CHAPTER 2

REVIEW OF LITERATURE
Besides the extreme halophiles, the moderate halophiles are also important group of microorganisms adapted to live in hyper saline habitats and constitute a heterogeneous group, which includes a great variety of bacteria (Manikandan et al., 2009; Ramesh et al., 2009). Moderately halophilic bacteria have the capabilities for exciting and promising applications and hence, could be among the most potential candidates, compared with other extremophiles. During the past decades, the studies on ecology, physiology, and taxonomy of halophilic organisms revealed an impressive diversity. Till now haloalkaliphiles were studied extensively for the microbiological classification and phylogeny; only limited attempts have been made to explore molecular basis of adaptation, enzymatic potential and their other biotechnological implications. The diversity of the halophilic, haloalkaliphilic and alkaliophilic microbes has been studied from the hyper saline and hyper alkaline environment.

Microbial species represent the largest species diversity on the Earth and their composition is of complex and dynamic nature (Torsvik and Ovreas, 2002). They provide an infinite source of gene sequences, encoding functional components of numerous catabolic pathways and regulation systems, of which many remain to be discovered. At present, only a minor fraction of the microorganisms on Earth have been explored (Burg, 2003). The development of new strategies for isolation, of uncultured microbes; particularly extremophiles, is a challenging issue for the scientists. It is of great value to make available the unexplored world of organisms, besides that, their investigation is likely to generate enough knowledge of many basics questions of biology towards harsh conditions.

Now, it has become clear that apart from soil and surface water, microbes colonized almost every noxious environment on the Earth, considered to be extreme (Horikoshi 2011a and b; Thomas and Dieckmann, 2002). Such extremophiles have specialized skill, rarely seen in nature (Eichler, 2001). Some of them, such as, thermoacidophiles, thermoalkaliphiles, thermohalophiles and haloalkaliphiles could sustain in more than one extremity and are known as poly-extremophiles. These groups of bacteria are mainly habituated from the fresh water to the (hyper) saline and alkaline environments, Dead Sea, saltern crystallizer ponds and other places saturated
with respect to sodium chloride. So far, large numbers of hyper saline environments have been studied for these bacteria from the ecology and diversity point of view. On the basis of the physical parameters and chemical compositions, hyper saline environments are mainly classified in to two; thalassohaline (arising from sea water and contain sodium chloride as the predominant salt) and athalassohaline (largely derived from the solution of evaporative deposits and contain different ion ratios (Grant, 1993; Madern et al., 2004).

2.1 Hyper saline and alkaline environments

Hyper saline environments can be expected to have a relatively simple ecosystem structure. The diversity of saline and hyper saline habitats with respect to their properties reflected in the great diversification of microbial communities adapted to prevailing conditions (Oren, 2002). Salt Lakes and other ecosystems with salt concentrations at or approaching saturation are, therefore, convenient model systems for studies in microbial ecology. As a result of natural and man-made global changes, hyper saline environments are increasing.

Hyper saline waters are defined as having salt concentrations greater than that of sea water (3.5%, w/v) (Grant et al., 1998). Several halophilic biotopes have been identified, including saline lakes; evaporate lagoon sediments and coastal salterns. Saline soils and the salt-excreting surfaces of animals are among the less explored habitats, but almost all hyper saline biotopes are thought to harbor significant populations of microorganisms (Grant et al., 1998).

2.1.1 Athalassohaline Environments

Athalassohaline environments are those, in which the ionic composition differs greatly from that of sea water and in which the salts are of non-marine proportion. The concentration of sea water leads to precipitation of NaCl, leaving a high concentration of potassium and magnesium salts. This point marks the upper limit of resistance of all biological forms (Das Sarma and Arora, 2001). Dead Sea, some alkaline Soda Lakes, carbonate springs, salterns brines and alkaline soil are the examples of thalassohaline environments.

2.1.2 Soda Lake

Soda Lakes, which represent stable and extremely productive aquatic ecosystems, exhibit ambient pH values around 10 or higher. Most of the alkaline Soda Lakes in
Africa, India, China and elsewhere with pH values of 11 and higher and salt concentrations exceeding 300 g/l are teeming with life (Oren, 2002). Soda Lakes are widely distributed; however, as a result of their inaccessibility, few such Lakes have been explored from the microbial diversity and ecological point of view.

2.1.3 The Dead Sea
The Dead Sea presents unique challenges to the halophilic microorganisms inhabiting it because of its peculiar ionic composition. The concentration of divalent cations (presently about 1.9M Mg\(^{2+}\) and 0.4M Ca\(^{2+}\)) is dominant over monovalent cations (1.6M Na\(^{+}\) and 0.14M K\(^{+}\)) and the pH is relatively low (about 6.0). Even such a hostile environment periodically supports dense microbial blooms (Oren, 1988). The Dead Sea in the Middle East is the largest hyper saline environment studied in great detail (DasSarma and Arora, 2001) (Table 2.1).

2.1.4 Carbonate springs
Carbonates rich springs and alkaline soils provide organic matter for diverse groups of heterotrophs, primarily alkaliphilic *Bacillus* spp. and several species of *Cynobacterium* are also normally abundant in such habitats. Decomposition of the protein and hydrolysis of urea leads to microbial ammonification at particular place resulting in high concentration of ammonia that raises pH of the habitat and encourages the growth of alkaliphiles (Horikoshi, 2011a and b).

2.1.5 Thalassohaline environment
Many hyper saline environments originated by evaporation of sea water are known as thalassohaline environments. Their salt composition is similar to that of sea water: sodium and chloride are the dominating ions, and the pH is near neutral to slightly alkaline. When evaporation proceeds, some changes occur in the ionic composition due to the precipitation of gypsum (CaSO\(_4\cdot2\)H\(_2\)O) and other minerals after their solubility has been exceeded (Oren *et al.*, 2005).

2.1.6 Salt Lakes and alkaline environments
There are two kinds of naturally occurring stable alkaline environments in the world; high Ca\(^{2+}\) (ground waters bearing high calcium hydroxide) and low Ca\(^{2+}\) environments (Soda Lakes and Soda deserts are dominated by sodium carbonate) (Grant, 1991 and 1992). Besides, natural hyper saline natural lakes, numerous
artificial solar lakes have been constructed for producing sea salts (Satyanarayana et al., 2005).

<table>
<thead>
<tr>
<th>Ion (g/l)</th>
<th>Sea water</th>
<th>Dead Sea</th>
<th>Great Salt Lake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>10.8</td>
<td>39.2</td>
<td>105.4</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.4</td>
<td>7.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.3</td>
<td>40.7</td>
<td>11.1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.4</td>
<td>16.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>19.6</td>
<td>212.4</td>
<td>181.4</td>
</tr>
<tr>
<td>Br⁻</td>
<td>0.1</td>
<td>5.1</td>
<td>0.2</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>2.7</td>
<td>0.5</td>
<td>27</td>
</tr>
<tr>
<td>HCO₃⁻/CO₃²⁻</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Total salinity</td>
<td>35.2</td>
<td>322.6</td>
<td>333.6</td>
</tr>
<tr>
<td>pH</td>
<td>8.2</td>
<td>5.9-6.3</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Table 2.1: Characteristics of (hyper) saline environments (Grant, 1992)

2.1.7 Solar salterns
Multi-pond solar salterns present a gradient of salinities, from sea water salinity to halite saturation. The salt concentration in each pond is kept relatively constant and microbial community densities are generally high. Although, salterns are superficially similar all over the world, they differ with respect to nutrient status and retention time of the water, depending on climatic conditions (Javor, 1983). NaCl saturated brines such as; saltern crystallizer ponds often display a bright red color due to the large numbers of pigmented microorganisms (Oren, 2002).

2.1.8 Sea water
When 1 cubic foot of sea water evaporates it yields about 2.2 pounds of salt but 1 cubic foot of fresh water from Lake Michigan contains only one-hundredth (0.01) of a pound of salt, thus, sea water is 220 times saltier than the fresh lake water. The salinity of ocean water varies as affected by many factors; melting of ice, inflow of river water, evaporation, rain, snowfall, wind, wave motion, and ocean currents that cause horizontal and vertical mixing of the saltwater. The saltiness of sea is due in part to the high water temperature, causing a high rate of evaporation. Sodium and chloride constitute 85% of the dissolved solids in sea water and account for the
characteristic salty taste. Certain constituents in sea water, such as calcium, magnesium, bicarbonate and silica are partly taken out of solution by biological organisms, chemical precipitation, or physical-chemical reactions. Thus, the sea contains many moderately halophilic or at least extremely halotolerant bacteria.

2.1.9 Saline and alkaline soils
The soil habitat is inherently inhomogeneous and wide range of salinities might be present in any saline soil (Grant, 1991). Saline soils appear to yield mostly halotolerant rather than halophilic microorganisms, presumably reflecting adaptation to periodic episodes of relatively high dilution (Ruiz-Garcia et al., 2005a and b). However, isolation of novel halophilic *Actinopolyspora* and *Nocardiopsis* species from salty soils in Death Valley (Calif.), Alicante, and Iraq (Al-Tai and Ruan, 1994) suggests that a wealth of interesting unknown halophilic microorganisms may be present in such saline soils.

2.1.10 Other saline habitats
Extensive microbiological studies have been carried out in the Antarctic, especially the cold saline lakes in the Vestfold Hills (East Antarctica) region and the saline soils of the Dry Valleys. The best studied is Organic Lake, a meromictic lake with a maximum depth of 7.5m. The lake is stratified, with salt concentrations increasing from 0.8 to 21%. Many strains of moderate halophiles, belonging to genera including *Halomonas, Flavobacterium* and *Cytophaga*, were isolated from the lake (Dobson et al., 1991; Franzmann et al., 1987). Many moderately halophilic bacteria have been isolated from salted fish, meat, and other food products (Onishi et al., 1980; Vilhelmsson et al., 1996). Besides, moderately halophilic bacteria may be found in some unusual environments, such as on desert plants (Simon et al., 1994) and desert animals (Deutch, 1994).

2.2 Halophiles, Alkaliphiles and Haloalkaliphiles: Diversity and Molecular Phylogeny
The diversity of an ecosystem is dependent on the physical characteristics of the environment, the diversity of species present, and the interactions that the species have with each other and with the environment. Environmental disturbance on a variety of temporal and spatial scales can affect the species richness and, consequently, the diversity of an ecosystem.
The traditional and classical methods of the classification are not sufficient to generate the evolutionary relationship between different groups. Ribosomal RNA is an ancient molecule, functionally constant, universally distributed and moderately well conserved across broad phylogenetic distances (Madigan et al., 1997). Moreover, there is no evidence of lateral gene transfer of rRNA genes between different species and therefore rRNA genes can bring true information regarding evolutionary relationships (Pace, 1997). 16S rRNA studies have shown to support a different but equally diverse population of halophilic, alkaliophilic and haloalkaliphilic bacteria and archaia (Jones et al., 1994).

To describe halophilic bacteria, several classifications or categories have been proposed according to their behavior towards salt, (Kushner, 1978; Vreeland, 1987; Ramos-Cormenzana, 1989). The most widely used is that of Kushner. According to him, one can distinguish between slight halophiles (many marine organisms; sea water contains about 3-5% (w/v) NaCl), moderate halophiles (optimal growth at 3–15% (w/v) salt), extreme halophiles (optimal growth at 25% (w/v) NaCl; *Halobacteria* and *Halococci*), and borderline extreme halophiles (requirement of at least 12% (w/v) salt). This diversity of halophilic and highly halotolerant microorganisms is expressed both at the phylogenetic level; halophiles are found in all three domains of life: Archaea, Bacteria, and Eucarya and at the physiological level, most modes of energy generation known in non-halophiles are also used by halophilic counterparts. Still, most microbiologists do not realize the true extent of the diversity of halophilic and halotolerant microorganisms in nature (Oren, 2002a; Guranthon et al., 2010).

### 2.2.1 Halophilic bacteria and archaea

Bacterial halophiles are abundant in environments such as salt lakes, saline soils, and salted food products (Oren, 1999). Further, they are also found in both; the aerobic branches (*Bacillus* and related organisms) and anaerobic branches. There is even an order, the *Halanaerobiales*, consisting of two families (the *Halanaerobiaceae* and the *Halobacteroidaceae*) that consist solely of halophilic anaerobic microorganisms (Oren, 2002b). DasSarma and Arora (2001) and Oren (2002c) have extensively reviewed the diversity and molecular ecology of the halophilic and extremely halophilic bacteria and archaia in different groups of microbes. The 250 million year old halotolerant bacteria, *Bacillus sphaericus*, was isolated from spore, extracted from
a salt crystal buried at more than 1,500 feet underground in Carlsbad (Vreeland et al., 1987).

2.2.2 Alkaliphiles
Alkaliphiles consist of two main physiological groups of microorganisms; alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH (>pH 9) and high salinity (up to 33% (w/v) NaCl) (Horikoshi, 2011). Horikoshi have spent the major part of his research career to investigate the physiology, ecology, taxonomy, enzymology, molecular biology and genetics of the alkaliphiles.

2.3 Haloalkaliphilic bacteria
Haloalkaliphiles possess special adaptation mechanisms for survival in highly saline and alkaline pH. These properties make them interesting not only for fundamental research but also for industrial application (Margesin and Schinner, 2001). So far, moderately haloalkaliphilic bacteria have been isolated from the different saline and alkaline environments (Ventosa et al., 1998; Xu et al., 2001; Xin et al., 2001; Zhang et al., 2002; Doronina et al., 2003a; 2003b; Hoover et al., 2003; Patel et al., 2005a; Dodia, 2005; Patel et al., 2006a; Dodia et al., 2008a and b; Nowlan et al., 2006).

2.4 Adaptation strategies
Life is based on organic chemistry and the mechanisms of such chemistry must be allowed to function for life to continue. Extremophiles adopt two distinct approaches to living within extreme environments; they adapt to function within the physical and chemical bounds of their environment or they maintain mesophilic conditions intracellularly, guarding against the external pressures. Among them, halophiles are an interesting class of extremophilic organisms that have adapted to harsh, hyper saline environments.

The organisms living in extreme conditions possess special adaptation strategies that make them interesting not only for fundamental research but also towards exploration of their applications, Horikoshi (2008). These organisms may hold secret for the origin of life, apart from that it will unfold many basic questions about the stability of
the macromolecules under extreme conditions. Therefore, their studies would provide important clues for adaptation under salinity.

To cope up with the high and often changing salinity of their environment, the aerobic halophilic bacteria, similar to all other microorganisms, need to balance their cytoplasm with the osmotic pressure exerted by the external medium (Oren, 2008; Oren, 2010). Osmotic balance can be achieved by the accumulation of salts, organic molecules or similar mechanism. Alternatively, the cell is able to control water movement in and out and maintain a hypo-osmotic state of their intracellular space.

The extremely halophilic archaea and bacteria adapt various strategies, viz. molar concentrations of chloride is pumped into the cells by co-transport with sodium ions and/or using the light-driven primary chloride pump halorhodopsin (Oren, 2010). Distribution of charged amino acids could also serve as one of the major approach.

### 2.4.1 Chloride Pumps

A very high requirement for chloride was demonstrated in two groups of bacteria; anaerobic *Halanaerobiales* and the aerobic extremely halophilic *Salinibacter rubber*, that accumulate inorganic salts intracellularly rather than using organic osmotic solutes. Thus, it becomes clear that chloride has specific functions in halo-adaptation in different groups of halophilic microorganisms (Müller and Oren, 2003).

### 2.4.2 Osmoregulation in bacteria

Osmoregulation is a fundamental phenomenon developed by bacteria, fungi, plants and animals to overcome osmotic stress. The most widely distributed strategy of response to hyperosmotic stress is the accumulation of compatible solutes, which protects the cells and allows growth. Adaptation of bacteria to high solute concentrations involves intracellular accumulation of organic compounds called osmolytes. Osmolytes are often referred to as compatible solutes because they can be accumulated to high intracellular concentrations without adversely affecting cellular processes it can be either taken up from the environment or synthesized *de novo*, and they act by counterbalancing external osmotic strength, thus preventing water loss from the cell and plasmolysis. Since the water permeability of the cytoplasmic membrane is high, imposed imbalances between turgor pressure and the osmolality gradient across the bacterial cell wall are short in duration. Bacteria respond to osmotic upshifts in three overlapping phases: dehydration (loss of some cell water);
adjustment of cytoplasmic solvent composition and rehydration and cellular remodeling.

2.4.3 Compatible solutes
The accumulation of organic solutes is a prerequisite for osmotic adjustment of all organisms. Archaea synthesize unusual solutes such as β-amino acids, \( \text{N}^E \)-acetyl-β-lysine, mannosylglycerate and di-\( \text{myo} \)-inositol phosphate. Among all of them, uptake of solutes such as glycine betaine is preferred over \textit{de novo} synthesis. Most interestingly, some solutes are not only produced in response to salt but also to temperature stress.

Glycine Betaine
The ability of the organism to survive both high salt concentrations and low temperatures is attributed mainly to the accumulation of the compatible solute glycine betaine. One of the most effective compatible solutes widely used by bacteria is glycine betaine, the \( \text{N} \)-trimethyl derivative of glycine, which can be accumulated intracellularly at high concentration through either synthesis or uptake or both. \textit{Bacillus subtilis} has been shown to possess three transport systems for glycine betaine: the secondary uptake system \textit{opuD} and two binding-protein-dependent transport systems, \textit{opuA} and \textit{opuC} (\textit{proU}).

Distribution of amino acids
The cell wall halophilic archaea \textit{Halobacterium} has a high proportion of the acidic amino acids; aspartate and glutamate as sodium salts. Interestingly, this sodium binding is essential to maintain the cell wall and dilution of the medium leads to repulsion between the free carboxylate groups leading to cell wall disintegration and cell lysis.

Molecular aspects of salinity
Marine microbes are known to play an essential role in the global cycling of nitrogen, carbon, oxygen, phosphorous, iron, sulfur and trace elements (Nada \textit{et al.}, 2011). Salinity tolerance comes from genes that limit the rate of salt uptake from the soil or water and the transport of salt throughout the plant, adjust the ionic and osmotic balance of cells in roots and shoots and regulate leaf development and the onset of senescence (Munns and Tester, 2008). However, very little progress has been made in this regard so far, as the gene expression pattern and analysis has been difficult. Most
of the sequenced culturable microorganisms from the deep-sea are Alteromonadales from the Gammaproteobacteria. Unique properties of sequenced deep-sea microbes are that they all have a high ratio of rRNA operon copies per genome size, and that their intergenic regions are larger than average (Lauro and Bartlett, 2008). These properties are characteristic of bacteria with an opportunistic lifestyle and a high degree of gene regulation to respond rapidly to environmental changes when searching for food.

Study of the molecular basis of osmoadaptation and its regulation in archaea is still in its infancy, but genomics and functional genome analyses combined with classical biochemistry shed light on the processes that confer osmoadaptation in archaea. Furthermore, they showed that betS is constitutively expressed, whereas BetS activity depends on posttranslational activation by high osmolarity and is most likely the emergency system transporting betaines for immediate osmotic protection. Many microorganisms possess two or more glycine betaine transport systems. Salmonella typhimurium, for example, possesses two genetically distinct pathways, a constitutive low affinity system (ProP) and an osmotically induced high-affinity system (ProU), while B. subtilis has three glycine betaine transport systems, OpuD, OpuA, and OpuC.

2.4.4 pH

Internal pH maintenance in alkaliphilic bacteria is achieved by both; active (sodium ion channels) and passive regulation (through cytoplasmic pools of polyamines and low membrane permeability). The pools of cytoplasmic polyamines are rich in amino acids with positively charged side groups (lysine, arginine and histidine).

The cell wall play a key role in protecting the cell from alkaline environments, hence in addition to peptidoglycan, alkaliphilic Bacillus sp. contains certain acidic polymers, such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, and phosphoric acid (Aono et al., 1999; Kitada et al., 2000). Some alkaliphilic bacteria, however, have developed sodium ion channels that actively drive the entry of protons across the membrane through $\text{H}^+/\text{Na}^+$ antiporters, thus decreasing the overall pH of the cytoplasm. Besides controlling protons, $\text{Na}^+$ dependent pH homeostasis requires reentry of $\text{Na}^+$ into the cell. $\text{Na}^+$ coupled solute symporter and $\text{Na}^+$-driven flagella rotation ensure a net sodium balance.
2.4.5 Light
Halophilic archaea live in shallow evaporation pond encounter with very high temperature and ultraviolet light. They have developed a special retinal pigments called carotenoid. These pigments provide protective barrier to the ultraviolet light. These pigments not only found in halophilic archaea (Lakatos et al., 2002) but also in haloalkaliphiles (Bivin and Stoeckenius, 1986).

2.5 Industrial and Biotechnological Relevance of Halophiles, Alkaliphiles and Haloalkaliphiles
Besides their important role in ecology of hyper saline environments, these groups of prokaryotes have received considerable interest because of their potential for use in various biotechnological and industrial applications, such as biomedical and chemical sciences, food, leather, laundry detergent and pharmaceutical industries (Rothschild and Mancinelli, 2001). Moreover, some archaeal metabolites, such as some proteins, extracellular enzymes, osmotically active substances (compatible solutes), exopolysaccharides and special lipids have potential industrial applications (Schiraldi, 2002). They appear to be a very good source of various biomolecules and can open the dimensions for the development of novel value based products because of unique properties, which can withstand at harsh environment.

2.5.1 Compatible solutes
Halophilic bacteria produce compatible solutes that maintain a positive water balance in the cell and are compatible with the cellular metabolism. These low molecular weight substances are excellent stabilizers for whole cells and biomolecules (Galinski, 1993; Da Costa et al., 1998; Welsh, 2000; Santos and Da Costa, 2002; Vargas et al., 2004). The stabilizing effect of mannosylglycerate (MG) and diglycerol phosphate is higher for several enzymes subjected to heating or freeze-drying to that of other stabilizers (Lamosa et al., 2000). Ectoine and derivatives have been patented as moisturizers in cosmetics (Montitsche et al., 2000), although the most promising application may be as stabilizers in the polymerase chain reaction (Sauer and Galinski, 1998).

2.5.2 Antimicrobial substances
The biological diversity of the marine environment, in particular, offers enormous scope for the discovery of novel natural products, several of which are potential
targets for biomedical developments (Austin, 1989; Fenical, 1997). Extremophiles have been recognized as valuable sources of novel bioproducts and this may well include antimicrobials (Horikoshi, 1993; Kokare et al., 2004; Fiedler et al., 2005). Halocins are bacteriocin-like proteins or peptides produced by many species of *Halobacteriaceae*, *Halofex mediterranii* and *Halofex gibbonsii*, which act against haloarchaea and haloalkaliphilic rods which suggests that these different archaeal kingdoms may share a common archaeal-specific target (Platas et al., 2002; Yun et al., 2003).

### 2.5.3 Bacteriorhodopsin

Certain extremely halophilic and haloalkaliphilic bacteria contain membrane bound retinal pigments called Bacteriorhodopsin (BR) and halorhodopsin (HR) (Lanyi, 1993). The applications comprise holography, special light modulators, artificial retina, neural network optical computing and volumetric and associative memories. Recently, cloning and functional expression of archaerhodopsin gene from *Halorubrum xinjiangense* was successfully achieved in *E. coli*, where the purple membrane was fabricated into films and photoelectric responses depending on the light-on and light-off stimuli were observed (Feng et al., 2006).

### 2.5.4 Biosurfactants

Biosurfactants enhance the remediation of oil-contaminated soil and water and have potential for pollution treatment in marine environments and coastal region (Banat et al., 2000). Biosurfactants from these extremophiles are also used for *in-situ* microbially enhanced oil recovery (MEOR) but the production cost is limiting factor for exploitation of these biosurfactants.

### 2.5.5 Exopolysaccharides

Halophilic exopolysaccharide (EPS) producers could be interesting source for MEOR, where polymers with appropriate properties act as emulsifiers and mobility controllers. The exopolymer polyg-D-glutamic acid (PGA) can be used as a biodegradable thickener, humectant, sustained release material, or drug carrier in the food or pharmaceutical industry (Kunioka, 1997). Hezayen et al., (2000) reported the first description of a PGA-producing extremely halophilic archaeon related to the genus *Natrialba*. 
2.5.6 Liposomes
Liposomes are used in medicines and cosmetics for the transport of compounds to specific target sites in the body. Liposomes prepared from polar archaeal glycerolipids are of special interest because of their adjuvant activities in mammals (Sprott et al., 2003).

2.5.7 Food Biotechnology
Halotolerant microorganisms play an important role in various fermentation processes, occurring in the presence of salt and producing various compounds that give characteristic taste, flavor and aroma to the resulting products. In the production of pickles (fermented cucumbers), brine strength is increased gradually from 5-15.9% (w/v) NaCl. Certain species of halophiles; *Halobacterium salinarum*, *Halococcus* sp., *Bacillus* sp., *Pseudomonads* and *Coryneform* bacteria are used in the production of an Asian (Thai) fish sauce, in which fish is fermented in concentrated brine (Thongthai and Suntinanalert 1991; 2001). Canthaxanthin is used in cosmetics to decrease the necessary exposure time in sunlight to acquire a tan and to intensify the tan as the compound attaches to the subcutaneous layer of fat (Margesin and Schinner, 2001).

2.5.8 Biological waste treatment
Degradation of aromatic compound
Relatively few reports have addressed the degradation of aromatic compounds under highly saline conditions by halophilic and haloalkaliphilic bacteria. The ability of halophiles/halotolerants to oxidize hydrocarbons in the presence of salt is useful for the biological treatment of saline ecosystems, which are contaminated with petroleum products (Margesin and Schinner, 2001). Several studies have demonstrated bacterial degradation of aromatic compounds in saline conditions (Piedad Diaz, et al., 2000; Mellado and Ventosa, 2003; Peyton et al., 2004). However, the ecological studies concerning the ability of these microorganisms to degrade different aromatic compounds are still in their infancy.

Development of a bioprocess
It is quite expensive and difficult to dispose the briny alkaline waste, produced after resin regeneration from the process of ion exchange. The biological removal of nitrate and subsequent reuse of these brines can potentially provide a cost-saving alternative to disposing of this waste product.
Bioplastics
Polyhydroxyalkanoates (PHA) is intracellularly accumulated bacterial storage compounds. Due to the unique characteristic of polyhydroxybutyrate (PHB), such as biodegradable thermopolyester that can be produced from renewable resources, and has properties similar to those of petroleum derived plastics (Lee, 1996; Steinb¨uchel and F¨uchtenbush, 1998). Production of PHB was very recently reported by a moderate halophile, Halomonas boliviensis LC-1, isolated from Bolivian highlands (Quillaguam´an et al., 2006).

2.6 Approaches for the study of haloalkaliphilic bacteria

2.6.1 Classical Approaches
The three major techniques for identification of bacteria are biochemical tests, fatty acid profiling, and DNA sequencing. Each technique has its strong points and weaknesses. Biochemical test-based identification systems are familiar to most microbiologists and require little training to operate. Systems range from strip cards for specific groups of bacteria to large plate arrays that may be automatically scanned for changes due to pH shifts or redox reactions. The strength of identification in enteric is generally quite good and the ease of use and cost per sample for identification is considerably less than for other molecular approaches.

2.6.2 Molecular approaches
DNA sequencing
DNA-based technology for the identification of bacteria typically uses only the 16S rRNA gene as the basis for identification. This technique has the advantage of being able to identify difficult-to-cultivate strains, and is growth and operator independent. As the 16S rRNA gene is highly conserved at the species level, speciation is commonly quite good, but as a result, subspecies and strain level differences are not shown.

16S rRNA sequencing
Molecular approaches based on 16S ribosomal RNA (rRNA) sequence analysis allow direct investigation of the community structure, diversity, and phylogeny of microorganisms in almost any environment, while quantification of the individual types of microorganisms or entire microbial communities may be addressed by nucleic acid hybridization techniques (Maidak et al., 1996).
To identify bacteria in sample material, ribosomal sequences are analyzed by transcribing ribosomal RNA into cDNA, which can then be cloned. Alternatively, extracted DNA can be used as a template to amplify ribosomal gene fragments with primers for universal sequences by PCR (Polymerase Chain Reaction). The PCR amplified fragments can be cloned as well. The result of both strategies is a clone library, containing ribosomal sequences as inserts. By sequencing individual inserts and comparing the obtained sequences with sequences present in databases, it is possible to identify the phylogenetic position of the corresponding bacteria without their cultivation. An alternative to this approach is the Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified gene fragments coding for rRNA (Muyzer et al., 1993). This technique allows the separation of partial 16S rDNA amplified fragments of identical length but different sequence due to their different melting behaviour in a gel system containing a gradient of denaturants. As a result, a band pattern is obtained, which reflects the complexity of the microbial community.

**FAME (Fatty acid microbial identification)**

The FAME (Sherlock System) identifies microorganisms based on gas chromatographic (GC) analysis of extracted microbial fatty acid methyl esters (FAMEs). Microbial fatty acid profiles are unique from one species to another, and this has allowed for the creation of very large microbial libraries. There is a large library with over 1,500 bacterial species, along with 200 species of yeast. A combination of features makes the system attractive for use in all fields of microbiology. These features include, but are not limited to: accurate identifications, large environmental libraries, the ability to perform presumptive “strain tracking” (for finding the source of a contaminant), high throughput, and a low cost per sample for consumables.

**2.7 Extremozymes**

The focus on industrial enzymes that can withstand harsh conditions has greatly increased over the past decade. Considerable efforts have been made to study extracellular salt-tolerant enzymes of the moderately halophilic and haloalkaliphilic bacteria, towards developing a new era in biotechnological processes. These enzymes include hydrolases (proteases, nucleases, lipases, phosphatases) and many polymer-degrading enzymes (amylases, cellulases and chitinases), viewed as important
candidates for various industries such as food, detergent, chemical, pharmaceutical, paper and pulp or waste-treatment, (Patel et al., 2006; Thumar and Singh, 2007; Arikan, 2008; Carvalho et al., 2008; Dodia et al., 2008a and 2008b; Ghorbel et al., 2008; Joshi et al., 2008; Boominidhan et al., 2009; Ramesh, 2009; Sorror, 2009; Sorror et al., 2009; Raj et al., 2010). Extremozymes have gained considerable attention in the various industrial communities and several products based on particularly proteases have been launched successfully in the market in past few years.

While the biotechnological applications of enzymes from extremophiles offer great horizons, it’s still long way to go to capture the opportunities. Nevertheless, in view of the great potential of biocatalysis, it is quite likely that new concepts will be developed resulting in the application of enzymes from extremophiles. Bacteria secrete variety of enzymes, many of them being commercially significant. Beside, the patterns of enzyme secretion and characteristics may also suggest on the population heterogeneity in a particular extreme habitat. Enzymes from extreme microbes have great potential for biocatalysis and biotransformation, due to their stability under number of extreme conditions.

Several microbes have been investigated for their ability to secrete these enzymes and over the years, Bacillus species have emerged as the key producers of extracellular proteases having potential applications in detergent, food, pharmaceutical, leather and chemical industries (Patel et al., 2005; Patel et al., 2006a; Patel et al., 2006b; Dodia et al., 2008a and 2008b; Joshi et al., 2008; Purohit and Singh, 2009; Siddhpura et al., 2010; Purohit and Singh, 2011). During recent years, there has been increasing emphasis on the search and development of enzymes with capabilities to function and maintain stability under multitude of extreme conditions.

The results indicated that different proteolytic bacteria release different amounts or activities of proteases (Dodia et al., 2006; 2008a; 2008b; Joshi et al., 2008; Purohit and Singh, 2009; Siddhpura et al., 2010; Purohit and Singh, 2011). The proteolytic bacterial communities may play a major role in determining the population dynamics in context with the available nutrition.

This is mainly due to the discovery of novel enzymes from extremophilic microorganisms. However, the ability to withstand the rigorous environments is not
sufficient for commercial success. In additions, number of other factors must also be considered and investigated.

Both the discovery of new extremophilic species and the determination of genome sequences provide a route to new enzymes, with the possibility that these will lead to novel applications. Of equal importance, protein engineering and directed evolution provide approaches to improve enzyme stability and modify specificity in ways that may not exist in the natural world (Takahashi et al., 2010; Sato et al., 2010).

### 2.7.1 Proteases

Proteases are degradative enzymes that catalyze the hydrolysis of the proteins. Proteases have occupied an important position with respect to their applications in both physiological and commercial context. They have been studied in great detail not only because they play important role in cellular metabolic processes but also for their pivotal role in industrial community. The quantity of proteases produced on a commercial scale worldwide is greater than any other enzymatic group of biotechnological relevance (Horikoshi et al., 2008; Horikoshi et al., 2010).

Since proteases are physiologically necessary for all living organisms, they are ubiquitously being found in a wide diversity of sources such as plants, animals and microorganisms. The inability of the plants and animals proteases to meet current demands had led to an increase interest in microbial proteases. Microbial proteases account for 40% of the total worldwide enzymes sales (Horikoshi, 2008) and around two third share of the commercial protease production in the world (Horikoshi, 2010). Many scientists have reviewed microbial proteases discussing their different aspects. The major studies are the selection of the microbes and fermentation of proteases, as well sources of microbial proteases and their possible functional role in nature. Different types of proteases and their commercial applications (Dodia et al., 2008a and b, Joshi et al., 2008; Thumar et al., 2008; Manikandan et al., 2009, Toyokawa et al., 2010) and the role of molecular biology in protease research (Ni et al., 2009; Zhang et al., 2008a and b). The bioindustrial viewpoints of microbial alkaline proteases from production to downstream processing, characterization and commercial applications have also been reviewed in some recent publications. In many cases, these enzymes retain their catalytic activity not only at elevated
temperatures but also in the presence of detergents or other denaturing agents (Dodia et al., 2008; Rasch et al., 2010; Manabe et al., 2010; Vijayanand et al., 2010).

2.7.2 Microbial proteases

Classification of Proteases

According to the nomenclature committee of the international union of biochemistry and molecular biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (IUBMB, 1992). Depending on the site of action, proteases are mainly subdivided into two major groups, i.e., exopeptidases (cleave the peptide bond proximal to the amino or carboxy termini of the substrate) and endopeptidases (cleave the peptide bonds distant from the termini of the substrate).

Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960). Table 2.2 describes the classification of the protease.

✓ Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Manni et al., 2008; Sorror et al., 2009). They are characterized by the requirement of a divalent metal ion for their activity. Entomopathogenic bacterium Photorhabdus Sp. Strain EK1, purification and characterization was carried by Sorror et al., 2009. Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I and (iv) Myxobacter II.

✓ Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Most aspartic proteases show maximal activity at pH 3 to 4 and have isoelectric points in the range of pH 3 - 4.5.
<table>
<thead>
<tr>
<th>Proteases</th>
<th>EC number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopeptidases</td>
<td>3.4.11</td>
</tr>
<tr>
<td>Dipeptidyl peptidase</td>
<td>3.4.14</td>
</tr>
<tr>
<td>Tripeptidyl peptidase</td>
<td>3.4.14</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>3.4.16–3.4.18</td>
</tr>
<tr>
<td>Serine type protease</td>
<td>3.4.16</td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>3.4.17</td>
</tr>
<tr>
<td>Cysteine type protease</td>
<td>3.4.18</td>
</tr>
<tr>
<td>Peptidyl dipeptidase</td>
<td>3.4.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteases</th>
<th>EC number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipeptidases</td>
<td>3.4.13</td>
</tr>
<tr>
<td>Omega peptidases</td>
<td>3.4.19</td>
</tr>
<tr>
<td>Endopeptidases</td>
<td>3.4.21–3.4.3</td>
</tr>
<tr>
<td>Serine protease</td>
<td>3.4.21</td>
</tr>
<tr>
<td>Cysteine protease</td>
<td>3.4.22</td>
</tr>
<tr>
<td>Aspartic protease</td>
<td>3.4.23</td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>3.4.24</td>
</tr>
<tr>
<td>Endopeptidases</td>
<td>3.4.99</td>
</tr>
</tbody>
</table>

Table 2.2: Classification and EC number of proteases (Hartley, 1960)

✓ **Cysteine/Thiol proteases**

The activity of all cysteine proteases depends on a catalytic site consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such as cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like (ii) trypsin-like with preference for cleavage at the arginine residue (iii) specific to glutamic acid (iv) others. Papain is the best-known cysteine protease.

✓ **Serine proteases**

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to these organisms. Serine proteases are subdivided into four classes; chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala–D-Ala peptidase A (SE).

Serine proteases are recognized by their irreversible inhibition by 3, 4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), DFP, phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Their molecular masses range between 18-35 kDa.

✓ **Serine alkaline proteases**

The alkaline serine proteases are the most important group of enzymes exploited commercially. It is produced by several bacteria, molds, yeasts, and fungi. They are
inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK. They hydrolyze a peptide bond, which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. Their molecular masses are in the range of 15 - 30 kDa.

✓ **Subtilisin**

The microbial proteases are generally secreted extracellularly for the purpose of scavenging nutrients and are specific for aromatic or hydrophobic residues such as tyrosine, phenylalanine and leucine. They are highly sensitive towards PMSF. There are two major classes of subtilisin as highlighted below.

**Subtilisin Carlsberg**

Gutenlberg and Ottesen (1952) discovered enzyme capable of converting ovalbumin to plakalbumin. This enzyme was known as subtilisin carlsberg. The source of this enzyme was *B. pumilis* and *B. licheniformis*. Subtilisin Carlsberg is widely used in detergents. Enzyme has a wide pH range 5.0-11.0 for stability. They are most active at pH 10.0 with molecular weight between 15-39 kDa (Gupta *et al.*, 2005). The Carlsberg enzyme has broader substrate specificity and does not depend on Ca$^{2+}$ for its stability.

**Subtilisin Novo or bacterial protease Nagase (BPN’)**

This alkaline serine protease was first purified and crystallized by Hagihara, (1958). It is usually present as a side activity in commercial preparation of *Bacillus α*-amylases.
2.8 Purification of the alkaline protease

As described above, proteases are among the most commercially exploited enzyme. A number of alkaline proteases have been purified and characterized. However, to reach the current industrial demand for the enzymes with specific features, further sources need to be explored. Generally, crude preparations of alkaline proteases are widely used in industries such as detergent; however, purification of the proteases is important for the better understanding of the structural and functional relationship (Purohit and Singh, 2011). Besides, many applications would require enzyme in homogeneity.

2.8.1 Enzyme concentration

After separating the culture from the biomass by filtration or centrifugation, the culture supernatant is concentrated by means of ultra-filtration (Kang et al., 1999; Smacchi et al., 1999), salting out by solid ammonium sulfate (Thumar and Singh, 2006; Reza et al., 2008; Purohit and Singh, 2011). Besides, salt precipitation solvent extraction methods using acetone (Kumar et al., 1999; Thangam et al., 2002) and ethanol are also effective. In order to purify the enzymes many chromatographic techniques can be used in different combinations.

2.8.2 Affinity Chromatography

Reports on the purification of alkaline proteases by different affinity chromatographic methods showed that an affinity adsorbent hydroxyapatite can be used to separate and purify the proteases from a Bacillus sp. (Dodia et al., 2008a and b; Gupta et al., 2005). However, the cost of enzyme supports and the labile nature of some affinity legends limit the used of this technique at large scale.

2.8.3 Ion Exchange Chromatography

Alkaline proteases are generally positively charged and thus could not bind to anion exchangers (Fujiwara et al., 1993; Kumar, 1999). However, cation exchangers can be a rational choice and the bound molecules are eluted from the column by an increasing salt or using pH gradient (Joshi et al., 2008). Positively charged proteins (cationic proteins) can be separated on negatively charged carboxymethyl-cellulose (CM-cellulose) columns. The adsorbed protein molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution.
2.8.4 Hydrophobic interaction chromatography
This approach exploits the variability of external hydrophobic amino acid residues on different proteins. These hydrophobic interactions are strengthened by high salt concentrations and higher temperatures, and are weakened by the presence of detergents or miscible organic solvents. Hydrophobic interactions are much more variable in behavior than ion exchangers and, thus, resolution is generally poor than ion exchange. The most commonly used hydrophobic adsorbents are octyl- (C$_8$-) and phenyl-substituted matrices.

2.8.5 Affinity precipitation
Affinity precipitation is a function of a soluble macromolecule (ligand polymer and macroligand) that has two functions: (1) it contains an affinity ligand (polyvalent macromolecule), and (2) it can be precipitated in many ways, i.e., by change in pH, temperature or ionic strength. After elution of proteins the polymer can be recycled.

2.8.6 Gel filtration
In addition to the above chromatographic techniques, gel filtration is used for rapid separation of macromolecules based on size. Recently, many new agarose based and more rigid and cross-linked gels, such as Sephacryl, Superose, Superdex and Toyopearl are also being used for purification purposes. They are generally used either in the early-to-middle stage of purification or in the final stages of purification (Joshi et al., 2008). An extreme halophilic bacterium Chromohalobacter sp. strain TVSP101 protease was purified using this chromatography to 180 fold with 22% yield (Vidyasagar et al., 2009). Major disadvantages of this method are the lower capacity for loading proteins and that the desired protein gets too diluted.

2.8.7 High-Pressure Liquid Chromatography
The resolving power of all of the column techniques can be improved substantially through high-pressure liquid chromatography (HPLC) giving high resolution as well as rapid separation. Halophilic archaeabacterium strain 172 P1 was purified by HPLC technique (Seno, 2009).

2.8.8 Aqueous two-phase systems
This technique has been applied for purification of alkaline proteases using mixtures of polyethylene glycol (PEG) and dextran or PEG and salts such as H$_3$PO$_4$, MgSO$_4$ (Sharma et al., 2007; Sinha et al., 1996; Hotha et al., 1997).
2.9 Properties of Alkaline Proteases

2.9.1 Influence of temperature and thermostability on protease activity and stability

Thermostable enzymes are of special interest for industrial applications due to their stability under typical operation conditions; such as high temperatures and wide pH range. The thermophilic proteases catalyze the reaction and maintain the stability at higher temperatures. In addition, higher temperatures can accelerate the reaction rates, increase the solubility of non-gaseous reactants and products and decrease the incidence of microbial contamination by mesophilic organisms. Many thermophiles, such as *Bacillus stearothermophilus*, *Thermus aquaticus*, *Bacillus licheniformis*, *Bacillus pumilus* and *Thermoanaerobacter yonseiensis*, produce a variety of thermostable extracellular proteases (Carvalho et al., 2008; Ueda et al., 2008; Wang et al., 2008; Zhang et al., 2008a and b; Toyokawa et al., 2008). It has been known that enzymes from thermophilic bacteria are unusually thermostable, while possessing other properties identical with enzymes found in mesophilic bacteria (Battestein and Macedo, 2007).

According to some reports, salt enhanced the thermostability of alkaline proteases. Similarly, Ca$^{2+}$ and Polyethylene glycol also plays a very important role in enhancing the temperature stability of the enzymes (Ghorbel et al., 2007; Dodia et al., 2008a and b, Manni et al., 2008). The sequencing, structure, and mutagenesis information accumulated during the last 20 years have confirmed that hydrophobicity (Luke 2007; Vielle et al., 2008; Berezovsky and Shakhnovich, 2008). Enzyme producing industries use cloning and expression as one of the approaches to obtain high quantity of desired proteins (Guo et al., 2008; Ni et al., 2009). Protein engineering could be considered as one of the important approaches to obtain improved biocatalysts. As an alternate to such modern but expensive and time consuming techniques, exploration of microbial resources from extreme environments can provide much needed biocatalytic platform (Reza et al., 2009).

While there are number of thermostable proteases reported from thermophilic organisms, similar citations from non-thermophilic organisms are quite rare (Ramesh et al., 2009). Search for thermostable enzymes from other groups of extremophiles
would be quite attractive in providing the biocatalysts with the abilities to function under multitudes of non-conventional conditions.

2.9.2 Salt adaptation of halophilic and haloalkaliphilic proteins
The enzymes from halophiles and haloalkaliphiles can not only withstand higher NaCl concentrations but actually its fundamental requirement for the function and stability of those enzymes. Stability and activity are strongly depend on protein dynamics, which is itself solvent environment dependent. Stability is needed to ensure the appropriate geometry for ligand binding, as well as to avoid denaturation, while flexibility is necessary to allow catalysis at a metabolically appropriate rate.

The understanding of the processes of protein folding, stability and solubility is of fundamental importance in basic molecular biology, and also in the development of methods in protein engineering. High salt concentration affects the conformational stability of proteins and in general the salt conditions that favor the solubility destabilize the folded form. Halophilic proteins have evolved specific mechanisms that allow them to be stable and soluble at high salt concentrations (Madern et al., 2000).

Negative charges on the halophilic proteins bind significant amounts of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. Halophilic proteins are distinguished from their non-halophilic homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and by maintaining soluble and active conformations in high concentrations of salt, for example, up to 5 M NaCl (Madern et al., 2000). The requirement of high salt concentration for the stabilization of halophilic enzymes, on the other hand, is due to a low affinity binding of the salt to specific sites on the surface of the folded polypeptide, thus stabilizing the active conformation of the protein. It has been proposed that salts exert charge screening, reducing electrostatic repulsion and enhancing hydrophobic interaction, favoring a compact folded structure of halophilic proteins (Karan et al., 2011)

2.9.3 Effect of salt on protease activity and stability
As discussed above, proteins from halophilic and haloalkaliphilic organisms require salt (NaCl/KCl) for their activity and stability. However, the requirement of salt was highly varied among them. Most of the halophilic proteins active and stable up to
4M, optimum being at 1-2M (Gimenez et al., 2000; Thumar and Singh, 2007; Dodia et al., 2008a and b; Joshi et al., 2008) and inactivated and denatured at concentrations below 1M NaCl or lost their activity in the absence of salt. In general for haloalkaliphiles; salt strongly increases enzyme activity, solubility, stability and thermal stability. Similar kind of trend has been also reported by many other proteins of halophilic origins. Alkaline protease from the novel haloalkaliphilic Bacillus sp. were active up to 0.2-0.5M NaCl and the decreased with the further increase of NaCl (Gupta et al., 2005; Patel et al., 2006b Dodia et al., 2008a; Joshi et al., 2008; Purohit and Singh, 2011).

2.9.4 Effect of pH
Protease activity is highly pH dependent and they are generally active in the range of pH 8-10 (Sanchez-porro et al., 2003; Hiraga et al., 2005; Gupta et al., 2005; Patel et al., 2006b; Dodia et al., 2006, Purohit and Singh, 2011). However, there are many examples where optimum pH was higher; pH 11, with broad range of activity from pH 8-11(Purohit and Singh, 2011). However, the pH optimum was quite low pH 7.5 for the two novel halotolerant extracellular proteases from Bacillus subtilis strain FP-133 (Setyorini et al., 2006) and pH 8 for alkaline protease from novel haloalkaliphilic Bacillus sp. (Patel et al., 2006b).

2.9.5 Metal ion requirement
Alkaline proteases require divalent cations, viz. $\text{Ca}^{2+}$, $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ or a combination of these cations for maximum activity. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Manni et al., 2008). Activity of an alkaline serine protease from Bacillus subtilis increased in the presence of $\text{Ca}^{2+}$, $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ (Bayoudh et al., 2000; Adinarayana et al., 2003; Gessesse et al., 2003).

2.9.6 Effect of surfactant and detergents
As discussed, the alkaline proteases are most commercial viable enzyme and widely applied for the detergent industries, due to its unique properties. More and more alkaline proteases were explored from varied categories of microbes and characterized from this point of view. Some alkaline proteases were stable with sodium dodecyl sulphate (SDS) and sodium linear alkyl benzene sulphonate (Joshi et al., 2008). The
alkaline protease from *Bacillus clausii* was highly stable with 5% SDS and 10% H$_2$O$_2$ (Joo *et al.*, 2003). The extracellular alkaline proteases from the haloalkaliphilic *Bacillus* sp. were stable in the presence of SDS, Triton X-100 and Tween-80 (Dodia, 2005; Patel *et al.*, 2006b; Dodia *et al.*, 2008b; Joshi *et al.*, 2008).

### 2.9.7 Protein folding

To have biologically active protein, it must fold into proper secondary and tertiary structures. These structures are held together by chemical interactions between the side chains of the amino acids, including; hydrogen bonds, hydrophobic interactions, and, at times, covalent bonds. Regardless of its function, a protein must be properly folded to carry out its biological role. Genes from extremophiles are being cloned in mesophilic bacteria to generate the protein in large amount. Fast and high-level expression of heterologous proteins in bacterial hosts often results in the accumulation of almost pure aggregates, inclusion bodies of the target protein. Hence, renaturation of the over expressed but wrongly folded proteins have gained considerable attention. While denaturation behaviors of alkaline proteases have been studied by few scientists (Kamatari *et al.*, 2003; Dodia *et al.*, 2008b). The process of protein folding is quite significant during cloning and over-expression, the over-expressed protein need to be identical and correctly folded. High level expression of recombinant protein produced in *E. coli* often forms aggregate, in insoluble fraction (Machida *et al.*, 1998; Fu *et al.*, 2003; Singh *et al.*, 2009; Yan *et al.*, 2009; Purohit *et al.*, 2008; Siddhpura *et al.*, 2010). In order to address the problem of inclusion bodies formation during over-expression various *in-vitro* and *in-vivo* strategies have been attempted (Yan *et al.*, 2009). The association of molecular chaperone is required for the stability and function of over-expressed protein (Singh *et al.*, 2009).

Alkaline protease from haloalkaliphilic *Bacillus* sp. was sensitive to urea denaturation and denatured within 30 min (Patel *et al.*, 2006b). However, this finding was in contrast with some of our own studies with other strains of haloalkaliphilic bacteria, where the extracellular proteases were highly resistant to urea denaturation and the resistance nature was salt dependent (Dodia, 2005; Dodia *et al.*, 2008; Purohit and Singh, 2011).

During the last several years, various *in-vitro* methods have been developed to obtain successful renaturation of the proteins. Among these approaches, gentle removal of
denaturant by modified dialysis (Maeda et al., 1995), a resin bound dialysis, rapid dilution method and folding in immobilized state by FPLC (Singh et al., 2002) have led to attractive options for protein folding. Another useful strategy to improve the refolding yield of proteins is to use small molecular weight additives; low concentrations of denaturants, polyethylene glycol, polyols and sugars in the refolding buffer (Baynes et al., 2005). The renaturation of urea-denatured alkaline protease from haloalkaliphilic Bacillus sp., in-vitro conditions was significantly enhanced at lower protein concentrations (Patel et al., 2006b). In contrast, the renaturation of another alkaline protease from haloalkaliphilic Bacillus sp., was not achieved by conventional dialysis and at even at lower protein concentrations (Dodia, 2005).

2.10 Applications of alkaline protease

As reviewed above, many alkaline proteases have stability in wide range of pH and many of them are thermophilic and halophilic in nature. These properties make them attractive candidates in enzyme market and account for major share of the enzyme market (Horikoshi, 2008). Major applications of alkaline protease are listed below.

2.10.1 Detergent industries

Alkaline proteases have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Microbial alkaline protease especially from the Bacillus sp. dominated commercial applications with a significant share of the detergent market (Horikoshi, 2008). The evaluation of detergent proteases is mainly dependent upon parameters such as the pH and ionic strength of the detergent solution, the washing temperature and pH, mechanical handling, level of soiling and the type of textile (Gupta et al., 2002a). Reports have been published on the compatibility of alkaline protease with detergent (Joshi et al., 2008; Raj et al., 2010).

2.10.2 Photographic industries

Alkaline proteases find potential application in the bioprocessing of used X-ray films for silver recovery. Used X-ray film contains approximately 1.5-2.0 % (by weight) silver in its gelatin layers (Kumar and Takagi, 1999). The enzymatic hydrolysis of the gelatin layers on the X-ray film allows the silver as well as the polyester film base, to be recycled. Alkaline proteases can also be used for silver recovery.
2.10.3. Medical usage
Alkaline proteases are also used for developing products of medical importance. Kudrya and Simonenko (1994) exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim and his coworkers (1998) have reported the use of alkaline protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity. Similarly, for medical usage Purification and characterization of a clostripain-like protease from a recombinant *Clostridium perfringens* culture was studied by, Sado *et al.*, (2010) and full-length protease domain of murine MMP-9 was expressed in *Drosophila* S2 cells by Rasch *et al.*, (2010).

2.10.4. Food industries
Alkaline proteases have broad substrate specificity and can hydrolyze proteins from plants, fishes, or animals to produce hydrolysates of well-defined peptide profile and high nutritional value. The commercial alkaline protease *Alcalase*, was used in the production of a less bitter hydrolysate (Adler-Nissen, 1986) and a debittered enzymatic whey protein hydrolysate which play an important role in blood pressure regulation, in infant food formulations and therapeutic dietary products (Neklyudov *et al.*, 2000).

2.10.5. Leather industry
The traditional methods of bating and dehairing of leather using sodium sulfide treatment creates a lots of environmental pollution problem, which contributes to 100% of sulfide and over 80% of the suspended solids in tannery effluents (Malathi and Chakraborty, 1991). Thus, the biotreament of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being ecofriendly (Boominadhan *et al.*, 2009). Alkaline proteases are eco-friendly enzymes that can be used as an alternate to chemical processes of pre-tanning operations in tanning industry (Jaswal *et al.*, 2009).

2.10.6. Enzymatic Cleansing of Contact Lenses
Several microbial enzymes from *Bacillus* sp., *Streptomyces* sp. and *Aspergillus* sp. were reported for cleansing of tear films and debris of contact lens. With the view of overcoming these drawbacks and to make the cleansing composition odorless and safe
i.e., not producing an allergic response or causing irritation to the eyes, bacterial proteases are gaining importance. Several reports are available on production of proteases from bacterial cultures and *Bacillus* sp. is the dominating organism (Tari *et al.*, 2007; Nilegaonkar *et al.*, 2007). Therefore, it is essential to explore bacterial protease based cleansing solutions for lens cleansing. Recently protease isolated from *Bacillus* sp. 158 has potential application in contact lens cleansing (Pawar *et al.*, 2009).

### 2.10.7 Fish Sauce Fermentation

Fish sauce is a popular seasoning in Southeast Asia, as typified by nam pla in Thailand, nuoc mam in Vietnam, and patis in the Philippines, and in Thailand, it is produced by mixing fish, such as anchovies with salts and fermenting for 6 to 12 months at room temperature. The fermentation liquid is rich in fish soluble proteins, peptides, and amino acids that are characterized by Umami taste (Curtis, 2009). They are produced during proteolytic degradation by endogenous proteases in the muscles or digestive tracts of fish, and various microorganisms exist in the fermentation broth (Taira *et al.*, 2007). Hence, microbial halotolerant proteases are considered to greatly contribute to fish sauce fermentation in the food industry. Thus the halotolerant proteinase from *B. licheniformis* RKK-04 is a key enzyme for fish sauce fermentation isolated from a fermented Thai fish sauce broth showing capable to digest the myosin heavy chain of fish protein completely can be used for fish sauce fermentation (Toyokawa *et al.*, 2010).

### 2.11 Cloning and Expression of Alkaline Proteases enzyme

Among the enzymes from extremophilic organisms, relatively limited awareness exists about enzymes from haloalkaliphilic bacteria. Extremozymes offer new opportunities for biocatalysis and biotransformations as a result of their extreme stability (Niehaus *et al.*, 1999). From recent work, major approaches to extending the range of applications of extremozymes have emerged. Both the discovery of new extremophilic species and the determination of genome sequences provide a route to new enzymes, with the possibility that these will lead to novel applications. Of equal importance, molecular gene cloning and over-expression of protein, protein engineering and directed evolution provide approaches to improve enzyme stability and modify specificity in ways that may not exist in the natural world (Colquhouna,
In the aspect of novel enzymology, the enzymes from extremophilic organisms are relatively less explored (Horikoshi, 2011). All that is known/ explored about the extremophilic enzymes is its character to work at relative high/ elevated temperatures. The well known examples are the Taq polymerases from various thermophilic organisms. In the past few decades, biocatalysts have been successfully exploited for the synthesis of complex drug intermediates, specialty chemicals and even commodity chemicals in the pharmaceutical, chemical and food industries. Recent advances in recombinant DNA technologies, high-throughput technologies, genomics and proteomics have fuelled the development of new catalysts and biocatalytic processes. In particular, gene cloning and directed evolution have emerged as powerful tools for biocatalyst engineering in order to develop enzymes with novel properties, even without requiring knowledge of the enzyme structure and catalytic mechanisms.

The approach of directed evolution has been reviewed several times by a number of researchers. Also very important is the cloning of these important genes which in turn code for extremophilic enzymes and novel proteins (Matsuo et al., 2001; Yan et al., 2009). Cloning of potential proteins, gradually leads to cloned gene is it’s over expressed form in stable host or suitable mesophilic host which produces desired protein in bulk quantities.

2.12 Expression Systems

2.12.1 Bacteriophage CE6

Expression can be induced from a host strain without a source of T7 RNA polymerase by infection with Bacteriophage CE6. CE6 is a lambda recombinant that carries the cloned polymerase gene under control of the phage pL and pI promoters, the cI857 thermolabile repressor, and the Sam7 lysis mutations (pET Manual, Novagen, USA). When CE6 infects an appropriate host, the newly made T7 RNA polymerase transcribes target DNA so actively that normal phage development cannot proceed with the transcription. Although this method is less convenient than induction of DE3 lysogens, it can be used if target gene products are too toxic to be maintained any other way. As, in a host system, there is no presence of T7 RNA polymerase before infection, so it should be possible to express any target DNA that can be cloned under control of a T7 promoter.
2.12.2 Induction of λDE3 Lysogens with IPTG
After a target plasmid is established in a λDE3 lysogen, expression of the target DNA is induced by the addition of IPTG to a growing culture. IPTG induction results in uniform, concentration-dependent entry into all cells in the population. A range of IPTG concentrations from 25 μM to 4 mM is required for target protein activity and solubility.

2.13 Recombinant Protein Purification
The methods chosen for protein purification depend on variable factors, including the properties of the protein of interest, its location and form within the cell, the vector, host strain background, and the intended application for the expressed protein. Culture conditions can also have a dramatic effect on solubility and localization of a given target protein. Many approaches can be used to purify target proteins expressed with the pET System. One advantage of the system is that in many cases the target protein accumulates to such high levels that it constitutes a high percentage of the total cell protein. Therefore, it is relatively straightforward to isolate the protein in two or three chromatographic steps by conventional methods (ion exchange, gel filtration, etc.).

Before purification or activity measurements of an expressed target protein, preliminary analysis of expression levels, cellular localization, and solubility of the target protein should be performed. The target protein may be found in any or all of the following fractions: soluble or insoluble cytoplasmic fractions, periplasm, or medium. Depending on the intended application, preferential localization to inclusion bodies, medium, or the periplasmic space can be advantageous for rapid purification by relatively simple procedures.

2.14 Solubilization and Refolding Proteins
A variety of methods have been published describing refolding of insoluble proteins (Kurucz et al., 1995; Burgess, 1996; Frankel et al., 1996; Rudolph et al., 1996; Mukhopadhyay, 2006; Machida et al., 2002; Singh et al., 2002; Vincentelli et al., 2004; Willis et al., 2005). Most protocols describe the isolation of insoluble inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The protein is then dialyzed or diluted into a non-denaturing buffer where refolding occurs. Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be empirically determined (Dodia et al.,
Optimal refolding conditions can be rapidly determined on a small scale by a matrix approach, in which variables, such as protein concentration, reducing agent, redox treatment, divalent cations, etc., are tested. Once the optimal concentrations are found, they can be applied to a larger scale solubilization and refolding of the target protein.

The protein refolding requires CAPS buffer at alkaline pH in combination with N-lauroylsarcosine to achieve solubility of the inclusion bodies, followed by dialysis in the presence of DTT to promote refolding. Depending on the target protein, expression conditions, and intended application, proteins solubilized from washed inclusion bodies may be >90% homogeneous and may not require further purification. In current procedures, purification under fully denaturing conditions (before refolding) is possible using fusion proteins and immobilized metal.

2.15 Purifying Target Proteins
Fusion proteins solubilized from inclusion bodies using 6M urea can be purified under partially denaturing conditions by dilution to 2M/1M urea prior to chromatography on the appropriate resin. Refolded fusion proteins can be affinity purified under native conditions using appropriate affinity tags (e.g., GST•Tag and T7•Tag).

2.16 Examples of Successful Cloning Approaches of Several Enzymes
With reference to halophilic proteins particularly, maintenance of stability and activity in high salt is major challenge (Ueda et al., 2008; Wang et al., 2008a and b). Most typical halophilic enzymes from extremely halophilic archaea and bacteria require high concentrations of salt for their activity and stability and are inactivated in Escherichia coli unless refolded in the presence of salts under in-vitro conditions. Recombinant DNA technology in conjunction with other molecular techniques is being used to improve and evolve enzymes and opening new opportunities for the construction of genetically modified microbial strains with the selected biocatalysis (Caralino et al., 2008). Many newer preparations, such as Durazym, Maxapem and Purafect, have been produced, using techniques of site-directed mutagenesis and/or random mutagenesis. Directed evolution has also paved the way to a great variety of subtilisin variants with better specificities and stability. Knowledge of full nucleotide sequences of the enzyme genes has facilitated the deduction of the primary structure of the encoded enzymes and in many cases, identification of various functional
regions. These sequences also serve as the basis for phylogenetic analysis of proteins and assist in predicting the secondary structure of proteins, leading to the understanding of structure and function relationship of the enzymes.

Several examples are available in literature where successful cloning and expression has been analyzed, the gene encoding a ferredoxin of nucleoside diphosphate kinase from a moderately halophilic eubacterium was cloned and protein was over expressed in *E. coli* (Matsuo *et al.*, 2001).

Some alkaline protease-encoding bacterial genes have been cloned and expressed in new hosts, the two major organisms for cloning and over-expression being *E. coli* and *B. subtilis*. The gene of a highly thermostable alkaline protease from an alkalophilic bacillus was cloned by PCR and nucleotide sequence was determined. Similarly, around 1242 base pair DNA fragment from *Bacillus halodurans* isolated from alkaline sediments coding for a potential protease was cloned and sequenced (Zang *et al.*, 2008a and b).

Earlier, to study gene expression in halophilic archaea, a reporter system was analyzed by β–glycosidase enzyme. The developments related to cloning and expression of the genes from halophilic organisms in heterologous hosts will certainly boost the number of enzyme-driven transformations in chemical, food, pharmaceutical and other industrial applications (Singh *et al.*, 2009).

The gene encoding the protease Nep from haloalkaliphilic archaeon *Natrialba magadii* was cloned and sequenced. The nep gene was expressed in *Escherichia coli* and *Haloferax volcanii* resulting in production of active Nep protease. The nep-encoded polypeptide had a molecular mass of 56.4 kDa, a pI of 3.77 and included a 121-amino acid propeptide not present in the mature Nep. The primary sequence of Nep was closely related to serine proteases of the subtilisin family from archaea and bacteria (50–85% similarity). The *Hfx. volcanii* synthesized protease was active in high salt, high pH and high DMSO (Rosana *et al.*, 2008). A homology search of the N-terminal amino acid sequence of the purified PseA protease revealed an exact match to a *P. aeruginosa* PST-01 protease gene, las B (Gupta *et al.*, 2008).
2.17 Metagenomics Aspects

Microbiology has experienced a transformation during the last 25 years that has altered microbiologists’ view of microorganisms and how to study them. The realization that most microorganisms cannot be grown readily in pure culture forced microbiologists to question their belief that the microbial world had been conquered. We were forced to replace this belief with an acknowledgment of the extent of our ignorance about the range of metabolic and organism diversity. In principle, any study that addresses all the individuals of community as a single genomic pool can be seen as an exercise in metagenomics.

Metagenomics allows to insight into specific physiological and ecological functions, metabolic variability of an environment. The vast majority of the biosphere’s genetic and metabolic diversity is currently locked up within the world’s microbial communities, containing a staggering number of yet uncharacterized microbial. It has become well accepted that the diversity of microorganisms represented in culture collections is highly skewed toward those taxa that are amenable to growing under laboratory conditions, making our discovery of microbial genes through cultivation-dependent conventional genome sequencing equally skewed. In the late 1980s, the direct analysis of rRNA gene sequences had also shown that the vast majority of microorganisms present in the environment had not been captured by culture-dependent methods. Even with the recent success of novel and high throughput culturing strategies, we are still unable to mimic most microbial environments sufficiently to induce growth of many environmentally relevant microbes.

Among the methods designed to gain access to the physiology and genetics of uncultured organisms, metagenomics, the genomic analysis of population of microorganisms, has emerged as a powerful centerpiece.

Direct isolation of genomic DNA from an environment circumvents culturing the organisms under study, and cloning of it into a cultured organism captures it for study and preservation. Advances have derived from sequence-based and functional analysis in samples from water and soil and associated with eukaryotic hosts. In a nutshell, metagenomics allows isolation of large portions of genomes which provide access to genes for protein-coding for biochemical pathways.
2.18 Extraction of Metagenome (Total DNA)

Metagenomic studies begin with the extraction of total DNA from a particular environment (Purohit and Singh, 2008). Metagenomics, the analysis of DNA isolated from environmental samples, has proved particularly useful for the knowledge of uncultured bacteria. In the core of the metagenomic approaches, establishing better DNA extraction techniques is of prime significance. Detecting the rare members of a microbial community is a challenge (Voget et al., 2003). However, it is equally important to know about a small number of microbes that play a critical role in the community. Improved DNA extraction techniques could help ensure that a metagenomic library adequately represents the entire community’s genome and has little or no contamination. Extending the analyses beyond the DNA sequence to study the proteins and metabolites (the products of cellular processes) generated by a community is critical for understanding how the microbial community operates and interacts within the habitat.

Among the key factors responsible for the success of metagenomics, the isolation of quality environmental DNA in appreciable amount from a given habitat holds significance (Raes, 2007). The isolation of total DNA appears to be of prime importance and a bottleneck step in metagenomic studies, as the extracted DNA should be of high quality to pursue molecular biological applications (Voget et al., 2003; Desai and Madamwar, 2007; Gilbert, 2010). Standardization of total DNA extraction technique is desirable as the composition of different habitats varies with respect to their matrix, organic and inorganic compounds and biotic factors (William, 1998). Improved DNA extraction techniques should also ensure a metagenomic library adequately representing the entire community’s genome without inhibitory substances (Santosa, 2001).

Mostly metagenomics projects currently focus on the microbes found in the sample environment that have smaller amounts of DNA, such as bacteria and other microbes which can live in extreme environments (Risenfeild et al., 2004; Sharma et al., 2007). During the last 10 years, number of protocols for DNA extraction from environmental sample have been reported (Kauffman et al., 2004; Handelmann et al., 2004) and commercial soil DNA extraction kits (Mo Bio, Maildegen, USA) are also available. These kits and most of the published methods have improved the original direct DNA
extraction procedures mainly in terms of DNA yield and quality. The protocols for isolating total DNA from environmental sample could be broadly classified as direct and indirect methods. The variability in the outcome among the methods is viewed with respect to the degree of shearing, purity and quantity of the extracted DNA (Desai and Madamwar, 2007).

2.19 Approaches and Techniques
The DNA is extracted from a sample followed by the construction of a genomic library containing pieces of the genomes of all the microbes. This metagenome can further be applicable by two approaches:

2.19.1 Sequence-Based Metagenomics
Entire information’s of genetic sequences could be determined by sequence-based approaches, which reflect DNA profile and addresses population heterogeneity in particular (Tringe et al., 2005; Glockner et al., 2010). Phylogenetic and large-insert metagenomic approaches, provide access to genetic information contained within microbial populations only known to us in the form of specific phylogenetic marker gene sequences (Rondon et al., 2000).

2.19.2 Function-Based Metagenomics
It explores and aims at the specific products from the microbes in a community. In function-based metagenomics, researchers screen metagenomic libraries for various functions, such as biocatalysts, vitamins or antibiotic production (Raes et al., 2007). Through this approach, scientists can search and identify the functions that are largely unknown. Similarly, recent technological advances enable to directly extract and identify novel proteins and metabolites (the products of cellular processes) from a microbial community (Jeffrey, 2010). Moreover, metagenomics analyses microbial communities as systems that have functional properties beyond individual genes or individual microbes (or even single-taxon populations). Metabolic cascades, for example, can be distributed over different members of multi-taxa communities, (Rajenderan and Gunashankar, 2008). Product or activity-driven metagenomic studies are often approached with a more applied perspective in view, exploring useful properties encoded within the metagenome (Raes et al., 2007).
2.19.3 Techniques
A wide range of techniques has been employed to gain access to metagenomes, among them shotgun analysis of community genomes is a rather simple exercise (Wooley, 2010). Metagenomics, however, has more to offer than merely providing lots of interesting DNA sequence data. It takes a non-traditional focus on the genomic resources of a dynamic microbial community, rather than on individual strains of microbes or individual genes and their functions. Community genomics perspectives aim to explore how horizontal transfer allows otherwise distantly related organisms. Therefore, metagenomic analyses of microbial communities focus on systems having functional properties beyond the individual genes or individual microbes (Jeroen et al., 2007). Through automated high-throughput methods, it’s possible to recover and sequence as many clones as necessary (Handelsman, 2004; Handelsman, 2005).

2.20 Metagenome Screening
An enormous variety of different biocatalysts or other functional products can be theoretically obtained using DNA extracted from a given environmental sample. An example for the impressive diversity of metagenome-encoded enzymes was provided by Diversa Corporation (San Diego). By fragmenting total DNA from an alkaline marine sample, cloning it into an expression vector, and screening for esterase/lipase activity in an easily cultivable host strain, 120 new enzymes were discovered, falling into 21 protein families (Miller, 2008). During the past five years, cloning of genes from the metagenome has become the most popular tool for cultivation-independent enzyme discovery, leading to the recovery of a range of new biocatalysts by academic and commercial institutions (Kennedy, 2008). While in almost all studies, E. coli was used as expression host, there is an example of cloning in a broad host range vector and expression in Streptomyces lividans (Tringe et al., 2003; Hugenholt, 2008). Vector systems used for the cloning of environmental DNA range from small-insert cloning vectors such as plasmids or phage vectors (up to 15 kb inserts) to bacterial artificial chromosomes (BACs) that can harbor as much as 100 kb fragments. While BAC vectors are usually applied when activities are targeted on the expression of large gene clusters (e.g. metabolite formation), small-insert libraries are usually prepared for the screening of single genes or small operons (Gilbert, 2010).
However, smaller cloned fragments necessitate larger gene banks required for a comprehensive and comparable coverage of the genetic information, which ultimately leads to more laborious screening procedures. For example, one amylase-expressing clone could be isolated per 450 clones screened using a BAC vector (Gilbert, 2010).

2.21. Molecular Tools Used In Metagenomics

Molecular tools developed during the past 20 years by molecular biologists have facilitated the extraction, cloning, screening and sequencing of genes and genomes. Many of these approaches have also allowed microbial ecologists to access and study the microbial diversity in its totality, regardless of our ability to culture organisms. This has opened the doors of unexplored domains of non-cultivable microbes, allowing unprecedented access to the world of natural products encoded by community genomes (Gilbert, 2010; Glockner et al., 2010).

The advent of culture-independent techniques has transformed the field of microbiology and microbial ecology in particular. PCR-based techniques allow the classification of microorganisms based on particular genetic markers and the profiling of complex microbial communities on the basis of sequence diversity (Bach, 2001).

The most commonly used marker for profiling bacterial communities is the 16S rRNA gene. The size of this gene (1.5kb) is large enough for reliable phylogenetic information. Hierarchal domain specific primers are designed, which can target broadly or with high specificity. Different functional genes can also be used in order to target specific groups of bacteria. Domain specific primers are designed on the basis of conserved residues of sequences.

One technique that is now routinely used is denaturing gradient gel electrophoresis (DGGE) and the analogous temperature gradient gel electrophoresis (TGGE) (William, 1998). It is a genetic fingerprinting technique that is used to separate individual sequences from a complex mixture. In principle, this means that DNA fragments of the same length are separated on the basis of differing sequences, even by a single base (Ercolini, 2004).

We have attempted to find 16S rRNA sequence/s of unculturables from saline soils of Coastal Gujarat, India by the DGGE protocols perfected in our laboratory. Towards this end, we aim to focus on identifying signature sequences of
halophiles/haloalkaliphiles; based on shotgun sequencing approaches and designing specific primers for halophiles/haloalkaliphilies (Siddhpura et al., 2010).

The results indicated that different proteolytic bacteria release different amounts or activities of proteases (Dodia et al., 2008a and b; Joshi et al., 2008; Purohit and Singh, 2008; Siddhpura et al., 2010). The proteolytic bacterial communities may play a major role in determining the population dynamics in context with the available nutrition. In the overall scenario of the secretion of extracellular proteins by the microbes in their surrounding, the recently published idea on the economic synthesis of the proteins/enzymes by the microbes assumes significance (Smith and Chapman, 2010). According to this proposed theme supported by the analysis of the data, the organisms spend minimum energy on the synthesis of extracellular proteins.

The widespread use of molecular techniques in studying microbial communities has greatly enhanced our understanding of microbial diversity and function in the natural environment and contributed to an explosion of novel commercially viable enzymes. Technological advances in sequencing and cloning methodologies as well as improvements in annotation and comparative sequence analysis, generate information for microbial ecologists. e. g. Natural products isolated from sponges are an important source of new biologically active compounds (Raes et al., 2007; Glockner, 2010). Metagenome of marine microbial communities have been shown to contain genes and gene clusters typical for the biosynthesis of biologically active natural products (Mitchell et al., 2000; Kauffman et al., 2004; Keneddy et al., 2007; Keneddy et al., 2008).

Combining metagenomic approaches with heterologous expression holds much promise for the sustainable exploitation of the chemical diversity present in the marine microbial community. A PCR-based method targeting a 59-base recombination site highlighted on the diverse bacterial taxonomic groups and that flanks gene cassettes are associated with integrons. The recovered gene cassettes contained complete open reading frames, most of which did not show homology to any database entry, and which potentially encode enzymes of biotechnological interest (Glockner, 2010). PCR-based cloning methods are also being employed to recover novel enzymes. In most cases, degenerate primers are used, hybridizing with
conserved regions that preferentially are located close to the extremities of the target genes (Liles, 2008; Ni et al., 2009).

We relied on similar approach for our own studies, where degenerate primers were designed for alkaline proteases by using bioinformatics tools. Designed sets of primers were specifically based on halophilic/haloalkaliphile alkaline proteases available from marine environment. We identified several such sequences and successfully cloned, over-expressed and characterized them in E. coli host system, our unpublished data (Singh et al., 2010a). The characteristic features of native and recombinant enzymes were studied, interestingly, we noticed that recombinant clones have maintained their nascent properties, specific activity of enzyme was found to be around five times higher activity than purified native enzymes.

However, this approach does not sound equally well in capturing functional attributes of sequences that share some sequence identity with already identified sequences (Craig and Venter 2004; Craig et al., 2009). Besides, expression-based identification of biocatalysts, large-scale shotgun sequencing projects and *in silico* identification of enzyme-coding regions are currently carried out, for instance by The Monterey Bay Coastal Ocean Microbial Observatory on marine picoplancton (Nakumura et al., 2009).

### 2.22 Gleaning Information out of the data: Bioinformatics and data analysis

Metagenomic approaches have the potential to generate enormously huge body of sequences. However, the knowledge gleaned from such studies is not proportional to the sequencing effort involved, and it depends on the bioinformatics interpretation of the informations.

### 2.23 Metagenomics: Commercial successes in Biotechnology

As the excitement about genetic access to the boundless realms of microbial diversity slowly gives way to the reality of tapping into this diversity, the usual challenge of heterologous gene expression needs to be addressed to turn metagenomic technologies into commercial successes, particularly in applications for which bulk enzyme or product quantities have to be produced at competitive prices.
Given that the majority of natural products are of microbial origin, and that the vast majority of microbial genomes have yet to be explored, it follows that microbial metagenomes contain a great economic potential. Due to their huge diversity and history as sources of commercially valuable molecules with agricultural, chemical, industrial, and pharmaceutical applications, soil environments have been the most common subjects of metagenome interrogation in this way. Functional screening methods potentially provide a means to discover new variants of functions of interest.

Metagenomics, together with in vitro evolution and high-throughput screening technologies, provides industry with an unprecedented chance to bring biomolecules into industrial application.

The goals of researchers venturing into the microbial metagenome vary from directed product discovery to total community characterization, and the phylogenetic complexity of the environments studied can range over orders of magnitude. Metagenomics has redefined the concept of a genome, and accelerated the rate of gene discovery. The potential for application of metagenomics to biotechnology seems endless.

A high-throughput pipeline has been constructed to provide high-performance computing to all researchers interested in using metagenomics (Morgan et al., 2010). The pipeline produces automated functional assignments of sequences in the metagenome by comparing both protein and nucleotide databases. Phylogenetic and functional summaries of the metagenomes are generated, and tools for comparative metagenomics are incorporated into the standard views.

2.24 Enzymes from solvent-tolerant microbes: A way towards non-aqueous enzymology

Biocatalysis under a water-restricted medium has undergone tremendous development during the last decade and numerous reactions have been introduced and optimized for synthetic applications (Carrea and Riva, 2000). By using enzymes in a solvent medium, it is now becoming possible to synthesize novel compounds which are difficult to synthesize conventionally and to obtain the biologically active enantiomer for which the racemic solution is either very complex or difficult (Karadzic et al., 2006; Klibanov, 2001). However, this necessitates efficient catalysis, and the stability
of the enzymes in organic solvents is a prerequisite. Enzymes, in general, get
denatured or give very low rates of reaction in solvent media because of the
unfolding, structural disfunctioning, and stripping of the essential water layer from the
enzyme molecule (Khare et al., 2000a, b; Klibanov, 2001; Vulfson et al., 2001).

The screening of solvent-stable enzymes in natural sources has come to be accepted
as a better and more promising approach than directed evolution, chemical
modification and protein engineering. Several solvent-tolerant microbial strains, some
of which produce solvent-stable enzymes, have been discovered. However, less
attention has been paid to their enzymes, which logically should be stable and
efficient catalysts for functioning in solvent media and which could be nature’s very
own toolbox for solvent-stable enzymes for applications in non-aqueous systems
(Fang et al., 2006; Ogino and Ishikawa, 2001; Takeda et al., 2006).

2.24.1 Microbial adaptation to solvents
It is necessary to look into the cellular toxicity of solvents for an understanding of the
generic adaptation mechanisms in this class of microbes. Solvent accumulation leads
to specific permeabilization of the cell membranes, leading to the leakage of ATP,
potassium and other ions, RNA, phospholipids, and proteins (Heipieper et al., 1991;
Ramos et al., 1997; Woldringh, 1973). Further, the membrane fluidity is also affected
by organic solvents (Sikkema et al., 1994). Several studies have shown that a
correlation exists between the solvent toxicity and its hydrophobicity, i.e. the log P ow
value (the partition coefficient of a given solvent in an equimolar mixture of octanol
and water) (Inoue and Horikoshi, 1991). Solvents in a log P range of 1–4 are more
water soluble and partition well to the membrane. Thus these solvents are much more
toxic in comparison to lipophilic solvents (log P > 4), which do not reach a high
membrane concentration owing to their low water solubility (Sardessai and Bhosle,
2004). Solvent-tolerant bacteria circumvent the solvents’ toxic effects by virtue of
various adaptations which have been excellently studied and reviewed (Heipieper et
al., 2007). Hence these are only briefly discussed here. Rigidification of the cell
membrane (a) a shift in the ratio of saturated to unsaturated fatty acids in cell
membrane (Mohammad et al., 2006). (b) Isomerization of the naturally synthesized
cis-isomer of an unsaturated fatty acid to the trans-isomer by an energy-independent,
periplasmic isomerase enzyme (Mohammad et al., 2006; Nielsen et al., 2005) (c)
Change in fatty acid composition; the phospholipids head group’s composition also
alters during solvent adaptation (Nielsen et al., 2005). (d) Changes in the composition of lipopolysaccharides (LPS), lipid–protein ratios, and outer membrane proteins (Pinkart et al., 1996; Ramos et al., 1997). These adaptations change the fluidity of the membrane and in this way suppress the effects of the solvents on membrane stability, biotransformation and degradation of toxic organic solvents.

2.24.2 Solvent-efflux pumps

Several solvent-efflux pumps involved in solvent tolerance in various bacteria have been described in the last few years and most of them belong to the RND (resistance/nodulation/cell division) family. Only a few efflux pumps for organic solvents, namely tolC, mar, rob, soxS and acrAB have been identified in Pseudomonas sp. (Kieboom et al., 1998; Li et al., 1998; Ramos et al., 1998) and E. coli (Asako et al., 1997; Kobayashi et al., 2001). Increase in cell size of P. putida and Enterobacter sp. adapt to toxic organic compounds by increasing their cell size. A bigger size reduces the relative surface and consequently reduces the attachable surface for toxic organic compounds. It is obvious that the functioning of solvent-efflux pumps is more effective, if the overall membrane surface is reduced. This leads to a reduction in the area that allows diffusion and partitioning of solvents into the membrane where they are recognized and excluded by the efflux-pump proteins (Neumann et al., 2005).

2.24.3 Enzymes from solvent-tolerant microbes

Solvent-tolerant microbes have been less studied from the perspective of non-aqueous enzymology. It is now becoming obvious that their enzymes display striking novel properties and that they attain a higher level of catalytic activity of their enzymes in organic solvents (Ogino and Ishikawa, 2001). Some of industrially important enzymes such as lipases, proteases, and amylases from solvent-tolerant microbes (Doukyu et al., 2003; 2007; Geok et al., 2003; Ghorbel et al., 2003; Gupta et al., 2005; Karadzic et al., 2004). Surprisingly, halophiles have also been noticed to exhibit the properties of solvent-tolerant enzymes. Examples are amylase from extremely halophilic archaea, Haloarcula sp. strain S-1 (Fukushima et al., 2005), and protease from the moderately halophilic bacterium Salinivibrio sp. strain AF-2004 (Heidari et al., 2007). The halotolerant actinomycetes derived alkaline proteases have also been reported having growth, activity and stability in the presence of solvents (Thumar and Singh, 2007a). This opens up the possibility of the availability of enzymes that have combinations of tolerances of extreme conditions, such as to salts and solvents, which
could be beneficial for industrial processes involving the use of high salt concentrations and hydrophobic organic solvents. The enzymes produced by the solvent-tolerant microbes that have been studied are mainly those wherein the reverse reactions are of industrial significance or the substrates are sparingly soluble in water but soluble in solvents.

The general features observed in enzymes from such sources include (i) better stability in hydrophobic solvents especially alkanes, (ii) monomeric proteins of molecular weight ranging from 20 kDa to 80 kDa, (iii) hydrophobic surfaces and the marked presence of disulphide bonds, (iv) hydrophobic interaction chromatography (HIC) or ion exchangers tend to work more selectively for their purification over conventional protocols, (v) most of the reported solvent stable proteases belong to the group of metalloproteases, and (vi) these have been mainly resourced from *Pseudomonas* and few from *Bacillus* sp. (Ogino *et al.*, 1994 and 1995; Geok *et al.*, 2003; Ghorbel *et al.*, 2003; Gupta *et al.*, 2005). It is interesting to note that their characteristics vary even for enzymes produced from one strain of *Pseudomonas* to another. The common attribute, however, is their stability in alkanes. In general, most of the reported enzymes from solvent-stable strains are stable-to-long-chain aliphatic hydrocarbons, benzene, toluene, and alcohols.

### 2.24.4 Solvent stable proteases

Proteases stable in organic solvents are desirable for effective peptide synthesis. Protease catalyzed synthesis has several advantages over chemical catalysis, e.g. regio- and stereo-selectivity, absence of racemization, lack of requirement of side chain protection and milder non-hazardous reaction conditions (Gill *et al.*, 1996; Klibanov, 1986; Rahman *et al.*, 2007). Several peptides, such as the analgesic dipeptide kyotorphin (Tyr-ArgS) (Jönsson *et al.*, 1996; Sareen *et al.*, 2004a and b) and aspartame (Eichhorn *et al.*, 1997), have been synthesized in aqueous or nonaqueous media using proteases. Subtilisin, thermolysin, and other proteolytic enzymes have been used in the presence of organic solvents as catalysts for peptide synthesis (Isowa and Ichikawa, 1998; Oka and Morihara, 1978; 1980; Pauchon *et al.*, 1993). But the rate of peptide synthesis is low in the presence of organic solvents because of denaturation or inactivation of the enzymes (Ogino *et al.*, 1999a; Vulfson *et al.*, 2001).
Recently it has also been shown that the amino acid residues located at the surface of the protein molecule played an important role in exhibiting the organic solvent tolerant nature of the protease (Gupta et al., 2007; Ogino et al., 2007). In general, these protease producers have been isolated from soil except in a few cases from fishing-industry wastewater (Ghorbel et al., 2003) and cutting oil used in industrial metal-working processes (Karadzic et al., 2004). Most of the reported solvent-stable proteases are mainly from *Pseudomonas* sp. In a few recent cases, *Bacillus* spp. have also been found to be endowed with solvent-stable proteases (Ghorbel et al., 2003; Sareen et al., 2004a and b). Both *Pseudomonas* and *Bacillus* proteases exhibit better stability towards hydrophobic solvents, especially alkanes (Ogino et al., 1995; Gupta and Khare, 2006a and b, Rahman et al., 2006 and 2007). However, in some cases, stability towards alcohols has also been reported (Ghorbel et al., 2003; Karadzic et al., 2004; Sana et al., 2006). These proteases are mainly being purified by using anion exchange and/or hydrophobic-interaction chromatography.

### 2.24.5 Biotechnological applications of solvent-tolerant microbes and their enzymes in bioremediation/ biotransformation

Apart from their enzymes, solvent tolerant bacteria can also be of vital importance in bioremediation/ biotransformation. The microbial transformation of hydrocarbons, soil remediation and waste-stream purification necessitates the survival and growth of microbes in toxic effluent (Kieboom et al., 1998). The persistence of many solvents in contaminated sites is indicative of the lack of natural systems that can efficiently degrade these compounds. Numerous problems appear in the application of biological systems to solvent treatment due to the toxic effects of organic solvents on the bacteria (Mohammad et al., 2006). Organic solvent tolerant bacteria can serve as an invaluable tool for such processes. The enzymes have been in use for bioconversions in two-phase systems for a very long time. The exploitation of solvent-tolerant bacteria for biotransformation in two phase fermentation systems has been recently reviewed by Heipieper et al., 2007.

### 2.24.6 Conclusions and future perspectives of solvent tolerant bacteria and enzymes

Biocatalysis in low water/solvent media has undergone tremendous development during the last decade and numerous new reactions have been introduced for synthetic applications. The stability and efficiency of enzymes in solvents, however, remains a
necessary prerequisite for such applications. The research efforts have been entwined with the development of new strategies to obtain solvent-stable enzymes. In this context, enzymes from solvent-tolerant microbes, with the requisite natural catabolic potential, seem to have a major advantage. Solvent-tolerant bacteria also show great promise for the future development of cost effective solvent bioconversion or remediation processes. Their ability to tolerate and mineralize high concentrations of toxic solvents widens the opportunities for biological treatments into areas traditionally served by chemical and physical techniques, which do not involve a pretreatment step for effluents so as to render them suitable for ‘normal’ biological conditions. These possibilities represent a future avenue of research for both microbiologists and enzymologists.