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Optimization of Alkalophilic Protease Production by *Pseudomonas aeruginosa* Isolated from the Gut of *Penaus monodon*

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Abstract: *Pseudomonas aeruginosa* isolated from the gut of marine coastal water shrimp (*Penaus monodon*) was tested for its ability to produce the protease enzyme. The effect of different production parameters such as pH, temperature, carbon source, nitrogen source, NaCl concentration and metal ions for protease production by the isolated bacterial strain were studied. Under optimized parameters maximum protease activity and specific activity were 500 U/ml and 161.12 U/mg respectively.

Key words: Alkaline protease · Bacterial enzyme · Optimization · Pseudomonas aeruginosa · Penaus monodon

INTRODUCTION

Proteases are several types based on their cleavage site and production of amino acid specificity such as serine protease, cysteine protease, aspartic protease and metallo protease. Proteases do not refer to a single enzyme but a mixture of enzymes including proteinases, peptidases and amidases [1]. The enzyme protease is one of the most important enzymes and it accounts for about 60% of the total worldwide sale in the market. The major microorganisms that produce industrial protease exist in bacteria such as genera *Clostridium*, *Bacillus*, *Pseudomonas* and fungi such as genera *Aspergillus*, *Mucor* and *Rhizopus* [2]. Bacterial protease is the most significant than other proteases [3]. *Pseudomonas* group is physiologically better adopted for environments with high concentrations of nutrients and NaCl [4].

Protease is one of the third largest groups of industrial enzymes and finds applications in bioremediation, detergents, leather, food and pharmaceutical industry [5]. Properties of this protease such as alkaline pH, thermo stability, solvent and detergent resistance make the enzyme useful for different applications. Proteolytic enzymes producers are also helpful for the health of the ecosystems of this earth as these microbes decompose the dead and decaying animal or plant tissues in water or land. They can create pollution free environment and they are responsible for the recycling of nutrients [6].

Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures. The activity and stability of the alkaline protease in a broader range of pH and salt would definitely make this enzyme an important candidate for various industrial applications [7]. The aim of this study was to optimize the culture conditions and evaluate the protease producing ability of *Pseudomonas aeruginosa* isolated from the gut of marine coastal waters shrimp *Paeneus monodon.*

MATERIALS AND METHODS

Isolation of Potential Protease Producing Microorganisms: The bacterium used in this study was isolated from the gut of marine coastal water shrimp *Paeneus monodon* which was collected from Rajakkamangalam, Kanyakumari District, Tamil nadu, India. The bacteria which produced maximum clear zone, on skim milk agar after 24h of incubation were used for

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further study. The bacterium was identified according to the standard key of Bergey's Manual of Determinative Bacteriology [8].

Culture Conditions for Protease Production: The tested organisms were inoculated onto a medium consisting of peptone - 1g; beef extract - 0.5g; NaCl - 0.5g; distilled water - 100ml; pH - 7 and incubated at 37°C for 24 h on a shaker. After incubation, the culture medium was centrifuged at 10,000 rpm at 15min to obtain the crude extract which was used as enzyme source for protease activity [9].

Protease Assay: The crude extract was estimated for protease activity as per the method of Kunitz [10] using casein as the substrate. Casein is the major milk protein, a macromolecule of amino acid linked by peptide bond. The peptide, mainly tyrosine liberated during proteolytic digestion was measured at 660 nm in UV-Vis spectrophotometer.

Effect of Ph on Protease Production: The effect of pH on protease production was determined by culturing the bacterium in the protease production media with different pH ranging from 4-10. The enzyme assay was carried out individually after 24 h of incubation at 35°C.

Effect of Temperature on Protease Production: The effect of temperature on protease production was studied by incubating the culture media with different temperatures such as 25, 30, 35 and 40°C. The enzyme assay was carried out individually after 24 h of incubation at 35°C.

Effect of NaCl Concentration on Protease Production: The effect of NaCl on protease production was studied by incorporating the medium with various concentration of NaCl ranging from 1.5 - 3.5%. The enzyme assay was carried out individually after 24 h of incubation at 35°C.

Effect of Carbon Sources on Protease Production: The effect of carbon source on protease production was determined in the production media with different carbon sources such as sucrose, glucose, maltose and starch. They were added separately at the concentration of 1%. The enzyme assay was carried out individually after 24 h of incubation at 35°C.

Effect of Nitrogen Sources on Protease Production: The protease production by the selected bacterium was optimized by supplementing different nitrogen sources such as beef extract, yeast extract, ammonium nitrate and ammonium sulphate that were added to the medium separately in a concentration of 0.5%. The enzyme assay was carried out individually after 24h of incubation at 35°C.

Effect of Metal Ions on Protease Production: The selection of suitable metal ion for protease production was determined by adding different metal ions such as $CaCl_2$, $MgSO_4$, $(Na_4)_2HPO_4$, K_2HPO_4 and KH_2PO_4 to the medium separately in a concentration of 0.01% and enzyme assay was carried out individually after 24 h of incubation at 35°C.

Mass Scale Culture: Mass scale cultivation processes was done in optimized parameters such as Glucose - 1%, Beef extract - 0.5%, NaCl - 3%, $CaCl_2$ - 0.01%, pH - 9 and shaking speed 150 rpm with these parameters *Pseudomonas aeruginosa* was grown in protease production broth incubated at 35°C for 24h. After mass cultivation the cell free extract was obtained by centrifugation. The cells were then harvested by centrifugation at 10,000 rpm for 15min and the supernatant was used for further assay.

RESULTS

Identification of Protease Positive Colony: Based on the morphological, physiological and biochemical characteristics, the protease positive colony was identified as *Pseudomonas aeruginosa* by following the standard keys of Bergey's Manual of Determinative Bacteriology [8].

Screening for Protease Production: The isolated bacterial strains were screened for protease producing ability on skim milk agar plates which forms a zone of hydrolysis on casein. Among the strains tested, *Pseudomonas aeruginosa* exhibited the largest zone of clearance (19mm) in skim milk agar medium. Hence it was selected for further experimental studies.

Effect of Ph on Protease Production: PH - 9 supported maximum production of protease (215.56 ± 4.22 U/ml) whereas lowest enzyme production was observed at pH - 4 (141.12 ± 4.11 U/ml) (Fig. 1).

Effect of Temperature on Protease Production: Temperature 35°C was found to be optimal at which maximum protease production (240.89 ± 4.56 U/ml) was obtained. Minimum activity (149.08 ± 4.12 U/ml) was observed at 25°C (Fig. 2).

Effect of Nacl on Protease Production: Maximum production of protease (344.97 ± 2.61 U/ml) was recorded at 3% NaCl whereas minimum activity (49.06 ± 4.09 U/ml) was found at 1.5% NaCl (Fig. 3).

Effect of Carbon Sources on Protease Production: Glucose showed the maximum protease activity (419.77 ± 4.62 U/ml), while sucrose showed the minimum protease activity (339.1 ± 3.68U/ml) (Fig. 4).

Effect of Nitrogen Sources on Protease Production: Beef extract showed the maximum activity (191.13 ± 3.21 U/ml) and the minimum protease activity was observed with ammonium nitrate (71.86 ± 3.55 U/ml) (Fig. 5).

Effect of Metal Ions on Protease Production: $CaCl_2$ showed the highest protease production (194.02 ± 4.50 U/ml), while $(Na_4)_2H_4PO_4$ showed the lowest enzyme activity (73.86 ± 4.45 U/ml) (Fig. 6).

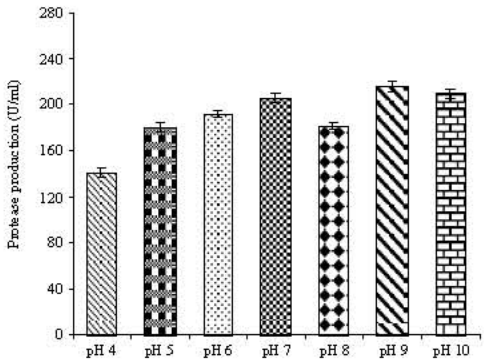

Fig. 1: Effect of pH on protease production

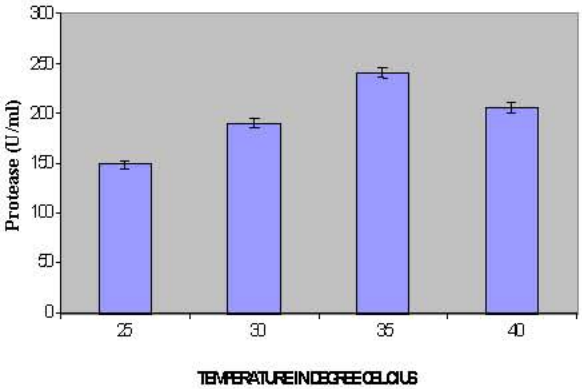

Fig. 2: Effect of temperature on protease production

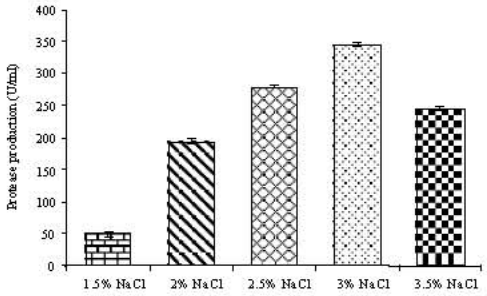

Fig. 3: Effect of NaCl on protease production

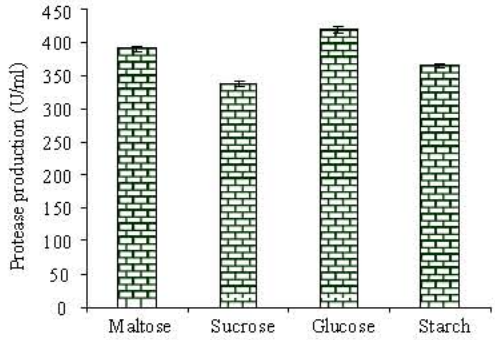

Fig. 4: Effect of carbon sources on protease production

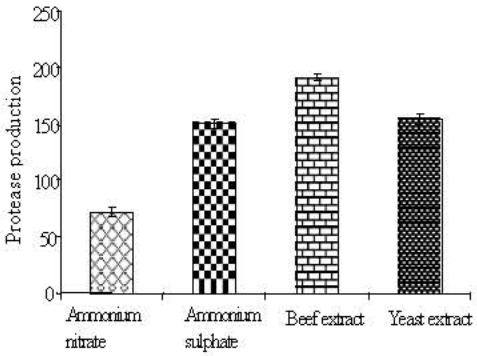

Fig. 5: Effect of nitrogen sources on protease production

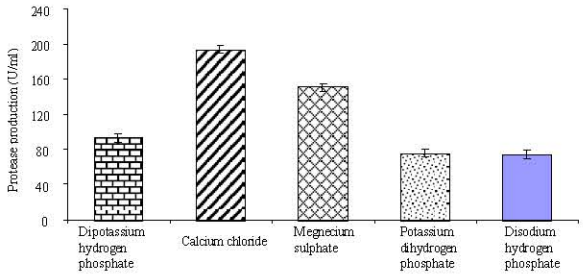

Fig. 6: Effect of metal ions on protease production

Mass Scale Culture: After mass cultivation, the cell free extract of *Pseudomonas aeruginosa* was obtained by centrifugation. Cell free extract showed maximum protease activity 500 U/ml and specific activity 161.12 U/mg.

DISCUSSION

The present study was designed to evaluate the protease producing ability of *Pseudomonas aereuginosa* isolated from the gut of marine coastal waters shrimp *Paeneus monodon*. Sugita *et al.* [11] has reported that gastro intestinal bacteria take part in the decomposition of nutrients and also provide the microorganisms with physiologically active material like enzymes, amino acids and vitamins. Another important aspect to be studied is the role of intestinal microflora in nutrition due to the release of extra cellular enzymes such as proteinases participating in the hydrolysis of complex biopolymers which is present in the feed [12]. Shrimp feeds contain a high amount of protein and proteolytic bacteria are likely to be the bacterial groups that will govern the quality of water and sediment in shrimp ponds and have a major influence on shrimp health. Proteolytic bacteria in shrimp cultivation play a major role by improving water quality and controlling shrimp disease [13]. Moreover many researchers reported that the *Pseudomonas sp.* is normal flora in shrimp ponds [14].

Protease from *P. aeruginosa* showed appreciable activity on the skim milk agar plates as indicated by the 19mm diameter of the zone of clearance in the present investigation. Similar results were reported by Khire and Pant [15] in *Bacillus sp.* and it was selected on the basis of clear zone around the wells on skim milk agar plates.

The effect of pH on the growth of *P. aeruginosa* showed that higher growth and maximum protease activity (215.56 ± 4.22 U/ml) was observed at pH - 9. As the protease active at higher pH it was confirmed as an alkaline protease. Similarly Borriss [16] reported maximum alkaline protease production at 9-13. Maximal proteolytic activity of strain K protease towards casein was observed at pH 7-9 [17].

The effect of temperature on protease production was observed in the range of 25°C to 40°C. The temperature 35°C was found to be optimum so that a maximum of 240.89 ± 4.56 U/ml protease activity was achieved. The alkaline protease production from *Pseudomonas fluorescens* isolated from a meat waste contaminated soil. The optimum conditions for protease production were 37°C, pH - 9 [18].

The effect of protease production by *Pseudomonas sp.* in the medium with various concentrations of NaCl (0-3.5%) were studied. Maximum enzyme activity of 344.97 ± 2.61 U/ml was observed at 3% of NaCl. Similar reports have reported by Porro *et al.* [19] production medium with 2% NaCl required for maximum protease production from *Pseudomonas sp.*

P. aeruginosa strain was grown in medium with different carbon sources such as maltose, glucose, sucrose and starch. Maximum enzyme activity (419.77 ± 4.62 U/ml) was noticed in glucose medium. Carbon source is a primary energy source, which have important role in the improvement of growth of the organisms. Similar reports were reported by Gassesse *et al.* [20] protease production in *Bacillus, Pseudofirmus* AL-89 increased in the presence of glucose.

Beef extract showed maximum activity among the different nitrogen sources (191.13 ± 3.21 U/ml). Saha and Bhattacharyya [21] reported in *Pseudomonas sp.* that the parent strain grew best in presence of beef extract, whereas the mutant strain in ammonium sulphate.

Among the metal ions calcium chloride showed higher protease production (194.02 ± 4.50 U/ml). Frikha *et al.* [22] reported that the production of protease by *Bacillus cereus* BG1 was absolutely calcium-dependant since no other divalent cations were able to induce enzyme synthesis. The $CaCl_2$ containing medium yielded a higher concentration of biomass and maximum protease production.

CONCLUSION

The data gathered in this study provides evidence for protease producing ability of the marine coastal water shrimp *Penaus monodon* gut bacterial isolate *Pseudomonas aeruginosa*. The production of protease and substrate interactions of bacterial strains in the *Penaus monodon* gut was also evident in this study. This study gives us a hint that the microbial wealth of

protease producing bacteria isolated from the *Penaus monodon* gut can be harnessed for biotechnological processes.

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