EXTREMOPHILES AS A SOURCE OF NOVEL ENZYMES FOR BIOTECHNOLOGICAL APPLICATIONS

by

ANSHU GUPTA

DEPARTMENT OF CHEMISTRY

Submitted

in fulfilment of the requirements of the Degree of

DOCTOR OF PHILOSOPHY

to the

INDIAN INSTITUTE OF TECHNOLOGY, DELHI

JANUARY 2006

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

S. K. Khare
Asst. Professor of Biochemistry
Department of Chemistry
Indian Institute of Technology, Delhi

Acknowledgements

THERE are a number of people without whom this thesis might have not been written, and to whom I am greatly indebted. It will not be possible for me to acknowledge my gratitude for the help that I received from all of them but I will still like to name a few people.

Dr. S. K. Khare: For introducing me to the world of science; for your competent guidance, untiring efforts, constant support and care during the course of study; for your enthusiasm and interest in research and for providing me all the necessary facilities to work on this thesis. You have always interacted with me at both the professional and the personal level. I have appreciated your ability to understand life, inside and outside microbial and biochemistry lab. Your constant encouragement helped me to develop confidence in myself. Without your initial momentum and continuous support, it would have been impossible for me to get where I stand.

Prof. M. N. Gupta: For initiating me into the exciting and enjoyable world of scientific research; for giving me the opportunity to work in your lab and never differentiating me from your own students; for your enthusiastic and infectious interest in research; for building confidence in me when I doubted myself; for helping me at any time and reminding me that I should concentrate on doing "good science"! It would not have been possible for me to work on this thesis without your kind help and support. Thank you, Sir for everything!

Prof. V. K. Nadir, Head, Department of Chemistry: For providing me all the necessary facilities.

Prof. H. P. Garg: For your advice and helpful suggestions and for always being there with a supporting and caring hand.

Dr. A. K. Panda, NII, New Delhi: For giving me the opportunity to work in your lab and fruitful guidance in the field of microbiology; Eshwari: for teaching me the techniques of microbiology.

Dr. S. P. S. Khanuja, CIMAP, Lucknow: For allowing me to work in your winter school, "Training on recent techniques in gene cloning, DNA analysis and functional genomics".

Prof. A. K. Tyagi, DUSC, New Delhi: For allowing me to work in your lab.

Dr. Sanjay Kapoor, *DUSC, New Delhi: For making my entry into the area of molecular biology; for being there with all the clarifications, guidance and support; for giving insightful comments and reviewing my one chapter on a very short notice;* ***Swati:*** *for guiding me to work on protease gene part of the work;* *Other lab members: for providing friendly environment in the lab, being ready to help whenever needed.*

Prof. S. P. Singh, *SU, Rajkot: For providing me the halophilic bacterial strain and for your encouragement.*

Dr. N. G. Ramesh, *IIT Delhi: For allowing me to use your lab facilities whenever needed and for your time-to-time help and encouragement.*

Dr. Shastry: *For introducing me to the beautiful world of solvent tolerant microbes and teaching me the technical skills.*

Dr. Rajni Singh: *For your help and advice on getting me started the research work and also for trying to teach me all the "tricks of the trade".*

Dr. Sandhya R. Shenoy: *For your words of wisdom and useful discussions on research and life.*

Dr. Ipsita Roy: *For initiating me into the world of proteins/enzymes purification that much easier and working with me with endurance; for dedicating your precious time and for your constructive criticism which always motivated me to improve.*

Ruchi Gaur: *For bearing with my short temper at times in the lab and being meek enough to listen to my most unfair words; for your homely and entertaining talks; for sharing all the responsibilities of the lab and news of the department and hostel; for proofreading my chapters. Also for not acknowledging me in your M. Tech thesis.*

Sonal: *For always being there to listen to all my grumblings and complaints; for providing me a shoulder to cry on and always being ready with a pain relieving cream; for trying to teach me the philosophy of life and for sharing your experience of the dissertation writing endeavor with me.*

Arunima: *For your phone calls to encourage me while writing this thesis.*

My research group *that has been a great source of inspiration and stimulation, as well as a enjoyable place; Dr. Sunita Teotia, Dr. Aparna Sharma, Dr. Hema Pant, Dr. Meryam Sardar, Dr. Shweta Sharma, Dr. Sulakshana Jain, Kalyani, Shweta, Smita, Reddy, Jita, Sohail, Aabir, Pradeep, Arvind and other past and present colleagues, specially Khalid, Sunita, Gorakh and Ramkaran: For your moral support, well-wishes, uninhibited help and teaching different aspects of research.*

My friends *Maneesha, Shobhit, Parul, Anamika, Rashmi, Geeta, Titto, Vipin, Garima, Pooja, Tokeer, Mital, Dr. Rajesh, Pinky, Reema and all other friends whose names are not listed here: For your support, help and care during my studies. I had always enjoyed your friendship and companionship that was invaluable to make me feel at home.*

All M. Sc., M. Tech. students and trainees *who have worked in our laboratory: For their help to learn me a lot about this work.*

I am thankful to Department of Chemistry, IIT Delhi and the Council of Scientific and Industrial Research (CSIR) Govt. of India for providing financial support.

My family, in-laws and relatives: *For always believing in me and being pride of me. Your support and encouragement over the years is sincerely appreciated.*

My parents, *to whom I owe everything I have achieved today: for giving me life in the first place, for giving me the greatest support of all; for your love, patience and encouragement to pursue my interests, even when the interests went to be separated and far away from each other; My sisters and brother* ***Ashwani:*** *For your love, invaluable support and care that you have always shown for me.*

......................and finally ***Hemant,*** *My greatest discovery, for standing by my side with encouragement, patience and love; for being the sunshine in my life during the dark autumn writing the thesis and giving me the strength to keep going, for listening to my complaints and frustrations; for always being there to scold me to eat something and for your hearty attempts to make sure that I had the most comfortable time while working on this thesis ....for everything!*

Most of all thanks to ***God - the Divine*** *who continues to make the impossible possible.*

Anshu Gupta.

(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, $CaCl_2$ (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* DH5α 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 $MnCl_2$ 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.

Table of Contents