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REVIEW

Potential for industrial products from the halophilic *Archaea*

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Abstract The halophilic *Archaea* are a group of microorganisms that have not been extensively considered for biotechnological applications. This review describes some of the enzymes and products and the potential applications of this unique group of microorganisms to various industrial processes. Specifically, the characteristics of the glycosyl hydrolases, lipases and esterases, proteases, biopolymers and surfactants, as well as some miscellaneous other activities will be described.

Keywords Halophiles · *Archaea* · Proteases · Amylases · Lipases/esterases · PHA

Introduction

The domain *Archaea* was first suggested by Woese et al. [101, 102] as one of the three domains of life. Within this domain is a separate branch for the halophilic *Archaea*. The current list of genera, as of this writing in 2010, is given in Table 1 along with the accepted three-letter code [22]. The haloarchaea are characterized as able to grow from around 8% (1.5 M) sodium chloride to approximately 36% (5 M) NaCl, which is at saturation for NaCl. They are Gram-negative, cocci or rods that are often pleomorphic, and they have at least one plasmid. In addition, they contain, as do all members of this domain, diether glycerolipids rather than fatty acid ester-linked glycerolipids. Most of the organisms also contain carotenoid pigments, but some are nonpigmented such as *Natrialb asiatica*, while others are alkaliphilic and are found in the group of genera belonging to the Natrono group because they grow in soda lakes or other high sodium carbonate environments. In general, the haloarchaea are at the upper end of the mesophiles growing over the temperature range of 15°C to roughly 45–50°C with optima around 37°C, but many of their enzymes retain activity well above this range [29]. Most of the members of this family use complex amino acids as their carbon and nitrogen sources, but some like the genera *Haloquadratum* and *Halosimplex* require simple carbon sources such as pyruvate and NH_4^+ as the nitrogen source [12, 99, respectively]. They all maintain their osmotic balance by concentrating up to 5 M KCl in their cytoplasm.

Because of the extremely high salt concentrations under which the haloarchaea live, they are often exposed to high levels of sunlight especially in solar salt evaporation ponds, reduced water activity because of the high salt content, and often oligotrophic nutrient conditions. The haloarchaea have developed proteins that are higher in the acidic amino acids and lower in the basic amino acids. These proteins, which frequently require high salt concentrations for optimal activity, were reviewed by Mevarech et al. [62]. The unusual composition of the proteins has also led to the recognition that at least some of the enzymes produced by haloarchaea will function in organic solvents. As early as 1969, Lanyi and coworkers reported that the catalase of *Halobacterium cutirubrum* (now *Hbt. salinarum* [97]) tolerated aqueous solutions of ethylene glycol, glycerol, and dimethyl sulfoxide (DMSO) (at up to 2, 4.5, and 2–5 M, respectively) without NaCl or other salts being present [55]. More recently, Usami et al. [96] examined two new strains of *Haloarcula* as well as two known strains, *Har. vallismortis* and *Har. argentinensis*, for their responses to organic solvents. The cultures were grown in test tubes containing complex media and the solvents were overlaid

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Table 1 List of current genera in the class Halobacteria as of November 2010 along with the accepted three-letter code [20]

Genus	Genus
Haladaptus Hap.	*Halorubrum Hrr.*
Halalkalicoccus Hac.	*Halosarcina Hsc.*
Haloarcula Har.	*Halosimplex Hsx*
Halobacterium Hbt.	*Halostagnicola Hst.*
Halobaculum Hbl.	*Haloterrigena Htg.*
Halobiforma Hbf.	*Halovivax Hvx.*
Halococcus Hcc.	*Natrialba Nab.*
Haloferax Hfx.	*Natrinema Nnm.*
Halogeometricum Hgm.	*Natronobacterium Nbt.*
Halomicrobium Hmc.	*Natronococcus Ncc.*
Halopiger Hpg.	*Natronolimnobius Nlm.*
Haloplanus Hpm.	*Natronomonas Nmn.*
Haloquadratum Hqr.	*Natronorubrum Nrr.*
Halorhabdus Hrd.	*Halopelagius Hpl.*

on them. All four cultures could grow in the presence of n-decane, n-nonane, and hexylether when 17% NaCl was also present. The two new strains OHF-1 and OHF-2 and *Har. vallismortis* could also grow in isooctane and cyclooctane with 25–27% NaCl in the medium [96].

Beginning in 2000 with the publication of the sequence of *Halobacterium* sp. NRC-1 [66], the chromosomes of several of the haloarchaea have been sequenced and can be found in the National Center for Biotechnology Information database [65]. Additionally, plasmids from several of the genera have also been sequenced. This is important because the plasmids of the haloarchaea are frequently almost as large as the chromosome and hence contain the genes for major metabolic pathways.

An earlier review of the haloarchaea and biotechnological applications included microbes from both domains, *Archaea*, and *Bacteria*. Those authors summarized the possible uses in such diverse areas as wastewater treatment, agriculture, bioplastics, and the medical field [59]. Much has happened in the intervening years, so this review describes some of the enzymes and products and the potential applications of this unique group of microorganisms to various industrial processes. Specifically, the characteristics of the glycosyl hydrolases, lipases and esterases, proteases, biopolymers and surfactants, as well as some miscellaneous other activities are discussed.

Glycosyl hydrolases

The glycoside hydrolases are a large group of enzymes related to carbohydrate metabolism. Many of these enzymes are excreted by the cells into their environment. All of the enzymes start with the EC 3.2.1.x numbering system and encompass, among others, the amylases, pullulanases, dextranases, hyaluronidases, xylosidases, and cellulases. While the EC system is based on function, these various enzymes have also been classified on the basis of their amino acid similarities [32, 33]. The archaeal extracellular α-amylases are included in group 13 along with members of the domain *Bacteria* (http://www.expasy.hcuge.ch/cgi-bin/lists?glycoside.txt). The α-amylases are currently most important in the food and detergent industries.

The first report of an amylase from haloarchaea was in 1969 by Nachum [Nachum and Bartholomew, Bacteriol Proc, p 137, 1969] who studied the amylase from *Hbt.* sp. This enzyme required high NaCl concentrations for activity and the increased concentrations of salt raised the optimum temperature of the enzyme. The amylase was stable for 6 days in 5% NaCl at 25°C, 15% NaCl 40°C; and 25% NaCl 55°C with all activity lost at 70°C [Nachum and Bartholomew Bacteriol Proc, p 137, 1969].

The next year a different type of amylase was reported from *Hbt. salinarum (*reported as *Hbt. halobium*) [28]. The *Hbt. halobium* amylase had optimal activity over the pH range of 6.4–6.6, calcium ions were not required, and the activity was optimal in 0.05% NaCl when in 0.01 M *β*-glycerophosphate buffer, pH 7, and at 55°C. Rapid inactivation occurred above 55°C. Although all activity was lost when dialyzed against distilled water, unlike other amylases, 90% of the activity was restored upon the addition of 0.25% NaCl or KCl. Starch was hydrolyzed to maltotriose, maltose, and glucose.

The haloalkaliphilic archaeon *Natronococcus* sp. strain Ah-36 excreted an amylase that resulted in maltotriose formation from starch [50]. The molecular mass was 74 kDa with maximal activity at pH 8.7 and 55°C when 2.5 M NaCl was present. Similar to the earlier amylase [Nachum and Bartholomew, Bacteriol Proc, p 137, 1969], this enzyme lost all activity at lower salt concentrations, however KCl, RbCl, and CsCl could partially spare the NaCl requirement but they had to be at 4–5 M levels. As seen earlier, rapid inactivation occurred at temperatures above 60°C. The dicarboxylic acids and their amides plus glycine accounted for 42.5% of the amino acids in the purified enzyme [50]. The gene for the amylase was cloned and expressed in *Haloferax volcanii* [51]. It was noted that the amylase gene encoded 504 amino acid residues, which is greater than the number of amino acids in the enzyme noted above implying that it might be a preenzyme. Indeed, the authors found a 43 amino acid residue signal protein which, when cleaved between Ala-43 and Arg-44, resulted in an active amylase of 461 amino acid residues with a molecular mass of the expressed enzyme of 51.4 kDa [51].

The circumneutral haloarchaeon *Hfx. mediterranei* also secretes an α-amylase when grown on ammonium acetate as carbon and nitrogen sources [70]. This amylase has a molecular mass of about 58 kDa with maximal activity at 3 M

NaCl. The optimum temperature was between 50 and 60°C, but unlike the amylases described so far, this amylase retained 65% of the maximum activity at 80°C. The optimum pH was 7–8 and EDTA inhibited all activity which could not be recovered, thus this is a metalloenzyme. It was specific for the 1–4 bond and did not attack 1–6 bonds such as found in pullulan. These authors continued to investigate this bacterium, concentrating on the intracellular amylases [71]. They also found that *Hfx. mediterranei* produced three intracellular amylases, AMY 1, AMY2, and AMY3, but AMY2 was found both intracellularly and extracellularly and none of these was produced in the absence of starch, which indicates that they are not constitutively expressed enzymes. While they studied all three enzymes, they examined AMY2 in greater detail noting that it had a very high molecular mass for an amylase of 116 kDa, which led the authors to believe it was a dimer. The extracellular amylase differed from the intracellular AMY 2 in that it had a molecular mass of 70 kDa and hydrolyzed larger saccharides than the intracellular AMY2. Optimal activity for AMY2 was at 1–3 M NaCl and pH 7. It remained active when stored in 1–4 M NaCl, but, as with other amylases, dialysis with distilled water completely inactivated it. It is possible that the extracellular amylase was derived from AMY2 [71].

Because haloarchaea thrive in environments with low water activity, it is not surprising that many of their enzymes are functional in organic, hydrophobic solvents. Such was the case for the α-amylase of *Haloarcula* sp. Strain S-1. The optimal salt concentration, temperature, and pH were 4.3 M, 50°C, and 7.0, respectively. However, the enzyme was stable and active in benzene, toluene, and chloroform. This latter spared the need for NaCl resulting in 20% of the optimal activity at less than 2 M NaCl while there was less than 10% activity without the chloroform (Fig. 1) when grown in just 2 M NaCl. EDTA inhibited the enzymatic activity with or without chloroform, so again this is a metallo amylase with a molecular mass of 70 kDa. Hydrophilic solvents inhibited activity [25]. It was also found that reverse micelles can stabilize halophilic enzymes for up to 45 days and still retain activity [72].

The α-amylase from *Har. hispanica* was investigated by Hutcheon et al. [37]. These authors found that the enzyme was very sensitive to lower pH values with an optimum at pH 6–6.5. The optimum temperature was 50°C, and the optimum salt concentration was from 4 to 5 M NaCl. However, unlike several other α-amylases, this enzyme retained 30% of its activity with no NaCl present. The results were similar when calcium ions were omitted from the buffer. Also interesting was the requirement for NaCl for the denaturation of the enzyme by urea, which perhaps implies that there is a change in the configuration when salt is present that makes it more susceptible to denaturation. These authors proved that the TAT pathway, discussed in the next section, is used for excretion of this α-amylase [37].

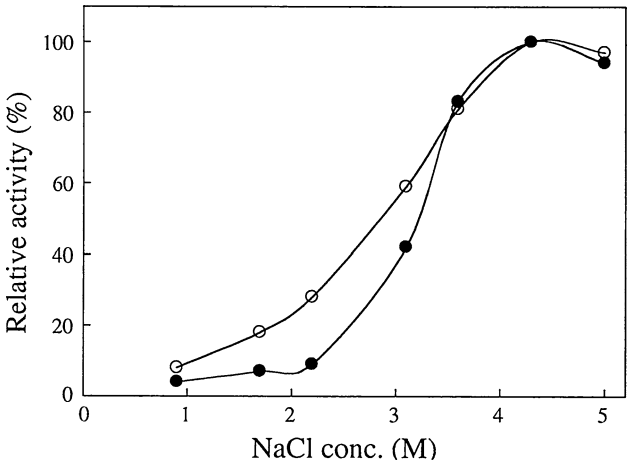

Fig. 1 Amylase activity of *Haloarcula* sp. Strain S-1 in the presence (*open circles*) and absence (*closed circles*) of chloroform at different salt concentrations [25]. Reprinted with kind permission of Elsevier

An interesting potential use of the α-amylases is in breadmaking. Rosell et al. [81] investigated the usual α-amylases and found that most of the fungal and bacterial enzymes were sensitive to the ingredients and additives. They did not look at the α-amylases of the haloarchaea. The α-amylases are important in preventing the retrogradation of amylopectin so breadmakers are interested in thermostable enzymes with a slightly acidic pH stability [81]. The mass production of haloarchaeal enzymes has been demonstrated by Bagai and Madamwar [4] who cross-linked *Hbt. salinarum* with 0.5% glutaraldehyde for stability and then encased the cells in calcium alginate beads. The culture continued to produce α-amylase for 45 days. Thus, the haloarchaeal α-amylases should prove applicable to the food industry.

The xylanases, EC 3.2.1.8 for the endo-β-xylanase and EC 3.2.1.7 for the β-xylosidases, have been partially purified from the haloarchaeon *Halorhabdus utahensis* [100]. Both types of activity were noted for this hemicelluose degrading haloarchaea. The β-xylanase was active over the salt range of near zero to 30% NaCl with the optimum at 5–15% NaCl. Despite dialysis against a phosphate buffer supplemented with 1% $MgSO_4 \cdot 7H_2O$, 55% of the activity remained, and two optima were observed, one at 55°C and the other at 70°C which indicates that two β-xylanases were present. This was confirmed by the zymogram which showed the presence of one enzyme at about 45 kDa and the other at about 65 kDa. The pH optimum was very sharp at 7.5. They also found that there were three stages of β-xylanase "activity": one which was unable to adsorb to xylan at high salt concentrations, one which adsorbed to xylan at high salinities but desorbed at low salinities (45% of the activity), and one which bound to xylan at low salinities. The β-xylosidase had salt, pH, and temperature optima of 5% NaCl, pH 6–7, and 65°C, respectively, and retained 83% of the initial activity when dialyzed in the same buffer. The

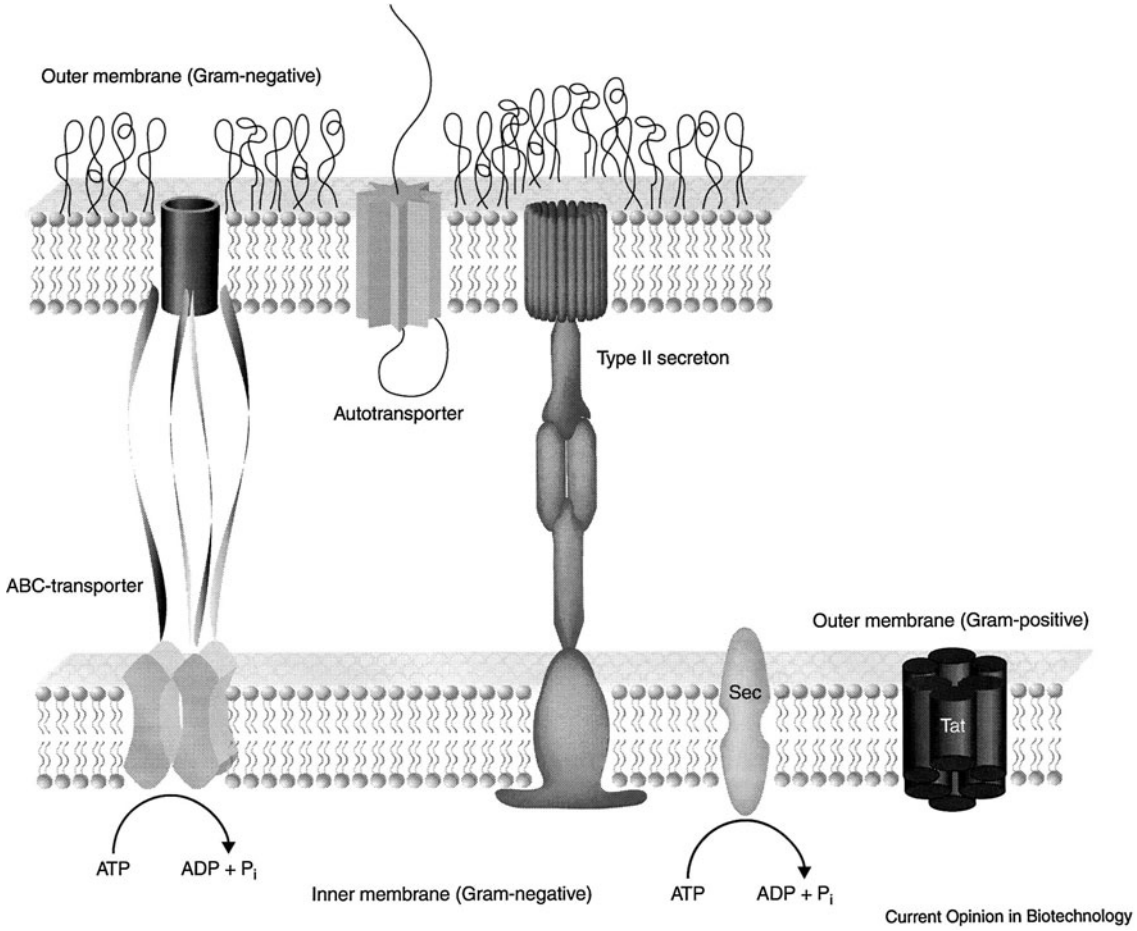

Fig. 2 Diagrammatic representation of lipase transport across Gram-negative outer and inner membranes using the ABC-transporter system [39]. Reprinted with kind permission of Elsevier

authors also found that approximately 48% of the β-xylanase was extracellular while 52% was cell wall/membrane bound. For the β-xylosidase, only 2% was extracellular (in the culture supernatant) while 87% was cell wall/membrane bound with the remainder in the periplasm [100].

Although not a member of the glycosidases, it is appropriate to mention here the work on the xylose dehydrogenase of *Har. marismortui* [40]. These enzymes catalyze the isomerization of glucose to fructose which is the final step in the production of sweeteners from starch. This 175-kDa xylose dehydrogenase was most active in 1 M NaCl or 1.5 M KCl and 100 mM $MgCl_2$. There was no mention of the temperature or pH optima. Thus it would seem worthwhile to consider the use of this dehydrogenase in conjunction with the amylases to obtain sweeteners from starch or maltose.

Lipases and esterases

There have been a number of reviews on microbial lipases and esterases but the halophilic *Archaea* are generally not mentioned [39]. Extracellular lipase activity is quite common among the haloarchaea with 5–15% of the haloarchaea isolated from the solar saltern in Eilat possessing true extracellular lipase activity on the hydrolysis of olive oil (Litchfield, unpublished data). It has been shown by Pohlschröder et al. [73, 80] that two secretory pathways for proteins exist in the haloarchaea. These are the SEC (secretory pathway which transports folded proteins) and the twin arginine pathway (TAT), which transports unfolded proteins. Because lipases are extracellular, it is likely that they use a secretion pathway similar to that of the Gram-negative bacteria, Fig. 2 [39].

Lipases can be either phospholipases, i.e., they either hydrolyze the phosphate moiety from phospholipids, or they can be active against either of the fatty acids on phospholipids or triglycerides. Bhatnagar et al. [8] screened strains of the halobacteria in an Algerian culture collection for activity against *p*-nitrophenyl butyrate and *p*-nitrophenyl palmitate. Most of the 35 strains displayed activity against both substrates, and 12% showed activity with olive oil. These authors selected a *Natronococcus* sp., an alkaliphilic halophile for further study, and found activity at 3 M

NaCl and 40°C but did not report on the salt, temperature, or pH requirements of the enzyme [8]. However, in a later paper they noted that 4 M NaCl was optimum for the partially purified lipase and that no activity occurred in the absence of NaCl [11]. The lipase was thermostable when incubated for 60 min at 50°C but retained only 50% activity at 80°C after 76 min. This *Natronococcus* lipase is a true lipase as it hydrolyzes olive oil and the active site contains serine [11].

Birbir et al. [9] studied 12 strains from the Tuzkoy salt mine in Turkey. Ten strains were positive for cellulase production, while all 12 exhibited activity against Tween 80. This again indicates that lipase and esterase activities are fairly common amongst the haloarchaea.

Isolates obtained from three salt lakes in Turkey were also screened for lipolytic activity [68]. A total of 118 strains was tested against both of the previously mentioned *p*-nitrophenyl substrates and 10% were positive. Cell-free extracts of five of the most active strains were examined for the effects of pH, temperature, and NaCl concentration on enzymatic activity. Most of the strains showed optimal activity at pH 8–9 for the esterase and pH 8 for the lipase activity. Sodium chloride had less of an effect on the esterase activity while lipase activity was optimal in the 3–4 M NaCl range. The esterases appeared to be more sensitive to temperature than the lipases as the esterases retained activity only in the 60–65°C range [68].

Crude extracts, both intracellular and extracellular, from *Har. marismortui* were assayed for esterase and lipase activities with both of the above *p*-nitrophenyl esters as well as two triglycerides [13]. In all cases, both lipase activity against the triglycerides and esterase activity against the *p*-nitrophenyl esters were significantly higher with the intracellular fractions. Esterase and lipase were optimally produced by 50–60 h of growth. Activity was found at two different salt concentrations, 0.5 M and 5 M NaCl, implying the presence of at least two different enzymes while 50% of the total activity was retained with no NaCl present. These results indicate either three enzymes or significant conformational changes that resulted in the maintenance of activity at multiple salt concentrations. Activities of the crude preparations were maximal at 45°C and inactive at 75°C [13].

Esterases are either extracellular as part of the general group of lipases or intracellular and involved in the hydrolysis of various types of carboxyl ester bonds. The carboxyl ester hydrolases of *Hbt.* sp. NRC-1 have been studied by Camacho et al. [14] and were found to be intracellular. Maximum production occurred after incubation for 40 h and 30–46°C and 3.5–5 M NaCl. The authors did not report whether the esterase required elevated salt concentrations to maintain activity.

These enzymes could be used in the production of specialized chemicals given the wide range of activities displayed. To date, though, there are no known direct industrial applications of any of these enzymes.

Proteases

Given that some of the first isolations of haloarchaea came from salted cod that had turned red due to their growth [6, 48], it is not surprising that there have been numerous studies on the proteases of the haloarchaea. One of the first studies of an extracellular protease from a haloarchaea was by Norberg and Hofsten [67]. Several strains of *Hbt. salinarium* and *Hbt. cutirubrum* (now classified as *Hbt. salinarum* [97]) were grown, and their cell-free culture supernatants were tested for activity against gelatin and casein. Two strains were studied further, and the authors noted that NaCl was required for activity and the enzyme(s) were more active with NaCl than KCl. They also tested a number of unnamed coccoid strains, which undoubtedly later formed the genus *Halococcus,* and found all of them to lack any extracellular proteolytic activity.

It was 14 years later before another study was reported on the *Hbt. halobium (salinarum)* proteases. Izotova et al. found a single band of proteolytic activity from affinity chromatography on bacitracin-Sepharose 4B in a buffer containing 25% isopropanol for the protease from *Hbt. halobium* P-353 (a mutant containing no gas vesicles). This enzyme had a molecular mass of 41 kDa and high specificity against azocasein and the synthetic peptide L-pyroglutamyl-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide. As with other haloproteases, the acidic amino acids and/or their amides accounted for 25% of the total amino acid residues. Unlike other haloarchaeal serine proteases (see below), this enzyme cleaved only one bond in the B chain of insulin and was inhibited by only 5% dimethylformamide (DMF) [38].

The serine proteases produced by *Hbt.* sp. strain TuA4 were purified 216-fold in a fast protein liquid chromatographic system. The authors used a combination of precipitation and column chromatography. The initial concentration of the supernatant was achieved by hollow fiber membrane filtration followed by precipitation of other proteins in a 10% PEG-6000 buffer containing 3 M NaCl. Acetone precipitation was performed next followed by purification on a CM-Sephadex column, and then columns filled with Mono Q HR 5/5 and a phenyl-Sephadex column [83]. These steps resulted in a 3% yield and a single band on SDS–PAGE electrophoresis. The molecular mass of the purified ESP4 was 56 kDa, and it maintained activity in 0.3 M NaCl-containing buffer [83].

Ryu et al. [82] studied the 66-kDa extracellular protease of *Hbt. halobium* ATCC 43214, which required 4 M NaCl for maximum activity. Interestingly, in 33% dimethylformamide

Table 2 Selected halocin characteristic

Halocin code number	Producer	Salt dependent	Thermal stability	Activity spectrum[a]
A4	Haloarchaean TuA4	No	> 1 week at boiling[b]	Broad/*Sulfolobus* spp.
C8	*Halobacterium* strain AS7902	No	>60 min at 100°C	Broad
G1	*Halobacterium* strain GRB	ND[c]	ND	Broad
H1	*Haloferax mediterranei* Ma 220	Yes	<50°C	Broad
H2	Haloarchaeon Gla22	ND	ND	Broad
H3	Haloarchaeon Gaa	ND	ND	Broad
H4	*Haloferax mediterranei R*4	Partially	<60°C	Broad
H5	Haloarchaeon Ma 220	ND	ND	Narrow
H6/H7[d]	*Haloferax gibbonsii Ma*2.39	No	<90°C	Broad
R1	*Halobacterium* strain GN 101	No	60°C	Broad/*Sulfolobus* spp. *Methanosarcina* spp.
S8	Haloarchaeon S8a	No	>24 h at boiling	Broad/*Sulfolobus* spp.

Data summarized from Shand and Leyva [86] with kind permission of Springer

[a] Activity spectrum is inhibition of haloarchaea

[b] This study was done at 2,113 m (7,000 ft); where water boils at 93°C

[c] Not determined

[d] H6 was first reported as a 32-Da protein, but is now a microhalocin of 3 kDa. H7 is now classified as halocin H6, but is produced by a halocin over-producing mutant of *Hfx. gibbonsii* Ma 239 renamed *Hfx. gibbonsii* SPH7

(DMF) the esterase activity of the enzyme was about 80 times higher than the amidase activity. At that time, DMF was used as the solvent in peptide synthesis so it was felt that this enzyme would be useful in such work, but this has not happened. The esterase activity is fairly broad with cleavage sites at phenylalanine, tyrosine, tryptophan, serine, glycine, and alanine esters. However, the cleavage site most involved contained glycine residues [82].

Further studies on this protease were concerned with the salt and solvent dependence of the enzyme. Kim and Dordick found that the protease was irreversibly inactivated at salt concentrations below 4 M NaCl. They also reported that the effect of several solvents was to salt-out the enzyme by making the protein more hydrophobic mimicking the salting-out by salts effect. Thus, a 42-fold increase in protein stability occurred with 40% dimethylsulfoxide at only 0.2 M NaCl. Tetrahydrofuran at 20% (v/v) concentration and DMF destabilized the protein at all salt concentrations tested [49].

Kamekura et al. [42, 43] described the extracellular proteases of a haloarchaeon designated strain 172P1, later classified as *Natrialba asiatica*. This bacterium produced three proteases as determined by column chromatography followed by ultracentrifugation, additional column chromatography, and slab gel PAGE electrophoresis. The first and smaller fraction was inactivated at less than 0.1 M NaCl while fraction 3 degraded to fraction 2 on standing. Thus fraction 2 was used for further study. This enzyme had maximal azocasein activity at 10% NaCl, while hydrolysis of synthetic substrates was maximal at 30% NaCl. Further studies revealed that it too was a serine protease. Later, the authors reported that they had identified the gene for this alkaline serine protease, which they named halolysin (Table 2) [44]. The protease has a molecular mass of 42 kDa and close homology to the thermitase of *Thermoactinomyces vulgaris*. The halolysin contained 411 amino acid residues and had a lone C-terminal extension of approximately 120 amino acids. The gene was isolated and cloned into *Hfx. volcanii* [44] to maximize production.

Hfx. mediterranei is a very versatile haloarchaeon. It also produces a halolysin, which Kamekura and coworkers named halolysin R4 (Table 2). It, too, is a serine protease as determined by its sensitivity to phenylmethanesulphonyl fluoride (PMSF), and the gene cluster for the protease was identified and cloned into *Hfx. volcanii.* This halolysin also has a 117 amino acid extension at the C-terminal which is essential for activity that is similar to the subtilisins [45].

Stepanov et al. [91] also investigated the serine protease of *Hfx. mediterranei* strain 1538, though at the time of publication it was listed as *Hbt. mediterranei strain* 1538. The serine protease was purified to electrophoretic homogeneity and found to have a molecular mass of 41 kDa and an exceptionally high p*I* of 7.5 for an archaeal protein with all of the acidic amino acids normally found in archaeal proteins, see below. This may have been a misprint, but so far no corrections have been reported. The inhibition caused by exposure to 0.15 mM PMSF and to 0.1 mM di-isopropyl fluorophosphate confirmed that this, too, was a serine protease. Maximal activity was achieved at pH 8–8.5 and required NaCl for activity. In 10% DMF and 4.5 M NaCl

only 30% of the activity remained. The pattern of hydrolysis on the B chain of insulin resulted in peptide cleavage similar to the subtilisins. Furthermore, the combined acidic amino acids and their amides accounted for one-fourth of the amino acids, which is typical of halophilic proteins in general [62].

Among the circumneutral pH haloarchaeal genera, to date the only other proteases that have been reported are in the genera *Halogeometricum*, *Natrialba*, and *Natrinema*. A thermostable, haloalkaliphilic serine protease from *Hgm borinquense* strain TSS101 was isolated and purified to electrophoretic purity [98]. This enzyme has a molecular mass of 86 kDa and maximum activity at pH 10 and 60°C; activity is increased 20% when 100 mM $CaCl_2$ is present. The requirement for NaCl showed 80% activity remaining at 15% NaCl, a maximum at 20% and a rapid decrease of 60% of the activity at 30% NaCl, Ten percent sucrose resulted in 100% activity in the absence of any NaCl, which is unusual. Other osmolytes such as mannitol (15%), glycerol (20%), or betaine (20%) also resulted in 100% relative activity in the absence of NaCl. This implies that an osmolyte is important for the stability of this protease and not the presence of the Na^+ or Cl^-. Maximum proteolytic activity was observed on 0.1% Azocoll followed by 0.4% Azocasein and 0.1% Casein (Hammerstein) [98]. Thus, this serine protease is substantially different from those described previously from members of the *Hfx*. genus and shows promise for industrial applications such as tanning.

Giménez et al. [27] also examined an extracellular protease from *Nab. magadii*. The molecular mass was 45 kDa and required 1.5 M NaCl to retain activity at 60°C. The enzyme was stable over the pH range of 6–12 pH units with an optimum at pH 8–10. It is also a chymotrypsin-type of protease. There was no cross-reaction with the antibodies to *Ncc. occultus*. Therefore, these authors concluded that this it is a different of protease.

The gene for an extracellular protease from *Natronomonas asiatica* sp. J7 was cloned into Hfx. *volcanii* [87]. The resulting protease SptA was studied and found to have properties that distinguished it from most of the haloarchaea proteases. When NaCl was removed, 20% of the activity was retained, but 60% enzymatic activity returned when 2.5 M NaCl was added back. This indicates that denaturation is not complete in the absence of NaCl. These authors found that the SptA protease was 72% similar to halolysin 172P1. This latter halosin had been found to remain active in 1.3% NaCl, so it is not unexpected that SptA retained activity at lower salt concentrations [43]. One of the more interesting aspects of this research on SptA was an investigation into how it is released from the cell. Shi et al. [87] tested this possibility by replacing the twin-arginine residues with twin-lysine residues and found that SptA was not secreted by *Hfx. volcanii* as it was in the wild-type. This demonstrates that the TAT pathway is important in haloarchaea and protein translocation.

The proteases from the alkaliphilic haloarchaea *Ncc. occultus* were also studied by De Castro and coworkers [84]. They initially concentrated on the intracellular proteolytic activities finding numerous bands on gel electrophoresis and gelatin zymography. Maximum activity occurred in late exponential phase with a sharp decline as the cells entered the stationary phase of growth. Starvation of the cells did not result in significant changes in the amount of proteolytic activity. The activity was greatest in 1 M NaCl whereas 2 M KCl was required to obtain 75% of the NaCl activity. As is common for the haloarchaea proteases, 60°C is the temperature optimum, and the proteases were inhibited by PMSF indicating again that these were all serine proteases [84].

An extracellular protease of *Ncc. occultus* was also studied the haloalkaliphile AH-6 also produced an extracellular serine protease with activity over the pH range 7.5–13 [16]. Maximum production during late exponential to early stationary growth [93]. Preliminary gelatin zymography revealed up to eight proteolytic bands in 3 M NaCl without preincubation and eight bands with preincubation for 7 days in 4 M betaine. All proteolytic activity was inhibited by PMSF. Further studies [19] showed that the intracellular autocatalytic proteolytic activity was the precursor to the extracellular activity. This extracellular protease was purified 328-fold, was active over a pH range of 5.5–12, and found to have a molecular mass of 130 kDa, which is larger than other haloarchaea proteases [94]. It may mean that this protease, while similar in thermal stability and salt requirements, is capable of autocatalytic activity extracellularly or is a dimer. With the broad pH range it should find application in several industrial processes.

In the continual search for inexpensive growth media, Gessesse [26] found that alkaliphilic bacteria could grow on chicken feathers and still produce extracellular proteases. Surely this is worth considering as an inexpensive medium component for the haloalkaliphilic archaea and their production of proteases. It would utilize a current waste product.

Biopolymers and surfactants

The production of extracellular polysaccharides (EPS) by haloarchaea has been recognized from the earliest days of cultivating these microorganisms because of the extremely slimy, mucoidy growth of many of the individual strains. It wasn't until 1988, however, that any of these exocellular polysaccharides was isolated and characterized [3]. *Hfx. mediterranei* ATCC 33500 produces extensive EPS when grown on a medium containing glucose, NH_4Cl, and KH_2PO_4. The polymer is a heteropolysaccharide that consists mainly of mannose but also includes glucose,

galactose, an unidentified sugar, amino acids, uronic acids, and sulfate [3]. Antón et al. [3] also reported the rheological properties showing a plasticity and high viscosity at low concentrations.

Although known to occur in the domain *Bacteria*, extracellular poly-γ-D-glutamate (PDG) is not common in the haloarchaea. However, PDG is produced by *Nab. aegyptiaca* strain 40. Its production was dependent on growth in media containing 20% (w/v) NaCl, and the maximum concentration occurred after 90 h of growth. This resulted in 470 mg PDG/L [34].

The first report of polyhydroxyalkanoates (PHA) in haloarchaea was presented by Fernandez-Castillo et al. [24] when they noted that both *Hfx. mediterranei* and *Hfx. volcanii* produced polyhydroxybutrate (PHB) at 17 and 7% dry wt, respectively. They also showed that the salt concentration made a major difference in the amount of PHB produced with maximum production of 35–38% dry weight occurring at 15–20% marine salts. Higher marine salt concentrations decreased these amounts by half [24]. PHB production by *Hfx. mediterranei* was further optimized to 67% of the dry cell mass with a yield of 6.7 g L^{-1}; with this organism, large intracellular granules are observed microscopically [78] (Fig. 3). The PHA from *Hfx. mediterranei* was fractionated and characterized by Don et al. [17]. They found that there were two co-polymers: one chain contains 10.75 mol percent of the 3-hydroxyvalerate, while the less prevalent chain (6.6% of the produced PHAs) has 12.3 mol percent of the 3-hydroxyvalerate. They did not mention whether this might be a reflection of the culture conditions such as temperature, carbon source, or NaCl concentration. Hezayen et al. [34] reported that PHA accumulation was significant at 10% NaCl which impaired the growth of their unnamed strain.

Recently, Xiang et al. [56] have studied the genetics of PHA production in the same strain of *Hfx. mediterranei*, mentioned above, with a view to understanding how this poly (*3*-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) is synthesized and eventually could be used to make biodegradable plastics. The key enzyme for this synthesis is the group of PHA synthetases which are grouped into four classes based on their substrate specificities and their protein subunits [92]. In *Hfx. mediterranei* both PhaC and PhaE are required for PHBV synthesis and constitute class III PHA synthetases [57]. Xiang's group also reported the production of class III PHA synthetases in *Har. marismortui* [30], *Har. hispanica* and *Hfx. volcanii* [31]. These authors noted that the gene FagG1 was a homologue of the PHA-specific acetoacetyl-CoA reductase PhaB found in bacteria in the domain *Bacteria*.

One of the limitations to using haloarchaea for production of biopolymers has been the relatively expensive medium for growth of the PHA producing organisms. Huang et al. [36] reported that *Hfx. mediterannei* strain ATCC 33500 when cultured in a fed-batch mode with extruded rice bran and extruded corn starch (1:8) resulted in 77.8 g PHA/L medium. Whey has also been used as the carbon source for PHA production by *Hfx. mediterranei* strain ATCC 33500 and two other haloarchaea and resulted in 5.5 g/l with a specific production rate of 9.1 mg g^{-1} h^{-1} by *Hfx. mediterranei.* This bacterium was considered the most promising of the three bacteria tested for industrial PHA production. Koller et al. [52] also noted the high quality of the PHA produced, its low melting temperature and high molecular mass with fairly inexpensive downstream processing when the organism was grown on whey.

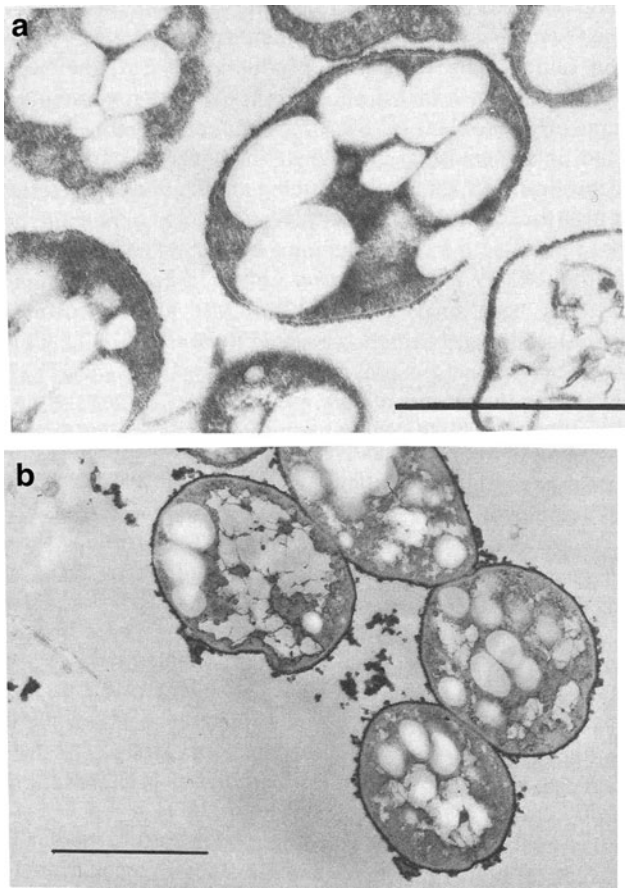

Fig. 3 Electron micrographs of thin sections of *Hfx mediterranei.* **a** Crystals of PHB in cells grown under optimal conditions for PHB production. **b** Ruthenium red-stained cells in which the extracellular polysaccharide (EPS) is the electron dense material. The *bar* represents 1 μm [78]. Reprinted with the kind permission of Springer

The PHA particles were described by Rehm [76] as nanoparticles resulting from polyester synthases. He noted that elongation of the alkanoate chains may occur by a mechanism similar to fatty acid synthesis [76]. If this proves to be true, it may explain the finding that the genes for a partial fatty acid synthesis pathway have been reported in *Hbt. salinarum* NRC1 [66] and might exist in other genera of the Halobacteriaceae especially those that produce PHAs.

A patent was issued to Escalona et al. in 1996 for the commercial production of PHAs from *Hfx. mediterranei* [21]. This organism is easily lysed in distilled water and the large PHA granules are then released for easy recovery [78], Fig. 3. With such ease of recovery and such large amounts of PHA's, it is surprising that this PHA has not seen more widespread applications.

Compared to the interest and research on biopolymers, studies on surfactant production by haloarchaea are very few. Cameotra and Makkar [15] in their review of biosurfactant production by extremophilic microorganisms, mentioned only three reports of biosurfactant production by halobacteria. However, biosurfactants have many industrial applications often involving hypersaline solutions/suspensions such as therapeutic antimicrobial agents, agricultural antimicrobials, uses in mining, personal care products, the food industry, application to oil tank cleaning and in oil recovery as well as bioremediation [5]. Most of the stated interest in halophilic synthesis of surfactants has been as emulsifying agents for remediation [7, 20], while Post and Al-Hajan [74] noted that the diphytanyl membrane lipids had surfactant properties and suggested that they may be used to enhance oil recovery.

Kebbouch-Gana et al. [47] followed up on the emulsification work of Post and Al-Harjan. Five isolated strains of haloarchaea were able to reduce their culture medium surface tension to below 40 mN m^{-1}, and strains A21 and D21 produced especially effective surfactants that stabilized diesel oil–water emulsions from pH 2 to 11 in the presence of 25–50% ethanol in the aqueous phase. They also found that the emulsion was stable over the range of 15–35% NaCl in the aqueous phase. Dilution with distilled water did not affect the emulsion. Strain D21 belongs to the genus *Haloarcula* while strain A21 is a member of the genus *Halovivax*. The authors partially characterized the chemical nature of the two surfactants with D21 producing a probable glycoprotein(s), and A21 producing a surfactant composed of sugar, protein, and lipid [47].

Other potential biotechnological uses

Several patents have been issued for the haloarchaea as reviewed by Litchfield [58].

In 1999, a patent was awarded Sprott et al. [89] for the use of liposomes from *Hbt. cutirubrum* (sic). The advantage of these liposomes is that they are resistant to phospholipases and could deliver drugs to various parts of the body. So far these halophilic liposomes have not seen any medical applications.

Marhuenda-Egea et al. [60] found that by making reverse micelles, they were able to maintain the enzymatic activity of an alkaline *p*-nitrophenylphosphate phosphatase from *Hbt. salinarum* in organic solvents such a 1-butanol. The activity of the enzyme was maintained even in 0.048–0.85 M NaCl solutions despite the fact that previous studies with the native enzyme showed only 15% activity at 1.2 M NaCl [10]. Other studies with haloarchaeal enzymes in solvents have been reviewed by Sellek and Chaudhuri [85]. Cross-linking in a proteinic matrix or immobilization with 0.5% glutaraldehyde of a halophilic aldolase has resulted in an increase in the temperature optimum from 55 to 65°C. This treatment also allowed the *Hfx mediterranei* enzyme to function in lower salt concentrations of 0.5 M KCl as compared with the normal 1–2 M NaCl concentration [18].

Another interesting application involves halophilic bacteriorhodopsin. The halobacteriorhodopsin was integrated into a complex soybean phospholipid, phospholipid antigen, ATP-ase (derived from mitochondria), and a pH sensitive dye as a nonliving energy generation system [63]. This liposome was fixed to a base plate with an antibody, and the amount of ATP present determined. Minoru et al. [63] suggested that this method would be useful for fieldwork. Patents have also been issued for the use of this pigment in solar energy conversion [2], photosensitive chromoprotein film [64], production of photocurrents when coupled to antibodies which oriented the bacteriorhodopsin [53], for power generation [79], and for its use in data storage systems [88].

Several experiments have been performed in which the haloarchaea were tested for their abilities to degrade pollutants. Most of these were conducted as mixed cultures except for the work of Emerson et al. [20] and Bertrand et al. [7] on hydrocarbon degradation. Other major studies have been performed by Kargi and Uygur [46] and Woolard and Irvine [103] for remediating saline wastewaters, Kulichevskya et al. [54] for oxidizing hydrocarbons, and the patent issued to Patzelt et al. [69] for the partial decomposition of halogenated hydrocarbons.

In the continuing search for new antibiotics, the halophilic halocins have in general been overlooked. Although first reported in 1982 by Rodriguez-Valera et al. [77], to date there has been little attention paid to this potentially interesting group of peptide antibiotics. One reason is that many of the purified halocins have not been active against the standard suite of bacteria: *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, B. cereus,* and *Sarcina luteus* [56].

Halocins are peptides that inhibit closely related haloarchaea and are seemingly an almost universal characteristic of the haloarchaeal rods, Table 2. Several of the halocins listed in Table 2 have also been found to be active proteolytic enzymes (see proteases section above), which might explain why they have not been considered for medical applications.

An examination of 68 strains of haloarchaeal rods showed that 67 of them exhibited inhibitory activity against

other haloarchaea [95]. The possibility of halophage activity causing this inhibition was ruled out, and all inhibition was the result of the presence of these small peptides. In an extensive review of these halophilic halocins (Table 2) Shand and Leyva [86] noted that halocin H7 has been shown by Meseguer to inhibit the Na+/H+ transporter in haloarchaea and in dogs [61]. Thus, the application of these peptide antibiotics may not be as antibiotics but as agents to control blood pressure and other osmotic functions in the body.

Finally, there has been interest in synthesizing polyisoprenoids for synthetic rubber, among other applications [90]. Recently, it has also been reported that isoprenoids can be used in the initial steps to the production of the anticancer drug Taxol [1]. To date, no one has reported utilization of the isoprenoid synthesis pathway of the halophilic *Archaea*. The membrane lipids in these organisms are composed of 4 or 5 linked isoprenoid molecules. Additionally, numerous squalenes are also found in the haolarchaea [41]. It has been known for some time that the isoprenoid structures are synthesized via the mevalonate pathway with three of the carbons arising from acetate [35]. Thus, it would seem that the lipids of these microorganisms are worth further investigation for industrial applications.

Conclusions

As mentioned in the introduction, the chromosomes of several of the haloarchaea have been sequenced and annotated. This led Falb et al. [23] to examine the metabolism of the halophilic archaea. They noted different haloarchaea had different pathways for folate biosynthesis and glycerol and pentose metabolism. As more strains are sequenced and annotated, such information should lead to greater applications of the haloarchaea in industrial settings such as biosynthesis and degradation of contaminated process waters/solvents.

Sodium chloride is generally required for growth by the haloarchaea at concentrations ranging from 1.5 to 4–5 M. These concentrations of salt have been a deterrent to the application of this group of haloarchaea to current industrial fermentation and biotransformation systems. This led Hezayen et al. [34] to develop a novel bioreactor for the production of poly(gamma-glutamic acid) from *Hal. mediterranei*. Such changes in fermentation equipment and downstream processing are monetary deterrents to applying halophilic enzymes to industrial needs. Some of the enzymes described in this paper, however, are extracellular which, once produced, benefit from ease of isolation and purification, especially those that do not require the higher salt concentrations to retain activity.

Additionally, by carefully manipulating the salt content of the growth medium or during enzyme purification, it is possible to lower the salt or pH optima for commercial use. In fact, it has been reported that many of the haloarchaea can grow at low salinities [75, Litchfield unpublished data]. One advantage of the high salt-containing media is that at salt concentrations over 12% media may not have to be sterilized saving time and money. Furthermore, enzymes which can function in greatly reduced water activity levels have been shown to function in various hydrophobic solvents, an advantage that may make the applications of haloarchaea of greater interest to industry.

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