STUDIES ON MODERATELY HALOPHILIC BACTERIA AND THEIR SALT AND SOLVENT STABLE PROTEASES

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by

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Dedicated to Ma and Baba

<u>CERTIFICATE</u>

This is to certify that the thesis entitled "**Studies on moderately halophilic bacteria and their salt and solvent stable proteases**" being submitted by **Ms. RAJESHWARI SINHA** to the Indian Institute of Technology Delhi for the award of the degree of ***Doctor of Philosophy*** in Chemistry is a record of bonafide research work carried out by her. Ms. Rajeshwari Sinha has worked under my guidance and supervision, and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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Professor of Biochemistry
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Date:
Place:

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Abstract

Halophiles are the class of extremophiles that grow and thrive in high salt concentrations. Their enzymes dubbed as “haloenzymes” play critical roles in regulation of cellular processes, maintain their structure and perform catalysis amidst saline milieu. They exhibit salt stability and resistance against solvent, salt or chaotropic agent induced denaturation. Research on halophiles has gained considerable attention in recent years, because they possess inherent clue of life evolution process and hold tremendous biotechnological potential owing to their novel enzymes and other biomolecules.

The thesis encompasses the detailed study of two halophilic protease producing microbes, *Virgibacillus* sp. EMB13 and *Bacillus* sp. EMB9 and their novel proteases. The highlight of the work is the stability of halophilic proteases in withstanding denaturing action of chaotropic reagents, solvents, nanoparticles, high temperature and extreme pH.

Protease producing halophilic microbial strains were isolated from the Somnath coast, Gujarat and Goan coast of India. Two isolates EMB9 and EMB13 with maximum zone of gelatin hydrolysis were selected for further studies. These were found to be related to *Virgibacillus* sp. and *Bacillus* sp. by biochemical tests and 16S rDNA sequencing.

Bacillus sp. EMB9 has been submitted to the Microbial Type Culture Collection (MTCC) with accession no. 11953.

The production optimization, purification and characterization of *Virgibacillus* sp. EMB13 protease were carried out. The protease production, optimized by one variable at a time approach, was enhanced by 2.7 folds yielding 270.0 U/ml protease. *Virgibacillus* sp. EMB13 produced five different extracellular proteases. The protease was partially purified by DEAE-cellulose ion-exchange and Sephadex G-75 gel filtration chromatography up to

4.2 folds and 3.1% yield. The purified preparation exhibited salt, pH, temperature, detergent and solvent stability. Circular dichroism studies showed that protease gained negative ellipticity with incorporation of salt and retained its native structure which may have been responsible for stability of the protease in salt and solvent.

The optimization of culture conditions for protease production from *Bacillus* sp. EMB9 was carried out by statistical approach using Response Surface Methodology. Ca^{2+} and NaCl were the most critical factors for improved protease yield. Under finally optimized conditions of (g/l): peptone, 5.0; gelatin, 9.5; NaCl, 62.5; $CaCl_2$, 0.3, yeast extract, 10.0; glucose, 5.0; pH 8.0; and inoculum size of 4.0% (v/v), at 30ºC with constant shaking at 150 rpm for 72 h, the production level reached a maximum of 369.0 U/ml.

Halophilic *Bacillus* sp. EMB9 protease was purified homogeneously using CM-cellulose chromatography to obtain 489 fold purity and 2.8% yield. The monomeric protease with molecular mass of 29.0 kDa was serine in nature and exhibited pH optimum 9.0, $t_{1/2}$ 190 min at 60°C and salt optimum 1% (w/v) NaCl. The protease was uniquely stable in hydrophilic solvents. The activity and stability of the purified protease were modulated differentially by Na^+/K^+ and Ca^{2+} ions. While Ca^{2+} ions prevented protease unfolding at room temperature, Na^+/K^+ ions assisted in refolding of the dialyzed enzyme.

Halophilic proteases exhibited greater stability and structural rigidity towards denaturants as compared to non-halophilic proteases. *Bacillus* sp. EMB9 protease was stable in 1.0 mM silver and zinc oxide nanoparticles, polar organic solvents (methanol, ethanol, propanol, t-butanol), urea (up to 8.0 M) and guanidium hydrochloride (up to 6.0 M). The secondary and tertiary structure of the native protease remained stabilized under these conditions as validated by CD and fluorescence spectral studies. Presence of NaCl also exerted a protective effect on dialyzed protease against chaotropism of solvents and urea/guanidium hydrochloride. Such stability amidst denaturing milieu may be attributed to the collective influence of higher proportion of negatively charged amino acid residues on protein surface, stabilization of protein secondary and tertiary structure and efficient charge screening of the protein surface by Ca^{2+} and Na^{+} ions.

Enzyme immobilization is generally known to enhance their stability and impart reusability. With this viewpoint, crude protease from *Bacillus* sp. EMB9 was immobilized on to silica nanoparticles with immobilization efficiency of 60% and immobilization yield of 80%. The immobilized preparation retained 80% of its original activity at 70°C while $t_{1/2}$ at 50°C showed 4 fold enhancement over that of free protease. Immobilized protease hydrolyzed whey proteins with high degree of hydrolysis. Other industrially relevant applications such as protein stain removal efficiency, hydrolyzed soyflour proteins and recovered silver from used X-Ray films were also explored.

The thesis also covers interaction of silver and zinc oxide nanoparticles with halophilic and non-halophilic bacterial cells. The effects of nanoparticles on their growth and viability indicated more pronounced nanotoxicity on Gram-negative bacteria as compared to Gram-positive. Increased resistance in Gram-positive cells was attributed to the presence of thicker peptidoglycan layer. Halophilic bacteria were more susceptible towards nanoparticle toxicity possibly due to the higher content of negatively charged cardiolipins on halophilic cell surface. SEM, TEM and EDX spectroscopy validated surface interactions between bacteria and nanoparticles with internalization and accumulation of nanoparticles. Silver nanoparticle induced changes in the intracellular bacterial proteome of halophilic *Bacillus* sp. EMB9 revealed global changes.

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