

**EXTREMOPHILES AS A SOURCE OF NOVEL
ENZYMES FOR BIOTECHNOLOGICAL
APPLICATIONS**

by

ANSHU GUPTA

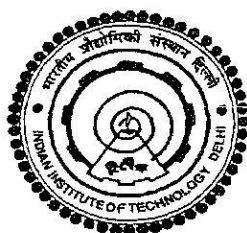
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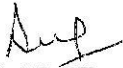
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CERTIFICATE

This is to certify that the thesis entitled "*Extremophiles as a Source of Novel Enzymes for Biotechnological Applications*" being submitted by **Ms. ANSHU GUPTA** 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. Gupta 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.

18 January, 2006


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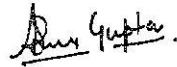
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(Anshu Gupta)
New Delhi

Dedicated to My Parents...

Abstract

THE thesis concerns the studies of proteolytic enzymes from extremophilic organisms. Two classes of extremophiles, solvent tolerant microbes and halophiles were selected for this purpose. The main part of the work deals with isolation of solvent tolerant microbes, their adaptive features imparting solvent stability, purification and characterization of solvent stable protease, molecular basis of solvent adaptation and their biotechnological implications.

Solvent tolerant bacteria are relatively new group of extremophilic microbes that thrive in the presence of high concentration of solvents. There is an increasing interest in their enzymes for use in non-aqueous enzymology. Four solvent tolerant bacteria were isolated from soil samples by cyclohexane enrichment in the medium. These were identified to be *Pseudomonas aeruginosa* strain PseA, *Enterobacter aerogenes* strain 3HC and *Serratia marcescens* strain 3HPC by biochemical tests and fatty acid methyl ester (FAME) analysis. One isolate i.e. strain 3HP could not be identified. Out of these four strains, *P. aeruginosa* strain PseA and *E. aerogenes* strain 3HC were selected for further studies. The strains were able to sustain and grow in a wide range of organic solvents but presence of solvent in culture media caused reduction in growth. Transmission electron micrographs showed convoluted cell membrane and accumulation of solvent in case of the cells grown in cyclohexane.

The *P. aeruginosa* strain PseA was found to be protease producer on skim-milk agar plates. It secreted a novel protease, which exhibited remarkable solvent stability. At 25% (v/v) solvent concentrations, protease retained most of its activity at least up to 10 days in most of the solvents. In case of benzene, n-decane and n-dodecane the stability was recorded up to 75% solvent.

Protease production from *P. aeruginosa* PseA was optimized by varying the culture conditions. Glycerol as carbon source (0.7%), casein (0.4%) and yeast extract (0.6%) as nitrogen sources, CaCl₂ (0.6 mM) as metal ion in culture media (pH 7.0), inoculated with 2.5% of 36 h grown mother culture and incubation for 24 h at 30°C with 250 rpm shaking were found to be the best culture conditions leading to maximum growth and protease production (1601 U ml⁻¹). This growth rate and protease production was comparable to other strains of *Pseudomonas* sp.

This protease was purified 11.6-fold with 60% recovery by combination of ion exchange and hydrophobic interaction chromatography using Q-Sepharose and Phenyl Sepharose 6 Fast Flow matrix respectively. The apparent molecular mass based on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was estimated to be 35 kDa. The purified enzyme was stable in the pH range of 6.0–9.0, the optimum being 8.0. The K_m and V_{max} towards caseinolytic activity were found to be 2.7 mg ml⁻¹ and 0.56 mg ml⁻¹ min⁻¹ respectively. The protease was most active at 60°C and characterized as a metalloprotease because of its sensitivity to EDTA and 1, 10-phenanthroline. It was tested positive for elastase activity towards elastin-orcein, thus appears to be an elastase, which is known as pseudolysin in other strains of *P.*

aeruginosa. The purified protease withstands range of detergents, surfactants and solvents.

To establish the structural features responsible for imparting solvent tolerance, the primary structure, conserved motif and hydropathy profile of PseA protease were investigated and compared with other well-known metallo-proteases. The N-terminal amino acid sequence of the purified *P. aeruginosa* PseA was determined and used to search NCBI database for its homologue. An exact match with a *P. aeruginosa* PST-01 protease gene, *lasB* was found. The c-DNA of PST-01 protease was used for designing the polymerase chain reaction (PCR) primers in order to clone the corresponding gene in *P. aeruginosa* strain PseA. The cloned DNA fragment contained a 1,494-bp open reading frame encoding a 498-amino-acid polypeptide (53.6 kDa).

The deduced protein contains a segment of first 23 residues as signal peptide (2.6 kDa) that is followed by an 18 kD propeptide (174 residues) and the 33 kDa mature enzyme (301 residues). The phylogenetic analysis of PseA protease with other known proteases revealed a tight sub grouping of *P. aeruginosa* proteases (elastases). It was found to contain a conserved HEXXH zinc-binding motif, confirming it to be a zinc metallopeptidase. The protein was highly hydrophobic and consisted of a large number of hydrophobic amino acids, which may be contributing to its solvent tolerance nature.

E. aerogenes 3HC strain obtained during the screening of solvent tolerant microbes was found to have an excellent property of mercury detoxification along with solvent stability. The bacterium was able to grow in the presence of mercury in culture media. Mercury level in the medium decreased with growth and transmission electron

micrographs showed accumulation of mercury inside the cells. To explore the genetic basis of metal/solvent resistance in *E. aerogenes*, its plasmid profile and transformation of *E. coli* DH5 α cells were initiated. The results indicated that mercury resistance might be plasmid mediated in *E. aerogenes*, whereas solvent tolerant property was not plasmid based. While going through these studies, a new technique for plasmid purification based on selective precipitation by a compaction agent MnCl₂ was developed.

A haloalkaliphilic bacteria (isolate Vel) related to *Bacillus pseudofirmus*, provided by Department of Biosciences, Saurashtra University, Rajkot, India, was studied for screening alkaline protease. The protease secreted by this bacterium was purified 10-fold with 82% yield by a single step method on Phenyl Sepharose 6 Fast Flow column. The apparent molecular mass on SDS-PAGE was estimated to be 29 kDa. The K_m and V_{max} towards caseinolytic activity were found to be 2 mg ml⁻¹ and 0.29 mg ml⁻¹ min⁻¹ respectively. The enzyme was active over the pH range of 8.5–12.0, the optimum being 10.0–11.0. The proteolytic activity was inhibited by PMSF, suggesting that the enzyme may belong to serine type protease. Interestingly, the activity was slightly enhanced with SDS (0.1%) and Triton X-100 (0.1%) but remained unaffected by Tween 80 (0.1%). The stability of the enzyme in the presence of detergent components and surfactant is particularly attractive for its application in detergent industries.

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