ENGINEERING OF PROTEASE FOR NON-AQUEOUS SOLVENTS

by

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Proteases - Non Aqueous Solvents
enzymes - microbial proteases
thermophilic organisms - organic solvents.

Dedicated to my
parents
to whom I am indebted
for
what I am

Certificate

This is to certify that the thesis entitled "**Engineering of protease for non-aqueous solvents**" being submitted by **Ms. Ritu Sareen** to the Indian Institute of Technology Delhi, for the award of the degree of "**Doctor of Philosophy**" is a record of bonafide research carried out by her, which has been prepared under my supervision in conformity with rules and regulations of the "Indian Institute of Technology Delhi". The research work and results presented in the thesis have not been submitted for any degree or diploma in any other University or Institute.

Prashant Mishra

Dr. Prashant Mishra

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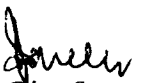

Ritu Sareen

ABSTRACT

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases are degradative enzymes, which catalyze the hydrolysis of proteins by cleaving the peptide bonds. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Microbial proteases have been extensively used in the food, dairy and detergent industries since ancient times. There is a renewed interest in proteases as targets for developing therapeutic agents against relentlessly spreading fatal diseases such as cancer, malaria, and AIDS. Advances in genetic manipulation of microorganisms by SDM of the cloned gene opens new possibilities for the introduction of predesigned changes, resulting in the production of tailor-made proteases with novel and desirable properties. The proteases isolated from extremophilic organisms are likely to mimic some of the unnatural properties of the enzymes that are desirable for their commercial applications. Though proteases have generally been associated with protein digestion, experiments providing evidence for the reverse of the hydrolysis has been demonstrated. The activities of the proteases in organic solvents though offer many advantages as better thermotolerance, novel activities but at the same time pose the problem of poor protease activity, stability in the non-aqueous milieu. This has been catered to by medium engineering and of late enzyme engineering approach. Though work has been done on this aspect with mesophilic subtilisin and *Pseudomonas* sp., but efforts have not been put to explore the extremophilic species in this respect.

In this thesis we report isolation of thermophilic *Bacillus licheniformis* RSP-09 from the hot water spring of Sohna, Haryana. The organism was screened for its tolerance to

organic solvent. Further a comparison was made between the mesophilic sp. of *Bacillus* viz *B. subtilis* NRRL B-543 and *B. licheniformis* NRRL-1001. The thermophilic strain proved to be better evolved for its tolerance towards organic solvent as compared to the mesophiles. The thermophilic *B. licheniformis* RSP-09 was randomly mutagenized using the chemical mutagen; Nitroso guanidine (NTG) resulting in *B. licheniformis* RSP-09-37 which showed improved tolerance to organic solvents. The protease secreted from *B. licheniformis* RSP-09-37 was checked for its tolerance towards organic solvents differing in their log *P* values. The protease displayed better tolerance for more hydrophobic organic solvents as compared to the hydrophilic ones. The protease was further purified with affinity chromatography using α-casein–agarose matrix that resulted in a purification fold of 86 and yield of 47%. The protease gene (*apr 46*) was isolated from the genomic DNA library using pUC 19 cloning vector and *Escherichia coli* JM 109 host cells. The gene was over expressed in *E. coli* BL-21 (DE 3) host cells using pET 29a expression vector. The gene was further evolved using the directed evolution technique of error–prone polymerase chain reaction (ep-PCR) for better synthetic activity towards kyotorphin. A screening method for the detection of the synthetic activity was also developed for the microtiter plates (MTP). The synthetic activity of RSPep 60-1-2, the protease obtained after the third round of ep-PCR was 1.2 folds of the protease obtained from *B. licheniformis* RSP-09-37.

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