

# Purification and Characterization of Protease Enzyme From Halophilic *Bacillus* sps

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## ABSTRACT

Proteases from halophilic microorganisms present the advantage of being stable at high salinities, constituting interesting enzymes from a biotechnological point of view. To maintain osmoregularity in saline environments the microorganisms adopt mainly two strategies, one followed by most moderately halophilic bacteria, accumulating organic compatible solutes in the cytoplasm, and the second followed by the halobacteria (extremely halophilic aerobic archaea), accumulating inorganic salts in the cytoplasm. Protease enzyme is one of the most important enzymes used in a number of industries. But normal bacteria can produce less amount of enzyme. If the halophilic bacteria use in the place of normal, the enzyme activity is high which depends upon the pH, temperature and concentration. So the protease enzyme is commercially important and tolerates the halophilic bacteria and has many potential for a number of applications. The present study provides the methods for the production of proteases by a representative organism from each of the two main groups inhabiting the saline habitats: the moderately halophilic bacterium *Bacterial* sp. The production process involves the culture of the microorganisms under optimal conditions for the production of the extracellular proteases, and the recovery and purification of the enzymes from the culture supernatant.

**Keywords:** Protease enzyme, Halophilic bacteria, Microbial and Biochemical assays, Effects.

## INTRODUCTION

Halophiles are extremophile organisms that can thrive if the enzyme working in the organism reaches a temperature of over 30<sup>0</sup>C while in environments with very high concentrations of salt. Some halophiles classified into the Archaea domain, there are also bacterial halophiles. Some well-known species give off a red color from carotenoid compounds. Such species contain the photosynthetic pigment bacteriorhodopsin. Halophiles are categorized slight, moderate or extreme, by the extent of their halotolerance. Halophiles can be found anywhere with a concentration of salt five times greater than the salt concentration of the ocean.

High salinity represents an extreme environment that relatively few organisms have been able to adapt to and occupy. Most halophilic and all halotolerant organisms expend energy to exclude salt from their cytoplasm to avoid protein aggregation ('salting out'). In order to survive the high salinities, halophiles employ two differing strategies to prevent desiccation through osmotic movement of water out

of their cytoplasm. Both strategies work by increasing the internal osmolarity of the cell. In the first organic compounds are accumulated in the cytoplasm – these osmoprotectants are known as compatible solutes. These can be synthesised or accumulated from the environment<sup>1</sup>. The most common compatible solutes are neutral or zwitterionic and include amino acids, sugars, polyols, betaines and ectoines, as well as derivatives of some of these compounds.



**Fig 1: Halophilic *Bacillus* sps**

The second, more radical, adaptation involves the selective influx of potassium ( $K^+$ ) ions into the cytoplasm. This adaptation is restricted to the moderately halophilic bacterial Order Halanerobiales, the extremely halophilic archaeal Family Halobacteriaceae and the extremely halophilic bacterium *Salinibacter ruber*<sup>2</sup>. The presence of this adaptation in three distinct evolutionary lineages suggests convergent evolution of this strategy, it being unlikely to be an ancient characteristic retained in only scattered groups or through massive lateral gene transfer<sup>3</sup>. The primary reason for this is that the entire intracellular machinery (enzymes, structural proteins, etc.) must be adapted to high salt levels, whereas in the compatible solute adaptation little or no adjustment is required to intracellular macromolecules – in fact, the compatible solutes often act as more general stress protectants as well as just osmoprotectants<sup>4</sup>



**Fig 2: Archaea bacteria from Marine water**

Haloarchaea, and particularly, the family Halobacteriaceae are members of the domain Archaea, and comprise the majority of the prokaryotic population in hypersaline environments<sup>3</sup>. There are currently 15

recognized genera in the family<sup>5</sup>. The domain Bacteria can comprise up to 25% of the prokaryotic community, but is more commonly a much lower percentage of the overall population<sup>6</sup>. At times, the alga *Dunaliella salina* can also proliferate in this environment<sup>6</sup>. At the protein level, the halophilic species are characterized by low hydrophobicity, over representation of acidic residues, under representation of Cys, lower propensities for helix formation and higher propensities for coil structure<sup>7</sup>. It is also evident that the core of these proteins is less hydrophobic, such as DHFR, which was found to have narrower  $\beta$ -strands<sup>8</sup>. At the DNA level, the halophiles exhibit distinct dinucleotide and codon usage<sup>9</sup>.

Proteases are the single class of enzymes which occupy a prominent position with respect in their applications in both domestic and commercial fields<sup>10</sup>. Proteolytic enzymes catalyze the cleavage of peptide bonds. They are degradative enzymes which catalyze the hydrolysis of proteins. Proteases<sup>11</sup> are divided into exo and endo peptidases based on their action at or away from the termini, respectively. They are classified into several types depending on the nature of functional group present in their active site<sup>12</sup>.

Proteases are mainly in the detergent and food industrial applications. In recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes<sup>13</sup>. They are also used in the pharmaceutical applications extensively<sup>14</sup>. Although proteases occur in all forms of living organisms like plants, animals, protozoans and microorganisms, the microbial proteases occupy a prominent position with wide applications owing to the limited space required for their cultivation, their ready susceptibility to genetic manipulation<sup>15</sup>.

### Materials and Methods:

Soil samples have been collected from the sea water salterns of Vizag, Andhra Pradesh, India. Halophilic bacteria were isolated from soil samples by spread and pour plate methods with Halophile media containing 1% peptone, 0.4% yeast extract, 10% (w/v) sodium chloride, 1.5% (w/v) agar in 1000 ml distilled water with pH 7.2 was sterilized by autoclaving. The sample was taken by following serial dilution and incubated for 24-48hs for growth of the colonies. Pure cultures obtained on isolation were maintained on H-agar media slants and were sub cultured every 15 days. Stock cultures of the isolates were further maintained on glycerol slants.

The organisms obtained on isolations were screened for proteolytic activity by performing casein hydrolysis test on sterile H-casein agar media plates. A loop full of 24 hr old culture of the organism from 10% H-agar media was inoculated at the centre of the solidified sterile casein agar plates. The plates were incubated at room temperature for 48hr to check proteolytic activity. After incubation, 2ml of 10% trichloroacetic acid was added to the casein plates for precipitation of proteins, so that zone of clearance can be observed in the areas where protein has been utilized. Among all these isolates one organism with profound proteolytic activity has been designated as Ydc.

### Protease Assay

The amount of protease produced was assayed by the casein Folin-Ciocalteu method in the presence of 10% NaCl (Hiraga et al., 2005). To 0.45ml of 1.0% casein solution containing 25mM Tris-HCl, pH 7.5, 10% (w/v) NaCl, 50 $\mu$ l of enzyme solution was added and incubated for 1hr. The reaction was stopped by adding 10% Trichloroacetic acid and the reaction mixture was centrifuged at 12,000 rpm for 10min. To

0.5ml of the supernatant, 2.5ml of the 0.2M sodium carbonate and 0.5ml of Folin-Ciocalteu reagent was added and incubated for 1 hr at 37°C for 20min. The optical density of the color developed was read at 660nm. One nit of enzyme activity is defined as the enzyme quantity that liberates 1µg of tyrosine per ml of the reaction mixture per minute. The amount of tyrosine produced in the test sample is estimated from the tyrosine standard graph. The selected isolate Ydc is cultivated in the halophilic broth medium at 300C at 120rpm for 48hrs. Morphological like Gram staining and biochemical characteristics such as IMViC, Catalase test, Gelatin Hydrolysis and starch hydrolysis test of the isolate have been used for the identification of the isolate. Purification of the protease by Ammonium sulfate fractionation, ion exchange chromatography and Electrophoresis, characterization of the protease enzyme by the effect of pH, temperature and concentration on the activity of the protease enzyme.

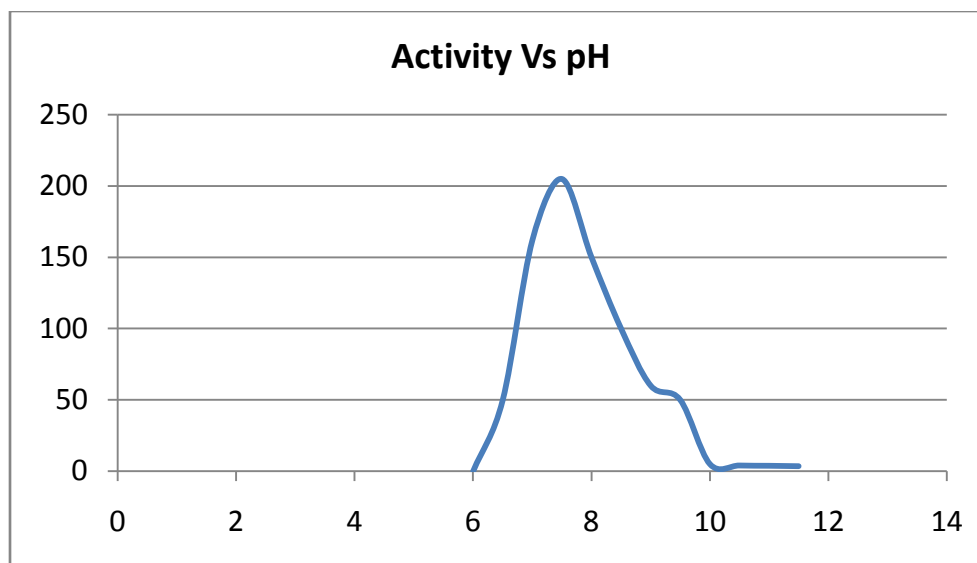


**Fig 3 : Isolation of protease bacteria**

## Results

When the study between the growth and protease production the Ydc is found to enter the stationary phase by 40<sup>th</sup> hour of growth. As the incubation time increases the biomass concentration increased experimentally. The increased trend is continued up to 32<sup>nd</sup> hour of incubation time. Further increase in incubation time shows the marginally improved biomass productivity up to 56 hr and the biomass production leveled off even though the incubation time increased until 120hr. The results indicating that, protease activity will be observed only till the end of exponential phase ceased at the beginning of stationary phase. The drastic decreases in protease activity beyond 56hr of incubation could be due to hydrolysis of protease.

The Active fraction obtained after ammonium sulfate loaded on to DEAE anion exchange column (2X20cm). After ion exchange chromatography the fraction eluted in 0.3M NaCl retained the protease activity. When crude proteins were electrophoresis on 10% polyacrylamide gels under native conditions about 10-12 bands were observed.



**Fig 4: The graph shows the protease activity Vs pH**

Effect of pH on the protease activity was studied by standard enzyme assay by using different buffers and protease activity was measured in nits. The enzyme was found to be active at pH in the range of 6.5-9.5 with an optimum activity at pH 7.5. Assay of the protease activity at different pH values the range of 6-11 has indicated 7.5 as the optimum pH.

The temperature tolerance and optimal temperature for the protease enzyme has been assessed by carrying out the assay at different temperatures. Activity of the enzyme has been shown at temperature within the range 25°C-55°C with an optimum activity at 35°C. Assay of the protease activity at different temperatures within the range of 0-60°C has indicated 35°C as temperature.

### Conclusion

The broad range of salt (0-25%), pH (6.5-9.5) and temperature (25°C-55°C) tolerance of this moderately halophilic protease makes it a commercially important enzyme with wide industrial applications. This enzyme has potential for applications in detergent formulations, fermentation of fish sauce and as an antifouling agent. To get the large amount of the protease the marine archaea and halophiles are highly useful for the future use.

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