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Original Research Article

Isolation and Characterization of Protease Producing Bacteria from Rhizosphere Soil and Optimization of Protease Production Parameters

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A B S T R A C T

Protease enzyme performs proteolysis, which is catabolism of protein by hydrolysis of the peptide bonds. Alkaline proteases have various applications in industrial products and processes such as detergents, food, pharmaceuticals and leather.For the alkaline protease production, a number of microbial strains were screened using skimmed milk agar media and gelatine hydrolysis method from different soil samples. Out of 20 bacterial strains screened, maximum protease producing strain has been selected. This strain has been identified as *Bacillus*, on the basis of morphological and biochemical characters using Bergey's Manual of Determinative Bacteriology. Different fermentation parameters such as media, optimum media pH, optimum incubation and temperature were tried to optimize for maximum production of enzyme from the source organism and Luria Bertani media with 3% casein, pH-11 at 37°C for 48hrs has been showing maximum enzyme production.

Keywords

Rhizosphere Soil, Protease Enzyme, *Bacillus*

Introduction

Proteases are enzymes that break the proteins to smaller peptides or amino acids. Proteases are of various types namely serine, cysteine, metallo aspartate and threonine. Proteases are generally found in plants, animals and micro-organisms. For industrial production of the proteases micro-organisms are preferred so that large scale production of enzymes can be achieved. *Bacillus*, *Aspergillus*, *Pseudomonas* etc. are the organisms that produce protease. Bacteria are most important alkaline protease producers with the genus *Bacillus* being the most prominent source, because of their ability to produce large amount of protease having significant proteolytic activity and stability at high pH and temperature (Rakesh Kumar and Ritika Vats, 2010). The production of microbial protease from bacteria depends on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermostability (Mukesh Kumar *et al.*, 2012). They conduct highly selective and specific modification of proteins i.e. zymogenic form of enzymes by limited proteolysis, blood clotting and lysis

of fibrin clots, processing and transport of secretory proteins across the membrane. They also play an important role in proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms. Their involvement in the life cycle of disease causing organisms has led to become a pharmaceutical agent against fatal diseases such as cancer and AIDS. Alkaline proteases alone account for 20% the world enzyme market with their predominant use in leather processing and detergent industries. It has been proved that alkaline protease from *Bacillus* can be used as a dehairing agent, indicating elastolytic, keratinolytic activities and also a low hydrolytic collagen activity (Agrawal *et al.*, 2012).

Materials and Methods

Collection of samples

Soil samples were collected from various geographical areas such as garden soil from Pune, laundry washing center soil (Shivajinagar, Pune) and soil from Kaas plateau of Satara district, Maharashtra, India (Location: 17.7201607°N 73.8227916°E). The soil was collected from 15cm below surface.

Isolation of alkaline protease producing bacteria

Each soil sample was serially diluted and spread plated on Skimmed milk agar plates (g/l) (skim milk powder-28, casein enzymic hydrolysate-5, yeast extract-2.5, dextrose-1, NaCl-0.5, agar-15) with pH 9.0 (using phosphate buffer) and incubated at 37°C for 48 hrs. The formation of clear zone around the colonies confirms the production of alkaline protease. The colonies that had formed a clear zone around the growth were considered as protease positive isolates. Protease production was further confirmed by Gelatine hydrolysis on nutrient gelatine agar medium containing Ingredients- Peptone-0.1% w/v, NaCl-0.5% w/v, agar-2% w/v, Gelatine- 10% w/v at pH 9 using 1N NaOH. Individual colonies were spot inoculated on Nutrient Gelatine agar plates and were incubated at 37°C for 48 hours. The colonies showing zone of clearance around the colony confirmed the protease production.

The positive isolates were further screened for better production of enzyme by assaying the protease activity in liquid culture using casein as substrate at 37°C. The strain which showed maximum activity was selected for further studies.

Characterization and identification of bacteria

The isolated bacteria was identified based on cellular morphology, growth condition, Grams staining, endospore staining and biochemical tests The preliminary characterization of the selected strain was done according Bergey's Manual of Determinative Bacteriology Biochemicals performed were namely Catalase enzyme production, Oxidase enzyme production., Glucose fermentation test, Growth at 3% to 13% NaCl concentration, Gelatinase enzyme production.

Quantification assay for alkaline protease (*Takami et al.,* 1990)

Preparation of crude enzyme extracts

150 ml of assay medium (LB + 1% Casein) in Phosphate buffer (0.05M) having pH 9 was inoculated with each isolate and incubated at 37°C for 48 hrs in rotary shaker. After incubation the broth cultures were subjected to centrifugation at 10,000

rpm for 10 minutes at 4°C in centrifuge. The supernatant obtained after centrifugation was used to determine the amount of the extracellular protease release into the assay medium (Sai Smita *et al.,* 2012).

Determination of proteolytic activity

The assay was carried out routinely in a mixture containing 0.5ml of crude enzyme solution and 2.5ml casein (1% w/v in 50mM Glycine- NaOH buffer (pH 9) solution.

After incubation of 1hour at 30°C in water bath(without incubating control), the reaction was terminated by addition of 2.5 ml 0.44M trichloroacetic acid (TCA) solution.

After 10 min the mixture was centrifuged at 7500rpm for 10min at 4°C. An aliquot of 0.5ml of supernatant was mixed with 2.5ml of 0.5M Na_2CO_3 and 0.5ml of 1:1 Folin-Ciocalteu's phenol solution in distilled water and kept for 30min at room temperature.

The optical densities of the solutions were determined with respect to sample blanks at 660nm. The tyrosine standard graph was prepared of concentration ranging from 0.01 mM to 0.1 mM to determine protease activity. One unit of enzyme activity is defined as µMole of tyrosine released from 1 ml crude enzyme in 1 hour incubation. **(**Takami *et al.,* 1990).

Standardization of fermentation parameters

Optimization of medium pH

LB was prepared in buffer with different, pH-3, pH-5, pH-9 & pH-11. Media were inoculated 1 % (v/v) inoculum and incubated at 37°C for 48 hrs.

Optimization of incubation temperature:

The effect of Temperature on alkaline protease production from the isolate I-A was determined.Media were inoculated 1 % (v/v) inoculum and incubated at 25°C, 37°C and 46°C for 48 hrs.

Induction studies

The effect of concentration of substrate casein on alkaline protease production from the isolate I-A was determined by growing the isolate in assay medium with different concentration of substrate casein (1%, 2%, 3% and 4%).

Results and Discussion

Isolation and screening

Morphologically different 20 strains were isolated from different soil samples. Isolated cultures were separately screened for their proteolytic activity. Eleven isolates showed proteolytic activity, as indicated by zone of clearance on skim milk agar and nutrient gelatine agar medium and were named as I-A, II-A….XIII.

Isolate I-A was selected for further studies as it exhibited largest zone of Gelatine hydrolysis amongst the 11 isolated strains. Figure 1 shows zone of clearance around the colony of the bacterial isolate (Gelatine hydrolysis).

Characterization and identification of bacteria

Strain I-A was found to be Gram positive and motile and belongs to Genus as *Bacillus*, on the basis of morphological and biochemical characters using Bergey's Manual of Determinative Bacteriology.

Table.1 Colony characters

Strain	**Size**	**Shape**	**Colour**	**elevation**
I-A	2mm	Circular	White	Low convex
Consistency	**Margin**	**Opacity**	**Gram character**	**Motility**
Sticky	Irregular	Opaque	Gm +ve Rods	Motile

Table.2 Biochemical tests

Strain	**Catalase**	**Oxidase**	**Glucose fermentation**	**NaCl Concentration**	**Endospore**	**Gelatinase**
I-A	+	+	+	Turbidity observed from 1% to 5%	+	+

+ presence of character
- Absence of character

Figure.1 Zone of gelatine hydrolysis around the colony of I-A on gelatine agar plate

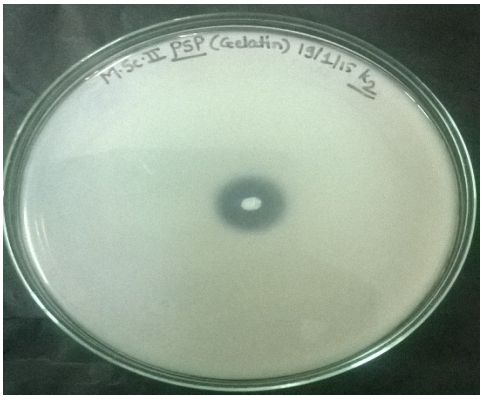

Figure.2 Effect of pH on protease production from Strain I-A

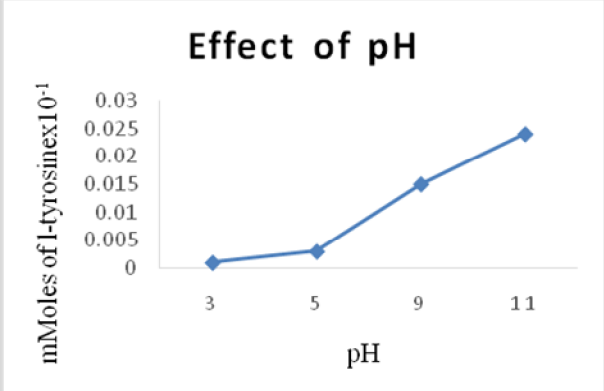

Figure.3 Effect of temperature on protease production from Strain I-A

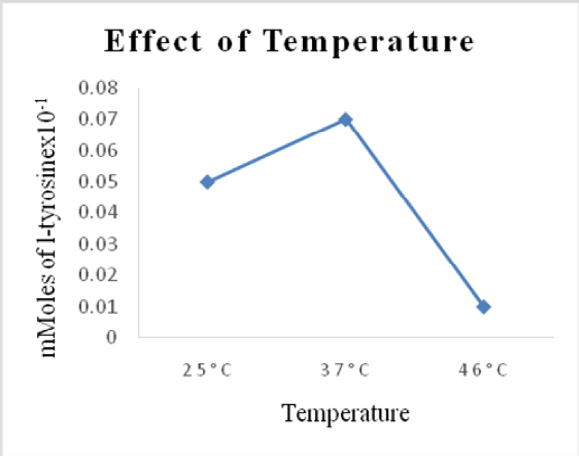

Figure.4 Effect of substrate concentration on protease production from Strain I-A

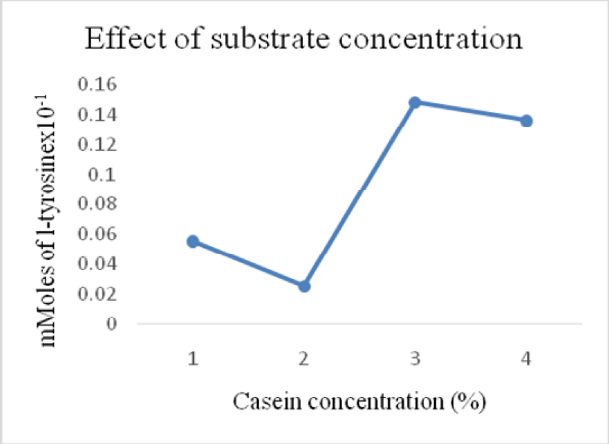

Table 1 and 2 shows the colony characters & biochemical characters of selected strain I-A.

Optimization of medium pH

The pH of the medium had significant effect on alkaline protease production by I-A. The production of protease increased with increase in pH of the medium. The optimum pH was for protease production was found to be 11 which resulted around 0.005 mM (5 µM) of tyrosine released per ml of crude enzyme extract (0.0025 mM per 0.5 ml of crude enzyme extract) that is 5 PU/ml (Figure 3).

Optimum pH for maximum enzyme activity was found to be 11 for the source organism which is a promising and prospective characteristic for its industrial application. The similar results were obtained by Khan *et al.* (2011) where maximum enzyme activity was seen at pH 11. There is report of Maximum protease production at pH-9.0 from *Bacillus* sp. MPTK 712 (Mukesh Kumar *et al.*, 2012). As per the reports by Sharma *et al.* (2014) *B. aryabhattai* K3

produced very low levels of alkaline protease at pH 6.0 while the production of protease increased with increase in pH of the medium and maximum enzyme activity was seen at pH 8.0.

Optimization of incubation temperature

The culture I-A was incubated at various temperatures to determine optimum temperature for alkaline protease production. The optimum temperature for protease production was found to be 37°C which resulted around 0.014 mM (14 µM) of tyrosine released per ml of crude enzyme extract (0.007 mM per 0.5 ml of crude enzyme extract) that is 14 PU/ml (Figure 4).

Optimum incubation temperature 37°C suggests that given isolate is mesophillic. Reports by Agrawal *et al.* (2012) say that optimum temperature for maximum protease activity from *Bacillus sp* was 40°C which is comparable with the incubation temperature for our isolate to give maximum enzyme production. *Bacillus* sp. MPTK 712 showed optimum incubation temperature 55°C (Mukesh Kumar *et al.*, 2012).

Induction studies (casein substrate concentration)

The alkaline protease production was enhanced with increasing concentrations of casein as a substrate. The optimum concentration of casein for maximum alkaline production was obtained to be 3%, where the protease activity of crude extract was 3PU/ml.

Various fermentation parameters such as media, optimum media pH, optimum incubation temperature and inducers were optimized for maximum production of enzyme from the Rhizosphere origin strain of Genus *Bacillus* and Luria Bertani media with 3% casein, pH-11 at 37 °C for 48hrs has been showing maximum alkaline protease enzyme production**.**

Future prospects

Further purification and characterization of the protease from source organism can achieved to test its suitability for other industrial applications. Studies may be extended in search of new and economic methods to enhance the protease production as well as immobilization methods can be tried.

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