzymes [17]. So it is desirable to search for new proteases with novel properties from as many different sources as possible. The aim of the present study was to isolate *Bacillus* sp from the soil and optimize the conditions for maximum production of extra cellular protease.

[II] MATERIALS AND METHODS

2.1 Isolation of microorganism

Soil samples were collected from different places in and around Bangalore University campus, Bangalore, India. About 1-5g of sample were suspended in sterilized distilled water and 0.1ml of this suspension was spread on a nutrient agar plate and incubated at 37^0C for 24 h. Microorganism which formed colonies on the plates, were examined microscopically. Well isolated rod shaped colonies were picked and further purified by repeated streaking on nutrient agar plates.

2.2 Screening for proteolytic activity and identification

The purified bacterial isolates were plated on the agar plates containing casein (1% w/v) and milk powder (1% w/v) and incubated at 37^0C for 48 h. The plates were flooded with 25% TCA (trichloro acetic acid) solution and incubated for 15 min at 45^0C. Depending on the clear zone of hydrolysis, strain KSM P-1 was selected for further experimental studies. It was maintained on nutrient agar slants at 4^0C and was subcultured at 1 week intervals. The isolated strain was identified based on cellular morphology, gram staining, motility and biochemical profile tests [18].

2.3 Production of proteases

The culture medium (90ml sterile broth) containing glucose (1.0g/l), peptone (10.0g/l), yeast extracts (0.2g/l), $CaCl_2$ (0.1g/l), K_2HPO_4 (0.5g/l) and $MgSO_4$ (0.1g/l) was inoculated with 10ml *Bacillus* KSM P-1 inoculum and incubated at 37^0C for 48 h in a shaking incubator (150rpm). After 48 h of incubation, the cells were harvested at 15000 rpm for 10 min and the clear crude supernatant was stored at 4^0C for further studies.

2.4 Protease assay

The enzyme was assayed in the reaction mixture containing 2.0ml of 0.5% casein solution in 0.1 M CO_3-HCO_3 buffer (9.0) and 1ml enzyme solution in a total volume of 3.0ml. The reaction were carried at 37^0C for 10 min and then terminated by adding 3.0 ml of 10% TCA and centrifuged at 10000 rpm for 15 min. One unit of protease activity is defined as the amount of enzyme required to liberate 1µmol tyrosine in 1 min under the standard assay conditions used.

2.5 Protein assay

Total protein of the cell free filtrate was determined by the Lowry's method [19]. Bovine serum albumin (250µg/ml) was used as a standard.

2.6 Effect of incubation period, temperature and pH on protease production

The effect of incubation period for protease production was determined by incubating production medium at different incubation period viz. 12, 24, 48, 72 and 96h. Optimum temperature for protease production was achieved by incubating the culture medium at 25- 65^0C by the increment of 10^0C for 48h. The pH of the

medium was adjusted before autoclaving from 5.0- 11.0 by the increment of 1.0 at 37^0C for 48h.

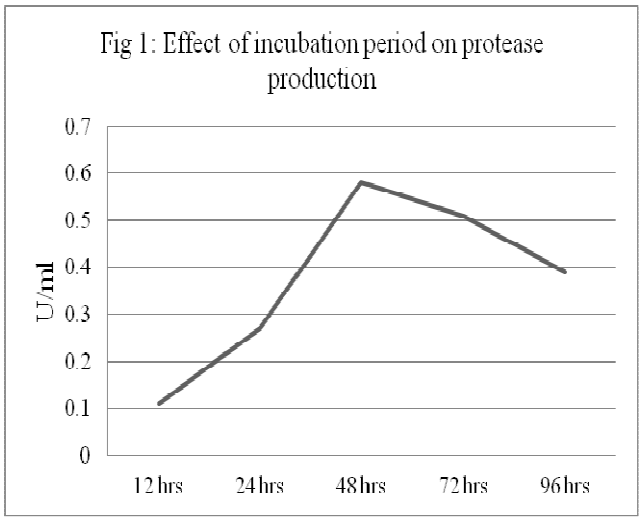
2.7 Effect of different carbon and nitrogen sources on protease production

The culture medium was added with different carbon source (2%) such as glucose, sucrose, maltose, lactose, mannitol and sorbitol for protease production. To study the effect of different nitrogen sources on protease production, nitrogen source (0.1%) such as ammonium chloride, ammonium sulphate, potassium nitrate, peptone, tryptone and yeast extract were used in culture medium.

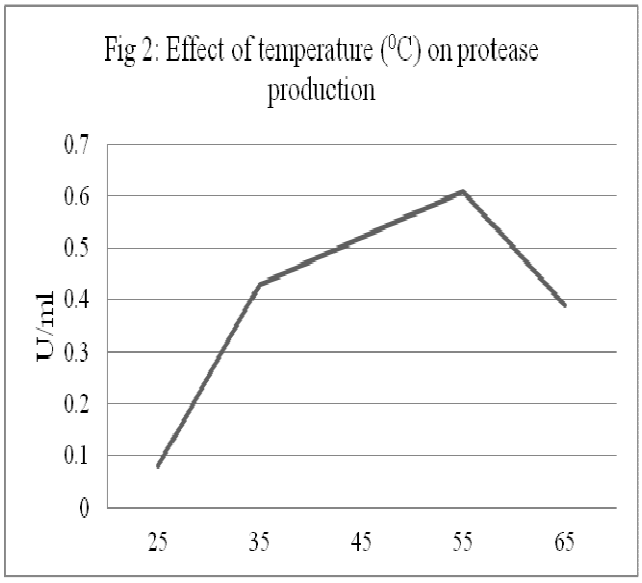
[III] RESULTS AND DISCUSSION

In the present study, the isolated bacteria were identified as *Bacillus* sp. by using morphological and biochemical characteristics [20]. The *Bacillus* isolates were then characterized for protease production by using the agar plates containing casein (1% w/v) and milk powder (1% w/v), incubated at 37^0C for 48 h. The proteolytic activity was detected by the presence of clear zone. It was found that *Bacillus* sp. KSM P-1 yielded the highest protease activity with a clear zone of hydrolysis.

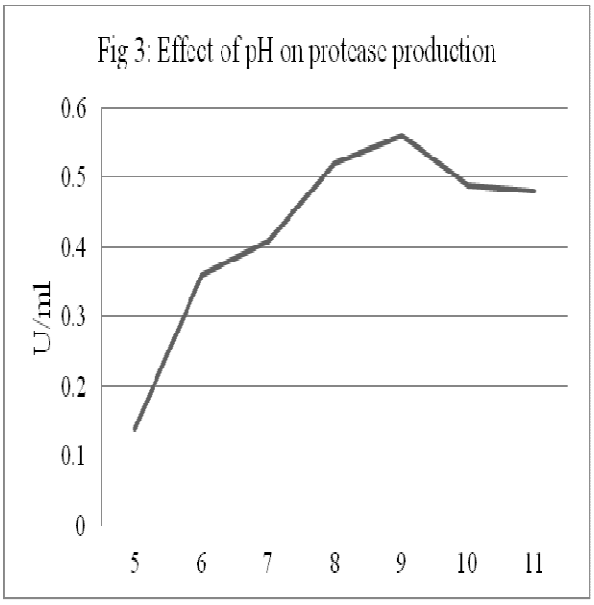
Incubation period plays an important role in the maximum production of enzymes. In the present study, maximum enzyme production was observed at 48h of incubation (Fig 1). The culture showed typical growth curve of bacteria with exponential starting after 12h and reaching the stationary phase at 48h of growth. Maximum protease was produced during the stationary phase. A gradual decrease in enzyme units was observed with increase in incubation period, clearly suggesting that the enzyme production is growth associated in nature. The maximum enzyme production was observed during continuous growth of the culture at the late exponential phase and early stationary phase of the growth and thereafter number of viable cells decreased due to depletion of readily available carbon source and other nutrients. These results are in accordance with observations made by Durhams [21], Gessesse [22] and Qadar et al., [23]. The subsequent decrease in the enzyme units could probably due to inactivation of enzyme by other constituent proteases [24].



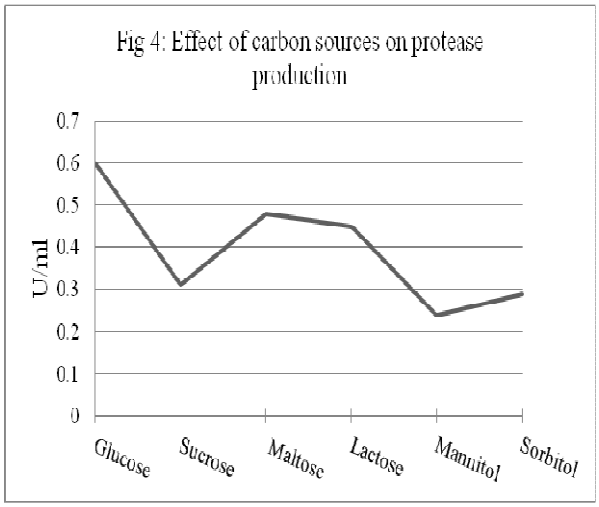
It was reported that maximum protease production was achieved at 47^0C [25], while 60^0C was the best temperature for *B. subtilis* PE-11 [26] and 37^0C was reported to be the best temperature for protease production for certain Bacillus sp. [27]. Qadar et al., have reported maximum protease production at 35^0C by using *Bacillus sp.* PCSIR EA-3 [28]. However, in the present study, the optimum temperature for protease enzyme activity was found to be at 55^0C (Fig 2).

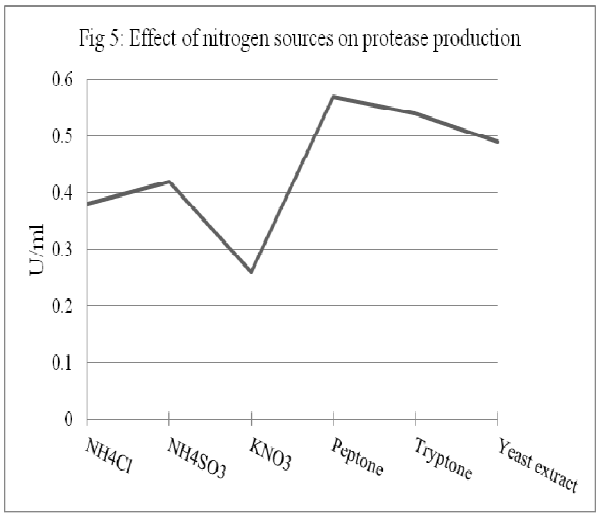


The optimum pH for protease activity was 9 although the enzyme was active in the pH range of 7- 12 (Fig 3). From a survey of literature it can be seen that the optimum pH range of alkaline proteases is generally between pH 9 to 11. Alkaline proteases of *Bacillus subtilis* PE-11 with similar properties have been reported by Adinarayana et al., [27]. These findings are in accordance with several earlier reports showing pH optimal of 9.0-10.5 for protease from *Bacillus* sp. by Durham [29], *Xanthomonas maltophila* by Debette [30] and *Vibrio metscnikovii* by Kwon et al., [31].



Among the various carbon sources used for protease production, glucose was found to be the best substrate, showing maximum enzyme activity of 0.60U/ml (Fig 4). This was followed by sucrose, maltose, mannitol, lactose and sorbitol. Similarly glucose was found to be the best carbon source for protease production by *Bacillus subtilis* [32] while starch was found to be best carbon source for protease production by *Bacillus licheniformis* S-40 [16].





Effect of various nitrogen sources on protease production in growth medium was also examined. It was observed that growth medium containing peptone produced maximum protease (Fig 5). This was followed by yeast extract, tryptone, ammonium sulphate and ammonium chloride. Protease activity was found minimum in potassium nitrate containing medium. Nilegaonkar et al., has used peptone, tryptone, yeast extract and casein as substrates for alkaline protease production [33]. Atalo and Gashe showed that yeast extract and peptone can induce the alkaline protease production in glucose medium [34].

In conclusion, *Bacillus* KSM P1 was able to produce protease enzyme on growth medium and showed optimum activity at pH 9 and temperature 55^0C. The best carbon and nitrogen source was found to be glucose and peptone. The genetic manipulation of this organism is required to increase productivity and then it can be used for protease production on industrial scale.

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