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Isolation, Identification, Partial Purification, Optimization and Characterization of Alkaline Protease from *Bacillus subtilis*

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ABSTRACT:
In the present study, 10 different microbial strains were isolated from the soil samples from different regions of Allahabad. Among the isolates the strain namely, *Bacillus subtilis* was selected for the alkaline protease production. The protease production efficiency of the organisms was measured with different environmental and nutritional parameters. The optimum fermentation conditions of production were pH 5, 7 & 11 and temperature 70^{o}C. Lactose, sodium nitrate and calcium chloride as good nutritional sources for producing higher yield of the enzyme.

Keywords: *Bacillus subtilis*, alkaline protease, screening, lactose, sodium nitrate and calcium chloride, etc.

[I] NTRODUCTION

Proteases are proteolytic enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. Proteases represent one of the three largest groups of industrially important enzymes [3]. Bacteria are the most dominant group of alkaline protease. Bacillus being the most relatively prominent and serve as an ideal source of these enzymes biotechnological importance [5, 9] because of their rapid growth and limited space required for their cultivation [1]. Among different types Acidic, neutral and alkaline proteases, alkaline protease plays very important role as most commonly used industrial enzyme in view of their activity and stability at alkaline pH. They are used in detergent formulations, food, pharmaceuticals, and leather, in film industry, medical use and by waste processing companies [2, [4-5], [6-7], 8, 10]. Multiple application of these enzymes stimulated interest to discover them with novel properties and considerable advancement of basic research into these enzymes. Proteases can be produced from wide diverse sources such as plants, animals and microorganisms. Protease can be produced by all microorganisms that produce a substantial amount of extracellular protease have been exploited commercially. Microbial proteases play a crucial role in numerous pathogenic processes mainly responsible for degradation of elastin, collagen, proteoglycons and also proteins that function in vivo host defence. Identification and characterization of microbial protease are prerequisite for understanding their role in pathogenesis.

[II] MATERIALS AND METHODS

2.1. Isolation and alkaline protease production

Soil samples from different regions of Allahabad were collected.1gm of soil sample was taken in

25ml conical flask containing 10 ml of sterilized water and contents were mixed well in vortex to get homogeneous suspension. The suspension is serially diluted 10^{-6} times and using streak plate technique the diluted samples are transferred to petri-dishes containing sterile skim milk agar medium. After inoculation the plates were incubated at 37°C for 24 hours. After incubation bacterial colonies appearing over skim milk agar medium were identified based on colony characteristics and their identities were confirmed through Gram staining methods and by a series of biochemical tests as prescribed by Bergley manual. For enzyme production, strain was cultured in 250 ml of Erlenmeyer flask containing 100 ml culture medium, which consists of Nutrient-Gelatin-1g/100ml, Glucose-1g/100ml, KH_2PO_4-0.05g/100ml, K_2HPO_4-0.05g/100ml, $CaCl_2$-0.05g/100ml. The inoculated medium was placed in a thermostatic orbital shaker for 72 hours at 37°C and 90 rpm. The culture was centrifuged at 10,000 rpm for 10 min to obtain crude enzyme [11].

2.2. Protease assay

For estimation of alkaline protease 0.5% (w/v) casein solution was used as a substrate. Casein solution and buffer solution (carbonate-bicarbonate buffer) was taken in test tube and further enzyme solution (cell extract) was also added in test tube and the reaction mixture thus prepared. The reaction was stop by adding 1% tri-chloro-acetic acid solution. Then small amount of aliquot and Na_2CO_3 was taken and few drops of folin's reagent (double diluted) was added in the test tube. After 30 minutes the reading was taken against blank in double beam spectrophotometer at 660nm.

2.3. Optimization of production parameters

2.3.1. Effect of Nutritional parameters

Nutritional parameters include carbon source, nitrogen source and metal ions. Firstly the respective carbon sources lactose, sucrose, fructose, maltose and dextrose were added as a sole source of nitrogen (1%, w/v). The different nitrogen sources gelatin, ammonium chloride, urea, ammonium nitrate and sodium nitrate were added as a sole source of carbon (1%, w/v). Different metal ions (Na, K, Ca, Mg and Zn) were added as metal ion source (1% w/v). The enzyme activity was monitored for all different nutritional parameters after 24 h growth at 37°C [11].

2.3.2. Effect of Environmental parameters

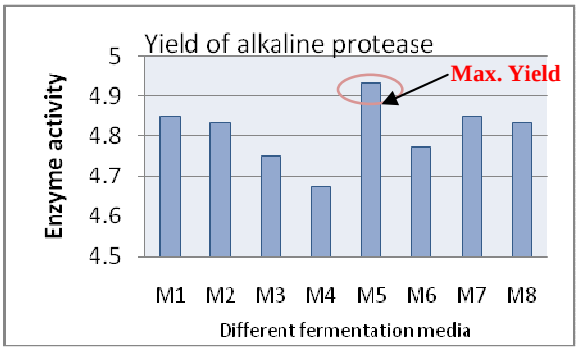
Environmental parameters include pH (3, 5, 7, 9 & 11) and temperature (40°C, 50°C, 60°C, 70°C & 80°C). The enzyme activity was monitored for all the above parameters in the respective ranges [11].

[III] RESULTS AND DISCUSSION

3.1. Screening of Bacteria and Estimation

Bacteria producing alkaline protease were isolated from soil by serial dilution techniques were identified through a series of biochemical tests as *Bacillus subtilis.* The bacterium was identified as protease producing by formation of clear zone around the bacterial colony on Skim milk agar media (Figure 1). The estimation of protease activity was done in eight different fermentation media via; casein as substrate and it was found that production media M5 was best for protease production (Graph 1).

Figure 1: Positive result of Casein hydrolysis Test (*Bacillus subtilis)*; (i) Clear zone around the growth due to production of protease enzyme (ii) Media Used: Skim Milk Agar Media

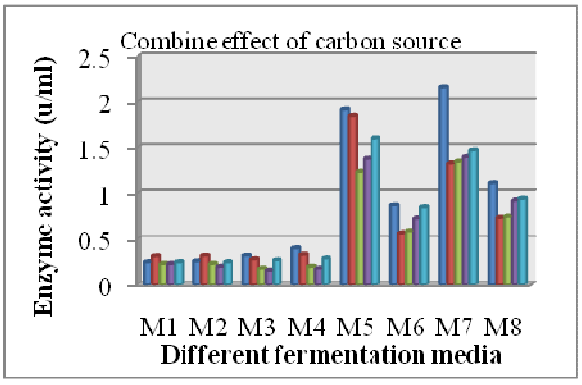


Graph 1: Estimation of alkaline protease produced from *Bacillus subtilis* (a) Maximum yield was observed in M5 fermentation media

3.2. Optimization of Nutrient parameters

3.2.1. Effect of carbon source

All the eight different fermentation media inoculated with *B. subtilis* were optimized by different carbon source to study the maximum activity of alkaline protease. The maximum activity was observed in M7 (2.17±0.76U/ml), M5 (1.85±0.57U/ml), M7 (1.35±0.47U/ml), M7 (1.40±0.53U/ml) and M5 (1.61±0.56U/ml) in lactose, sucrose, fructose, maltose and dextrose as carbon source respectively. Among these highest activity was observed in lactose in M7 (2.17±0.76U/ml) media (Graph 2). Increased yield of alkaline protease production were reported by several other researchers who used different sugars such as lactose, maltose, sucrose and fructose [12].

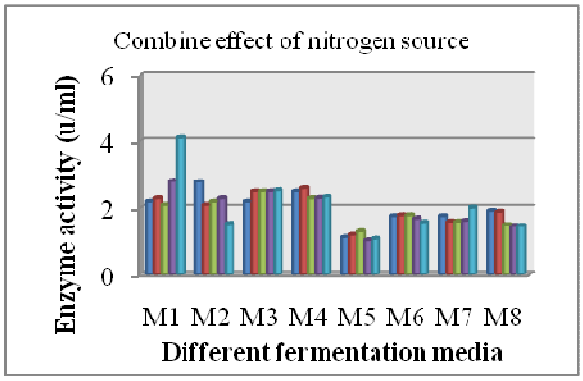


Graph 2: Optimization of alkaline protease production from *B. subtilis* in different carbon source (lactose, sucrose, fructose, maltose & dextrose) (a) Maximum yield was observed in M7 viz. lactose (b) Minimum yield in M3 viz. maltose.

Effect of lactose on yield of alkaline protease inoculated with *B. subtilis*
Effect of sucrose on yield of alkaline protease inoculated with *B. subtilis*
Effect of fructose on yield of alkaline protease inoculated with *B. subtilis*
Effect of maltose on yield of alkaline protease inoculated with *B. subtilis*
Effect of dextrose on yield of alkaline protease inoculated with *B. subtilis*

3.2.2. Effect of nitrogen source

All the eight different fermentation media inoculated with *B. subtilis* were optimized by different nitrogen source to study the maximum activity of alkaline protease. The maximum activity was observed in M2 (2.8±0.51U/ml), M4 (2.6±0.47U/ml), M3 (2.5±0.42U/ml), M1 (2.82±60U/ml) and M1 (4.11±0.94U/ml) in gelatin, ammonium chloride, urea, ammonium nitrate and sodium nitrate as nitrogen source respectively. Among these highest activity was observed in sodium nitrate in M1 (4.11±0.94U/ml) media (Graph 3). Similar observations were noticed in case of alkaline protease production by *Bacillus* sp. [13].

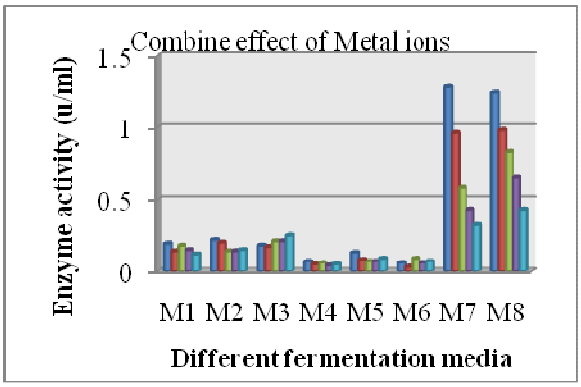


Graph 3: Optimization of alkaline protease production from *B. subtilis* in different nitrogen source (gelatin, ammonium chloride, urea, ammonium nitrate and sodium nitrate) (a) Maximum yield was observed in M1 viz. sodium nitrate (b) Minimum yield in M5 viz. ammonium nitrate.

Effect of gelatin on yield of alkaline protease inoculated with *B. subtilis*
Effect of ammonium chloride on yield of alkaline protease inoculated with *B. subtilis*
Effect of urea on yield of alkaline protease inoculated with *B. subtilis*
Effect of ammonium nitrate on yield of alkaline protease inoculated with *B. subtilis*
Effect of sodium nitrate on yield of alkaline protease inoculated with *B. subtilis*

3.2.3. Effect of metal ions

All the eight different fermentation media inoculated with *B. subtilis* were optimized by different metal ions to study the maximum activity of alkaline protease. The maximum activity was observed in M2 (0.207±0.52U/ml), M2 (0.16±0.40U/ml), M1(0.37±0.28U/ml), M3 (0.25±0.21U/ml) and M3 (0.20±0.13U/ml) in sodium chloride, potassium chloride, calcium chloride, magnesium chloride and zinc chloride as metal ion source respectively. Among these highest activity was observed in calcium chloride in M1 (0.37±0.28U/ml) media (Graph 4). It was reported that, some bacteria and fungi showed maximum enzyme production with metal ions such as Ca^{2+}, Mg^{2+} supplemented medium [14].



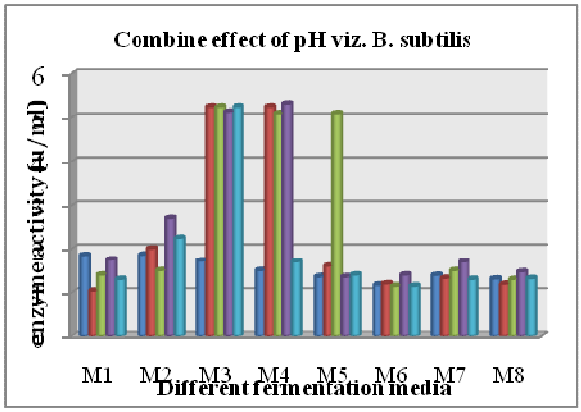
Graph 4: Optimization of alkaline protease production from *B. subtilis* in different nitrogen source (sodium chloride, potassium chloride, calcium chloride, magnesium chloride and zinc chloride) (a) Maximum yield was observed in M1 viz. calcium chloride (b) Minimum yield in M7&4 viz. magnesium chloride & zinc chloride.

- Effect of sodium chloride on yield of alkaline protease inoculated with *B. subtilis*
- Effect of potassium chloride on yield of alkaline protease inoculated with *B. subtilis*
- Effect of calcium chloride on yield of alkaline protease inoculated with *B. subtilis*
- Effect of magnesium chloride on yield of alkaline protease inoculated with *B. subtilis*
- Effect of zinc chloride on yield of alkaline protease inoculated with *B. subtilis*

3.3. Optimization of Environmental parameters

3.3.1. Effect of pH

All the eight different fermentation media inoculated with *Bacillus subtilis* were optimized at different pH range to study the maximum activity of alkaline protease. The maximum activity was observed in M2 (1.86±0.26U/ml), M3&4 (5.25±1.8U/ml), M3 (5.25±1.9U/ml), M4 (5.32±1.6U/ml) and M3 (5.25±1.3U/ml) at pH3, pH5, pH7, pH9 and pH11 respectively. Among these maximum activities was observed at pH5, 9 & 11 in M3 and M4 (5.25±1.9U/ml) media (Graph 5). Similar result was reported in case of *Beauveria feline* was optimal active at pH 7.0 for the protease production [15].



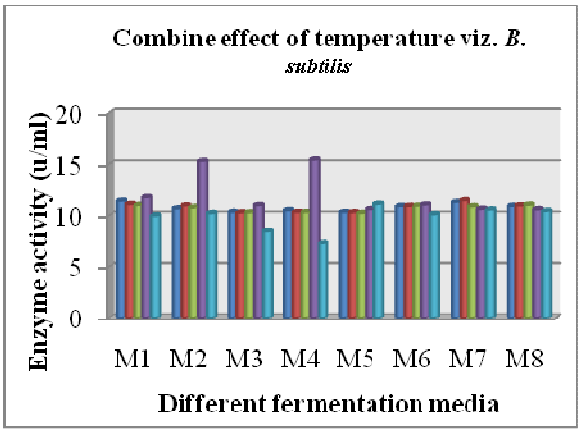
Graph 5: Optimization of alkaline protease production from *B. subtilis* at different pH (3, 5, 7, 9 &11) (a) Maximum yield was observed in M3&4 at pH 5, 7& 11. (b) Minimum yield in M1 at pH5.

- Effect of pH 3 on yield of alkaline protease inoculated with *B. subtilis*
- Effect of pH 5 on yield of alkaline protease inoculated with *B. subtilis*
- Effect of pH 7 on yield of alkaline protease inoculated with *B. subtilis*
- Effect of pH 9 on yield of alkaline protease inoculated with *B. subtilis*
- Effect of pH 11 on yield of alkaline protease inoculated with *B. subtilis*

3.3.2. Effect of temperature

All the eight different fermentation media inoculated with *B. subtilis* were optimized at different temperature range to study the maximum activity of alkaline protease. The maximum activity was observed in M1 (11.41±0.40U/ml), M7 (11.46±0.42U/ml), M7 (11.86±0.32U/ml), M4 (15.43±2.1U/ml) and M5 (11.12±2.1U/ml) at 40°C, 50°C, 60°C, 70°C and 80°C respectively. Among these highest activity

was observed at 60°C in M4 (15.43±2.1U/ml) media (Graph 6). Similar result was reported that the maximum alkaline protease production by marine bacterium *Roseobacter* sp after 48h of incubation at 60°C [16].



Graph 6: Optimization of alkaline protease production from B. subtilis at different temperature (40°C, 50°C, 60°C, 70°C & 80°C) (a) Maximum yield was observed in M4 at 70°C (b) Minimum yield in M4 at 80°C.

- Effect of 40°C on yield of alkaline protease inoculated with *B. subtilis*
- Effect of 50°C on yield of alkaline protease inoculated with *B. subtilis*
- Effect of 60°C on yield of alkaline protease inoculated with *B. subtilis*
- Effect of 70°C on yield of alkaline protease inoculated with *B. subtilis*
- Effect of 80°C on yield of alkaline protease inoculated with *B. subtilis*

[IV] CONCLUSION

Bacillus subtilis can be used profitably for production of alkaline protease to meet the present day demand of the industrial sector. Lactose, sodium nitrate and calcium chloride best nutritional parameters for an enhanced enzyme production with optimum range of pH (5, 7 & 11) and temperature (70°C).

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