Summary

Microorganisms have successfully colonized saline environments, which represent the majority of the biosphere on Earth. They have evolved special mechanisms to overcome the life-endangering influence of high salt concentrations. Halophilic adaptation includes a complex range of structural and functional adaptations at the level of all cellular constituents, such as membranes, proteins and mechanisms to avoid the destructive effect of salt accumulation. These strategies offer multiple biotechnological applications of salt-adapted organisms and their products in various fields.

Halophilic microorganisms, usually defined as salt-loving organisms that inhibit hypersaline environments, show considerable growth at salt concentrations >2.0 – 30.0%. Halophiles are involved in centuries-old processes such as the manufacturing of solar salt from seawater and the production of traditional fermented foods. Two biotechnological processes involving halophiles are highly successful: the production of β-carotene by the green alga Dunaliella and the production of ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinemethylcarboxylic acid), used as a stabilizer for enzymes and now also applied in cosmetic products, from moderately halophilic bacteria. The potential use of bacteriorhodopsin, the retinal protein proton pump of Halobacterium, is being explored in optoelectronic devices and photochemical processes and may well lead to commercial applications in the near future. Demand for salt-tolerant enzymes in current manufacturing or a related process is limited. Other possible uses of halophilic microorganisms such as treatment of saline and hypersaline wastewaters, and the production of exopolysaccharides, poly-β-hydroxyalkanoate bioplastics and biofuel are being investigated.

In the present study, nine halotolerant bacterial strains were isolated from a rock salt mine, the strains were collected from twenty meters inside the mine. Morphological studies showed that all the strains were Gram positive rods except for the strain JPBW-1 that was
Gram positive, coccus. Salt tolerance analysis of all the strains revealed that all were halotolerant rather than halophilic because they were tolerating salt but their growth was best when grown in the absence of salt. The strains JPBW-1 and JPBW-9 were extremely halotolerant, while, rest of the strains were moderately halotolerant. Identification of these strains was done on the basis of 16S rRNA sequences. BlastN analysis of the partial 16S rRNA gene sequences revealed that all the strains are bacteria belonging to the phylum Firmicutes. A total of nine strains were identified comprising one strain of Staphylococcus arlettae (JPBW1), three strains (JPBW-4, JPBW-5, JPBW-10) of Bacillus licheniformis, one strain (JPBW-6) of Bacillus amyloliquefaciens and four strains (JPBW-2, JPDW-7, JPDW-8, JPBW-9) of Bacillus subtilis.

Retrieval of halotolerance proteins from different organisms showed that the predominant family of proteins involved in halotolerance among halophiles was of regulators of ion transporters (29.0 %) followed by transporters (24.0%). Computational analysis of halotolerance proteins from halo- and non-halotolerant organisms was done to infer signature residues conferring halotolerance. The comparison of pI in homologs of haotolerance genes in extreme halophiles (EH), moderate halophiles (MH), slight halophiles (SH) and non-halophiles (NH) revealed that EH have lowest pI followed by MH. The halotolerance proteins of EH showed more acidic nature in comparison to proteins of MH, SH and NH. Specifically, transporters, regulators of ion transporters and molecular chaperones are more acidic in comparison to the proteins for salt toxicity targets and osmotic tolerance proteins (e.g. proteins involved in production of compatible solutes). Comparision of amino acids composition at the surface of proteins showed that the proteins in EH have abundance of acidic amino acids at the surface in comparison to MH, SH and NH. There was an enormous increase of non-polar amino acids at the surface of proteins in EH in comparison to MH, SH and NH. The occurrence of non-polar amino acids at the surface of EH was a distinctive
observation. Non-polar amino acids are generally abundant in anti-freeze proteins, thus, the extremophilic microorganisms that have the ability to adapt to one extreme also have the capability to adapt to other extremes.

The exploration of isolated halotolerant strains for halotolerance genes, through comparative genomics, resulted in the detection of fourteen genes for halotolerance. These genes included, 2 transporters: \( \text{Li}^+ \text{Na}^+ \text{P-ATPase} \) (\textit{ena}1), halotolerance protein (\textit{hal}1) and, 3 genes belonging to regulators of ion transporters: halotolerance proteins (\textit{hal}4, \textit{hal}5) and auto-inhibited \( \text{Ca}^{2+} \text{ATPase} \) gene (\textit{aca}4), 3 molecular chaperones: heat shock proteins \textit{dna}K, \textit{gro}EL and \textit{gro}ES, 3 genes belonging to osmotic tolerance: glyceraldehyde-3-phosphate dehydrogenase (\textit{gpd}1), cysteine synthase K (\textit{cys}K) and ectoin biosynthesis (\textit{ect}B) and 3 genes belonging to salt toxicity targets: elongation initiation factor 1A (\textit{eif}-IA), 3'(2'),5'-bisphosphate nucleotidase (\textit{tol}1) and halotolerance protein (\textit{hal}2).

The mining of halotolerant strains for halotolerance genes revealed that most of the strains are having useful genes, which can be cloned to full length and used in the development of transgenics for various agricultural crops and can also be used in engineering microbes for various industrial applications. Halophilic/halotolerant bacteria constitute an excellent model for the molecular study of the osmoregulatory mechanisms that permit them to grow over a wide range of salt concentrations. This aspect has very exciting potentialities, like for instance, their possible application in agriculture to construct salt-resistant plants carrying prokaryotic genes encoding enzymes for the synthesis of osmoprotective compounds.

A considerable amount of effort has been devoted to the study of extracellular salt-tolerant enzymes of the moderately halophilic bacteria, especially toward the use of such enzymes in biotechnological processes. The present study also explored screening, partial purification and preliminary activity analysis of thermo-halotolerant lipase and \( \alpha \)-amylase
enzymes in the halotolerant strains. Lipase was detected in strains, JPBW-1, JPBW-5, JPBW-9 and amylase in all the strains except JPBW-1. Thermo-halotolerant lipase was detected for the first time in *S. arlettae* JPBW-1. Most of the enzymes currently in industrial use lack activities at high temperature and salt concentration (e.g. in leather industry). Lipase production by *S. arlettae* showed characteristic growth pattern that varied with variation in NaCl concentration. Culture survived in log phase for 3.0 h in control, 5.0% and 10.0% (w/v) NaCl supplemented media, while growth rate was very low in the presence of 19.0% (w/v) NaCl, thereby, confirming the halotolerant nature of bacteria. The presence of lipid source, pH, temperature and salinity of the growth medium appeared to be the most critical factors for lipase production by *S. arlettae*. Lipase produced by *S. arlettae* JPBW-1 showed activity in a wide pH range from 4.0 to 11.0 with maximum activity at pH 8.0 during 3.0h of culture growth. The salinity of the medium strongly influenced the lipase production with increase in activity with NaCl up to 5.0% after which it declined. Reduction in lipase activity beyond 5.0% w/v NaCl concentration may be due to its structural instability in high salt concentration. The lipase produced in our study from *S. arlettae* was active at wider temperature range of 40.0 - 90.0°C with maximum activity at 50.0°C. Optimum temperature for lipase activity obtained from *Staphylococcus epidermidis* was reported to be 50.0°C (Esakkiraj *et al.*, 2010). The range of optimum temperature for bacterial lipases has been reported to be 30.0 - 60.0°C (de Guzman, 2008). The enzyme (lipase from *S. arlettae* JPBW-1) identified in the current study has shown better activities than most of the commercially available enzymes from Indian manufacturers. The presence of such enzymes will open up new avenues for their applications in different commercial sectors and in economization of bioprocesses. The study assumes significance in the ability of the halotolerant bacterium to survive in a wide range of salinity and yield optimum levels of extracellular thermo-halotolerant lipase.
Growth and enzymes (lipase and α-amylase) production by *B. subtilis* JPBW-9 showed characteristic growth pattern that varied with variations in NaCl concentration. Culture survived in log phase for 3.0h in control, 5.0% and 10.0% (w/v) NaCl supplemented media, whereas the growth rate was almost half at 5.0% (w/v) NaCl. In the presence of 10.0% and 19.0% (w/v) NaCl, growth rate was almost negligible, confirming the halotolerant nature of bacteria. Lipase from *B. subtilis* JPBW-9 has a broad pH range with higher activity in the alkaline conditions, and optimum pH 10.0. Lipase activity was seen at temperature range of 30.0 – 80.0°C, while there was no activity at temperatures beyond this range. Highest lipase activity in the presence of salt (NaCl) was observed in substrate containing 3.0% w/v NaCl at pH 10.0 and temperature 40.0°C. The enzyme was active in NaCl concentration range of 1.0-8.0%.

The pH optimum for α-amylase activity was observed at pH 8.0, with wide activity range of pH 4.0 11.0. Maximum α-amylase activity was observed at 70.0°C and pH 8.0, whereas activity was also observed in the temperature range of 40.0 - 90.0°C. Maximum α-amylase activity in the presence of salt was observed at 4.0% (w/v) NaCl at pH 8.0 and temperature 50.0°C. In the presence of salt, α-amylase showed activity in the range of 1.0 - 10.0% (w/v) NaCl. It is reported that α-amylases from halotolerant bacteria maintain their stability both in the presence and absence of salt (Setati, 2010).

Preliminary optimization of enzyme parameters showed that these enzymes have potential for their applications in different commercial sectors and in economization of bioprocesses.

The stability of halotolerant lipases at extremes of NaCl, as well as their ability to function optimally at elevated temperatures make them attractive candidates for industrial processes. Similarly, α-amylases are attractive candidates for hydrolysis of starch in industrial processes which are commonly performed at low water activity such as the production of
syrups and also in the treatment of saline water or waste water solutions containing starch residues in the presence of high salt. Moreover, the enzymes derived from halophiles make excellent additives for laundry detergents.

One of the advantages of the enzymes (lipase and α-amylase) isolated in the current study is their stability at a very high temperature (90.0°C) and activity at 19.0% salt concentration which makes these enzymes suitable candidates for application in processes requiring high temperature and salt concentration. The enzymes isolated in the present study have advantage over halophilic proteins, as these are not inactivated in the absence of salt.