

## Review

# Diversity and industrial potential of hydrolase-producing halophilic/halotolerant eubacteria

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Accepted 21 December, 2009

**Halophilic and halotolerant eubacteria have been isolated from different marine and hypersaline environments. Halophilic eubacteria also occur in environments typified by more than one soda lakes which are both hypersaline and extremely alkaline. These organisms have been shown to produce a wide array of hydrolytic enzymes including proteases, amylases, xylanases, cellulases as well as lipases and DNases. These enzymes are commonly applied in the production of fermented food and food supplements, in animal feed, laundry detergents and textile industries. Several studies have shown that enzymes derived from halophilic and halotolerant eubacteria are not only halostable but may also be thermostable and alkalistable. This extremophilicity make the enzymes suitable candidates in various fields of biotechnology and may even open up new application opportunities.**

**Key words:** Halophilic/halotolerant, Hydrolases, biodiversity, alkaliphilic, proteases, amylases, xylanases, cellulases.

## INTRODUCTION

Hypersaline environments are widely distributed on the earth's continent where they exist either as natural water bodies such as permanent saline lakes, ephemeral salt pans and salt marshes, or as artificial solar salterns which comprise a series of interconnecting evaporation and crystallizer ponds constructed for the production of salt, potash and soda. Hypersaline environments can be divided into two broad categories. These are the thalassohaline and athalassohaline. Thalassohaline water bodies have similar salt composition to seawater with sodium and chloride being the dominant ions; common examples include the Great Salt Lake in Utah, playas, brine springs from underground salt deposits and solar salterns (Litchfield and Gillevet, 2002). In contrast, athalassohaline water bodies such as the Dead sea, Lake Magadi in Kenya, Wadi Natrun in Egypt, the soda lakes of Antarctica and Big Soda Lake and Mono Lake in California are dominated by potassium, magnesium, or sodium (Oren, 2002; Litchfield and Gillevet, 2002). Hypersaline water bodies are commonly 9-10 times more concentrated than sea water, which is generally defined as having 3.5% (w/v) dissolved salts. Both natural and artificial hypersaline environments harbour remarkably high and diverse microbial cell densities. Microorganisms that thrive in these environments have been broadly

classified into halophilic microorganism (that is, require salt for their viability) and halotolerant microorganisms which are able to grow in the absence as well as in the presence of NaCl. Halophiles can be further divided into slight halophiles that grow optimally in 3% (w/v) total salt, moderate halophiles [optimal growth at 3 - 15% (w/v) salt] and extreme halophiles that grow optimally at 25% (w/v) salt (Ventosa et al., 1998). Eubacteria are mainly represented within the halotolerant, slight halophiles and moderate halophiles, with only a few genera been shown to be extremely halophilic. To adapt to saline conditions, these bacteria have developed various strategies to maintain cell structure and function. These include the accumulation of osmolytes such as ectoine and hydroxyectoine, glycine and betaine (Vargas et al., 2008). While most research performed on hypersaline environments has focused on the microbial diversity and ecology of these environments, there is growing interest in the extracellular hydrolytic enzymes from moderately halophilic bacteria. Most halophilic hydrolase producers have been assigned to the family Halomonadaceae and were shown to produce industrially relevant enzymes such as cellulases, amylases, xylanases, proteases and lipases (Sánchez-Porro et al., 2003a; Govender et al., 2009; Rohban et al., 2009). It is generally believed that

while these halophilic enzymes perform the same enzyme function as their non-halophilic counterparts, they can catalyse such reactions under different conditions, such as high salt environments. In addition, some of the enzymes derived from bacterial strains that were isolated from soda lakes and solar salterns originating from athalassohaline environments could display polyextremophilicity due to their adaptation to high salt and alkaline pH typical of soda lakes. Consequently, these bacteria would be an excellent source of enzymes that exhibit optimal activities at various ranges of salt concentrations and pH.

### ECOLOGICAL DISTRIBUTION OF HYDROLASE-PRODUCING 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-independent 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 (Nordberg 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*, *Chromohalobacter* and *Halomonas* (Sánchez-Porro et al., 2003b; Rohban et al., 2009). Amylases, lipases, proteases, xyla-

nases and cellulases are widely distributed amongst halophilic bacteria. While this could be unexpected, it is understandable since most natural and artificial hypersaline environments are open system with an influx and presence of plant and animal matter at any given time. Consequently, the microbial population in these environments can be expected to harbour the machinery to exploit the nutrient resources present in their surroundings.

### OSMOADAPTATION IN HALOPHILIC BACTERIA

Hypersaline environments are characterized by high but variable osmotic strength and microorganisms present in these environments must be able to adapt to the changes in osmolarity. Most halophilic and halotolerant bacteria maintain viability in these environments by accumulating low-molecular weight water-soluble organic compounds commonly referred to as compatible solutes to counteract the deleterious effect of high salinity on cell physiology and loss of cell water (Louis and Galinski, 1997; Cánovas et al., 1998; Bursy et al., 2008). Both Gram-positive (e.g. *Marinococcus halophilus*, *Streptomyces coelicolor*, *Nesterenkonia halobia*) and Gram-negative bacteria (e.g. *Halomonas elongata*, *Chromohalobacter salexigenes*) are known to accumulate ectoines as the predominant class of osmolytes while other compounds such as glycine and betaine are only accumulated in small amounts (Louis and Galinski, 1997). These compounds are synthesized *de novo* or may be taken up from the external environment and they can be amassed by the cell in very high concentrations to provide osmotic balance without affecting essential cellular functions (Vargas et al., 2008; Bursy et al., 2008). Several studies have shown that osmolytes such as ectoines may serve as general stress protectants as they are produced both in response to salt and heat stresses (Bursy et al., 2008; Vargas et al., 2008). The ectoine synthesis pathway has been extensively studied and the ectoine synthesis gene cluster (*ectABC*) was found to be highly conserved among the ectoine-producing bacteria (Calderón et al., 2004; Vargas et al., 2008). The genes *ectA*, *ectB* and *ectC* encode the enzymes diaminobutyric acid acetyl transferase, diaminobutyric acid transaminase and ectoine synthase, respectively, which, altogether constitute the ectoine biosynthetic pathway (Calderón et al., 2004; Kuhlmann and Bermer, 2002; Vargas et al., 2008). While ectoine is generally thought to serve as an osmoprotectant, it has also been reported to play a critical role in stabilizing proteins and supporting correct folding of polypeptides under denaturing conditions (Bursy et al., 2008).

### 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 polyextremophilic. 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.

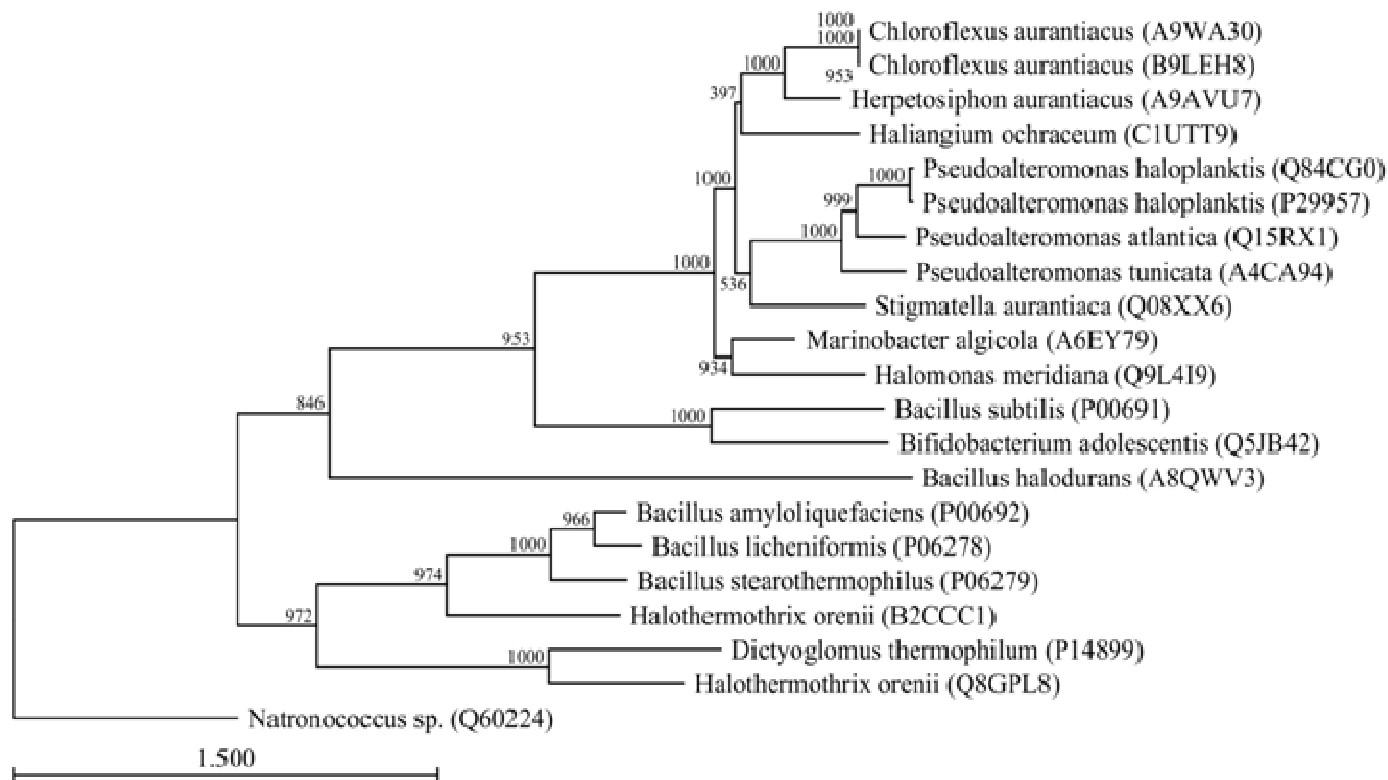
### $\alpha$ -Amylases

Amylases are a class of hydrolases which catalyse the degradation of starch polymers to produce dextrans and different gluco-oligosaccharides of variable lengths. Amylases are widely employed in different biotechnological applications including the food industry where they are used extensively in bread and baking industry to improve the volume of dough, colour and crumb softness. Amylases are also applied in detergents to promote stain removal and are utilised in the paper and pulp industry for the modification of starches for coated paper (Gupta et al., 2003). Halophilic amylases, commonly cyclomalto-dextrinases (EC: 3.2.1.54), have been produced from bacteria such as *Micrococcus halobius* (Onishi and Sonoda, 1979), *Halomonas meridiana* (Coronado et al., 2000a), *Halobacillus* sp. (Amoozegar et al., 2003), *Halothermothrix orenii* (Mitjs and Patel, 2002; Tan et al., 2008), *Streptomyces* sp. (Chakraborty et al., 2009) as well as *Chromohalobacter* sp. (Prakash et al., 2009a). These enzymes generally display broad pH optima and stability and they remain active at temperatures above 50°C. For instance, the amylase from *Halobacillus* and *Chromohalobacter* species were found to be stable at pH 7 – 10. Some of the enzymes such as the *Chromohalobacter* amylase maintain their stability in the presence and absence of NaCl. Halophilic amylases display molecular weights ranging between 50-75 kDa. The stability of these enzymes at extremes of pH and NaCl, as well as their ability to function optimally at elevated temperatures make them attractive candidates for hydrolysis of starch in industrial processes which are commonly performed at low water activity such as the production of syrups and also in the treatment of saline water or waste water solutions containing starch residues in the presence of high salt (Margesin and Schinner, 2001). In addition, some of the halophilic enzymes such as the amylase from a marine *Streptomyces* sp. remain stable in the presence of commercial detergents and would therefore, be attractive additives in laundry detergents (Chakraborty et al., 2009). Currently, only a few halophilic amylase encoding genes have been sequenced. Phylogenetic analysis shows that the amylase from the moderate halophile *H. meridiana* clusters together with amylases from marine bacteria in a

distinct clade away from other extremophilic amylases (Figure 1). The enzyme was reported to display 55 and 53% identity to the amylases from *Pseudoalteromonas haloplanktis* and *Aeromonas hydrophila*, respectively (Coronado et al., 2000b). In contrast, the amylase from the thermophilic, moderately halophilic anaerobic bacterium *H. orenii* display high homology with thermophilic amylases from *Dictyoglomus thermophilum* and *Bacillus* species, although it has narrow substrate specificity as it does not hydrolyse substrates such as pullulan and cyclodextrins (Mitjs and Patel, 2002). The *H. orenii* amylase also lacks transferase activity which means that it can perform the same catalytic reactions as the thermophilic amylases but will most probably generate a different range of products (Tan et al., 2008).

### Proteases

Microbial proteases are one of the most extensively studied enzymes and they are widely applied in industrial processes. They are commonly used as additives in laundry detergents, food processing, pharmaceuticals, leather and diagnostic reagents, waste management as well as silver recovery (Amoozegar et al., 2007; Karbalaee-Heidari et al., 2009). Halophilic proteases have been isolated and characterized from several bacterial species including *Bacillus* sp. (Kamekura and Onishi, 1974; Kumar et al., 2004; Setyorini et al., 2006; Shivanand and Jayaraman, 2009), *Pseudoaltermonas* sp. (Sanchez-Porro et al., 2003b), *Salinivobrio* sp. (Amoozegar et al., 2007), *Salicola* sp. (Moreno et al., 2009), *Halobacillus* spp. (Namwong et al., 2006; Karbalaee-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 soy sauce (Setyorini et al., 2006). Moreover, the enzymes derived from halophiles make excellent additives for



**Figure 1.** Neighbour-joining phylogenetic tree inferred from alignment of  $\alpha$ -amylase protein sequences of selected extremophilic bacteria. The protein sequence of the haloalkaliphilic archaeon *Natronococcus amylolyticus* was used as the outgroup. Numbers at the nodes indicate the level of bootstrap support on 1000 resamplings. The numbers in parentheses are accession numbers.

laundry detergent as most of them are either alkalitolerant 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).

### Xylanases

Xylanases play a pivotal role in the degradation of xylan. They are widely used in the baking industry to improve the properties of dough, and also for the past two decades the potential use of xylanases in biobleaching of paper and pulp has been growing perpetually (Mamo et al., 2009). However, efficient application of xylanases in biobleaching requires them to be alkaliphilic and thermotolerant. Halophilic organisms are the most likely source of enzymes with such properties although research in this arena is currently limited. Only a few halophilic/halotolerant xylanases have been described. They include enzymes derived from marine and hypersaline bacteria such as *Glaciecola mesophila* (Guo et al., 2009) and *Chromohalobacter* sp. (Prakash et al., 2009) and *Nesterenkonia* sp. (Govender et al., 2009). Some of these enzymes display stability at wide pH (6-11), remain active at temperatures above 60°C and may display an

absolute requirement for NaCl (Wejse et al., 2003; Guo et al., 2009; Prakash et al., 2009).

Cellulases are mainly applied in textile industry for biopolishing of fabrics and production of stonewashed denims, as well as in laundry detergents for fabric softening and brightening (Aygan and Arikan, 2008). Interest in cellulases is also increasing in the production of bio-ethanol as the enzymes are used to hydrolyse pretreated cellulosic materials to fermentable sugars (Wang et al., 2009). Currently, halophilic and halotolerant cellulases derived from *Bacillus* sp. (Aygan et al., 2008), *Salinivibrio* sp. (Wang et al., 2009) and metagenome library (Voget et al., 2006) have been characterized. The enzymes were reported to be thermostable, halostable and alkalostable, making them ideal candidates for various industrial applications.

### ENZYME HALOPHILISM

The enzymes described above are typically secreted into the extracellular environment throughout the growth cycle of halophilic bacteria in the presence of suitable substrates which would act as indirect inducers of respective genes. It can therefore, be expected and has been proven that these enzymes are adapted to

functioning under high NaCl concentrations. Halophilic enzymes remain highly stable under these conditions while most non-halophilic enzymes often aggregate and become non-functional. It is generally believed that this stability is governed by the high acidic amino acid content of halophilic enzymes (Ventosa et al., 1998). Acidic amino acid residues have a high water binding capacity when they are deprotonated and can thus form a solvation shell on the surface of the proteins and keep them hydrated under high salt conditions (Mamo et al., 2009). The common characteristics of halophilic enzymes include: (i) optimal activity at high NaCl concentrations, (ii) higher reversibility to denaturing stresses, (iii) higher stability in the presence of NaCl and (iv) slow mobility on SDS-PAGE due to lower SDS binding (Tokunaga et al., 2008).

## CONCLUSION AND FUTURE PROSPECTS

Halophilic and halotolerant bacteria secrete a wide range of hydrolytic enzymes into their surrounding environment. Several of these enzymes which include amylases, proteases, xylanases and cellulases display polyextremophilic properties. They are generally haloalkaliphilic and thermotolerant which renders them amenable to an array of industrial processes, normally performed at extreme conditions of temperature and pH. However, only a limited number of these enzymes have been well characterized and only a few of them are exploited commercially, mainly because research has largely focused on microbial diversity in hypersaline environments rather than the industrial potential of halophiles. In order to fully reap the benefits of newly described bacterial species, it is necessary to understand their metabolic and physiological properties. This will allow the generation of valuable information and the definition of the repertoire of extreme enzymes that has the potential to open new biotechnological applications. Therefore, it is necessary to expedite research on the sequence analyses, expression and characterization of halophilic enzymes so that the potential of these enzymes for industrial applications can be explored.

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