

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

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AND THEIR SALT AND SOLVENT STABLE
PROTEASES**

by

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Submitted

in fulfillment of the requirements of the Degree of
DOCTOR OF PHILOSOPHY

to the



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Dedicated to Ma and Baba

CERTIFICATE

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.

Date:
Place:

Dr. S. K. Khare
Professor of Biochemistry
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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 30°C 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.

Table of Contents

Certificate	i
Acknowledgements	ii
Abstract	v
Table of Contents	viii
List of Figures	xviii
List of Tables	xxiv

CHAPTER 1 INTRODUCTION

1.1 Rationale of the thesis	1
1.2 Objectives	3
1.3 Scope of the thesis	3
1.4 Review of literature	6
1.4.1 Halophiles: Life under salinity	6
1.4.2 Phylogenetic diversity in halophiles	8
1.4.3 Adaptive features in halophiles to cope with saline environment	12
1.4.4 Adaptations in halophilic proteins	15
1.4.5 Industrial relevance of halophiles	18
1.4.6 Enzymes from halophiles	23
1.4.7 Proteases	26
1.4.8 Microbial sources of proteases	27
1.4.9 Proteases from halophilic microorganisms	27
1.4.10 Protease production from halophilic microorganisms	30
1.4.11 Purification of halophilic proteases	35
1.4.12 Properties of halophilic proteases	42
1.4.13 Molecular characterization of halophilic protease genes	70
1.4.14 Application of halophilic proteases	73

CHAPTER 2 CHARACTERIZATION OF PROTEASE PRODUCING HALOPHILIC MICROORGANISMS

2.1	Introduction	77
2.2	Materials and Methods	79
2.2.1	Materials	79
2.2.2	Isolation and identification of protease producing halophilic microorganism	79
2.2.3	Biochemical and physiological characterization of the isolates	80
2.2.4	Identification by 16S rDNA analysis	80
2.2.5	Scanning and Transmission Electron Microscopy	81
2.2.6	Protease assay	81
2.2.7	Cell growth and protease production	82
2.2.8	Preliminary characterization of crude halophilic proteases	83
2.3	Results and Discussion	84
2.3.1	Protease producer halophiles	84
2.3.2	Biochemical and physiological characterization	85
2.3.3	Identification by 16S rDNA sequencing	87
2.3.4	Scanning and transmission electron microscopy	88
2.3.5	Growth kinetics and protease production	90
2.3.6	Preliminary characterization of halophilic proteases	93
2.4	Conclusions	94

CHAPTER 3 PRODUCTION, PURIFICATION AND CHARACTERIZATION OF PROTEASE FROM HALOPHILIC *VIRGIBACILLUS* SP. EMB13

3.1	Introduction	96
3.2	Materials and Methods	97
3.2.1	Materials	97
3.2.2	Microorganism	98
3.2.3	Inoculum preparation	98

3.2.4	Growth and protease production by <i>Virgibacillus</i> sp. EMB13	98
3.2.5	Protease assay and protein estimation	99
3.2.6	Optimization of protease production	100
3.2.7	Purification of <i>Virgibacillus</i> sp. protease	100
3.2.8	Polyacrylamide gel electrophoresis and zymography of purified protease	101
3.2.9	Characterization of <i>Virgibacillus</i> sp. protease	101
3.2.9.1	Determination of temperature, pH optimum and stability	101
3.2.9.2	Effect of NaCl and other metal ions	102
3.2.9.3	Determination of K_m and V_{max}	102
3.2.9.4	Effect of additives	102
3.2.9.5	Protease stability in organic solvents	103
3.2.9.6	Stability in detergents, oxidants and bleaching agents	103
3.2.10	Circular dichroism spectroscopy	104
3.2.11	Washing efficiency of the protease	104
3.3	Results and Discussion	105
3.3.1	Protease production from <i>Virgibacillus</i> sp. EMB13	105
3.3.2	Activity staining of the protease	107
3.3.3	Purification of <i>Virgibacillus</i> sp. protease	108
3.3.4	Characterization of partially purified protease	111
3.3.4.1	Enzymatic properties	112
3.3.4.2	Effect of salt on activity and stability of protease	113
3.3.4.3	Organic solvent stability of protease	115
3.3.4.4	Compatibility with detergents, oxidants and bleaching agents	119
3.3.5	Washing efficiency of the protease	120
3.4	Conclusions	121

CHAPTER 4 OPTIMIZATION OF CULTURE CONDITIONS FOR PROTEASE PRODUCTION BY *BACILLUS* SP. EMB9

4.1	Introduction	122
4.2	Materials and Methods	123
4.2.1	Materials	123
4.2.2	Microorganism	124
4.2.3	Inoculum preparation	124
4.2.4	Growth and protease production by <i>Bacillus</i> sp. EMB9	124
4.2.5	Protease assay	124
4.2.6	Optimization of protease production	125
4.2.6.1	Plackett-Burman design	125
4.2.6.2	Response Surface Methodology	125
4.2.6.3	Validation of the model	126
4.2.7	Growth and protease production under optimized conditions	126
4.3	Results and Discussion	126
4.3.1	Production of <i>Bacillus</i> sp. EMB9 protease	126
4.3.2	Growth of <i>Bacillus</i> sp. and protease production under optimized conditions	133
4.4	Conclusions	135

CHAPTER 5 PURIFICATION AND CHARACTERIZATION OF *BACILLUS* SP. EMB9 PROTEASE

5.1	Introduction	136
5.2	Materials and Methods	137
5.2.1	Materials	137
5.2.2	Microorganism	137
5.2.3	Inoculum preparation	137
5.2.4	Protease assay and protein estimation	137
5.2.5	Protease production	138

5.2.6	Protease purification	138
5.2.7	Polyacrylamide gel electrophoresis and activity staining of purified protease	139
5.2.8	Characterization of purified protease	139
5.2.8.1	Enzymatic properties	139
5.2.8.2	Kinetic parameters for casein hydrolysis	140
5.2.8.3	Thermodynamic parameters for casein hydrolysis	140
5.2.8.4	Effect of NaCl, KCl and CaCl ₂ on dialyzed protease	141
5.2.8.5	Protease stability in organic solvents	141
5.2.8.6	Effect of surfactants and detergents	141
5.2.9	Circular dichroism spectroscopy	142
5.2.10	Fluorescence measurements	142
5.3	Results and Discussion	143
5.3.1	Purification of <i>Bacillus</i> sp. protease	143
5.3.2	Instability of purified protease: protective effect of Ca ²⁺	145
5.3.3	Structural basis of calcium dependent stability of purified protease	147
5.3.4	Characterization of the purified protease	150
5.3.4.1	Substrate specificity and enzymatic properties	150
5.3.4.2	Kinetic and thermodynamic properties	150
5.3.4.3	Effect of salt and additives on protease activity	152
5.3.5	Effect of NaCl and KCl on reactivation of dialyzed protease	153
5.3.6	Organic solvent stability	156
5.3.7	Detergent compatibility	157
5.4	Conclusions	158

CHAPTER 6A DIFFERENTIAL INTERACTION OF HALOPHILIC AND NON-HALOPHILIC PROTEASES WITH NANOPARTICLES

6A.1 Introduction	160
6A.2 Material and Methods	162
6A.2.1 Materials	162
6A.2.2 Microorganism	162
6A.2.3 Protease assay	162
6A.2.4 Protease production	163
6A.2.5 Protease purification	163
6A.2.6 Circular Dichroism spectroscopy	163
6A.2.7 Fluorescence measurements	163
6A.2.8 Fourier Transform Infrared (FTIR) spectroscopy	163
6A.2.9 Effect of nanoparticles on structure and activity of proteases	164
6A.3 Results and Discussion	164
6A.3.1 Purification of <i>Bacillus</i> sp. EMB9 protease	164
6A.3.2 Effect of nanoparticles on protease activity	164
6A.3.3 Conformational changes in nanoparticle treated proteases	166
6A.4 Conclusions	174

CHAPTER 6B STRUCTURAL CHANGES IN *BACILLUS* SP. EMB9 PROTEASE IN RESPONSE TO CHAOTROPIC AGENTS

6B.1 Introduction	175
6B.2 Material and Methods	177
6B.2.1 Materials	177
6B.2.2 Microorganism	177
6B.2.3 Protease assay	178
6B.2.4 Protease production	178
6B.2.5 Protease purification	178
6B.2.6 Denaturation studies	178

6B.2.7	CD spectroscopy	179
6B.2.8	Fluorescence measurements	179
6B.2.9	Differential Scanning Calorimetry measurements	179
6B.2.10	ANS fluorescence spectroscopy	180
6B.3	Results and Discussion	180
6B.3.1	Purification of <i>Bacillus</i> sp. EMB9 protease	180
6B.3.2	Effect of varying concentration of denaturants	180
6B.3.3	Denaturation kinetics in 8 M urea	183
6B.3.4	Structural basis of stability of halophilic protease	185
6B.3.5	DSC measurements	190
6B.3.6	ANS fluorescence spectroscopy	191
6B.3.7	Protective role of NaCl against denaturation in halophilic protease	192
6B.4	Conclusions	195
 CHAPTER 6C EFFECT OF ORGANIC SOLVENTS ON THE STRUCTURE AND ACTIVITY OF HALOPHILIC <i>BACILLUS</i> SP. EMB9 PROTEASE		
6C.1	Introduction	197
6C.2	Material and Methods	199
6C.2.1	Materials	199
6C.2.2	Microorganism	200
6C.2.3	Protease assay	200
6C.2.4	Protease production	200
6C.2.5	Protease purification	200
6C.2.6	Protease stability in organic solvents	200
6C.2.7	Effect of organic solvents on secondary and tertiary structure of protease	201
6C.2.8	Effect of NaCl on organic solvent stability of halophilic protease	201

6C.2.9	CD spectroscopy	202
6C.2.10	Fluorescence measurements	202
6C.3	Results and Discussion	202
6C.3.1	Purification of <i>Bacillus</i> sp. EMB9 protease	202
6C.3.2	Effect of polar organic solvents on activity of halophilic <i>Bacillus</i> sp. protease	203
6C.3.3	Effect of organic solvents on secondary structure	206
6C.3.4	Effect of organic solvents on tertiary structure	210
6C.3.5	Protective effect of salt against solvent induced denaturation	212
6C.4	Conclusions	217
 CHAPTER 7 IMMOBILIZATION OF <i>BACILLUS</i> SP. EMB9 PROTEASE ON SILICA NANOPARTICLES AND APPLICATIONS		
7.1	Introduction	218
7.2	Materials and Methods	220
7.2.1	Materials	220
7.2.2	Halophilic protease	220
7.2.3	Protease assay	221
7.2.4	Activation of functionalized silicon oxide	221
7.2.5	Immobilization of EMB9 protease	221
7.2.6	Optimization of immobilization conditions	222
7.2.7	SEM, TEM and FTIR	222
7.2.8	Characterization of free and immobilized protease	223
7.2.9	Reusability of the immobilized protease	224
7.2.10	Applications of the free protease	224
	7.2.10.1 Destaining efficiency	224
	7.2.10.2 Enzymatic hydrolysis of defatted soyflour	224
	7.2.10.3 Determination of Degree of hydrolysis and protein concentration	225
	7.2.10.4 Sodium Dodecyl Sulphate-Polyacrylamide	

	Gel Electrophoresis	225
	7.2.10.5 Hydrolysis of gelatin and release of silver from used X-Ray films	226
	7.2.11 Application of immobilized protease	226
	7.2.11.1 Whey protein hydrolysis	226
	7.2.11.2 Determination of Degree of hydrolysis	227
7.3	Results and Discussion	227
7.3.1	Immobilization of protease on silica nanoparticle	227
7.3.2	Optimization of immobilization conditions	229
7.3.3	SEM, TEM and FTIR analysis	230
7.3.4	Characterization of immobilized protease	234
	7.3.4.1 pH and temperature optimum	234
	7.3.4.2 pH and temperature stability	236
	7.3.4.3 Kinetic properties of the immobilized protease	237
	7.3.4.4 Reusability of the immobilized protease	238
7.3.5	Applications of halophilic protease	239
	7.3.5.1 Washing efficiency	239
	7.3.5.2 Soyflour protein hydrolysis by halophilic protease	239
	7.3.5.3 Enzymatic recovery of silver from used X-Ray films	242
7.3.6	Applications of immobilized protease in hydrolysis of whey protein	244
7.4	Conclusions	247

CHAPTER 8 COMPARATIVE NANOTOXIC EFFECT OF ZnO AND Ag NANOPARTICLES ON HALOPHILIC AND NON-HALOPHILIC BACTERIAL CELLS

8.1	Introduction	249
8.2	Materials and Methods	251
8.2.1	Materials	251

8.2.2	Microorganisms	252
8.2.3	Inoculum	252
8.2.4	Microbial growth and culture	252
8.2.5	Preparation of nanoparticle suspensions	253
8.2.6	Tests for antibacterial activity	253
8.2.7	Scanning electron microscopy	254
8.2.8	Transmission electron microscopy	254
8.2.9	Energy dispersive X-Ray spectroscopy	254
8.2.10	Trizol based sample preparation for 2D electrophoresis	254
8.2.11	2D gel electrophoresis and analysis of protein expression levels	256
8.3	Results and Discussion	257
8.3.1	Effect on non-halophilic Gram-negative <i>Enterobacter</i> sp.	257
8.3.2	Effect on non-halophilic Gram-positive <i>Bacillus subtilis</i>	264
8.3.3	Effect on halophilic Gram-negative <i>Marinobacter</i> sp.	268
8.3.4	Effect on Gram-positive Halophilic bacterium EMB4	270
8.3.5	Effect on halophilic Gram-positive <i>Bacillus</i> sp. EMB9	272
8.3.6	Proteomic analysis of <i>Bacillus</i> sp. EMB9 cells in absence and presence of silver nanoparticles	274
8.4	Conclusions	278
	Overall Conclusions	280
	Future Perspectives	284
	References	286
	List of Publications	352
	Brief Bio-Data	356

List of Figures

Figure 1.1	Pink halite (rock salt) crystals from Searles Lake, California, USA	7
Figure 1.2	Major sites in the world where halophilic microflora predominates	9
Figure 1.3	Satellite image of the Sambhar Salt Lake, Rajasthan, India	12
Figure 1.4	Structure of <i>Haloferax mediterranei</i> glucose dehydrogenase	16
Figure 2.1	Halophilic isolates showing proteolytic activity (zone of hydrolysis) on gelatin agar plates	84
Figure 2.2	Phylogenetic inference based on 16S rDNA analysis of halophilic isolates	87
Figure 2.3	Scanning and transmission electron micrographs of the <i>Virgibacillus</i> sp. EMB13 and <i>Bacillus</i> sp. EMB9 cells grown in nutrient broth medium	89
Figure 2.4	Growth curve and protease production profile of <i>Virgibacillus</i> sp. EMB13 and <i>Bacillus</i> sp. EMB9	91
Figure 3.1	Growth of <i>Virgibacillus</i> sp. EMB13 and protease production under optimized media conditions	106
Figure 3.2	Purification of <i>Virgibacillus</i> sp. EMB13 protease	109
Figure 3.3	Gel electrophoresis and activity staining of partially purified <i>Virgibacillus</i> sp. EMB13 protease	110
Figure 3.4	Effect of NaCl on the activity of the dialyzed protease preparation	114
Figure 3.5	Far UV-CD spectra of <i>Virgibacillus</i> sp. EMB13 protease in absence and presence of NaCl	115
Figure 3.6	Organic solvent stability of <i>Virgibacillus</i> sp. protease	116
Figure 3.7	Far UV-CD spectra for the effect of hexane on the secondary structure of <i>Virgibacillus</i> sp. EMB13 protease	118
Figure 3.8	Stability and compatibility of EMB13 protease in the presence of various commercial detergents	119

Figure 3.9	Washing efficiency of <i>Virgibacillus</i> sp. EMB13 protease towards blood stain	120
Figure 3.10	K/S values of stained cloth subjected to washing by EMB13 protease	120
Figure 4.1	Response surface 3-D contour plot for protease activity as response variables for <i>Bacillus</i> sp. EMB9	131
Figure 4.2	Perturbation plot for the production of halophilic protease by <i>Bacillus</i> sp. EMB9	132
Figure 4.3	Growth and protease production by <i>Bacillus</i> sp. EMB9 under optimized condition	134
Figure 5.1	Purification of <i>Bacillus</i> sp. EMB9 protease by CM-cellulose ion exchange chromatography	143
Figure 5.2	Gel electrophoresis and activity staining of purified <i>Bacillus</i> sp. EMB9 protease	144
Figure 5.3	Effect of calcium on the activity of the purified protease	146
Figure 5.4A	Variation in the secondary structure of EMB9 protease with time	148
Figure 5.4B	Effect of Ca^{2+} on secondary structure of purified EMB9 protease	148
Figure 5.5	Effect of Ca^{2+} on tertiary structure of purified EMB9 protease	149
Figure 5.6	Effect of Na^+ , K^+ and Ca^{2+} on activity and stability of dialyzed protease	153
Figure 5.7	Effect of salts on secondary and tertiary structure of dialyzed EMB9 protease	155
Figure 5.8	Effect of organic solvents on the stability of the protease	156
Figure 5.9	Stability of the <i>Bacillus</i> sp. EMB9 protease in commercial detergents	158
Figure 6A.1	Effect of nanoparticles on activity of halophilic and non-halophilic proteases	165
Figure 6A.2	Effect of nanoparticles on secondary structure of non-halophilic <i>B. licheniformis</i> protease	167
Figure 6A.3	Effect of nanoparticles on secondary structure of halophilic <i>Bacillus</i> sp. EMB9 protease	167

Figure 6A.4	FTIR spectra of <i>Bacillus</i> sp. EMB9 protease before and after interaction with nanoparticles	169
Figure 6A.5	Effect of nanoparticles on tertiary structure of halophilic <i>Bacillus</i> sp. EMB9 protease	171
Figure 6A.6	Effect of varying concentration of ZnO nanoparticles on fluorescence spectra of halophilic protease	172
Figure 6B.1	Effect of varying concentration of denaturants on proteolytic activity	181
Figure 6B.2	Effect of 8.0 M urea on proteolytic activity as a function of time	183
Figure 6B.3	Far UV-CD spectra of halophilic <i>Bacillus</i> sp. protease in absence and presence of 8.0 M urea after 6 h	186
Figure 6B.4	Far UV-CD spectra of non-halophilic protease (subtilisin Carlsberg) in absence and presence of 8 M urea after 6 h	187
Figure 6B.5	Fluorescence spectra of halophilic <i>Bacillus</i> sp. protease in absence and presence of 8.0 M urea after 6 h	188
Figure 6B.6	Fluorescence spectra of non-halophilic protease (subtilisin Carlsberg) in absence and presence of 8.0 M urea after 6 h	189
Figure 6B.7	DSC curves for <i>Bacillus</i> sp. EMB9 protease in absence and presence of 8.0 M urea	190
Figure 6B.8	Effect of ANS on EMB9 protease in absence and presence of urea	192
Figure 6B.9	Denaturation kinetics of halophilic <i>Bacillus</i> sp. EMB9 protease in 8.0 M urea and 6.0 M GdmCl	193
Figure 6B.10	Effect of NaCl concentrations on denaturation kinetics of halophilic <i>Bacillus</i> sp. EMB9 protease in 8.0 M urea after 2 h	194
Figure 6C.1	Effect of polar organic solvents on the activity of the halophilic <i>Bacillus</i> sp. EMB9 protease	203
Figure 6C.2	Far UV-CD spectra of halophilic <i>Bacillus</i> sp. EMB9 protease in absence and presence of hydrophilic organic solvents after 6 h	206
Figure 6C.3	Far UV-CD spectra of halophilic <i>Bacillus</i> sp. EMB9 protease in absence and presence of hydrophobic organic solvents	207
Figure 6C.4	Percent negative ellipticity of the protease in different organic solvents	208

Figure 6C.5	Far UV-CD spectra of non-halophilic subtilisin Carlsberg in absence and presence of organic solvents after 6 h	210
Figure 6C.6	Fluorescence spectra of halophilic <i>Bacillus</i> sp. EMB9 protease in absence and presence of organic solvents after 6 h	211
Figure 6C.7	Effect of organic solvents on intrinsic fluorescence spectra of EMB9 protease	212
Figure 6C.8	Far UV-CD spectra of dialyzed halophilic <i>Bacillus</i> sp. EMB9 protease in absence and presence of n-dodecane after 2 h	214
Figure 6C.9	Fluorescence spectra of dialyzed halophilic <i>Bacillus</i> sp. EMB9 protease in absence and presence of methanol after 2 h	215
Figure 7.1	Schematic representation of activation of functionalized silica nanoparticles followed by protease immobilization onto it by covalent coupling	228
Figure 7.2	Scanning electron micrograph images of protease immobilized silica nanoparticles	231
Figure 7.3	Transmission electron micrograph images of protease immobilized silica nanoparticles	231
Figure 7.4	FTIR spectra of <i>Bacillus</i> sp. EMB9 protease immobilized on silica nanoparticles	233
Figure 7.5	Effect of pH and temperature on activity of <i>Bacillus</i> sp. EMB9 protease	235
Figure 7.6	Effect of temperature on stability of <i>Bacillus</i> sp. EMB9 protease	236
Figure 7.7	Operational stability of <i>Bacillus</i> sp. EMB9 protease immobilized on silica nanoparticles	238
Figure 7.8	Blood stain removal by <i>Bacillus</i> sp. EMB9 protease	239
Figure 7.9A	Degree of hydrolysis of protein in defatted soyflour by halophilic <i>Bacillus</i> sp. EMB9 protease	240
Figure 7.9B	Comparative degree of hydrolysis of defatted soyflour protein by commercial and halophilic protease	240
Figure 7.10	SDS-PAGE profile of untreated and protease treated soy flour	241

Figure 7.11	Enzymatic recovery of silver from used X-Ray films	243
Figure 7.12	Milk whey hydrolysis by immobilized EMB9 protease	244
Figure 7.13	Degree of hydrolysis of protein in milk whey by halophilic <i>Bacillus</i> sp. EMB9 protease	245
Figure 7.15	SDS-PAGE profile of untreated and treated milk whey with immobilized protease	246
Figure 8.1	Effect of nanoparticle on growth and viability of Gram-negative <i>Enterobacter</i> sp.	258
Figure 8.2	Effect of Ag on cell viability of <i>Enterobacter</i> sp.	259
Figure 8.3	Scanning electron micrograph images of <i>E. aerogenes</i> cells grown in absence and presence of 5.0 mM ZnO and 2.0 mM Ag nanoparticles	260
Figure 8.4	Transmission electron micrograph images of <i>E. aerogenes</i> cells grown in presence of 5.0 mM ZnO nanoparticles	260
Figure 8.5	EDX spectroscopy for <i>E.aerogenes</i> cells grown in absence and presence of 5.0 mM ZnO and 2.0 mM Ag nanoparticles	261
Figure 8.6	Effect of nanoparticle on growth and viability of Gram-positive <i>Bacillus</i> sp.	264
Figure 8.7	Scanning electron micrograph images of <i>Bacillus subtilis</i> cells grown in absence and presence of 10.0 mM ZnO and Ag nanoparticles	266
Figure 8.8	EDX spectroscopy for <i>B. subtilis</i> cells, grown in absence and presence of 10.0 mM ZnO and Ag nanoparticles	267
Figure 8.9	Effect of ZnO and Ag bulk and nanoparticles on growth of halophilic Gram-negative <i>Marinobacter</i> sp.	269
Figure 8.10	Transmission electron micrograph images of halophilic Gram-negative <i>Marinobacter</i> sp. grown in absence and presence of nanoparticles	270
Figure 8.11	Effect of ZnO and Ag bulk and nanoparticles on growth of Gram-positive Halophilic bacterium EMB4	271

Figure 8.12	Transmission electron micrograph images of Gram-positive Halophilic bacterium EMB4 grown in absence and presence of nanoparticles	272
Figure 8.13	Effect of 1.0 mM ZnO and Ag nanoparticles on growth of halophilic Gram-positive <i>Bacillus</i> sp. EMB9	273
Figure 8.14	Scanning and transmission electron micrographs of the <i>Bacillus</i> sp. EMB9 cells grown in absence and presence of silver nanoparticles	274
Figure 8.15	Two dimensional gel electrophoresis analysis of intracellular proteome from <i>Bacillus</i> sp. EMB9 cells grown in absence and presence of nanoparticles	275

List of Tables

Table 1.1	Common halophilic bacteria: their saline habitat and salt requirements	11
Table 1.2	Industrially important enzymes from halophiles	25
Table 1.3	Major industrial applications of proteases	26
Table 1.4	Some recently reported halophilic protease producers	29
Table 1.5	Protease production in halophiles	32
Table 1.6	Purification strategies for various halophilic proteases	36
Table 1.7	Important characteristics of intracellular and membrane bound halophilic proteases	44
Table 1.8	Important characteristics of extracellular halophilic proteases	46
Table 1.9	Kinetic properties of halophilic proteases	55
Table 1.10	Organic solvent stability in halophilic proteases	60
Table 1.11	Effect of salt on activity and structure of halophilic enzymes	65
Table 1.12	Molecular studies on halophilic proteases	71
Table 2.1	Biochemical and physiological characteristics of halophilic isolates	86
Table 2.2	Preliminary characterization of crude proteases from <i>Virgibacillus</i> sp. EMB13 and <i>Bacillus</i> sp. EMB9 proteases	93
Table 3.1	Purification of <i>Virgibacillus</i> sp. EMB13 protease	110
Table 3.2	Enzymatic properties of EMB13 protease	112
Table 4.1	Plackett-Burman experimental design and observed responses	127
Table 4.2	Regression coefficient values obtained after Plackett–Burman data analysis	127
Table 4.3	Experimental design generated by Response Surface Methodology	128
Table 4.4	Analysis of Variance for the quadratic model generated by CCD for optimization of protease production	129
Table 5.1	Purification of <i>Bacillus</i> sp. EMB9 protease	144
Table 5.2	Enzymatic properties of purified <i>Bacillus</i> sp. EMB9 protease	151
Table 5.3	Effect of additives on <i>Bacillus</i> sp. EMB9 protease activity	152

Table 6C.1	Half-life of <i>Bacillus</i> sp. EMB9 protease in polar organic solvents	204
Table 6C.2	Residual protease activity in presence of organic solvents	213
Table 6C.3	Maximum emission wavelength (λ_{\max}) in presence of organic solvents: with and without salt.	216
Table 7.1	Immobilization of protease by adsorption and covalent coupling to silica nanoparticles	228
Table 7.2	Optimized conditions for protease immobilization on silica nanoparticles	230
Table 7.3	Thermal stability of EMB9 protease at different temperatures	237
Table 7.4	Kinetic properties of EMB9 protease at different temperatures	237
Table 8.1	Characteristics of differently expressed intracellular proteins of <i>Bacillus</i> sp. EMB9 grown in presence of Ag nanoparticles	276