3.1 Distribution of Extremophiles:

Extremophiles are bizarre microorganisms that can grow and thrive in extreme environments, which were formerly considered too hostile to support life. The extreme conditions may be high or low temperature, high or low pH, high salinity, high metal concentrations, very low nutrient content, very low water activity, high radiation, high pressure and low oxygen tension. Some extremophiles are subject to multiple stress conditions. Extremophiles are structurally adapted at the molecular level to withstand these harsh conditions. Currently, only 1–2% of the microorganisms on the Earth have been commercially exploited and amongst these there are only a few examples of extremophiles the biocatalysts, called extremozymes, produced by these microorganisms, are proteins that function under extreme conditions. Due to their extreme stability, extremozymes offer new opportunities for biocatalysts and biotransformation. Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes. However, the renewed interest that is currently emerging as a result of new developments in the cultivation and production of extremophiles and success in the cloning and expression of their genes in mesophilic hosts will increase the biocatalytic applications of extremozymes (Madigan et al., 1999)

3.1.1 Extremozymes

Enzymes from extreme microbes have great potential for biocatalysis and biotransformations, due to their stability under number of extreme conditions. Various approaches include discovery of new extremophilic species with novel biocatalytic features and search for unique and novel gene sequences from the total environmental genome pool. The enzymes from extremophilic organisms, particularly halophilic and haloalkaliphilic counterparts are relatively less explored. Major interests have focused on the studies of extremophilic enzymes with respect to their ability to work at higher
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temperatures. However, one of the most important characters to be explored and developed would be their ability to act under alkaline conditions with high salt concentrations.

With increasing emphasis on extremozymes during the recent years, the microbial enzymes are being looked to replace chemical catalysts in transformations and manufacturing processes. Among the biocatalysts, proteases constitute one of the most important groups of industrial enzymes and account for about 60% of the total worldwide commercial enzyme (Horikoshi, 2008), two-third of them being obtained from microbial sources (Ramesh et al., 2009).

Extensive global research efforts have revealed the novel diversity of extremophilic microbes. They grow under extreme environmental conditions that are hostile to most organisms. Extremophilic microorganisms are adapted to thrive in such hostile environments (Vijayanand et al., 2010). As a result of adaptations to extreme environment, extremophiles have evolved unique properties which can be of biotechnological and commercial significance. The organisms living in such dual extreme environment possess special adaptation strategies that make them interesting not only for fundamental research but also towards exploration for industrial applications (Margesin et al., 2001). Extremophiles have been proven to be a rich source of biological information (Vijayanand et al., 2010). Extremozymes, the enzymes isolated from extremophiles are now replacing the harsh chemical catalysts in many industries, including manufacturing of chemicals, textiles, pharmaceuticals, detergents, food, paper and agricultural chemicals (Mehta et al., 2006). They include protease, amylase, cellulase, xylanase, keratinase and other enzymes that have numerous applications in many industrial processes. These enzymes are adapted to extreme environments and as a result, they are unusually stable. They are therefore suitable candidates for applications in industrial processes that are performed under harsh conditions, such as high temperatures or in the presence of organic solvents or high ionic strength. For the same reasons, they are also ideal molecules for investigations aimed at elucidating the mechanisms of chemico-physical stability of proteins (Schiraldi et al., 2002). With increasing emphasis on environmental protection the use of enzymes particularly from extremophiles has gained considerable attention during last several years. The increasing industrial demands for biocatalysts that can cope with industrial process conditions, has led to the considerable efforts for the search for such enzymes. Despite the
fact that to date more than 3000 different enzymes have been identified and many of these have found their way into biotechnological and commercial applications, the present enzyme tool box is still not sufficient to meet the complete demands. A major reason for this is that many available enzymes do not withstand industrial reaction conditions. As a result, the characterization of microorganisms that are able to thrive in extreme environments has received a great deal of attention. Such extremophiles act as a valuable source of novel enzymes (Sanchez-Porro et al., 2003; Cojoc et al., 2009).

3.2 HALOPHILISM

Salt is essential for all life forms on earth, with excess salinity representing a common stress condition. Halophiles are organisms that require more than 0.2 $M$ NaCl for their growth and can resist the effects of osmotic stress. Halophiles are found in nearly all major microbial clades, including prokaryotic (Bacteria and Archaea) and eukaryotic forms. They are classified as slight halophiles when they grow optimally at 0.2–0.85$M$ (2–5%) NaCl, as moderate halophiles when they grow at 0.85–3.4 $M$ (5–20%) NaCl, and as extreme halophiles when they grow at 3.4–5.1 $M$ (20–30%) NaCl (DasSarma et al., 2005). At lower salinities, members of the Eukarya and Bacteria dominate populations, while members of the Archaea dominate at higher salinities. They have adapted to thrive in ecological niches in saline and hypersaline environments. As a result, these microorganisms produce unique enzymes and metabolites able to develop biological activity in conditions in which their counterparts could not be functional. These properties could be exploited for the development of additional bio industrial processes designed basically on the optimal conditions of these biomolecules. Two fundamentally different approaches are involved in order to cope with osmotic challenges associated with life in saline environments. In the case of halophilic Bacteria, the cytoplasm contains low concentrations of salt compared with surrounding environment, but it has high organic solute levels. The halophilic Archaea (halobacteria), on the other hand, have developed an entire biochemistry that functions at saturating salt concentrations (Madigan et al., 1999).

3.3 Halophile-Definition and Classification

Halophile (salt-lover) can be defined as microorganisms that require salt to grow. They are a type of extremophile organism. The name comes from the Greek word for
"salt-loving. Halophiles can be found anywhere with a concentration of salt five times greater than the salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds (Ventosa et al., 2010). Microorganisms in this area were defined by Kushner.

An important review by Larsen, 1962 outlined a scheme that has relevance today. Nonhalophiles are those microorganisms that grow best below 2 % salt. Slight, moderate, and extreme halophiles are those that grow best in media containing 2 to 5 %, 5 to 20 %, and 20 to 30 %, respectively. Kushner, 1968 then distinguished between obligate moderate halophiles and obligate extreme halophiles, which require 0.5 to 3.5M and 3.0M to saturated salinities, respectively. Kushner, 1978 later added a definition for borderline extreme halophiles that grow best at 0.5 to 2.5M salinity.

**Classification of Halophiles**


<table>
<thead>
<tr>
<th>Category</th>
<th>Salt concentration</th>
<th>Optima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non halophile</td>
<td>0–1.0</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Slight halophile</td>
<td>0.2–2.0</td>
<td>0.2–0.</td>
</tr>
<tr>
<td>Moderate halophile</td>
<td>0.4–3.5</td>
<td>0.5–2.0</td>
</tr>
<tr>
<td>Borderline extreme halophile</td>
<td>1.4–4.0</td>
<td>2.0–3.0</td>
</tr>
<tr>
<td>Extreme halophile</td>
<td>2.0–5.00</td>
<td>&gt;3.0</td>
</tr>
<tr>
<td>Halotolerant</td>
<td>0–&gt;1.0</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Haloversatile</td>
<td>0–&gt;3.0</td>
<td>0.2–0.5</td>
</tr>
</tbody>
</table>

**3.4 Ecological distribution of halophiles**

Halophiles are found in many saline environments; the most important are hypersaline waters and soils, but the latter are much less studied. They can also be isolated from salt or salt deposits and from a variety of salted products, from salted fish or meats to fermented foods, as well as other materials such as salted animal hides. Hypersaline waters, those with higher concentrations of salt than sea water, can be divided into thalassohaline, which have a marine origin, if they have a composition similar to that of sea water, or athalassohaline, if their composition reflects the composition of the surrounding geology, topography and climatic conditions, often particularly influenced by
the dissolution of mineral deposits; thus the composition of such waters varies widely (Rodriguez-Valera, 1988; Grant, 1990). Typical examples of thalassohaline water systems are solar salterns, used for the natural evaporation of sea water for the production of salt. They are excellent models for the study of halophiles, providing a series of ponds with different salinities, from sea water to salt saturation (Rodriguez-Valera, 1988; Grant, 1990). Typical examples of athalassohaline waters that have been studied in more detail are the Dead Sea, Great Salt Lake, some cold hypersaline lakes in Antarctica or alkaline lakes, particularly East African lakes, like Lake Magadi or helakes of Wadi Natrun (Rodriguez-Valera, 1988; Javor, 1989; Grant, 1990).

3.5 Hyper saline 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 seawater (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 harbour significant populations of microorganisms (Grant et al., 1998).

3.5.1 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.
3.5.2 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 (CaSO4·2H2O) and other minerals after their solubility has been exceeded (Oren et al., 2005).

3.5.3 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).

3.6 Phylogenetic Diversity of Halophiles

Halophilic and highly halotolerant microorganisms can be found in each of the three domains of life: Archaea, Bacteria, and Eucarya. The domain Bacteria contains many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic subgroups. Many of these have been reviewed by Ventosa et al. (1998). Within the lineages of the Gram-positive Bacteria (Firmicutes), halophiles are found both...
within the aerobic branches (Bacillus and related organisms) and within the anaerobic branches. There is even an order, the Halanaerobiales, consisting of two families (the Halanaerobiaceae and the Halobacteroidaceae) that consists solely of halophilic anaerobic microorganisms (Rosenberg et al., and Rainey et al., 1995).

3.7 Osmoadaptation of halophilic microorganisms

Within the group of extremophiles, halophilic microorganisms developed specific cellular mechanisms to protect their interior processes and chemistry against the ruling environmental stress. Their natural saline habitats mainly change ionic strength by weather reliant desiccation and irrigation. Such osmotic changes strongly affect the microorganisms by osmotic gradient between cell turgor and environment, and can lead to cell damage and death. To counteract this destructive influence, the halophilic microorganisms use short and long-term adaptations, providing best possible flexibility at shock situations and thus increasing survivability.

3.7.1 Salt in Strategy

The short-term osmoadaptation is mainly based on raising or decreasing the salt concentration in the cytoplasm by thermodynamical adjustment (Figure a). This mechanism was first discovered in halophilic archaea and named as salt-in strategy. Thereby, the organism accumulates or release counter ions, such like Cl and K+, in or from the cytoplasm by proton motive force in order to maintain the ionic gradient, until the turgor is isosmotical with their environment. In particular, chloride was found to have specific functions for haloadaptation. However, the salt-in strategy requires specific adaptation of compounds included in metabolic and genetic regulation, and seems to be restricted to archaea, Haloanaerobiales and Salinibacter ruber. 

Salt-in strategy. The organism transports ions to balance the osmotic gradient and thus avoiding water efflux and desiccation

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3.7.2 Salt-Out Strategy

Long-term osmoadaptation can be apportioned into evolutionary modifications and uptake or release of organic osmolytes. Since halophiles are able to live in high salinities, the cytoplasm has to deal with the similar osmolarities and internal function needs to be maintained. High ion concentrations disturb the metabolism. Hence, enzymes and proteins of halophilic microorganisms are modified to remain active, soluble and stable at these conditions. Even, the distribution of internal amino acids is adjusted to the ionic strength to avoid precipitation and to improve the availability. The surface of proteins from halophiles are generally more negative than those of non-halophiles and demonstrates higher hydrophilic properties. In addition, the cell membrane, which forms a barrier between the cytoplasm and the environment, adjusts to the external salinity by alterations of membrane composition and lipid conformations. However, the most investigated and discusses osmoadaptation is the organic osmolyte mechanism. The microorganisms accumulate compatible solutes inside the cells in response to osmotic stress. This step within osmoregulation is also named salt-out strategy, as the prior accumulation ions (salt-in strategy) are replaced by these osmolytes (Figure b).

**Figure b**

*Salt-out strategy - The organism synthesizes compatible solutes de novo or/and transport them into the cell by high affine transporters*

These compounds are highly soluble in water and do not interfere with the metabolism even in high cytoplasmic concentrations. The osmolytes are quasi compatible with the cell metabolism, which is reflected in the naming of the compound class. Compatible solutes are synthesized by *de novo* or uptaken directly from the environment by highly affine transporters, which is energetically preferable. Thus the *de novo* synthesis is repressed when external osmolytes are available. In general, the highly water-soluble compatible solutes fall into the chemical categories: (1) zwitterionic solutes, (2) non
charged solutes (3) anionic solutes. The wide distribution of these compounds has been reviewed currently for prokaryotes. Compatible solutes are able to stabilize proteins, nucleic acids and even whole cell membranes against osmotic and temperature stress, providing an advantage when living in extreme habitats. For example, protein denaturation is abated at high ionic strength. The mode of action is suggested to be thermodynamically founded and described by the preferential exclusion model, which is the most popular explanation model by now. By exclusion of the compatible solutes from the hydrate envelope of the protein, the protein is forced to remain in the native structure. In addition, some compatible solutes were found to protect against UV radiation by hydration effect.

3.8 Physiology of Halophiles

3.8.1 Internal ion Concentrations

To cope 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. Osmotic balance can be achieved by the accumulation of salts, organic molecules, or a combination thereof. A fourth possibility, that the cell is able to control water movement in and out and maintain a hypoosmotic state of their intracellular space, has been proposed for S. costicola and H. elongata (Shindler et al., 1977; Vreeland, 1987 and Vreeland et al., 1983).

The intracellular abundance of individual ions in a halophile an be summarised as:

i. Sodium - The apparent intracellular Na\(^+\) concentrations are often far too high to enable the generally salt-sensitive cytoplasmic enzymes to be active. However, the assessment of the true intracellular Na\(^+\) concentration is problematic, as discussed above. In addition, Na\(^+\) and other ions may be bound to the outer cell layers, in amounts increasing with external salinity (Imhoff, 1993).

ii. Potassium - In most halophilic bacteria, K\(^+\) is accumulated to a few tenths of 1 M (Imhoff, 1993). H. elongata seems to be an exception, with K\(^+\) concentrations as low as 20 mM (Vreeland et al., 1983). In any case, in contrast to some archaeal halophiles, cytoplasmic potassium contributes relatively little to the achievement of an osmotic balance.
iii. Magnesium - Intracellular Mg$^{2+}$ concentrations have been determined only seldom in halophilic bacteria. In *H. elongate* grown in medium containing 24 mM Mg$^{2+}$, the intracellular Mg concentrations varied from 9 to 23 mM depending on the growth conditions (Vreeland *et al.*, 1983), which is not particularly high compared to 30 mM in *Escherichia coli* and 102 mM *Halobacterium salinarum* (*cutirubrum*) (De Me´dicis, 1986a).

iv. Calcium - *H. elongata*, growing in media with 0.7 mM Ca$^{2+}$ and in the presence of 0.05 to 3.4 M NaCl, was reported to contain between 3.1 and 12 mM Ca$^{2+}$ intracellularly, most of it probably bound (Vreeland, 1993).

v. Manganese - A single measurement of intracellular Mn$^{2+}$ concentrations in *S. costicola* gave a value of 0.6 mM Mn$^{2+}$/kg of cell water (De Me´dicis *et al.*, 1986b).

vi. Chloride - Estimations of intracellular chloride concentrations within cells of moderately halophilic bacteria are greatly variable, from relatively low values (55 and 139 mM in *H. halodenitrificans* and *S. costicola*, respectively, grown in 1 M NaCl) (Christian and Waltho., 1962.) to values as high as 0.7 to 1 M in “*P. halosaccharolytica*” grown at NaCl concentrations between 1 and 3 M (Masui and Wada., 1973). The measured intracellular Cl$^{2+}$ concentrations are in most cases much lower than the combined Na$^+$ and K$^+$ concentrations (Imhoff, 1993).

3.8.2 *Nutritional Requirements of Halophiles-Growth Media*  
Specialized media and conditions are used to enrich for microbes from specific biogeochemical guilds, anaerobes, and alkaliophiles. Extreme halophiles are predominantly archaea and are cultured at warmer temperatures (37°C) with salinities of 20 % or more. Hypersaline media can be divided into complex media that include organic components for which exact chemical formulae are not known and defined media where all components can be described by chemical formulae. There is a wide range of organic ingredients used for hypersaline media, the most popular of which are yeast extract, peptone, tryptone, and casamino acids (v.i.). The predominant salt is nearly always is NaCl. Additional
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salts are often constituted like seawater, since the bulk of hypersaline research has been done in marine solar salterns or other thalassohaline environments. Extreme halophile media often have elevated levels of magnesium, particularly for Dead Sea isolates. The source of water used for hypersaline media preparation varies, with some media based on natural waters from the sea or hypersaline lakes (Volcani, 1944; Madeley et al., 1967; Oren, 1983a,b; Paterek and Smith, 1985; Franzmann et al., 1987; Yu and Kawamura, 1987; Wais 1988).

General Chart of Media Composition for Halophiles

<table>
<thead>
<tr>
<th>Component</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>250</td>
<td>125</td>
<td>234</td>
<td>250</td>
<td>220</td>
<td>200</td>
</tr>
<tr>
<td>KCl</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K_2SO_4</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KNO_3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>K_2HPO_4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH_4)_2SO_4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>MgSO_4.7H_2O</td>
<td>20</td>
<td>-</td>
<td>29</td>
<td>-</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>MgCl_2.6H_2O</td>
<td>-</td>
<td>50</td>
<td>19.5</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CaCl_2.6H_2O</td>
<td>-</td>
<td>0.12</td>
<td>1.1</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>NaBr</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaHCO_3</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FeCl_2</td>
<td>0.023</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Na-Citrate</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Tryptone/Peptone</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


Microbial growth in media of high salinity is often slow, so it is not unusual to maintain cultures for weeks rather than days. Evaporation from liquid cultures, especially shake-flasks at elevated temperatures, can be problematic and lead to changes in salinity with time and even salt precipitation. Agar plates already present a relatively dry environment, so the addition of high salt exacerbates potential limitations. It is prudent to wrap plates in plastic paraffin film to retain moisture. Another general consideration when
working with hypersaline cultures is that the appearance and growth habit of microbial isolates can change depending on salinity. For *Halobacterium* and some halococci, red pigmentation is increased at higher salinities (Kushner, 1993). In contrast, *Haloferax* may be more highly pigmented at lower salinities and colorless at high salinities (Rodriguez-Valera *et al.*, 1980; Kushwaha *et al.*, 1982). Colonies that are less highly colored, appearing cream or yellow, can exhibit more subtle changes in color at different salinities. Colonies may become smaller or mucoidy with increasing salinity. Cells may be smaller at higher salinities, often falling in the submicron range, making staining protocols more difficult. In addition, classic staining and biochemical tests have to be modified for higher salinities and may not be as consistent.

### 3.8.2.1 Magnesium

Requirements for Mg salts are broad among halotolerant and halophilic microorganisms. While Mg$^{2+}$ is a common component of microbiological media, haloarchaea are generally considered to require higher concentrations of Mg$^{2+}$ for growth. However, not all do and the growth of some halophilic and halotolerant microbes is inhibited by higher Mg$^{2+}$ concentrations (Soliman and Trüper, 1982; Juez, 1988). *Halobacterium curtirubrum* required at least 0.1M Mg$^{2+}$ for growth, helping cells maintain normal morphology at lower (2.5 M) salinities (Boring *et al.*, 1963). While slow growth was observed at Mg$^{2+}$ concentrations of 0.01–0.025 M, maximum growth occurred in the range of 0.1–0.5Mg$^{2+}$ for *Halobacterium halobium, Pseudomonas cutirubra, P. salinaria,* and *Sarcina littoralis* (Brown and Gibbons, 1955). Magnesium salts of chloride, nitrate, and sulfate were equally effective.

### 3.8.2.2 Potassium

KCl is typically added up to 2 %, to media that mimic concentrated seawater (Rodriguez-Valera *et al.*, 1980; Caton *et al.*, 2004; Caton *et al.*, 2009), and is likely a component of media based on natural brines. *Halobacterium halobium, Pseudomonas salinaria, P. cutirubra,* and *Sarcina littoralis* failed to grow in media that did not contain K$^+$ (Brown and Gibbons, 1955). Maximum growth was seen at 1–3 mM, but was not inhibited at 3M KCl.
3.8.2.3 Sulfur, Phosphate, and Nitrogen

Complex media typically rely on organic materials as sources of sulfur and nitrogen. In defined media, these can be supplied as inorganic chemicals or as amino acids. Nitrogen can be provided as NH4Cl, NaNO3, or (NH4)2SO4. The amino acids asparagine, cysteine, glutamate, glutamine, and histidine have been used (Flannery and Kennedy, 1962; Forsyth and Kushner, 1970; Grey and Fitt, 1976; Yu and Kawamura, 1987; Kauri et al., 1990).

3.8.2.4 Minor and Trace Salts

Iron is often added to both defined and complex hypersaline media as chloride, citrate, or as a double salt with ammonium sulphate. The influential medium of Sehgal and Gibbons (1960) is often supplemented with FeCl2 (Boring et al., 1963; Kushner and Bayley, 1963). Iron has been shown to be essential for growth of halophilic and halotolerant microbes at concentrations similar to those used for other bacteria and archaea (Brown and Gibbons, 1955).

3.8.3 Environmental Conditions

Generally haloarchaea grow best above room temperature. Most laboratories use 37°C (Abram and Gibbons, 1960; Boring et al., 1963; Dundas et al., 1963; Ducharme et al., 1972; Matheson et al., 1976; Tomlinson and Hochstein, 1986; Montero et al., 1988; Yu and Kawamura, 1987; Kamekura and Dyall-Smith, 1995). Halophilic and halotolerant bacteria are often grown at room temperature or at a slightly elevated temperature (30 °C) (Forsyth and Kushner, 1970; Vreeland et al., 1980; Oren, 1983a, Caton et al., 2004). Most halophilic and halotolerant microbes isolated to date are neutrophiles, growing best in media with pHs from 6.8 to 7.5 (Brown and Gibbons, 1955; Abram and Gibbons, 1960; Flannery and Kennedy, 1962; Tomlinson and Hochstein, 1972a,b; Mullakhanbhai and Larsen, 1975;; Vreeland et al., 2002; Caton et al., 2004). Most of the halotolerant and halophilic microbes studied to date are aerobes, although anaerobes are known (Oren, 1983a, 1986; Mathrani and Boone, 1985; Tomlinson et al., 1986; Zhilina, 1997;). A challenge for aerobic organisms in hypersaline systems is that oxygen solubility decreases with increasing salinity, dropping by half at 10 % salinity and by over 80 % at 30 % salinity. Therefore, more vigorous aeration should be considered when culturing at higher salinities.
Bioprospecting of haloprotease producing bacteria from Tuticorin salt pan and its application as antifouling agent

3.9 Biotechnological Potential of Halophiles

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. Halophilic microorganisms produce stable enzymes (including many hydrolytic enzymes such as DNases, lipases, amylases, gelatinises, and proteases) capable of functioning under high concentrations of salt which leads to precipitation or denaturation of most proteins. Most halophilic enzymes are inactivated and denatured at concentrations of NaCl below 1M. 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, nuclease, lipases, phosphatases) and many polymer degrading enzymes (amylases, cellulases and chitinases), viewed as important candidates. 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, nuclease, 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., 2005a; Thumar and Singh, 2009; Arikán, 2008; Carvalho et al., 2008; Dodia et al., 2008a and 2008b; Ghorbel et al., 2008; Joshi et al., 2008; Boominadhan et al., 2009; Ramesh, 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.

Many extreme and moderate halophiles have been isolated and investigated for possible biotechnological applications. These include the production of β-carotene, polyhydroxy enzymes and compatible solutes, enhanced oil recovery and degradation of toxic chemicals that can pollute hyper saline habitats. Additionally, halophiles produce exozymes such as amylases, proteases and nucleases of potential commercial values. Halophilic proteins are distinguished from their homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and by maintaining soluble and active conformations in high concentrations of salt up to 5 M NaCl. There are number of enzymes of this type produced by some halophilic micro-organisms that have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions (Vidhyasagar et al., 2009).

Halophilic Bacteria are metabolically more versatile than the Archaea, and their enzymatic activities are more diverse. Moreover, most haloarchaeal enzymes require at least 10–15% salt both for stability and activity, while bacterial enzymes generally do not show such a strict salt requirement. Extensive research has therefore been done on the properties of the enzymes from halophilic and halotolerant Bacteria and their possible applications (Kamekura, 1985). The common denominator for all moderately halophilic bacteria is their requirement for salt and their ability to tolerate high salt concentrations. Salt requirement and tolerance are highly variable among the different species. Moreover, these parameters are by no means constant, since they may vary according to the growth temperature and the nature of the nutrients available (Kushner, 1978). Salt requirement and tolerance may be temperature dependent. In certain halophilic archaea such as *Haloferax volcanii*, the minimum and optimum salt concentrations shifted to higher values with increasing temperature (Mullakhanbhai, and Larsen, 1975), and a similar phenomenon was observed in halophilic bacteria as well. Thus, the optimum salt concentration for growth of *H. halophila* at 32 and 42°C was 7.5%, whereas the optimal concentration for growth at 22°C was 5% (Quesada, et al., 1987). *H. elongata* grew in complex medium at 20 and 30°C at salt concentrations between 0.05 and 3.4 M. At 40°C, no growth was
obtained at 0.05 M, but growth was possible between 0.375 and 4.5 M. In defined medium with glucose and alanine as organic nutrients, salt tolerance was decreased, growth occurred within a narrower salt range than in complex medium, and a higher salt concentration was needed for optimum growth (Vreeland et al., 1993).

Most moderate halophiles have more demanding nutritional requirements at high salt concentrations. Complex media stimulate growth at high salt concentrations. The effect may be due to the presence of compatible solutes or their precursors that can be accumulated or to the fact that other growth factors may be synthesized more slowly under the high-salt conditions (Kamekura et al., 1985). Thus, the salt tolerance of *S. costicola* in defined medium could be extended by including 2% sodium glutamate (Javor, 1989), and its growth in 4 M (but not in 3 M) salt required the presence of nutrients such as glycine betaine (Russell and Kogut, 1985).

### 3.9.1 Halophilic Enzymes

The primary interest in enzymes from extremophiles stems from their activity under unique conditions. There has been extensive research into how key metabolic enzymes from these organisms can be exploited for industrial use. Halophilic enzymes are of particular interest because they are active in environments with low water activity. They are thought to remain active by having a predominance of negatively charged residues on the solvent-exposed surfaces of the protein. These negative charges attract water molecules and thereby keep the proteins hydrated so that they do not precipitate. It has also been shown that the hydrogen bonds formed between the negative side chains and the water molecules lead to the formation of a stable hydration shell (Balasubramanian et al., 2002).

### 3.9.2 Halophilic Hydrolases

There has been growing interest in scientific research on salt tolerant enzymes derived from halophilic bacteria due to the potential industrial application of these enzymes. It is generally believed and has been proven that many halophilic enzymes are poly extremophilic. These enzymes not only remain active and stable in high salt environments but are often also thermotolerant and alkaliphilic (Moreno et al., 2009).
These properties made halophilic enzymes attractive for various biotechnological applications as they would be able to catalyze reactions under harsh conditions typical of many industrial processes.

3.9.3 Ecological Distribution of Hydrolase Producing Halophilic Bacteria

Hypersaline environments maintain remarkably high microbial cell densities and are biologically very productive ecosystems. Various culture-dependent and nutritional analyses carried in tandem with molecular culture dependent techniques have been used to characterize the microbial communities in hypersaline environments. Halophilic and extremely halotolerant microorganisms are present in each of the three domains of life: archaea, bacteria and eukarya (Oren, 2002). The domain bacteria typically contains many types of halophilic and halotolerant microorganisms that spread over a large number of phylogenetic subgroups. Most of these fall in the family Halomonadaceae (class Gammaproteobacteria, order Oceanospirillales) and they are moderate rather than extreme halophiles (Oren, 2002). Research on hydrolytic enzymes from halophilic organisms was pioneered by Nordberg and Hofsten at the end of the 1960s (Norberg and Hofsten, 1969). Since then, a considerable amount of effort has been dedicated towards the evaluation of extracellular salt-tolerant enzymes of the moderately halophilic bacteria and the use of such enzymes in biotechnological processes (Ventosa et al., 1998).

Several researchers have screened halophilic bacteria from different hypersaline environments through direct plating on agar media amended with substrates specific for enzymes of interest. A wide variety of bacteria that secrete extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNases, pullulanases and xylanases have been isolated and characterized (Sánchez-Porro et al., 2003b; Rohban et al., 2009; Govender et al., 2009). Greater hydrolytic activity is commonly observed amongst Gram-positive moderately halophilic bacteria than Gram-negative bacteria. Most of the Gram-positive bacteria belong to the Bacillus group including Salibacillus, Halobacillus, Oceanobacillus, Gracilibacillus, Virgibacillus, Thalassobacillus and Piscibacillus (Sánchez-Porro et al., 2003b; Rohban et al., 2009). Hydrolase-producing Gram-negative bacteria commonly comprise species of Salinivibrio, Chromohalobacteria and Halomonas (Sánchez-Porro et al., 2003b; Rohban et al., 2009).
3.10 Proteases

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzyme. Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. These are degradative enzymes that catalyze the cleavage of peptide bonds leading to total hydrolysis of proteins. These proteases represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sales in the world. These enzymes have numerous applications in the industrial production of different items including detergents, foods, pharmaceuticals, leathers and diagnostic reagents. These enzymes have also been used for waste management and silver recovery (Gupta et al., 2002). Recently a considerable attention has been given to the enzymes produced by halophilic microorganisms and their biotechnological potentials (Ventosa, 2004; Ventosa et al., 1998).

The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Gimenez et al., 2000). Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Litchfied et al., 2002).
3.10.1 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).

Classification

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (International Union of Biochemistry). 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).

Classification of proteases and their peptide bond cleavage (Rao et al., 1998)

<table>
<thead>
<tr>
<th>Type of protease enzyme</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exopeptidase</td>
<td>●↓ o-o-o-o-o</td>
</tr>
<tr>
<td>Amino peptidase</td>
<td>●-●↓ o-o-o-o</td>
</tr>
<tr>
<td>Dipeptidyl peptidase</td>
<td>●-●-●↓ o-o</td>
</tr>
<tr>
<td>Tripeptidyl peptidase</td>
<td>o-o-o-o-o-●</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>o-o-o-o-o-●</td>
</tr>
<tr>
<td>Serine type protease</td>
<td>o-o-o-o-o-●</td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>o-o-o-o-o-●</td>
</tr>
<tr>
<td>Cysteine type protease</td>
<td>o-o-o-o-●-●</td>
</tr>
<tr>
<td>Peptidyl dipeptidases</td>
<td>●-●</td>
</tr>
<tr>
<td>Dipeptidases</td>
<td>*-●↓ o-o-●</td>
</tr>
<tr>
<td>Endopeptidases</td>
<td>-----o-o-o-●</td>
</tr>
</tbody>
</table>

Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.
3.10.1.1 Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the amino (N) or carboxy (C) terminus, they are further classified as aminoand carboxypeptidases, respectively.

3.10.1.2 Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds within the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases and (iv) metalloproteases. Each family of peptidases has been assigned code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively.

3.10.1.3 Aminopeptidases

Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi. In general, aminopeptidases are intracellular enzymes, except for a single report on an extracellular aminopeptidase produced by A. oryzae.

3.10.1.4 Carboxypeptidase

The carboxypeptidases act at C terminus of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes (Rao et al., 1998).

3.10.1.5 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 the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their
structural similarities, serine proteases have been grouped into 20 families, which have been further, subdivided into about six clans with common ancestors. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. The isoelectric points of serine proteases are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases. Serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragmentS.

3.10.1.6 Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (Barett, 1995), and have been placed in clan AA. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like enzymes produced by Aspergillus, Penicillium, Rhizopus, and Neurospora and (ii) rennin-like enzymes produced by Endothia and Mucor spp. The mechanism of aspartic proteases involves general acid-base catalysis with lytic water molecule that directly participates in the reaction. (Janssen et al., 1994).

3.10.1.7 Cysteine/thiol proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differ among the families. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or 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, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic
pH. They are susceptible to sulfhydryl agents such as p-CMB but are unaffected by DFP and metal-chelating agents. Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of serine proteases.

3.10.1.8 Metalloproteases

Metalloproteases are the most diverse of the catalytic types of protease. They are characterized by the requirement for a divalent metal ion for their activity and can be inactivated by dialysis or by the addition of chelating agents. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Hibbs et al., 1985; Okada et al., 1986). About 30 families of metalloproteases have been recognized, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases.

3.11 Halophilic Proteases:

Extreme halophiles act as an ideal source of extremozymes (halozymes) with extreme stability, and the application of these enzymes as biocatalysts is attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials (Vijayanand et al., 2010). Most significantly halophiles secrete a wide range of extracellular hydrolytic enzymes such as nucleases, proteases, amylases, cellulases, lipases and xylanases of potential commercial values (Alqueres et al., 2007).

Halozymes are distinguished from their homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and maintaining soluble and active conformations in high concentrations of salt up to 25% concentration (W/V) (Hough and Danson, 1999; Nascimento and Martins, 2004). This feature of halophilic microorganisms has showed several biotechnological applications (Ghosh et al., 2010). Halophilic proteases are active and highly stable in high salt concentrations which inhibit or even denature many conventional enzymes of non-halophilic organisms (Norberg and Hofsten, 1969).
3.11.1 Optimisation of Haloprotease

Halophilic proteases have been isolated and characterized from several bacterial species including *Bacillus* sp. (Kamekura and Onishi, 1974; Setyorini et al., 2006; Shivanand and Jayaraman, 2009), *Pseudoaltermonas* sp. (Sanchez-Porro et al., 2003), *Salinivibrio* sp. (Amoozegar et al., 2007), *Salicola* sp. (Moreno et al., 2009), *Halobacillus* spp. (Namwong et al., 2006; Karbalaei-Heidari et al., 2009), *Filobacillus* sp. (Hiraga et al., 2005), *Chromohalobacter* sp. (Vidyasagar et al., 2009), *Nesterenkonia* sp. (Bakhtiar et al., 2005) and *Virgibacillus* sp. (Sinsuwan et al., 2009). These enzymes display optimal activity in the presence of NaCl and maintain stability over a wide pH range (pH 5-10). In addition, the enzymes were active at temperatures of 40–75°C. While some of the enzymes display an absolute requirement of NaCl for activation, the protease from *Chromohalobacter* was reported to retain 100% stability in the absence of NaCl (Vidyasagar et al., 2009). In addition, some of the enzymes may display polyextremophilicity. For instance, the enzymes may be haloalkaliphilic (Gupta et al., 2005) or halothermophilic (Vidyasagar et al., 2009). Consequently, halophilic and halotolerant bacteria harbour a pool of proteases that will be more suitable for application in food production processes that are performed under saline conditions but can also be applied in saline free systems. For instance, saline fermentation processes involved in the production of various protein rich foods including processing of fish and meat-based products and the production of soysauce (Setyorini et al., 2006). Moreover, the enzymes derived from halophiles make excellent additives for laundry detergent as most of them are either alkali tolerant or alkaliphilic. Some proteases such as those from *Nesterenkonia* species have been reported to display unique substrate specificities which might open up new application opportunities (Bakhtiar et al., 2005).

In 2006 Vidyasagar et al., investigated that a novel haloalkaliphilic, thermostable serine protease was purified from the extreme halophilic archaeon, *Halogeometricum borinquense* strain TSS101. The enzyme optimized by using various parameters showed the highest activity at 60 °C and pH 10.0 in 20% NaCl. The enzyme had high activity over the pH range from 6.0 to 10.0. The enzyme exhibited relatively high thermal stability, retaining 80% of its activity after 1 h at 90 °C. Thermostability increased in the presence of Ca$^{2+}$. The stability of the enzyme was maintained in 10% sucrose and in the absence of
NaCl. The activity was completely inhibited by ZnCl₂ (2 mM), 0.1% SDS and PMSF (1mM). The protease was active and retained 100% of its activity in 10% (v/v) DMSO, DMF, ethanol and acetone.

In 2013 Santos et al. investigated the ability of the moderately halophilic bacterium *Halobacillus blutaparonensis* (strain M9), a novel species, to release proteolytic enzymes. This bacterial strain abundantly proliferated in Luria–Bertani broth supplemented with 2.5% NaCl as well as secreted proteases to the extracellular environment. The production of proteases occurred in bacterial cells grown under different concentrations of salt, ranging from 0.5% to 10% NaCl, in a similar way. The proteases secreted by *H. blutaparonensis* presented the following properties: (i) molecular masses ranging from 30 to 80 kDa, (ii) better hydrolytic activities under neutral–alkaline pH range, (iii) enzymatic stability in the presence of salt (up to 20% NaCl) and organic solvents (e.g., ether, isooctane and cyclohexane).

### 3.11.2 Purification of Halophilic Protease Enzyme

In industrial applications, use of crude enzyme is preferred over purified preparation. This is to avoid the cost of purification and make the processes commercially viable. The applications of crude proteases have been highlighted in several processes, for instance, the crude protease from the extreme halophilic archaea *Halobacterium* sp. SP1 (Akolkar et al., 2008) that is stable over a broad pH range and high salt concentration has been effectively used in order to accelerate the fish sauce fermentation process (Akolkar et al., 2010). Joshi et al. (2007) described the production of alkaline serine protease stable in the presence of NaCl, SDS and acetone by a moderately halotolerant strain of *Bacillus cereus* (designated MTCC 6840) isolated from Lake Nainital, Uttarakhand State, India. Many haloarchaea secrete proteolytic enzymes which enable the degradation of proteins and peptides in the natural environment, several of these extracellular serine protease have been purified and characterized. These include proteases of 40 – 66 kDa isolated from neutrophilic haloarchaea including strains of *Halobacterium salinarum* (*Halobacterium halobium*) (Norberg and Hofsten, 1969; Izotova et al., 1993, Schmitt et al., 1990; Ryu et al., 1994) *Natrialba asiatica* 172PI (Kamekura and Seno et al., 1990) and *Halofex mediterranei* 1538 (Stepanov et al., 1992).
Duong et al., (1981) reported that Halophilic protease in culture fluids of a moderately halophilic marine Pseudomonas sp. (A-14) was purified by ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration through Sephadex G-200. The enzyme was purified to homogeneity, as judged by polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be 120 000. The optimum pH for activity was 8.0. The enzyme had maximal activity at 18% NaCl concentration.

In 2006 Vidyasagar et al., investigated that an extreme halophilic bacterium was isolated from solar saltern samples and identified based on biochemical tests and 16S rRNA sequencing as Chromohalobacter sp. strain TVSP101. The halophilic protease was purified using ultrafiltration, ethanol precipitation, hydrophobic interaction column chromatography and gel permeation chromatography to 180 fold with 22% yield. The molecular mass of the protease determined by SDS PAGE was 66 kDa.

In 2006, Vidyasagar et al., isolated Halogeometricum sp. TSS101 from salt samples of Tuticorin, India and screened for the secretion of protease on gelatin and casein plates containing 20% NaCl. The archaeon was grown aerobically in a 250 ml flask containing 50 ml of (w/v) NaCl 20%; MgCl2 1%; KCl 0.5%; trisodium citrate 0.3%; and peptone 1%; pH 7.2 at 40°C on rotary shaker. The production of enzyme was investigated at various pH, temperatures, NaCl concentrations, metal ions and different carbon and nitrogen sources. The partially purified protease had activity in a broad pH range (7.0–10.0) with optimum activity a pH 10 and a temperature (60°C). The enzyme was thermostable and retained 70% initial activity at 80°C. Maximum protease production occurred at 40°C in a medium containing 20% NaCl (w/v) and 1% skim milk powder after 84 h in shaking culture. Enzyme secretion was observed at a broad pH range of 7.0–10.0.

In 2006, a novel haloalkaliphilic, thermostable serine protease was purified from the extreme halophilic archaeon, Halogeometricum borinquense strain TSS101 by Vidyasagar et al.,. The protease was isolated from a stationary phase culture and then purified by dialysis, chromatographic methods and obtained 116-fold with 18% yield purified enzyme and characterized biochemically.
In 2013, Nigam et al., isolated Alkaline proteases producing halophilic bacteria isolated from Sambar Lake, Rajasthan were investigated for keratinolytic activity. The aim of this study was to optimize culture conditions for maximum enzyme production as well as characterization of proteases with keratinolytic action. Casein was used as sole carbon and nitrogen source for alkaline protease production at 37°C and 150 rpm. Higher keratinolytic activity was obtained when the production medium was supplemented with chicken feathers at a concentration of 1g/L. Optimum pH and temperature for maximum activity was found to be 9.0 and 50°C respectively. The enzyme is stable at alkaline pH (8-9). It has high thermostability at 50°C and lost 25% of its keratinolytic activity after 8 h of incubation at 50°C.

Jothi Basu (2015) characterised a halophilic protease produced by an extremely halophilic bacteria Methylophaga sp., JS4 isolated from a hypersaline environment. The enzyme production by the strain JS4 was found to optimized at 40 °C, pH-7 (Neutral pH), after 72 hours of incubation at 150 rpm in the presence of 15% NaCl (W/V), Soya bean flour (Nitrogen source) and Sucrose (Carbon source).

3.11.3 Characterisation of Haloprotease

Mahnaaz Shahbazi et al., (2012) isolated an extracellular protease producing novel strain Salinivibrio sp. MS-7 from Maharlou salt lake (a hypersaline lake) in Iran. The enzyme revealed a monomeric structure with a relative molecular mass of 21KDa. The maximum caseinolytic activity of the enzyme was observed at 50°C, pH 8.0 and -0.5 M NaCl with s high tolerance to salt concentrations of upto 3M. The $K_m$ and $V_{max}$ values of MS-2 purified protease derived from Lineweaver-Burk plot was 1.14mg/ml and 7.24U respectively using casein as substrate. The activity increased rapidly above 30°C and maximum protease activity was found to be 50°C, followed by a thermal inactivation above 60°C. The protease was active in the pH range of 5-11 with an optimum at Ph 8.0. Among the metal ions tested, Ba$^{2+}$ and Ca$^{2+}$ ions were particularly effective in activating the enzyme causing 58% and 31% stimulation, respectively. The enzyme was very stable in the presence of high concentrations of NaCl (1 to 3M) after incubation for 60 min and the enzymes full activity remained in 3M NaCl.
3.12 Halobacillus sp

The genus Halobacillus, with the type species *Halobacillus halophilus*, was established and described by Spring and co-workers in 1996 (Claus et al., 1983; Spring et al., 1996). So far, nine species of this genus have been identified (Amoozegar et al., 2003a, b; Yoon et al., 2003, 2004, 2005; Liu et al., 2005). The increasing number of publications on bio-applications and other aspects of *Halobacillus* and the large number of 16S rRNA gene sequences deposited in databases for unidentified species reflect the wide distribution of these bacteria and their considerable scientific interest (Burja et al., 1999; Pinar et al., 2001; Yang et al., 2002; Rivadeneyra et al., 2004). *Halobacillus halophilus* is an aerobic, rod-shaped, motile and endospore-forming Gram-positive bacterium of the low GC branch (Claus et al., 1983; Spring et al., 1996). It is a moderate halophile that grows optimal between 0.5 and 2.0 M NaCl but even tolerates NaCl concentrations up to 3.0 M with a growth rate of 38% of its optimum, indicating effective mechanisms to respond to a broad range of salinities. Halobacillus was proposed with two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*, as well as *Sporosarcina halophila*, which was transferred to the genus as *Halobacillus halophilus* (Spring et al., 1996). Since then, no further Halobacillus species have been proposed. Jung-Hoon Yoon et al., (2003) isolated a strain named HSL-3T from a salt lake near Hwajinpo beach on the East Sea in Korea by the dilution-plating technique on marine agar 2216. Based on the biochemical characterisation and 16Sr RNA sequencing the strain HSL-3T was placed in the genus *Halobacillus* as a novel species, for which the name *Halobacillus salinus* sp. nov. was proposed.

*Halobacillus halophilus* also requires magnesium and sodium ions for growth and has the outstanding feature that growth is strictly chloride ion dependent (Roeßler and Müller, 1998).

The evolutionary success of a moderate halophile may depend on its ability to tolerate a wide range of salinities. It has been known for some time that *H. halophilus* accumulates compatible solutes to maintain its turgor in hyperosmotic conditions. The genes and pathways for the biosynthesis of the major solutes and their regulation have been identified in the emerging genome sequence. *Halobacillus halophilus* has an
interesting switch in its osmolyte strategy: at salinities ranging from 0.8 to 2 M NaCl, glutamine and glutamate are the predominant solutes whereas proline dominates at higher salinities (Saum and Müller, 2007). In addition, *H. halophilus* switches from proline to ectoine production at high salinities and stationary growth phase (Saum and Müller, 2008a). Furthermore, it produces *N*-acetyl-lysine and *N*-acetyl-ornithine in minor amounts and it can take up glycine betaine from the medium (Roeßler and Müller, 2001). Chloride is directly involved in regulation of compatible *Halobacillus halophilus* not only requires Na⁺ and Cl⁻ for growth but also Mg²⁺ in high amounts (50 mM for optimal growth) which is a rather unique feature. It is equipped with at least three different transport systems for Mg²⁺: a P-type ATPase (*Hbhal_1098*), one MgtE-type (*Hbhal_2330*) and one CorA-type transporter (*Hbhal_2746*). *Halobacillus halophilus* is a chemoorganoheterotrophic bacterium with great nutritional versatility. It is able to hydrolyse complex substrates such casein, gelatin, starch and pullulan (Claus *et al*., 1983) Namwong *et al.*, (2006) isolated a halophilic bacterium from fish sauce which was classified and named as Halobacillus sp. SR5-3. The ability of the isolate SR5-3 to secrete extracellular protease was detected by growing the organism in JCM no 377 medium incorporated with 1% skimmed milk powder and incubated at 37 °C for 7 days. The proteinase enzyme produced by the isolate SR5-3 was found to be a serine protease of molecular weight 43KDa and showed optimal activity at 50 °C and pH 9-10 in 20% NaCl.

Santos (2013) isolated a novel strain of Halobacillus named *Halobacillus blutaparonensis* strain M9, that was able to secrete extracellular proteases. The proteases secreted by *H. blutaparonensis* presented the following properties: (i) molecular masses ranging from 30 to 80 kDa, (ii) better hydrolytic activities under neutral-alkaline pH range, (iii) expression modulated according to the culture age, (iv) susceptibility to phenylmethylsulphonyl fluoride, classifying them as serine-type proteases, (v) specific cleavage over the chymotrypsin substrate, and (vi) enzymatic stability in the presence of salt (up to 20% NaCl) and organic solvents (e.g., ether, isoctane and cyclohexane).

### 3.13 Biopotential of Halophilic Protease

Natural and artificial substrates in the marine environment are quickly colonized by microfoulers and macrofoulers (Raikin, 2004). Both micro- and macrofouling in the world
Review of Literature

Oceans cause huge material and economic losses in maintenance of mariculture facilities, shipping facilities, vessels, and seawater pipelines (Fusetani, 2004). Over the past three decades, fouling on ships’ hulls has mainly been controlled by biocidal antifouling paints based on copper and tributyltin (TBT) (Townsin, 2003). The performance of these paints is usually boosted by the addition of co-biocides, particularly algicides. A global ban on the use of TBT from 2008 and the environmental impact of all current antifouling biocides is constantly under review. As a consequence, novel, environmentally benign methods to control biofouling are actively being sought (Yebra et al., 2004). It has been shown that bacterial chemical compounds inhibit larval settlement and may be used as ‘environmentally friendly’ antifoulants for protection against marine biofouling. A number of ‘candidate’ technologies, that are still experimental in nature are currently being investigated amongst which is the incorporation into coatings of enzymes, which are now commercially available. The rationale behind this approach is that enzymes of appropriate specificity may prevent biofouling by hydrolysing the various protein, glycoprotein and polysaccharide adhesive polymers produced by all fouling organisms during settlement, adhesion and colonization of surfaces in the marine environment. When focusing on hydrolysing organic compounds involved in marine biofilm, proteases in commercial enzymatic preparations seem to be good antibiofilm agent candidates due to their (i) availability (ii) biodegradability (iii) weak toxicity. They may inhibit the adhesion by degrading involved biofilm matrix molecules instead of killing marine micro and macroorganisms. In the natural environment microorganisms can easily biodegrade enzymes to amino acids and finally to carbon dioxide and water; therefore, it is generally accepted that enzymes are environment-friendly agents. Stability of enzymes in seawater and solvent-based paints limits commercial application of enzymes as antifouling agents. It has been shown that some enzymes (i.e., halophilic proteases) are quite stable in organic solvents (Klibanov, 2001).

3.13.1 Concept of Biofouling

Marine environment is the storehouse of all the aggressive corrosion factors along with wide variety of the marine micro- and macro organisms responsible for biofouling. Fouling is the undesirable accumulation of the deposits on the equipment surface that reduces its utility and service life. Biofouling, the colonization of submerged surfaces by
living organisms such as bacteria, algae, and invertebrates is a widespread global phenomenon and causes serious operational problems and huge economic losses every year. For inhibiting the fouling by marine organisms, earlier people were using plates of zinc, lead, copper etc. in wooden ship hulls, then mercury, lead, arsenic in coating as antifouling agent, and now a days organochemical compounds, copper compounds (Cu$_2$O and its salts) and organotin compounds as the most effective antifouling agent. Due to their toxic effects and negative impact on non target marine organisms, most of them have been banned and rests are under consideration. Mostly organotin antifouling paint belongs to the TBT group. Widely used antifouling TBT compounds are bis (tributyltin) oxide, tributyltin fluoride, organotin copolymers i.e. tributyl tin methacrylate and triphenyltin formulations. TBT can have a single active ingredient or two/more active ingredient or a combination of TBT and Cu compounds. But these TBT based antifouling coatings are responsible for some marine environmental problems due its toxic nature. As an example, it has been shown that extremely low concentrations of tributyltin moiety (TBT) cause defective shell growth in the oyster Crassostrea gigas (20 ng/l) and imposex, development of male characteristics in female genitalia, in the dog-whelk Nucella sp. (1 ng/l) (Swain, 1999). Malformations have been observed in many other species and the International Maritime Organization (IMO) also reports accumulation in mammals and debilitation of the immunological defences in fishes. These facts forced the development of national
regulations in countries all over the world. Most recently antifouling agents from marine organisms like coral, red algae, bacteria, bryozoans, sea grass, species of sea pansy etc and from their metabolites have been extracted. Synthetic fibers and hydrolytic enzymes, which are eco-friendly; with antifouling property; and its remedies have been reviewed.

3.13.2 Mechanism of Biofouling

Marine fouling is typically described as following four stages of ecosystem development. The chemistry of biofilm formation describes the initial steps prior to colonization. Within the first minute the van der Waals interaction causes the submerged surface to be covered with a conditioning film of organic polymers. In the next 24 hours, this layer allows the process of bacterial adhesion to occur, with both diatoms and bacteria (e.g. *Vibro alginolyticus*, *Pseudomonas putrefaciens*) attaching, initiating the formation of biofilm. By the end of the first week, the rich nutrients and ease of attachment in the biofilm allow secondary colonizers of spores of macro algae (e.g. *Enteromorpha intestinalis*, *ulothrix*) and protozoans (e.g. *Vorticella*, *Zoothmnium Sp.*) to attach themselves. Within 2 to 3 weeks the tertiary colonizers -the macro fouler have attached including tunicates, mollusks and sessile cnidarians.

Biofilm is a bacterial community which adheres onto biotic and abiotic surfaces and is embedded in a polymeric matrix composed mainly of polysaccharides, proteins, nucleic acids and mineral ions (Flemming & Wingender, 2001; Sutherland, 2001; Allison, 2003; Branda et al., 2005). Biofilm develops in many fields: health (nosocomial infection), industry (food industry, pulp and paper industry, textile industry, wastewater treatment), equipment in natural water (vessels, pipelines, harbour facilities, aquaculture equipment, marine sensors, cooling water system. Marine biofilms have an influence on marine macroorganisms where adhesion leads to fouling (Holmströrm et al., 1992; Wieczorek and Todd, 1998; Qian et al., 2003). The latter induces critical material biodamages and economic problems. To fight against fouling, one strategy is to control biofilm development, one of the first steps of fouling adhesion, by physical or chemical technology. Chemical techniques use biocide products such as metal compounds (tin, copper), oxidant compounds (chloride, bromide, ozone) or synthetic non oxidant compounds (algicide, bactericide, fungicide usually used in agriculture). It has been shown that antifouling paints with organostannic agents are very toxic for marine flora and fauna.
Review of Literature

Bioprospecting of haloprotease producing bacteria from Tuticorin salt pan and its application as antifouling agent

(Alzieu, 1998). Legislation on the use of biocides used is becoming more and more restrictive which control their use, especially by banishing organostannic compounds in antifouling paints. Therefore, it is essential today to look for environmentally friendly antibiofilm agents.

The biofilm matrix is mainly composed of water (97%) and extracellular polymeric substances (EPS) made up of polysaccharides, but also proteins and nucleic acids, lipids, mineral ions and various cellular debris (Sutherland, 2001). It fulfils different functions such as adhesive foundation, structural integrity, bacteria protection and intercellular communication (Allison 2003; Branda et al., 2005). When focusing on hydrolysing organic compounds involved in marine biofilm, hydrolases in commercial enzymatic preparations seem to be good antibiofilm agent candidates due to their (i) availability, some are already produced at an industrial scale; (ii) biodegradability, unlike to the long persistence of organometallic compounds; (iii) weak toxicity, contrary to oxidase enzymes. They may inhibit the adhesion by degrading involved biofilm matrix molecules instead of killing marine micro and macroorganisms.

3.13.3 Halophilic Protease as an Antifouling Agent

Biofouling in the marine environment is initiated by a conditioning film consisting of organic compounds followed by the initial colonizers – bacteria and microalgae – on which macroscopic algae and invertebrates settle and develop a complex community (Abarzua and Jakubowski, 1995). Traditional approaches to marine biofouling control through application of antifouling paints rely on the release of toxins that kill attaching organisms. Recent efforts have been directed towards developing environmentally friendly alternatives, which include modification of surface structure and chemistry to obtain non-stick surfaces, or replacing environmentally persistent toxins with naturally derived, degradable repellent compounds or enzymes (Yebra et al., 2004). Whereas traditional antifouling paints rely on cytotoxic effects, new environmentally safe alternatives aim to interfere with the adhesion and/or growth of the fouling organisms and biofouling research has two avenues namely:

1) Modification of surface structure and chemistry to obtain non-stick surfaces that minimize adhesion strength of the settling organisms(Holland et al., 2004; Ista et al., 2004; Yebra et al., 2004), and
2) Replacement of biocides with non-toxic alternatives (Armstrong et al., 2000; Krug, 2006) where have been proposed as a viable solution. (Pettitt et al., 2004; Poulsen and Kragh, 2002; Schneider and Allermann, 2003);

The idea of using enzymes for antifouling coatings reaches back as far as to 1983 and the concept has received increased interest in recent years (Dobretsov et al., 2007; Leroy et al., 2008; Olsen et al., 2007).

### 3.14 Antifouling

Anti-fouling is the process of removing or preventing these accumulations from forming. In industrial processes, bio-dispersants can be used to control biofouling. In less controlled environments, organisms are killed or repelled with coatings using biocides, thermal treatments or pulses of energy. Nontoxic mechanical strategies that prevent organisms from attaching include choosing a material or coating with a slippery surface, creation of an ultra-low fouling surface with the use of zwitterions, or creation of nanoscale surface topologies similar to the skin of sharks and dolphins which only offer poor anchor points. (Yebra et al., 2004)

A general idea of non-toxic coatings. (Coating represented here as light pea green layer.) They prevent proteins and microorganisms from attaching which prevents large organisms such as barnacles from attaching. Larger organisms require a biofilm to attach, which is composed of proteins, polysaccharides, and microorganisms.
Some organisms can secrete enzymes or metabolites to inhibit the growth of their competitors. These secretions have low-toxicity and are biodegradable, and have received much attention in recent years. Researchers have attempted to extract high concentrations of these secretions to use for biological antifouling (Abarzua et al., 1999). The application of enzymes as antifouling agents has been successfully investigated recently. Many types of enzymes, such as oxidoreductases, transferases, hydrolase, lyase, isomerase, and ligase, have been reported to have antifouling capabilities (Jakob et al., 2008, Dobretsov et al., 2007). From the perspective of enzymatic antifouling technology, biofouling problems are caused by the formation and reproduction of biofilms and the adhesion of spores and larvae of macroorganisms. Therefore, the functions of enzymes for antifouling applications can be divided into the following four categories: degradation of adhesives used for settlement, disruption of the biofilm matrix, generation of deterrents/biocides, and interference with intercellular communication.

3.14.1 Insilico analysis on Polyphosphate Kinase for Biofilm formation:

Polyphosphate kinase (PPK) in bacteria plays a crucial role in helping them adapt to stringent conditions of low nutritional availability thus making it a good target for antibacterials and antifungals. It is associated with pathogenicity, motility and drug resistance through quorum sensing, regulation of error-prone replication and biofilm formations (Rashid et al., 2000). In spite of this critical role, to best of our knowledge no in-silico work has been carried out to develop PPK as an antibiotic target. Study concentrated on virtual screening of PPK to carry out against all the potential molecule with pharmacological action present in the database. Screening results were further refined by interaction maps based on its scoring function. Resulting molecule from database has been found to have significant affinity towards PPK active binding site indicating its therapeutic relevance and functional relevance (Saurav et al., 2013).

3.14.2 Strategies for enzyme-based antifouling

The problems related to fouling are caused by one of two types of events. Either by the settlement of individual organisms or by the proliferation of settled organisms. The former relates to macrofouling larvae (e.g. barnacle cyprids to juvenile and adult barnacles) and spores (e.g. Ulva zoospores to plants), while the latter concerns
microfouling, e.g. bacteria and diatoms. Strategies to prevent biofouling can thus basically interfere with the first contact between organisms and surfaces or stop settled organisms from developing to problematic levels, although one strategy does not necessarily exclude the other. Enzymes may affect settlement and adhesion in four different ways. Firstly, they may attack the adhesive of settling organisms, thus preventing the settlement event. Secondly, enzymes may degrade the polymers in the biofilm matrix formed by proliferating, settled organisms. Thirdly, enzymes may catalyse the release of antifouling compounds from the surface. These compounds may be non-toxic, but since the compounds are produced in-situ, they can be much less stable than conventional biocides, which should eliminate the bioaccumulation of harmful chemicals. Finally, the intercellular communication during colonization of a surface may be obstructed by specific enzymes.

Proteins quantitatively constitute as important part of the biofilm matrix as do polysaccharides, and they may be as important for the chemical architecture. Similar to the prevention of adhesion, proteases can therefore be very efficient in breaking down the matrix, which has been observed for a strain of Pseudoalteromonas (Leroy et al., 2008).

Halophilic archaea produce proteases with unique property such as stability at saturating concentrations of NaCl which can have novel applications (Margesin and Schinner, 2001). Some recent applications of these enzymes can be in antifouling coating preparations and peptide synthesis in organic solvents. Antifouling coatings are used to control biofouling caused by the adhesion of organisms such as bacteria, algae and barnacles to any surface in marine environment. The recent legislature of the International Maritime Organization imposing a complete ban on the use of tin based antifouling paints by 2008 has led to the necessity of evolving environmental friendly antifouling formulations of easily biodegradable material (Kanthak and Bernstorff, 1999). A patent in 2005 has reported the application of halophilic proteases, which being non-toxic and effective at low concentrations compared to conventional tri-n-butyl tin (TBT), form a potential substitute. Halo archaeal proteases thus can be a better alternative for use in antifouling coatings provided they show solvent tolerance. Ruiz and De Castro (2007) have recently reported organic solvent tolerant protease from haloalkaliphilic archaea Natrialba magadii.,
Dobretsov et al., (2007) have reported that protease and trypsin individually incorporated in water soluble paint matrix inhibited biofouling in field experiment. A comparison between bacterial biofilms inducing and inhibiting, respectively, bryozoan larval settlement showed that the settlement inducing biofilms did not produce proteolytic enzymes, while the majority of the settlement inhibiting biofilms did express proteases. It has been shown that bacterial strains can be cultivated in large amounts and produce diverse chemical compounds under optimal conditions which inhibit larval settlement and may be used as environmentally friendly antifoulants for protection against marine biofouling. All these factors make bacteria an important source of antifouling compounds. In the natural environment microorganisms can easily biodegradable enzymes to aminoacids and finally to carbon dioxide and water, therefore it is generally accepted that enzymes are environment friendly agents. Stability of enzymes in seawater and solvent based paints limits commercial application of enzyme as antifouling agents. Dobretsov et al., 2007 reported that protease from P. issachenkonni remained active in seawater for 14 days at high salt concentration of about 2M and in acetone. In addition the enzyme has an optimum temperature (about 30ºC) and pH (about 8) close to those observed at tropical waters. All these facts suggest that the protease from P. issachenkonni can be widely used for seawater antifouling applications.

Halophilic proteases are non-toxic and can serve as effective alternative for such applications compared to tin and copper. One of the major advantages of halophilic proteases, they are used as an antifouling coatings as economic, nontoxic and effective alternative to traditional chemical. High salt concentration reduces water activity, feature common with organic solvents which facilitates halophilic protease to tolerate organic solvents. There are several advantages that apply to biocatalyst in organic media: higher solubility of hydrophobic species reduced microbial contamination and reduced water activity which alters the hydrolytic equilibrium. This condition if often used for peptide synthesis using proteases as catalysts. However, one disadvantages of using organic solvents in biocatalysts is that enzymes are easily inactivated. As halophilic extremozymes are best suited to function under harsh conditions, they offer the possibility of extending the activity of biocatalysts.