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HALOPHILIC ARCHAEA AND BACTERIA AS A SOURCE OF EXTRACELLULAR HYDROLYTIC ENZYMES

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1. Introduction

Halophiles constitute an important group of extremophilic microorganisms adapted to live and thrive in diverse hypersaline niches such as solar salterns, brines, hypersaline soils and lakes, as well as in salty foods (Horikoshi and Grant, 1998). Thanks to the application of novel phylogenetic and taxonomic approaches, the number of recognized species of halophilic microorganisms has increased tremendously during the past years (Kamekura, 1998; Madigan and Oren, 1999; Oren, 2002). The predominant groups of halophilic microorganisms inhabiting these hypersaline environments are moderately halophilic and extremely halophilic Bacteria and Archaea. The extreme halophiles show optimal growth in media containing 15 to 30% NaCl and are represented mainly by the halophilic aerobic Archaea or halobacteria (Grant et al., 2001), although some extremely halophilic Bacteria have been described as well, including some phototrophic species and the novel bacterium *Salinibacter ruber* (Antón et al., 2002). Moderately halophilic bacteria constitute a heterogeneous physiological group including a variety of Gram-positive and Gram-negative bacteria, which grow optimally in media containing 3 to 15% NaCl, although they can also grow above and below this range of salt concentrations (Ventosa et al., 1998).

Halophiles have developed two different adaptive strategies to cope with the osmotic pressure induced by the high NaCl concentration of the medium (Madigan and Oren, 1999; Oren, 2002). The halobacteria and some extremely halophilic Bacteria accumulate inorganic ions in the cytoplasm (K^+, Na^+, Cl^-) to balance the osmotic pressure of the medium, and they have developed specific proteins that are stable and active in the presence of salts. In contrast, moderate halophiles accumulate in the cytoplasm high amounts of specific organic osmolytes, which function as osmoprotectants, providing osmotic balance without interfering with the normal metabolism of the cell (Nieto and Vargas, 2002).

During the recent years, halophilic microorganisms have been explored for their biotechnological potential in different fields (Mellado and Ventosa, 2003). The applications range from the use of different products such as compatible solutes, biopolymers or carotenoids in a variety of industries or the use of these microorganisms in environmental bioremediation processes. Besides, being intrinsically stable and active at high salt concentrations, halophilic enzymes offer important potential

biotechnological applications, such as food processing, environmental bioremediation and biosynthetic processes.

To understand the molecular adaptation to extreme salinity of these enzymes is essential for the development of novel and more efficient protein engineering strategies. These molecular mechanisms have been most extensively studied in enzymes isolated from extremely halophilic Archaea (Madern et al., 2000; Mevarech et al., 2000). However, the enzymes from moderate halophiles present also a great biotechnological interest, since they are stable without salt and active in the presence of high salt concentrations (Ventosa et al., 1998).

The determination of the 3-D structure of some proteins from extreme halophiles has allowed the performance of comparative analyses between halophilic proteins and their non-halophilic homologs in order to understand the relationships between structure and particular biochemical and biophysical properties (Bieger et al., 2003; Dym et al., 1995; Frolow et al., 1996; Pieper et al., 1998; Richard et al., 2000; Yamada et al., 2002). Halophilic proteins, in general, present a significant excess of acidic amino acid residues over basic residues located mainly at the protein surface (Christian and Waltho, 1962; Danson and Hough, 1997; Ginzburg et al., 1970). These surface-exposed acidic residues have been proposed to help in organizing the solvation shell which surrounds the protein (Fukuchi et al., 2003).

The interest in these salt-adapted enzymes has increased considerably in the last few years, and a variety of extracellular enzymes from halophilic microorganisms have been isolated and characterized, aiming at expansion of their commercial applications. Moreover, the application of novel approaches to search for enzymes from natural environments without first isolating the organisms producing them is opening a new perspective for discovering industrial enzymes from hypersaline sources (Rees et al., 2003a, 2003b; Streit and Schmitz, 2004).

In this review, we will describe the properties of a number of these extracellular enzymes produced by halophilic microorganisms, and we will consider some aspects related to their potential biotechnological applications.

2. Halobacteria

Halobacteria (haloarchaea) constitute a well-defined group of Archaea. The main peculiarity of these microoorganisms is their ability to grow in hypersaline environments (>10% NaCl). They play an important role in the ecology of such extreme environments, representing and excellent example of adaptation to habitats with high salinities.

Halobacteria are included in the family Halobacteriaceae and at present are represented by more than 60 species grouped in 18 different genera: *Halobacterium*, *Haloarcula*, *Halobaculum*, *Halobiforma*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halomicrobium*, *Halorhabdus*, *Halorubrum*, *Halosimplex*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas* and *Natronorubrum*.

From the non-halophilic group of Archaea, several extracellular enzymes have been isolated and characterized. However, due to the fact that the common protein separation methods are ineffective at molar salt concentrations, extracellular hydrolases from halophilic Archaea have been less investigated and not many have been purified,

characterized and their genes cloned. These enzymes function under extremely high salts conditions, and they often lose their activities at low ionic strength (Madern et al., 2000). The purification methods of halophilic proteases produced by halobacteria were reviewed by Kamekura (1995).

Several early studies demonstrated the production of extracellular enzymes by halobacteria, such as proteases (Norberg and Hofsten, 1969) or lipases (Gonzalez and Gutierrez, 1970). Good and Hartman (1970) described the properties of an amylase produced by *Halobacterium salinarum* (*halobium*). The optimal activity of the enzyme was determined at 1% NaCl, but it remained active up to 23% NaCl. Hydrolysis products from amylose were maltose, maltotriose and glucose. However, further studies concerning the purification of this enzyme were not carried out.

The first extracellular hydrolase that was purified from an extremely halophilic archaeon, *Halobacterium salinarum* (*halobium*), was a serine protease. The protein was characterized by the irreversible loss of activity at NaCl concentrations lower than 2 M. This protease consists of one polypeptide chain with a molecular mass of 41 kDa and characteristically enriched in the acidic amino acids aspartic acid and glutamic acid (Izotova et al., 1983).

Another early study was carried out by Oren (1983) who described an extracellular thermophilic amyloglucosidase that degraded starch to glucose, produced by *Halorubrum sodomense*, an archaeon isolated from the Dead Sea. This enzyme showed optimal activity at pH 7.5 and 65ºC in the presence of 1.4 M NaCl, and 75ºC in the presence of 3.9 M NaCl. Besides it required high salt concentrations for activity (at least 0.5 M NaCl and up to 4 M NaCl) (Oren, 1983). The enzyme was purified and it was determined that it was a dimer with two different subunits of 72 and 82 kDa, respectively (Chaga and Porath, 1993).

Of the three extracellular proteases produced by the archaeon *Natrialba asiatica* (formerly designated strain 172 P1), one was purified and studied in detail. This enzyme is a serine protease, named halolysin 172P1. The enzyme is a thermophilic and halophilic protease, showing optimal activity at 75-80ºC and 25% NaCl. Its optimal pH is 10.7. Its molecular mass was estimated as 44-460 kDa by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Kamekura and Seno, 1990). Further studies allowed the molecular cloning and sequencing of the gene encoding halolysin 172P1 (Kamekura et al., 1992). The deduced amino acid sequence showed that this halolysin consists of 411 amino acids and its molecular mass is 41,963 Da. The highest homology was found with a thermitase from *Thermoactinomyces vulgaris*. It is interesting to note that this halolysin has a long C-terminal extension of approximately 120 amino acids, which was not previously found in any other extracellular subtilisin type serine proteases. The construction of a shuttle vector permitted expression of the protease gene *hly* in another halophilic archaeon, *Haloferax volcanii* (Kamekura et al., 1992).

Species of the genus *Natronoccoccus* are haloalkaliphilic Archaea that require an alkaline pH as well as a high salt concentration for growth. Yu (1991) described the features of a protease produced by *Natronobacterium* sp. strain A2. The maximal activity of the purified enzyme was at pH 9.0, 50ºC and in the presence of 1 M NaCl. *Natronoccoccus* sp. strain Ah-36, lately named as *Natronococcus amylolyticus*, produced an extracellular amylase when grown in a medium containing starch. The addition of 0.1% glucose to the medium completely inhibited the production of the

enzyme (Kobayashi et al., 1992). This amylase was purified following several steps: ethanol precipitation, hydroxylapatite chromatography, hydrophobic chromatography and gel filtration using Sephacryl S-200. Its molecular mass, as estimated by SDS-PAGE, was 74 kDa. The amylase was active in the range of pH 6.0 to 8.6, with only 30% loss of activity at pH 10.5. The enzyme shows optimal activity at 55ºC and 2.5 M NaCl; no activity was detected below 1.0 M. The gene encoding this protein has been cloned; it is 1,512 bp long, and the encoded protein includes a signal peptide of 43 amino acids. The amylase produced by *Natronococcus* sp. shows 30% identical amino acids with other amylases. The gene has been expressed in *Haloferax volcanii* (Kobayashi et al., 1994).

A serine protease secreted by *Haloferax mediterranei* was studied by Stepanov and co-workers in 1992. Its activity increases linearly with NaCl concentrations over the range 2-5 M. The enzyme has a molecular mass of 41 kDa and a pI of 7.5. The N-terminal sequence revealed that it belongs to the subtilisin family. *Haloferax mediterranei* also produced the halolysin R4. The gene encoding this halophilic protein has been cloned and expressed in *Haloferax volcanii*. The deduced amino acid sequence (403 amino acids) showed the highest similarity to the halolysin 172P1 produced by *Natrialba asiatica*. It is remarkable that both halolysins have long C-terminal extensions of 117 and 123 amino acids. This region is essential for the proteinase activity, since the removal of this tail region from halolysin R4 abolished proteinase activity (Kamekura et al., 1996).

Ryu and co-workers (1994) isolated and partially purified an extracellular protease from the extreme halophile *Halobacterium salinarum* presenting a molecular mass of 66 kDa. A dramatic decrease of the enzyme activity was observed when the NaCl concentration was lowered from 4 to 0 M.

Studies of gene expression in haloarchaea have been greatly hindered by the lack of a convenient reporter gene. As a first step in the development of a reporter system for gene expression in halophilic Archaea, a β-galactosidase was purified from *Haloferax lucentense* (Holmes et al., 1997). The enzyme was optimally active at 4 M NaCl. It cleaves several β-galactosidase substrates such as ONP-Gal, X-Gal and lactulose. The entire β-galactosidase gene (designated *bgaH*) was cloned, corresponding to a protein of 74.6 kDa. This gene, *bgaH*, has been used as a reporter gene for promoter analyses in *Halobacterium salinarum* (Patenge et al., 2000).

An extracellular protease from *Natrialba magadii* was purified from stationary-phase cultures. The native molecular mass of the enzyme determined by gel filtration was 45 kDa. The enzyme was purified using ethanol precipitation, affinity chromatography (Bacitracin-Sepharose 4B) and gel filtration (Sephacryl S-200). The highest enzyme activity was obtained at 60ºC, but it was not stable under these conditions, so 45ºC was determined as the optimum temperature. Maximal protease activity was measured at concentrations of 1-1.5 M NaCl or KCl and at pH values between pH 8 and 10. This protease degraded large proteins such as gelatine and casein. The enzyme was activated by thiol-reducing agents such as DTT and 2-mercaptoethanol. However, it was inhibited by SDS, urea and guanidine-HCl (Giménez et al., 2000).

A preliminary characterization of secreted proteolytic enzymes occurring in the haloalkaliphile *Natronococcus occultus*, which lives in environments with 3-4 M NaCl and pH values of 10-11, was reported by Studdert and co-workers (1997). A serine

protease, designated EP, was purified from *N. occultus* stationary phase culture medium (328-fold, yield 19%), and characterized at the biochemical level (Studdert et al., 2001). The protease was purified using ethanol precipitation and a bacitracin-Sepharose 4B affinity column. The maximal azocaseinolytic activity was obtained at 1 M NaCl or KCl. The protease EP retained 100% of its activity for at least 7 days in 3 M NaCl or KCl at 4ºC; however, at concentrations lower than 0.5 M the enzyme loses its activity (Studdert et al., 2001).

An α-amylase produced by the halophilic archaeon *Haloferax mediterranei* has been purified and characterized (Pérez-Pomares et al., 2003). This microorganism is able to grow in a minimal medium containing ammonium acetate as carbon and nitrogen source. When the medium is enriched with starch, α-amylase is excreted. The enzyme is monomeric with a molecular mass of around 58 kDa, as determined by SDS-PAGE. The optimal salt concentration for activity was 3 M NaCl, and very close activities were also reached at higher salt concentrations. No activity was measured in the absence of salt; however, the presence of a NaCl concentration of 1 M was enough to observe activity. The stability increased at higher salt concentrations, reaching half-life times of 83 days at 4 M NaCl (Pérez-Pomares et al., 2003).

Two enzymes, β-xylanase and β-xylosidase, are produced when the extremely halophilic archaeon *Halorhabdus utahensis* was cultured aerobically at 30ºC. β-Xylanase activity was nearly equally stable at 0.05% NaCl and 27% NaCl at 30ºC, whereas β-xylosidase activity was equally stable at 0.5% NaCl and 25% NaCl (Wainø and Ingvorsen, 2003).

Table 1 summarizes the properties of the extracellular enzymes produced by halobacteria that have been studied and characterized in detail.

TABLE 1. Selected extracellular hydrolytic enzymes produced by extremely halophilic Archaea.

Enzyme	Microorganism	NaCl for optimal activity	Molecular mass (kDa)	Reference
Amylase	*Halobacterium salinarum*	1%	ND	Good and Hartman,1970
Serine protease	*Halobacterium salinarum*	-	ND	Izotova et al., 1983
Amyloglucosidase	*Halorubrum sodomense*	7.5%	72 82	Oren, 1983; Chaga and Porath, 1993
Serine protease	*Natrialba asiatica*	10-15%	41.9	Kamekura and Seno, 1990; Kamekura et al., 1992
Protease	*Natronobacterium* sp.	5,5%	ND	Yu, 1991
Amylase	*Natronococcus amylolyticus*	15%	74	Kobayashi et al., 1992, 1994
Protease	*Haloferax mediterranei*	-	41	Stepanov et al., 1992
Protease	*Halobacterium salinarum*	23%	ND	Ryu et al., 1994
β-galactosidase	*Haloferax lucentense*	23%	180	Holmes et al., 1997
Serine protease	*Natrialba magadii*	6-9%	45	Giménez et al., 2000
Serine protease	*Natronococcus occultus*	6%	130	Studdert et al., 2001
Amylase	*Haloferax mediterranei*	17%	58	Pérez-Pomares et al., 2003
β-Xylanase	*Halorhabdus utahensis*	5-15%	45	Wainø and Ingvorsen, 2003
β-Xylosidase	*Halorhabdus utahensis*	5%	67	Wainø and Ingvorsen, 2003

ND, not determined.

3. Moderately Halophilic Bacteria

Several authors have previously reviewed the production of extracellular enzymes by moderately halophilic bacteria (Kamekura, 1986; Mellado et al., 2004; Sánchez-Porro et al., 2004; Ventosa et al., 1998). In this review we will follow a chronological order and describe the main features of the currently reported extracellular hydrolases produced by moderately halophilic bacteria.

The pioneering studies on extracellular enzymes produced by moderately halophilic bacteria were carried out by Onishi and Kamekura in Japan. They were intrigued by the fact that enzymes from such bacteria showed activity in media with high salt concentrations and that most of them were inactive in the absence of salt (Kushner, 1968). During the early studies in the 1970s several extracellular enzymes were described, although they were not studied at the molecular level.

Onishi (1972) reported the production of a halophilic amylase by a moderately halophilic bacterium obtained from unrefined salt, designated as *Micrococcus* sp. 28-3 (ATCC 21727). A more detailed taxonomic study showed that this bacterium constituted a new species, and it was described as *Micrococcus halobius* (Onishi and Kamekura, 1972), and lately was transferred to a new genus as *Nesterenkonia halobia* (Stackebrandt et al., 1995). This organism was able to grow optimally in complex media with 1 to 3 M NaCl, but it did not grow without added NaCl. The highest amount of amylase was produced in media containing starch with 1 to 2 M NaCl after incubation for 2 days. Amylase production was markedly repressed by the addition of glucose. Maximal activity of the crude enzyme was observed at pH 6 to 7 in 1.4 to 2 M NaCl or KCl at 50ºC; in the absence of high concentrations of NaCl or KCl, the enzyme was denatured. It was depended on a divalent metal ion such as Ca^{2+} for activity and stability (Onishi, 1972). Several years later this amylase was purified from culture filtrate to an electrophoretically homogeneous state by glycogen-complex formation, diethylaminoethyl-cellulose chromatography and Bio-Gel P-200 gel filtration (Onishi and Sonoda, 1979). The purified enzyme was maximally active at pH 6 to 7 in 0.25 M NaCl or 0.75 M KCl at 50 to 55ºC. The activity was lost by dialysis against distilled water. The molecular mass of the amylase was estimated to be 89 kDa. Hydrolysis of amylose, soluble starch, and glycogen produced maltose, maltotriose and maltotetraose, with lesser amount of glucose (Onishi and Sonoda, 1979).

Another interesting early study was carried out by Kamekura and Onishi (1974a), who described the properties of a halophilic nuclease (nuclease H) produced by the moderately halophilic bacterium *Micrococcus varians* subsp. *halophilus*, isolated from soy sauce mash. Similarly to *Nesterenkonia halobia*, this organism was unable to grow without NaCl and grew optimally in media with 1 to 3 M NaCl. Maximum nuclease production was obtained between 2 and 4 M NaCl. It is interesting to note that this enzyme was the first hydrolase produced by a moderately halophilic bacterium that was purified biochemically. The enzyme showed both deoxyribonuclease and ribonuclease activities, but phosphomonoesterase and phosphodiesterase activities were not detected. The nuclease activity of the purified enzyme was maximal at 2.9 M NaCl or 2.1 M KCl, pH 8.0 and 43ºC. The enzyme was inactivated by dialysis against low salt buffer. The molecular mass of the enzyme was 99 kDa and it had a high excess of acidic amino acids over basic amino acids (Kamekura and Onishi, 1978a). The production of the extracellular nuclease was completely inhibited by addition of more than 40 mM

$MgSO_4$ to a complex medium with 2 M NaCl. This inhibition of enzyme production was accompanied by flocculation of the cells (Kamekura and Onishi, 1976), and the extracellular enzymes produced were fully adsorbed on the surface of the flocculated cells (Kamekura and Onishi, 1978b).

A simple medium for the commercial production of nuclease H in a fermentor has been described (Kamekura and Onishi, 1979). The medium is composed of 0.7% ammonium sulfate, 1% glucose, minerals, three vitamins, and 2 M NaCl at a pH of 7.5-8.0. Since this enzyme degraded RNA and DNA to produce 5´-mononucleotides exonucleolytically, it was suggested that it could be used for the production of 5'-guanylic acid (5´-GMP) and 5´-inosinic acid (5´-IMP), flavouring agents which are produced commercially by enzymatic degradation of RNA or direct fermentation. Kamekura et al. (1982) reported the production of 5´-GMP from RNA extracted from commercial dry yeast, by using supernatants from broth cultures of *M. varians* subsp. *halophilus*. Kamekura and Onishi (1983) examined the effects of anions and cations on nuclease H production and the stability of the enzyme in the presence of different salts. Based on these studies, a bioreactor with a column of flocculated cells of *M. varians* subsp. *halophilus* was designed for the production of 5´-nucleotides from RNA, based on immobilization of the nuclease H on the surface (Onishi et al., 1988, 1991).

A second extracellular enzyme produced by *M. varians* subsp. *halophilus*, a halophilic 5´-nucleotidase, has been partially purified (Onishi et al., 1984). This enzyme showed maximal activity at 2 M NaCl or 2.5 M KCl and 0.1 mM Co^{2+} or 0.1 mM Mn^{2+}. Finally, an amylase produced by this moderately halophilic bacterium has been purified and characterized (Kobayashi et al., 1986). The production of this amylase was highest in medium with 2 M NaCl with maltose as an inducer. The enzyme had two components with molecular masses of 86 and 60 kDa, and showed optimal activity at pH 6-7, 55ºC (in absence of $CaCl_2$) or 60ºC (with 50 mM $CaCl_2$) and 0.75 to 1 M NaCl or KCl (Kobayashi et al., 1986).

Other early studies focused on the production of a protease by the moderately halophilic *Bacillus* sp. no. 21-1 isolated from unrefined salt (Kamekura and Onishi, 1974b) and of an amylase produced by *Acinetobacter* sp. isolated from sea sand (Onishi and Hidaka, 1978). *Bacillus* sp. no. 21-1 grew optimally in media with 1-2 M NaCl, while the production of the enzyme was optimal at 1 M NaCl; addition of 2 M KCl markedly depressed protease formation. Maximal enzyme activity was obtained at 0.5 M NaCl and 0.75 M KCl, and almost no activity was observed at 3 M NaCl (Kamekura and Onishi, 1974b). No further studies concerning the purification and properties of this protease have been reported. Two different amylases were purified from the culture filtrate of *Acinetobacter* sp. to an electrophoretically homogeneous state by glycogen-complex formation, DEAE-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration. Both amylases showed maximal activity in 0.2 to 0.6 M NaCl or KCl, at pH 7.0 and 50-55ºC. The end products of starch hydrolysis were maltose and maltotriose (Onishi and Hidaka, 1978).

The first extracellular protease that was studied at the biochemical level was produced by an unidentified moderately halophilic bacterium, designated *Pseudomonas* sp. strain A-14. The purified enzyme had a molecular mass of 12,000 Da and showed optimal activity at 18% NaCl and pH 8.0 (Van Qua et al., 1981).

Onishi and coworkers (1983) reported the production of another extracellular nuclease by a moderate halophile, *Bacillus* sp. N23-2 (ATCC 49085), isolated from

rotting wood on the seashore in Nauru. This bacterium was lately classified as a new *Bacillus* species, *B. halophilus* (Ventosa et al., 1989). Nuclease was produced when cultivated aerobically in media containing 1 to 2 M NaCl. The enzyme was purified from the culture filtrate to an electrophoretically homogeneous state by ethanol precipitation, DEAE-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration. The purified enzyme consisted of two isomers of the same molecular mass (138 kDa), but with different charges, and showed both RNase and DNase activities. Its maximal activity was in the presence of 1.4 to 3.2 M NaCl or 2.3 to 3.2 M KCl. The activity was lost by dialysis against water and low-salt buffer, but it was protected when 10 mM Ca^{2+} was added to the dialysis buffer. Besides, the enzyme exhibited maximal activity at pH 8.5 and at 50ºC on DNA and at 60ºC on RNA. It attacked RNA and DNA exonucleolytically, producing 5´-mononucleotides (Onishi et al., 1983). This nuclease shows similar characteristics to the nuclease H produced by *Micrococcus varians* subsp. *halophilus* (Kamekura and Onishi, 1974a, 1978a), especially with respect to halophilic properties and the mode of action on RNA and DNA; however, the *B. halophilus* nuclease required 3.5 M NaCl or 10 mM Ca^{2+} for stability and Mg^{2+} and Ca^{2+} (10:1) for maximal activity, whereas the nuclease H produced by *M. varians* subsp. *halophilus* required 2 M NaCl, 10 mM Mg^{2+}, or Mn^{2+}, but not Ca^{2+}, for stability (Kamekura and Onishi, 1978a; Onishi et al., 1983).

Khire (1994) described the production of an halophilic amylase by *Micrococcus* sp. strain 4 that showed optimal activity at pH 7.5, 50ºC and 1 M NaCl.

Coronado and coworkers (2000a; 2000b) studied an extracellular amylase designated as amylase H, produced by *Halomonas meridiana* DSM 5425. Maximal amylase production was achieved toward the end of the exponential phase in a medium with 0.5% starch and 5% total salts, in the absence of glucose. Activity was optimal at 10% NaCl, pH 7.0 (being also relatively stable under alkaline conditions) and at 37ºC. However, considerable amylase activity was detected even at 30% salts. The amylase hydrolyzed starch to form maltose and maltotriose as major products; maltose was not hydrolyzed. These data indicated an α-amylase activity (Coronado et al., 2000a). The gene encoding this amylase, *amyH*, was cloned by functional complementation of a *Tn1732*-induced mutant deficient in extracellular amylase activity. It encodes a 457-amino acid residue protein with a deduced molecular mass of 50 kDa which shows high sequence similarity to α-amylases from Gram-positive and Gram-negative bacteria. Besides, the amyH protein contains the four regions highly conserved in amylases. *H. meridiana* amylase H is a very acidic protein, similar to many other enzymes produced by halophilic microorganisms (Coronado et al., 2000b).

The study of Coronado et al. (2000b) was the first to clone a gene encoding for an extracellular enzyme produced by a moderate halophile. The *amyH* gene was found to be functional in another moderately halophilic bacterium, *Halomonas elongata*, and, when cloned in a multicopy vector, also in *Escherichia coli.* This is thus the first enzyme from a moderately halophilic bacterium that has been cloned and expressed in a non-halophilic host, opening new possibilities from a biotechnological point of view. Coronado and coworkers also achieved the heterologous expression of the *Bacillus licheniformis* thermostable α-amylase gene in two moderately halophilic bacteria: *Halomonas meridiana* and *Halomonas elongata*. This indicated that the secretion machinery of both halophiles was able to recognize and properly cleave the signal

peptide of the *Bacillus* amylase, and to secrete the enzyme to the extracellular medium (Coronado et al., 2000b).

An interesting study has been reported on the cloning, sequencing and expression of an α-amylase gene, *amyA*, from the thermophilic, moderately halophilic, anaerobic bacterium *Halothermothrix orenii* (Mijts and Patel, 2002). The gene encoded a 515 residue protein composed of a 25 amino acid putative signal peptide and a 490 amino acid mature protein. Greatest homology was to the *Bacillus megaterium* group of α-amylases. The *amyA* gene was expressed in *E. coli* as a hexahistidine-tagged enzyme and was purified and biochemically characterized. The purified recombinant enzyme was optimally active in 5% NaCl (showing a significant activity up to 25% NaCl) at pH 7.5 and 65ºC. It required NaCl and $CaCl_2$ for optimum activity and thermostability. However, the relative high proportion of acidic amino acids typically observed for many enzymes from halophiles was absent in this enzyme (Mijts and Patel, 2002). This amylase was the first extracellular enzyme produced by a moderately halophilic bacterium that has been studied crystallographically. The recombinant amyA protein crystallizes in the orthorhombic space (Li et al., 2002). A second α-amylase was found to be produced by *H. orenii*, and its structure has been determined by X-ray crystallography (Tan et al., 2003). The amylase amyB is a 599-residue protein, and is active up to 10% NaCl. The purified recombinant amyB protein crystallizes in the monoclinic space (Tan et al., 2003).

Recently, another extracellular amylase has been described, produced by the moderately halophilic *Halobacillus* sp. strain MA-2, isolated from saline soil in Iran (Amoozegar et al., 2003a). This organism has been proposed as a new species and named *Halobacillus karajensis* (Amoozegar et al., 2003b). Maximum amylase production was exhibited on starch media when 15% Na_2SO_4 or 10% NaCl were added. Optimal amylase activity was obtained in medium containing 5% NaCl at pH 7.5-8.5 and 50ºC (Amoozegar et al., 2003a). Further characterization of this enzyme at the molecular level has not yet been reported.

In order to isolate moderately halophilic bacteria producing extracellular hydrolytic enzymes, a screening was carried out in different hypersaline environments in the south of Spain. A total of 122 moderately halophilic bacteria were isolated. In contrast to culture collection strains that belong to previously described species that show very low hydrolase activities, environmental isolates produced a great variety of hydrolases such as amylases, DNases, lipases, proteases and pullulanases. These strains were identified as members of the genera: *Salinivibrio*, *Halomonas*, *Chromohalobacter*, *Bacillus-Salibacillus*, *Salinicoccus* and *Marinococcus* (Sánchez-Porro et al., 2003a).

A protease, designated as protease CP1, produced by *Pseudoalteromonas ruthenica* CP76, isolated in the screening, has been purified and characterized, and the gene encoding the protein has been cloned and expressed. This enzyme showed optimal activity at 55ºC and pH 8.5, and tolerated a wide range of NaCl concentrations (0 to 4 M NaCl). The most interesting features of this enzyme are its moderate thermoactivity (optimal activity at 55ºC), its activity over a wide range of pH values (6-10), and, specially, its salt tolerance (optimal activity at 7.5% total salt). The protease was purified using Q-Sepharose column chromatography and Superdex S-200 gel filtration. The purified protease has a molecular mass of 38 kDa by SDS-PAGE (Sánchez-Porro et al., 2003b). The gene has been recently cloned by inverse PCR, and shows a 69%

similarity to metalloprotease I of *Alteromonas* sp. O-7 (Sánchez-Porro et al., unpublished results).

Another enzyme under study that was selected during the preliminary screening is an extracellular lipase, designated SL1, produced by an isolate that was classified as a new species of the genus *Marinobacter*, *M. lipolyticus* (Martín et al., 2003). Different methods of purification have been used in order to purify this enzyme. Best results were obtained using octyl gel as hydrophobic support, but purification is as yet incomplete. We have constructed a gene library that allowed us to isolate the gene encoding this enzyme. This gene shows high similarity with α/β-hydrolase genes (Martín et al., unpublished results). These two extracellular enzymes are still under study; the currently available data suggest that they could have future biotechnological applications.

Table 2 includes the features of the extracellular enzymes produced by moderately halophilic bacteria that have been investigated up to date.

TABLE 2. Extracellular enzymes produced by moderately halophilic microorganisms with potential biotechnological applications.

Enzyme	Microorganism	NaCl for optimal activity	Molecular mass (kDa)	References
Amylase	*Nesterenkonia halobia*	0.25 M	89	Onishi,1972; Onishi and Sonoda, 1979
Nuclease H	*Micrococcus varians* subsp. *halophilus*	2.9 M	99	Kamekura and Onishi, 1974a, 1978a
5´-Nucleotidase	*Micrococcus varians* subsp. *halophilus*	2 M	ND	Onishi et al., 1984
Amylase	*Micrococcus varians* subsp. *halophilus*	0.75-1 M	86 60	Kabayashi et al., 1986
Protease	*Bacillus* sp.	0.5 M	ND	Kamekura and Onishi, 1974b
Amylase	*Acinetobacter* sp.	0.2-0.6 M	55 65	Onishi and Hidaka, 1978
Protease	*Pseudomonas* sp.	18%	12	Van Qua et al., 1981
Nuclease	*Bacillus halophilus*	1.4-3.2 M	138	Onishi et al., 1983
Amylase	*Micrococcus* sp.	1 M	ND	Khire, 1994
Amylase H	*Halomonas meridiana*	10%	50	Coronado et al., 2000a, 2000b
Amylase A	*Halothermothrix orenii*	5%	ND	Mijts and Patel, 2002
Amylase	*Halobacillus karajensis*	5 %	ND	Amoozegar et al., 2003a
Serine protease	*Pseudoalteromonas ruthenica*	10%		Sánchez-Porro et al., 2003b
Lipase	*Marinobacter lipolyticus*	ND	ND	Martín et al., 2003; This study

ND, not determined.

4. Uncultured Microorganisms - Enzymes from Environmental DNA Libraries

A large fraction of the microorganisms present in the environment has not been cultured due to difficulties in enriching and isolating them, and these are not accessible for biotechnology or basic research. It is estimated that less than 1% of the prokaryotic species have been cultivated and characterized (Amann et al., 1995). A new technology

called "metagenomics" has been developed trying to overcome this bottleneck in the biotechnological industry. Metagenomics is a new field which tries to assess and exploit the complex genomes of microorganisms found in nature (Knietsch et al., 2003; Streit and Schmitz 2004). The technique is based on the isolation and purification of DNA directly from environmental samples, followed by the construction of DNA libraries in suitable cloning vectors and host strains (Streit and Schmitz, 2004). This approach has yielded an increasing number of DNA sequences which can be used for novel biotechnological and pharmaceutical applications. A variety of enzyme classes have been isolated from cloned environmental DNA including hydrolases such as lipases, proteases, amylases, cellulases and others; nevertheless the list of enzyme activities studied by this method is still rather small (Healy et al., 1995; Henne et al., 2000; Richardson et al., 2002). The classical methodology to isolate new enzymes by enrichment and screening of a wide variety of microorganisms for the desired activity is still currently widely used, although the biodiversity of an enrichment culture will not be as rich as the original environmental source because of the nonculturability of many organisms (Rees et al., 2003b).

The biomass that can be retrieved from environmental samples is generally low, and large amounts of samples are needed to obtain sufficient DNA for cloning (Béjà et al., 2000). *E. coli* is still the preferred host for cloning and expression of any metagenome-derived genes, although other Gram-negative host are being used as well. Recently, large clone libraries have been screened and hundreds of thousands of clones have been analyzed to detect active ones (Henne et al., 2000; Majernik et al., 2001). It is expected that in the future the number of genes identified by this technique will exceed the number of those identified by the classical approach.

Genomic DNA libraries were made from DNA isolated directly from the Kenyan soda lakes, Lake Elmenteita and Crater Lake. Crater Lake clones expressing a cellulase activity and Lake Elmentetia clones expressing a lipase/esterase activity were identified and sequenced (Rees et al., 2003a). More recently, two genomic DNA libraries were made from enrichments obtained from lake sediment and soda soil obtained from the extremely saline and alkaline lakes of the Wadi el Natrun in the Libyan Desert (Egypt). A novel cellulase activity was identified in this environment and characterized. The lake cellulase ORF encoded a protein of 1,118 amino acids. It showed a high similarity with other bacterial endoglucanases, being most similar to a glucanase from *Xanthomonas campestris*. The soda soil cellulase was most closely related to an endo-glucanase from *Fibrobacter succinogenes* (Grant et al., 2004). It is noteworthy that the enrichment cultures obtained in this study showed presence of a wide diversity of species, often most closely related to as yet uncultured microorganisms. This could demonstrate that DNA from enrichment cultures, as well as DNA extracted directly from environmental samples, can be a valuable source of novel enzymes and biologically active compounds that would be very important for future biotechnological applications (Grant et al., 2004).

5. Summary and Future Perspectives

In this chapter we reviewed the properties of a number of extracellular enzymes produced by extremely halophilic Archaea and moderately halophilic Bacteria. These

include amylases, proteases, nucleases, lipases, etc. Besides, new enzymes from genomic DNA obtained from environmental samples have also been reported. In contrast to other extremophilic microorganisms such as the thermophiles or hyperthermophiles, the number of studies on extracellular enzymes from halophiles is very small. Very few enzymes have been studied at the molecular level, and only two have been crystallized and studied by X-ray crystallography. In the future we must expect more extensive studies focused on the biochemical and molecular features of new enzymes produced by halophiles, as well as on other enzymes that have not been previously reported such as xylanases, cellulases or pullulanases that could show novel features with respect to those produced by non-halophilic microorganisms. The extensive data on the complete genomes of microorganisms will help to understand and compare the features of these enzymes.

One interesting aspect that requires special attention is the study of the molecular mechanisms that enable the adaptation of moderately halophilic microorganisms which are able to grow in hypersaline environments as well as in media with low salt concentrations and the adaptation of their enzymes to function in a wide range of salt concentrations. The biotechnological application of enzymes from halophiles would require an in-depth study concerning their features, activity and stability under extreme conditions. Further molecular studies should enable their cloning and expression in non-halophilic hosts. Our studies showed that genes encoding enzymes from moderately halophilic bacteria can be successfully cloned and expressed in *E. coli.* Besides, enzymes from other microorganisms have been also expressed in halophiles (Coronado et al., 2000b; Frillingos et al., 2000). One important aspect that will require extensive studies and that constitutes a bottleneck is the secretion of the enzymes to the extracellular medium at high rates as to warrant an important production level.

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