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Production and Optimization of Extracellular Alkaline Proteases from *Bacillus* Sp. Isolated From Marine Soil

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ABSTRACT

The production of extracellular alkaline protease by producing *Bacillus* sp. was studied and the production parameters were optimized. Various bacterial samples were isolated from different marine soil and the isolates were positive on skim milk agar (1%) and thus were selected as protease producing strain. Among them, maximum protease producing strain was identified by Bergey's manual of determinative bacteriology. The growth conditions for protease production by identified *Bacillus* sp. were optimized with different fermentation period, pH, temperature, carbon and nitrogen sources. Among the different carbon sources, sucrose was found to be best source for the *Bacillus* isolate. Tryptone was found to be the optimum nitrogen source for protease enzyme production by the test isolate. The *Bacillus* sp. produced maximum amount of protease of 28.2 Uml^{-1} under the optimized conditions which was increased two folds than the unmodified ones.

Keywords: *Bacillus* sp, Alkaline protease, Optimization

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INTRODUCTION

Enzymes are delicate protein molecules necessary for life [1]. 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 [2]. Proteases are the hydrolytic enzymes act on the proteins. There are different types of protease depends on their active site or the structure of active site. Among them, alkaline proteases have widest application in various industries due to their biochemical diversity. Proteases of commercial importance are produced from microbial, animal and plant sources [3], but proteases from microbial sources are preferred to the enzymes from plant and animal sources since they posses almost all characteristics desired for their biotechnological applications [4] and 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 [5, 6]. From plants papain and ficin are important proteases, trypsin and chemotrypsin from animals and alkaline proteases from microorganisms like *Bacillus* sp., Microbacterium [7, 8], *Penicillium* sp., *Aspergillus* sp. etc. [9, 10]. Among these, *Bacillus* sp. are specific to produce extracellular and alkaline proteases. 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 subtilis* 3411[14], *Bacillus licheniformis* [15].

As there is increase in demand of industrial protease, so it is desirable to get new sources of protease with maximum production. At the same time proteases from marine microorganisms are currently receiving increase attention due to their inherent stability at different values of pH, temperature and salinity [14,16,17].Thus this present study was aimed to isolate the protease producing strain and to optimize its nutritional factors like optimum carbon and nitrogen sources and different cultural conditions such as temperature, pH and fermentation time.

MATERIALS AND METHODS

Collection and isolation of sample

Soil samples were collected from different places of Marina Beach, Tamil Nadu, India. About 1g of each soil sample was incubated with 5ml of sterile distilled water for 10min. The about 100µl of suspension was spreaded on the nutrient agar plate and incubated for 24h at 37°C.

Screening and identification of protease producing bacteria

The individual colony was screened for protease production using 1% skim milk agar plate. The plates were incubated for 24h at 37°C and then flooded with 10% tannic acid solution and incubated for 24h at room temperature. Based on the largest clear zone of

hydrolysis, the bacterial strain was selected for further use. The selected strain was identified based on the gram staining, morphology and biochemical test [18].

Protease production

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 of identified bacterial inoculum and incubated at 37°C for 24 h in a shaking incubator (150rpm). After 24 h of incubation, the cells were harvested at 15000 rpm for 10 min and the clear crude supernatant was stored at 40°C for further studies [19].

Protease assay

Alkaline protease activity was determined by the standard assay [20]. The reaction mixture contained 5 ml of casein (prepared in 50 mM of Tris buffer, pH 8.0) and an aliquot of 1.0 ml of the enzyme solution and incubated for 30 min. The reaction was stopped by adding 5 ml of trichloroacetic acid solution (TCA) (0.11M). After 30 minutes the mixture was filtered. 2ml of the filtrate was added to 5.0 ml of 0.5 M sodium carbonate and 1.0 ml of Folin - Ciocalteu's phenol reagent and kept for 30 minutes at 37°C. The optical densities of the solutions were read against the sample blank at 660 nm using UV - Visible Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per ml per min under assay conditions.

Total protein content

The total protein concentration of cell free filtrate was determined by the Lowry method using bovine serum albumin used as a standard [21].

Optimization of protease production

Effect of carbon source on protease production

To study the efficacy of various carbon sources, the medium was supplemented independently with 1% of glucose, maltose, sucrose, lactose, mannitol. The individual medium was sterilized, inoculated test sample and incubated at 37°C for 24h. After the incubation, cultures were centrifuged and supernatant was used for protease assay.

Effect of nitrogen source on protease production

For the optimization of nitrogen source, various nitrogen sources (1%) like ammonium chloride, yeast extract, ammonium sulphate, potassium nitrate, tryptone were used and incubated for 24h and the supernatant was used for protease activity.

Effect of temperature on protease production

The effect of temperature on protease production was studied using the temperature ranges from 35°C - 75°C. The optimum temperature was found out by inoculating the test bacteria in the optimized media with different temperature and the protease assay was done after 24h.

Effect of pH on protease production

To determine the optimum temperature and pH for protease production, the bacterium was inoculated in optimized medium at pH (5-10) for 24h. After the incubation, the cultures were harvested, centrifuged and supernatant was used for protease assay.

Effect of incubation period on protease production

The test organism was grown in optimized and unoptimized protease producing medium. It was incubated at 37°C for 24, 48, 72, 96 and 120 h in an orbital shaker incubator. The contents were then centrifuged at 10,000 rpm at 4°C for 15 min and protease activity was checked in the cell free extract for both the types.

RESULTS AND DISCUSSION

Screening and identification of protease producing bacteria

A total of ten bacterial samples isolated from marine soil were plated on 1% skim milk agar, 5 of them showed positive towards protease production by exhibiting as diameter of clear zone in mm. The maximum protease producing strain was selected depending on the highest clear zone. It was found that the bacterial strain BT4 showed largest zone of clearance of 21mm and this strain was selected for further use. Based on the morphological studies and biochemical test, the selected strain was identified as *Bacillus* sp. BT4 which has the highest protease activity.

Effect of carbon source on protease activity

Result shows that different carbon sources have different effect on production of alkaline extracellular protease by *Bacillus* sp. BT4. Among the various carbon sources tested, sucrose was found the optimum source (Figure 1).

Effect of nitrogen source on protease activity

The selection of nitrogen source has the influence the protease production. Figure 2 shows the different nitrogen sources used for the production of enzyme. Tryptone was found best nitrogen source for *Bacillus* sp. BT-4 while the other nitrogen sources repressed the production.

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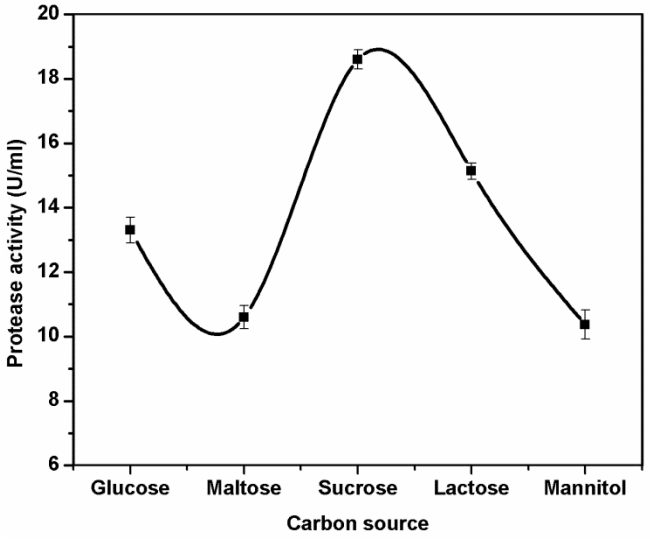

Figure 1. Optimization of carbon source

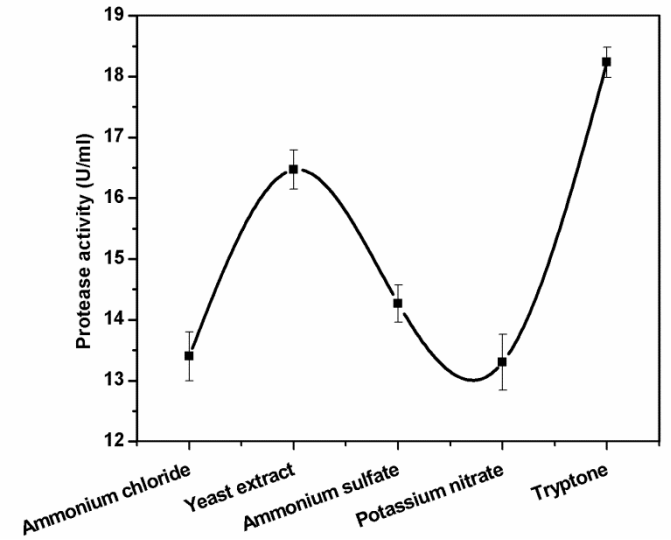

Figure 2. Optimization of nitrogen source

Effect of temperature and pH for enzyme production

The impact of temperature on enzyme production is highly important. Among the various temperature of incubation, the optimum temperature was found at 55°C where the minimum amount of protease production was found at 35°C (Figure 3). Alkaline protease production by microbial strains strongly depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production [22]. In the present study the protease production was active in the range of 7.0-10.0, but the optimum pH was 9.0 (Figure 4). As the enzyme is active in alkaline condition, indicating its potential use in detergent formulations [23].

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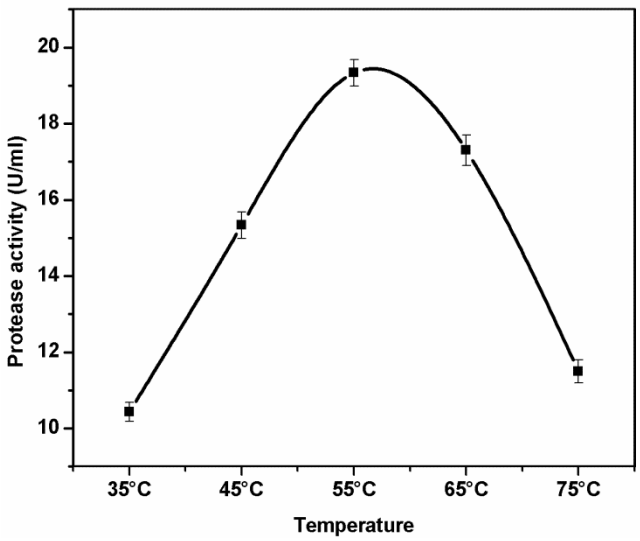

Figure 3. Optimization of temperature

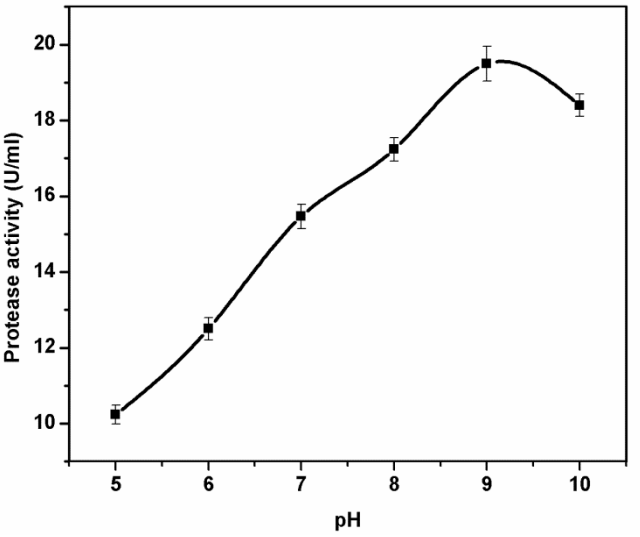

Figure 4. Optimization of pH

Effect of incubation period on enzyme production

Incubation period plays an important role production of enzymes. Here the strain *Bacillus* sp. BT4 was incubated in the protease producing media for different production period. Figure 5 shows the impact of various incubation time on protease production for the modified and unmodified medium. The maximum protease activity was observed in 48h for both medium. After reaching the optimum period, the enzyme activity was decreased. The subsequent decrease in the enzyme units could probably due to inactivation of enzyme by other constituent proteases [24]. The modified media was produced highest yield of protease of 28.2 Uml^{-1} with all the optimized factors. This production was increased approximately two folds than the unmodified media.

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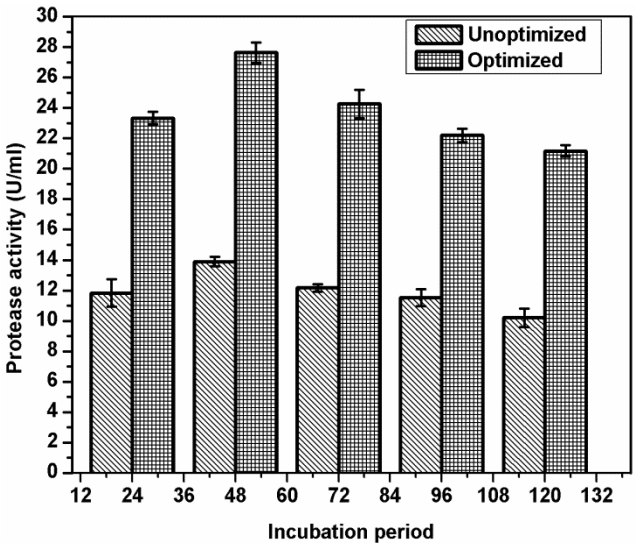

Figure 5. Optimization of incubation period

CONCLUSION

From these studies, it is evident that the isolated *Bacillus* sp. from marine soil has the potential to produce protease enzyme. The composition of medium and culture conditions plays a crucial role in the production of fermented, which include carbon source, nitrogen source, temperature, pH, incubation period. This *Bacillus* sp. showed optimum activity at 55°C, pH 9, 48h incubation period with sucrose and tryptone as optimum carbon and nitrogen source respectively. Further this enzyme can be purified and used for various industries.

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