CHAPTER 1

Introduction
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A variety of microbes inhabit extreme environments. Extremophiles are micro-organisms adapted to survive in adverse environmental conditions, such as high temperature, pH, pressure, salt concentration, low temperature, pH, nutrient concentration and water availability. There are many environments around the world that are extreme. These are geothermal areas with high temperature, polar regions with temperatures around the freezing point of water, deep oceans with very high pressure, and acid or alkaline springs with low or high pH, respectively. The realization that extreme environments harbour different kinds of prokaryote lineages has resulted in a complete reassessment of our concept of microbial evolution and has given considerable impetus to extremophile research (Horikoshi and Grant, 1991). During the last two decades, rapidly growing research activities have focused on the elucidation of basic rules that govern these extreme microorganisms. The extremophilic microorganisms provide a unique resource for a variety of biomolecules such as enzymes and compounds with high potential for applications in the biotechnology industry. Furthermore, the finding of novel biocatalysts will allow the development of more efficient and environment-friendly industrial processes.

Halophiles are organisms adapted to thrive in extreme conditions of salinity with distribution in hypersaline environments, such as solar salterns, the Great Salt Lake, the Dead Sea, as well as in rock salt mines (DasSarma et al., 2001). Hypersaline habitats are common throughout the world, but extremely hypersaline habitats are rather rare. Most hypersaline environments are derived from the evaporation of sea water, encouraged by restricted flow, high temperatures, low rainfall, low humidity and high wind speed. Different marine salterns have been studied throughout the world, including salterns in Alicante, Spain; Mexico, San Diego and Newark, California; Bonaire, the Netherlands; Eilat, Israel and Margherita di Savoia, Italy, etc.
(Litchfield and Oren, 2001). Hypersaline environments have been reservoirs for the long-term evolution of specifically adapted microorganisms. Halophiles have been mainly isolated from saline lakes, such as the Great Salt Lake in Utah (salinity, >2.6 M) and from evaporitic lagoons and coastal salterns with NaCl concentrations between 1.0 and 2.6 M (Grant et al., 1998). Saline soils are less explored and constitute less stable biotopes than hypersaline waters because they are subjected to periodic significant dilution during rainy periods. Alkaline soda lakes in Africa, India, China and elsewhere with pH values of 11.0 and higher salt concentrations exceeding 300.0 g/l are teeming with life (Oren, 2002). Some microorganisms may even simultaneously withstand high salt concentrations (200.0 g/l) coupled with high temperatures up to 68.0°C (Cayol et al., 1994).

For many years there has been a considerable interest in a group of microorganisms known as halophiles. Although the early studies on halophiles were based on strains isolated from salted foods because they may contaminate or spoil these products, the majority of recent studies concerning this group of microorganisms have been carried out in hypersaline lakes as well as ponds of marine salterns. The aspects that attracted the interest of researchers were mainly those related to their physiological adaptation to highly saline concentrations and their ecology (Kushner and Kamekura, 1998; Ventosa et al., 1998). Due to the importance in biotechnology, the isolation of new strains from hyper-saline ecosystems is continuing. There are several reports of microbial isolation from highly saline environments. Vreeland and Hual (1991) isolated many strains from brines with a range of salt tolerances, 17.0 - 32.0% (w/v) NaCl. Al-Zabran et al. (2002) isolated halotolerant Nocardiopsis sp. and a halophilic Saccharomonospora sp. from salt marsh soil in Kuwait. Both the microorganisms showed keratinase activities in the culture filtrate in the presence of feather meal as sole source of carbon.
and nitrogen. During the study of prokaryotic biodiversity in El Golea salt lake, in Algerian Sahara, extreme halophilic microorganisms were isolated from a site containing 290.0 g/l NaCl (Hacene et al., 2004). Birbiir et al. (2004) isolated twelve archaecal strains belonging to the genera *Halobacterium*, *Haloarcula*, *Natrinema* and *Halorubrum*, from salt mine in Central Anatolia, Turkey. The isolated strains produced hydrolytic enzymes like gelatinase, caseinase, amylase, cellulase and lipase. One hundred and sixty-five halophilic archaea were isolated from three different types of hypersaline lakes (Erliannor, Shangmatala and Xilin Soda Lake) in Inner Mongolia (Pau et al., 2006). Joshi et al. (2007) isolated 14 bacterial moderate halophiles from alkaline Lonar Lake, Maharashtra, India, which were capable of producing enzymes like amylase and lipase. Isolation of chromium resistant moderately halophilic bacterial strains (9.0% salt tolerant) from Palar river basin, Vellore are also reported (Sundar et al., 2010). These studies included culture-dependent approaches, leading to the isolation and characterization of many novel types of halophiles and new information on the abundance and geographic distribution of the known types, as well as culture-independent studies based on sequencing of DNA recovered from the environment.

Studies on isolation of microorganisms from rock salt mine have been done in the past. For example, Norton et al. (1993) isolated seven haloarchaecal strains from rock salt in Winsford salt mine in Cheshire, England belonging to the genus *Halorubrum* and *Haloarcula*. Cojoc et al. (2009) have also described the extracellular hydrolytic activities of halophilic bacteria isolated from subterranean rock salt crystal. Forty-seven bacterial strains were isolated from Winsford salt mine (UK), growing upto 30.0% (w/v) NaCl (Grant et al., 1998).

There are several ways in which microbes from the hypersaline environments are of interest. First, they represent a reservoir of microbes that could be used in an array of
biotechnological processes like in bioremediation of oil-polluted saline ecosystems. Some halophiles are used for the bioremediation of pollutants by halogenated organic compounds. *Lactobacillus plantarum* was used for the fermentation of pickles and sauerkraut (Pikuta *et al.*, 2006). Secondly, the halophiles represent a reservoir of genes/enzymes that are able to facilitate tolerance to extremely high concentrations of salt.

Halophiles are also a good source of genes for a variety of compatible solutes that can function under salty conditions. These solutes protect biomolecules and whole cells against denaturation caused by heating, freezing, desiccation, or chemical agents (Margeziu *et al.*, 2001). This property has attracted commercial attention. Compatible solutes can be used as chemical chaperones for protein folding, cryoprotectants of microorganisms, cosmeceuticals, and pharmaceuticals (Lentzen and Schwarz, 2006). Ectoine gene *ectABC* of the halophilic bacterium *Chromohalobacter salexigens* was introduced into *Escherichia coli* to synthesize ectoine continuously in less-aggressive media (Schubert *et al.*, 2007). 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-pyrimidinecarboxylic acid), used as a stabilizer for enzymes and now also applied in cosmetic products, from moderately halophilic bacteria. Halophiles produce biocatalysts that are functional under extreme conditions, that have resulted in several novel applications of enzymes in industrial processes. The industrial applications of enzymes that can withstand harsh saline conditions have greatly increased over the past decade. During recent years, these halophiles have been considered of great interest because of their
biotechnological potential, notably for producing enzymes (amylases, proteases, lipases and nucleases) of industrial interest (Cojoc et al., 2009).

Rock salt occurrences are limited in India the only rock salt mines is located in Himachal Pradesh. This sites, to the best of our knowledge, is the sole deposits of rock salts in India that need to be explored for microbial flora and their invaluable genes, enzymes and compounds.

One reason for the interest in halophiles is the need to understand the biochemical mechanisms involved under extreme conditions. Occurrence of novel and stable molecules in halophiles make them valuable for the future biotechnology pursuits. The potential use of bacteriorhodopsin, the retinal protein proton pump of Halobacterium, in optoelectronic devices and photochemical processes is being explored, and may well lead to commercial applications in the near future. Another potential promising future application of halophiles could be the enhancement of drought tolerance or salt tolerance of transgenic plants (Yoshida, 2002). The discovery of genes for salinity tolerance plants will provide the basis for effective genetic engineering strategies, leading to greater stress tolerance in economically important crops.

In order to shed light onto life in halophiles in environments like rock salt mines, the present study was conducted. The purpose was to explore the microbes harbouring the rock salt mine at Darang in Himachal. The inhabitants of rock salt mine were isolated and characterised with a view to provide insight on the possibility to use different halophiles as a source of extremophilic genes/enzymes in biotechnological processes. For the identification of the strains a PCR approach was used with universal bacterial 16S rRNA primers on the genomic DNA of halophilic strains.

The proteins from completely sequenced halophiles/non-halophiles were analysed and compared amongst each other. The gene function and protein properties analysis was performed
by comparative analysis. Seeing the importance of halotolerance genes in biotechnology we wanted to find out whether industrially significant genes are present in these halophiles. To achieve this we have searched halotolerance genes known from other prokaryotes and eukaryotes and designed primers from conserved region of these, then the primers were used in PCR analysis.

Enzymes from halophiles have potential in multiple areas, either by using the enzymes themselves, or by using them as sources to modify mesophile-derived enzymes. The fastidious growth conditions for halophiles means that it is often economically advantageous to express the gene in a more tractable host organism such as *E. coli*. Halophiles are the most expected source of halotolerant enzymes because not only are their enzymes salt-tolerant, but several of them are also thermotolerant. Industrial processes are carried out under specific physical and chemical conditions which cannot always be adjusted to the optimal values required for the activity of the available enzymes. For example, amylase produced by a *Bacillus* sp. is stable at 60.0°C and 5.0 M NaCl and could be used in the treatment of effluents containing starchy or cellulosic residues (Khitre and Pant, 1992). For that reason, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature.

In spite of all this, halophilic/halotolerant bacteria have not yet been used extensively for biotechnological purposes. Further work on these relatively little-studied microorganisms may be expected to bring dividends in the form of insight on the relation of internal and external solute concentrations, and on the state of cell-associated ions within the cytoplasm. The halophilic bacteria pose specific questions to the scientists, many of them related to their adaptability to a wide range of salinities. Studies carried out, especially during the last decade, have increased our current knowledge about different aspects of halophilic bacteria, such as their physiology,
ecology, taxonomy or phylogenetic relationships with other microorganisms, and, to a lesser extent, their genetics. Besides, there are several fields in which their industrial applications are more promising, and, as in the case of other extremophilic microorganisms, they have an important biotechnological potential as a source of compatible solutes, enzymes, and other compounds of industrial interest.

The current study was undertaken to isolate and characterize microorganisms, adapted to high salt concentrations in the rock salt mine for detecting the presence of useful genes and enzymes with the following objectives:

**OBJECTIVES**

1) Identification and characterization of strains isolated from a rock salt mine in Darang, H P, India

2) Computational analysis of halotolerance proteins to infer their sequence signatures

3) Identification of halotolerance genes in halotolerant bacterial strains through comparative genomics

4) Screening halotolerant bacterial strains for important enzymes (lipase and α-amylase)