

# Haloarcula Marismortui, Eighty-Four Years after its Discovery in the Dead Sea, Review

David Isidoro Camacho Córdova

Departamento de Ing. Química, CUCEI, Universidad de Guadalajara.

Guadalajara, Jalisco, Mexico

Rosa María Camacho Ruíz

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. CIATEJ

Guadalajara, Jalisco, Mexico

Juan Carlos Mateos Díaz

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. CIATEJ

Guadalajara, Jalisco, Mexico

Jesús Antonio Córdova López

Departamento de Química, CUCEI, Universidad de Guadalajara.

Guadalajara, Jalisco, Mexico

Jorge Alberto Rodríguez González

Centro de Investigación y Asistencia en Tecnología y

Diseño del Estado de Jalisco A.C. CIATEJ Guadalajara,  
Jalisco, Mexico

**Abstract**— *Haloarcula marismortui* is one of the few halophilic microorganisms from the Archaea domain to have been thoroughly studied. This microorganism has been considered as a model for many researcher groups to understand how it is able to proliferate at extremely high salt concentration. Such studies include the purification of the first haloarchaeal enzyme, the estimation of intracellular salt concentrations, the first crystal structure for haloarchaeal ribosome, the sequencing of the second haloarchaeal genome, the sizeable (4.27 Mb) and incredibly complex genome architecture, consisting of nine replicons, and many more examples that continue to providing interest insight into this microorganism. This review discusses and analyzes essential publications concerning *Haloarcula marismortui* to be taken account for future works with other halophilic microorganisms and an additional effort is made to collect physiological characteristics and biotechnological applications of this organism.

**Keywords**—*Haloarcula marismortui*, *Halobacterium marismortui*, *Halobacterium of the Dead Sea*, ATCC 43049<sup>T</sup>, *Halophilic archaea*.

## INTRODUCTION

Archaea is a domain which comprises numerous microorganisms having the ability to survive extreme conditions for life, called “extremophiles” [1]. Archaea, more than other organisms, are exposed to a variety of stressors in their natural habitats: physical (high and low temperatures, and radiation), mechanical (high and low pressures and shearing) and chemical (sudden shift from anaerobiosis to aerobiosis or vice versa; high and low pH, high osmolarity and salinity) [2]. The halophiles can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement on NaCl to live. In particular, extremely

halophilic archaea are well adapted to proliferate in media containing NaCl concentrations close to saturation. Halophiles are disseminated over the world, in hyper-saline environments, such as natural hypersaline brines in arid, coastal and deep-sea locations as well as in artificial salterns used as source of salts from the sea [3]. The use of halophiles for industrial purposes is expanding. Many potential uses have been proposed and the number of patents issued has increased. Due to the necessity for more environmentally friendly technologies, halophilic microorganisms are perceived as a source for enzymes [4],  $\beta$ -carotene and ectoine (used as a stabilizer for enzymes and for cosmetic products) and they are also utilized to produce traditional fermented foods [5].

*Haloarcula marismortui* is a halophilic archaeon isolated from the Dead Sea by Elazari-Volcani in the 1930s [6], whose genome was sequenced in 2004 [7]. Different biomolecules isolated from *Haloarcula marismortui* (lipids, carotenoids, bacterioruberins, rhodopsins, polymers, halocins and proteins) reveals it as an extremely important research topic.

## I. TIME-LAPSE

In the 1930s, Elazari-Volcani isolated a novel strain of the genus *Halobacterium* described as “*Halobacterium marismortui*” from the Dead Sea. However, this strain was never deposited in a culture collection and is believed to be lost. During the 1960s, a new *Halobacterium* strain was isolated from the Dead Sea by Ginzburg *et al* [8]. This strain was originally referred as “*Halobacterium of the Dead Sea*”. In 1975, the International Code of Nomenclature of Bacteria proposed the name *Haloarcula marismortui* (Volcani) sp. nov., nom. rev [6]. Between 1986 and 1988, the isolated strain by M. Ginzburg was deposited in the American Type

Culture Collection by L. I. Hochstein (ATCC 43049)[9]. In 1990, Oren *et al* compiled valuable physiological information about *Haloarcula marismortui*.

## II. GENOME

In 2004, Baliga *et al* reported the complete sequence of the 4.27 Mb genome of *Haloarcula marismortui*, which is one of the largest known genomes from the archaea domain, only surpassed by *Methanosarcina barkeri* (4.86 Mb) and *Methanosarcina acetivorans* (5.75 Mb) [10]. *Haloarcula marismortui* is the second sequenced genome from halophilic archaea. The genome is organized into nine circular replicons of varying G-C compositions with an average of 57% [7]. This G-C composition is similar to other halophilic archaea such as *Haloferax volcanii* (average of 55%) [11] and *Halobacterium* sp. NRC-1 (average of 58%) [12]. Furthermore, 1,310 proteins identified from mid-log and late-log phase of *Haloarcula marismortui* cultures were analyzed in metabolic and cellular process networks after being exposed to environmental stresses by integrative genomics and proteomics analyzes [13].

## III. PHYSIOLOGICAL STUDIES

Physiological studies of *H. marismortui* have been performed and the results are summarized in Table I. *H. marismortui* catabolizes sugars by glycolysis and Entner-Doudoroff pathways and some amino acids. *H. marismortui* is a facultative aerobe, requiring growth factors; chemoorganotroph, oxidase and catalase positive. It can grow anaerobically with nitrate as an electron acceptor. It utilizes a range of organic compounds as sole carbon and energy sources (glucose, fructose, sucrose, glycerol, acetate, succinate and malate). It produces organic acids from glucose, fructose, ribose, xylose, maltose, sucrose, mannitol, sorbitol and glycerol. It hydrolyzes starch very slowly and does not produce indole. It is susceptible to bacitracin and novobiocin [6, 14].

TABLE I. PHYSIOLOGICAL CHARACTERISTICS OF *HALOARCULA MARISMORTUI*.

Morphology	Flat disk-shape	[14]
	Pleomorphic, flat and disk-shape	[6]
Mode of motility	Rarely observed, motile cells rotate around their axis	[14]
	Nonmotility (but rarely cells are observed to rotate around their axis)	[6]
	Motility (helicity flagellar filaments)	[17]
Size	1-2 and 2-3 $\mu$ m	[6]
Intracellular ions	Na <sup>+</sup> 1.2-3M, K <sup>+</sup> 3.77-5.5M, Cl <sup>-</sup> 2.3-4.2M	[8]
Sensitivity to antibiotics	Bacitracin and novobiocin	[7, 17]
Starch digestion	Positive	[14]
	Slow	[6]
Utilization of carbon sources	Glucose, fructose, ribose, alpha-methylglucoside, maltose, sucrose, manitol, sorbitol, glycerol, acetate, pyruvate, succinate and malate	[7, 17]

Furthermore, optimal culture conditions (temperature, pH, salt concentration and composition media) for *H. marismortui* are showed in Table II. The optimal incubation temperature, pH and salt concentration ranges reported for *H. marismortui* cultures are 40-50°C, as the family Halobacteriaceae that need for optimal growth slightly elevated temperatures (38–45°C) [3, 15] 7.0-7.4, and 3.4-5.1 M, respectively. This last feature denotes the marked halophilic character of *H. marismortui*. Differences in biomass concentration values of *H. marismortui* reported by several research groups, could be explained by the different tested culture conditions (aeration, light, agitation, temperature, pH, type and concentration of nutrients in the culture media) [16].

TABLE II. OPTIMAL CULTURE CONDITIONS FOR *HALOARCULA MARISMORTUI*.

Temperature (°C)	NaCl (M)	pH	Aeration (vvm)	Ref
40-50	3.4-3.9	7.0	*	[14]
42.5	3.6	7.4	1	[18]
Culture media composition (g/L)				
NaCl, 234; MgSO <sub>4</sub> .7H <sub>2</sub> O, 53.2; CaCl <sub>2</sub> 2H <sub>2</sub> O, 0.1; MnCl <sub>2</sub> , 0.02mg; KCl, 0.51; K <sub>3</sub> (PO <sub>4</sub> ), 0.31; yeast extract, 10. OD <sub>400-465nm</sub> of 0.2 (at 37°C).				[8]
NaCl, 206; MgSO <sub>4</sub> .7H <sub>2</sub> O, 36; KCl, 0.373; CaCl <sub>2</sub> .2H <sub>2</sub> O, 0.5; MnCl <sub>2</sub> , 0.013 mg; yeast extract, 5.0. Doubling time of 4.5-5 h (at 40°C).				[14]
NaCl, 250; MgSO <sub>4</sub> .7H <sub>2</sub> O, 20; trisodium citrate, 3; KCl, 2; tryptone, 5; yeast extract, 3; 0.1 ml of oligoelements. OD <sub>600 nm</sub> of 3.3 (at 37°C).				[18]
NaCl, 125; MgCl <sub>2</sub> .6H <sub>2</sub> O, 160; K <sub>2</sub> SO <sub>4</sub> , 5; CaCl <sub>2</sub> .2H <sub>2</sub> O, 0.1; yeast extract, 1; casamino-acids, 1; starch, 2. Cultures were performed at 160 rpm and 37°C, during five days.				[20]

\* Cultures were made using Erlenmeyer flasks

## IV. BIOTECHNOLOGICAL APPLICATIONS OF *HALOARCULA MARISMORTUI*

A few studies have been performed on the use of halophilic microorganisms for industrial waste treatment. *Haloarcula* sp. IRU1 produced polyhydroxybutyrate (PHB) using petrochemical wastes and a saline culture medium [21]. *H. marismortui* MTCC 1596 produced PHB, using vinasses (ethanol industry liquid waste) and a saline culture medium. In that study, *H. marismortui* cells accumulated 23 and 30% of the PHB (cell dry weight), using 10% of raw or pre-treated (with activated carbon) vinasses, respectively [22]. One unconventional application using *H. marismortui* IR is to cure salted anchovies. The fermented anchovies exhibited low loads of Staphylococci, Enterobacteriaceae and lactic acid bacteria. They also had a reduced content of histamine and an improved organoleptic acceptance [23]. Remarkably, industrial residual waters from the manufacture of cheese, corn flour, fish and other processes, using high salt concentrations, could be utilized as carbon and nitrogen sources for the production of biomass and metabolites of archaea.

## V. BACTERIORUBERIN AND RHODOPSIN

Habitats, where haloarchaea are plentiful, are hot, dry and exposed to high levels of solar radiation. Therefore, it is not surprising that haloarchaea contain pigments with potential applications in photochemical processes. One of the major types of pigments produced by haloarchaea are carotenoids, including  $\alpha$ -bacterioruberin and other derivatives with carbon chains above 50. These compounds are often found in large concentrations in the membranes of archaea [19]. Rhodopsin is a membrane protein occupying nearly 75% of the cell surface area, forming a hexagonally symmetric purple membrane composed of three identical protomers [24]. Different physiological functions have been identified for nearly 5,000 retinal-binding photoreceptor rhodopsins. The greatest number of rhodopsins observed in a single archaeon is six. This discovering was recently found in *H. marismortui*. The six-rhodopsin system showed a more diverse absorbance spectral distribution than any previously known rhodopsin system [25].

## VI. LIPIDS

Lipid composition in *H. marismortui* was first investigated in 1980 by Evans *et al* [26]. They found that it consisted of 86% polar lipids and 14% of nonpolar lipids. Four major polar lipids were detected, all derivatives of 2,3-di-O-phytanyl-*sn*-glycerol: i) a novel glycolipid, 2,3-di-O-phytanyl-1-O-beta-D-glucopyranosyl-(1'-6')-O-alpha-D-mannopyranosyl-(1'-2')-O-alpha-D-glucopyranosyl-*sn*-glycerol (11 mol%); ii) phosphatidylglycerol (11 mol%); iii) phosphatidylglycerophosphate (62 mol%); iv) phosphatidylglycerosulfate (17 mol%). In addition, a minor glycolipid (less than 1 mol%) was detected and partially characterized. Trace levels of two unidentified glycolipids and two unidentified phospholipids were also detected. The nonpolar lipids in *H. marismortui* consisted mostly of squalenes, vitamin MK-8 and bacterioruberins with traces of beta-carotene, lycopene and retinal [26]. De Souza *et al* also analyzed the lipid composition of *H. marismortui*, finding abundant phytanyl diether phospholipids, namely archaetidic acid (AA), archaetidyl-glycerol (AG), archaetidylglycerosulfate (AGS), archaetidylglycerophosphate methyl ester (AGP-Me) and triglycosyl archaeol (TGA) [27].

Because archaea are considered as safe, pigments and lipid derivatives are waiting for cosmetic and food applications. The use of carotenoids as antioxidants and natural cell protectors is another promising application. However, archaeal pigments have not been exploited at the same level as algae and yeast carotenoids, for instance, *Dunaliella* spp., *Haematococcus pluvialis*, *Chlorococcum* spp. and *Xanthophyllomyces dendrorhous*.

## VII. POLYMERS

Kirk and Ginzburg (1972) reported two strains of *Halobacterium* sp. (namely *H. marismortui*), producers of polyhydroxybutyrate (PHB) granules, which were surrounded by a 40 Å membrane [28]. An interesting research combining genetics, design of culture media and microorganism stress to

promote PHB synthesis, was later published [29]. Production of PHB was optimized by limiting oxygen, nitrogen, phosphorous or sulfur sources in the culture media. In that work, sodium acetate was also identified as a convenient carbon source for PHB accumulation. *H. marismortui* accumulated a maximum of 105 µg PHB/L culture under any nutrient limitation [30]. PHB accumulation of *H. marismortui*, using different carbon sources is an unexplored field; however, studies for other halophilic microorganisms showed the following results: 1) *Halobacterium mediterranei* accumulated 38% (w/w) of PHB at 15% of NaCl utilizing glucose as carbon source [31]. 2) *P. oleovorans* accumulated 25.3, 49 and 33% (w/w) of PHAs using octane, nonanoate and nonanol as carbon sources, respectively [32].

## VIII. HALOCINS (HALOPHILIC BACTERIOCINES OR ARCHAEOCINS)

Halocines are proteins of ecological significance in saline ponds, often synthesized in NaCl saturated solutions by species from the Halobacteriaceae family, to compete with other microbial species to colonize the same ecological niche. Some strains synthesize halocines inhibiting a broad range of test organisms, while others have a narrow specificity. Although, *H. marismortui* produce halocines, the inhibition of test organisms have not been reported [33]. The potential of halocines from 68 archaea was evaluated with 22 bacteria, finding large competitive advantages of archaea over bacteria in habitats containing high salt concentrations. The fact that archaea and bacteria co-exist in the same niche is very interesting, suggesting that hyper-saline environments around the world have similar relations between bacteria and archaea. However, when other bacteria (collected from distant sites) are exposed to halocines, a high toxic effect is observed. Consequently, potential antimicrobials could be discovered by analyzing the secreted metabolites by archaea [34].

## IX. ENZYMES

Malate dehydrogenase from *H. marismortui* is the best-known halophilic enzyme. Furthermore, important advances have been carried out with other enzymes of *H. marismortui* such as 2Fe-ferredoxin, catalase-peroxidase and esterases. A few other enzymes have been briefly studied. It is worth noting that a general characteristic for halophilic proteins has been established to explain their high solubility and flexibility at high salt concentrations: The highly negative surface charge establishes tight interactions with water (charge-dipole) [35].

Malate dehydrogenase from *H. marismortui* (HmaMDH, EC1.1.1.37) was the first enzyme from haloarchaea to be purified [36] and the first to be successfully expressed in *E. coli* and recovered as a functional enzyme [37]. The amino acid composition analysis revealed an excess of 10.4 mol % of negatively charged amino acids [38]. HmaMDH is soluble and active in saturated NaCl or KCl solutions but it unfolds in solutions containing less than 2.5 M of NaCl or KCl. A novel stabilization model was proposed from experiments involving

the interactions of HmaMDH with different salts solutions (potassium phosphate,  $MgCl_2$ , NaCl and KCl)[39]. The resistance to denaturation of a mutant HmaMDH was compared to that of the wild-type protein, employing NaCl and KCl solutions, and incubation temperatures of 4°C and 25°C for 24 h [40]. Thermal deactivation of HmaMDH at low salt concentrations (0.35-0.5 M  $CaCl_2$  and 0.05-0.8  $MgCl_2$ ) was different from that occurring at high concentrations (0.5-1.0 M  $CaCl_2$  and 0.8-1.5 M  $MgCl_2$ ). At low salt concentrations, protein stability decreases as temperature increases. At high salt concentrations, stability as a function of temperature showed a bell-shaped behavior, reaching a maximum at 4°C [41].

A 2Fe-ferredoxin from *H. marismortui* was purified and their amino acid composition revealed an extremely high excess of acidic amino acid residues: Forty four glutamate and aspartate residues compared to six lysines and arginines, as well as a high content of aromatic amino acids [42].

A heme protein, exhibiting both peroxidase and catalase activities, showed optimal pH values of 6.0 for peroxidase activity (assayed in absence of NaCl) and 7.5 for catalase activity (assayed with 1 M NaCl)[43]. The gene open reading frame corresponded to 731 amino acid residues and the calculated molecular mass of the mature protein was 81,253.65 Da [18].

Despite the importance of the industrial applications of lipases and esterases, a few papers have researched the presence of them in *H. marismortui*. The first work reported the capability of *H. marismortui* to synthesize esterases and lipases, and the effect of physicochemical culture conditions on the growth and production of enzymes. Esterases were active at different salt concentrations, showing two optimal activities (at 0.5 and 5 M NaCl), which suggested the presence of two isoenzymes. Esterase and lipase activities were maximal at 45°C and inactive at 75°C [18]. In a second work, the *H. marismortui* encoding gene for esterase (HmaLipC) was cloned and functionally expressed in *E. coli* BL21-DE3. The recombinant protein (HmaEST) was biochemically characterized, exhibiting a preference to hydrolyze esters of short chain fatty acids and monoesters. Esterase activity was optimum at 3 M KCl and no activity was detected in the absence of salt [44]. In a third work, HmaLipC was cloned and functionally expressed in *E. coli* BL21-DE3. This esterase had an optimum activity at pH 9.5, 45°C and 3.4 M NaCl or 3 M KCl. Interestingly, when this enzyme was incubated at extreme salt concentrations (low or high values), ester hydrolysis (*p*-nitrophenyl acetate) was barely detectable; however, enzyme quickly recovered its hydrolytic activity as salt concentration was adjusted close to optimal values and incubated for 3–5 h. The reversibility on the recovery and loss of enzymatic activity by changing salt concentrations was a distinctive feature of this halophilic enzyme [45].

The encoding gene for nitrate and nitrite reductases from *H. marismortui* was cloned and sequenced [46]. The purified enzyme showed a nitrite reductase activity which enhanced as salt concentration increased, reaching a maximum activity (960  $mM NO_2 \text{ min}^{-1} mg^{-1}$ ) at 2 M NaCl [47].

Glutamate dehydrogenase was purified and the molecular mass calculated (212 kDa). The enzyme is stabilized by

sulfate ions at lower concentrations than chloride ions. The optimal salt concentration seemed to be dependent on the ionic strength, since the optimum activity with sodium sulfate occurred at 0.45 M, while with sodium chloride occurred at 1.1-1.35 M[48].

*H. marismortui* produced alkaline phosphatase when the inorganic phosphate was depleted in the culture medium. This inducible extracellular enzyme is a glycoprotein whose molecular mass is 160 kDa, as estimated by SDS-PAGE. Enzyme activity was maximal at pH 8.5, requiring  $CaCl_2$  and NaCl, and it was inhibited by phosphate [49].

Activity and stability of the purified seryl-tRNA synthetase in KCl, NaCl and  $MgCl_2$  solutions were characterized. A structural model was proposed by comparison of wild and mutant (expressed in *E. coli*) seryl-tRNA synthetases, which illustrates the high negative-charge density of the surface of the hyperhalophilic enzyme [50].

The expression of ADP and AMP -forming acetyl-CoA synthetases was investigated with glucose and acetate as substrates [51]. ADP-forming acetyl-CoA synthetase was purified and characterized as a salt dependent enzyme. The encoding gene was identified and functionally expressed in *E. coli*. The recombinant enzyme was reactivated from inclusion bodies by solubilization and refolding in the presence of salts [52]. The purified AMP-forming acetyl-CoA synthetase had a molecular mass of 72-kDa and an optimum activity at 41°C, pH 7.5 and KCl concentration of 1-1.5 M, whereas NaCl concentration had no effect on enzyme activity. Using the N-terminal amino acid sequence, an open reading frame coding for a 74 kDa protein was identified in the genome of *H. marismortui* and then expressed in *E. coli*. The recombinant enzyme was reactivated from inclusion bodies, by solubilization in urea and refolding in the presence of salts (2 M KCl) [53].

The uses of haloarchaeal alcohol dehydrogenases are of increasing interest as industrial biocatalyst. The purified alcohol dehydrogenase catalyzed optimally at 2 M KCl for the preferred interconversion between alcohols to aldehydes and alcohols to ketones [54].

D-xylose dehydrogenase was recovered from *H. marismortui* cultures, purified and characterized. The coding gene for D-xylose dehydrogenase was then expressed in *E. coli* and the recombinant enzyme was reactivated from inclusion bodies by treatment with urea and refolded in the presence of salts. The enzyme catalyzed oxidation of xylose with both  $NADP^+$  and  $NAD^+$  as coenzymes [55].

#### X. STUDIES OF AMINOACID COMPOSITION OF *H. MARISMORTUI* ENZYMES

Bioinformatics studies of extreme halophilic protein surfaces have revealed a high number of acidic residues (glutamate and aspartate) compared with nonhalophilic counterparts [56]. Acidic residues bind water by ion-dipole interactions to form a solvation shell at the protein surface, thus allowing the proteins to remain soluble and properly folded at high salt concentrations [57]. This feature prevent denaturation, aggregation and precipitation that usually results when nonhalophilic proteins are exposed to high salt concentrations [3]. Table III resumes the amino acid

composition of different enzymes, deduced from the *H. marismortui* genes, using web tools, which were classified as acid, basic and non-polar.

TABLE III. CLASSIFICATION AND COMPOSITION OF AMINO ACID RESIDUES OF DIFFERENT ENZYMES OF *H. MARISMORTUI*

Enzyme (gene)	Amino acids (percentage composition)			
	Acid D/E	BasicK/R/ H	Nonpolar A/L/T/P	Ref
<b>MDH</b> (trnAC270 6)	35/27 (20.4)	8/15/7 (9.8)	28/20/15/1 1 (24.3)	[36]
<b>CPx</b> (trnAC201 8)	81/70 (20.7)	24/41/11 (10.4)	72/62/41/4 5 (30.2)	[58]
<b>EST, LipC</b> (trnAC008 8)	35/20 (16.8)	3/16/10 (8.9)	41/26/22/2 8 (35.8)	[44, 45]
<b>NRG</b> (trnAC119 9)	103/79 (19.1)	28/51/28 (11.2)	59/64/61/5 4 (25.0)	[46]
<b>NRH</b> (trnAC120 0)	32/43 (20.9)	14/22/8 (12.2)	16/20/13/2 6 (21.0)	[46]
<b>SRS</b> (trnAC280 8)	48/62 (23.9)	15/35/11 (21.9)	27/45/13/2 3 (23.5)	[50]
<b>ACS</b> (trnAC322 8)	74/73 (22.1)	20/27/12 (8.9)	51/43/40/3 6 (25.6)	[51]
<b>ADH</b> (pNG7278 )	33/28 (15.9)	4/26/12 (10.9)	35/26/21/1 9 (26.3)	[54]
<b>XDH</b> (trnAC303 4)	35/39 (20.5)	6/15/13 (9.6)	39/26/26/1 7 (29.9)	[55]
<b>APH</b> (trnAC027 3)	81/56 (16.8)	18/32/11 (8.2)	68/50/62/3 8 (29.4)	[49]
<b>FD</b> (trnAC252 6)	20/15 (22.2)	4/6/2 (7.6)	13/10/4/6 (20.8)	[41]
<b>Media (%)</b>	(19.9)	(10.9)	(26.5)	

The aminoacid composition of different enzymes were inferred from the *H. marismortui* genes, using the web tools: [http://www.genome.jp/dbget-bin/www\\_bget?genome:T00211](http://www.genome.jp/dbget-bin/www_bget?genome:T00211). **MDH** is Malate dehydrogenase; **CPx** Catalase-peroxidase; **EST** and **LipC** esterases; **NRG** Nitrate reductase G; **NRH** Nitrite reductase H; **SRS** Seryl-tRNA synthetase; **ACS** AMP-forming acetyl-CoA synthetase; **ADH** alcohol dehydrogenase; **XDH** D-Xylose Dehydrogenase; **APH** Alkaline phosphatase; **FD** 2Fe-ferredoxin. Data represent the number of total aminoacids and the composition of acid, basic and nonpolar aminoacids in the proteins is given in parenthesis.

The average percentage composition of acid aminoacids (AAA) of different enzymes of *H. marismortui* is 19.9%, with the AAA highest content of 23.9% for seryl-tRNA synthetases (HmaSRS) and the AAA lowest content of 15.9% for Alcohol dehydrogenase (HmaADH).

The average percentage composition of basic aminoacids (BAA) is 10.9%, with the highest content of 21.9% for HmaSRS and the lowest content of 7.6% for 2Fe-ferredoxin (HmaFD). On the other hand, the average percentage composition of nonpolar aminoacids (NPAA) is 26.5%, with the highest content of 35.8% for esterase and the lowest content of 21% for nitrate and nitrite reductases (HmaNRG and HmaNRH). A recent comparison between *H. marismortui* esterase (HmaEST) and *Alicyclobacillus acidocaldarius* esterase (EST2, isolated from a hot and acidic spring in Yellowstone National Park, USA) revealed that AAA content in HmaEST was almost 2-fold higher than EST2 (a non-halophilic esterase), suggesting that the proportion of acidic residues in a protein is positively correlated with the degree of halophilicity of an enzyme [44]. Furthermore, the composition of aminoacids in globular proteins (nonhalophilic) was calculated as follow: 11.76% of AAA, 12.99% of BAA and 27.28% of NPAA. Some differences were clearly distinguished: halophilic proteins have a high content of AAA compared to globular proteins. Composition of BAA and NPAA in halophilic proteins are slightly different (1%) compared to globular proteins [59]. Thus, the haloadaptation strategy seems to be related to a modification of protein surface with an increase of negatively charged residues. On the surface, halophilic proteins possess a large number of AAA and a low BAA residues; while inside protein the aminoacid composition is not significantly different between halophilic and nonhalophilic proteins [60].

#### XI. THE ROLE OF SALT IN ACTIVITY AND STABILITY OF *H. MARISMORTUI* ENZYMES

Large number of studies have been undertaken to investigate the role of salt in the *H. marismortui* enzyme activities. Some of these results are summarized in Table IV.

The results provide evidences of the salt dependence of proteins for activity. In general, a high salt concentration is required for optimal activity. The majority of the studies indicate loss of enzyme activity upon salt removal. Exceptionally, some *H. marismortui* enzymes may not require salt to be catalytically active. Per example: The peroxidase exhibited a relative activity of 55% and 42%, respectively for NaCl and  $(\text{NH}_4)_2\text{SO}_4$  concentrations close to 0 M [42]. The esterase exhibited an activity of 55% in absence of salt in the reaction medium [18]. The nitrate and nitrite reductases showed 18% of relative activity in salt concentration close to 0 M [47]. The Alkaline phosphatase showed 10% and 29 % of relative activity in concentrations of  $\text{CaCl}_2$  and KCl, near to 0 M [49]. Interestingly, salt dependence for amylase activity of *Haloarcula hispanica* was not observed, remaining completely active upon salt removal [61]. The nucleoside diphosphate kinase from *Halobacterium salinarum* was catalytically active under low and high salt concentrations. It is worth noting that this enzyme is a

hexamer when is incubated at high-salt concentrations, which dissociates into dimers without denaturation at low salt concentrations. This conversion seems to be the mechanism to withstand nonsaline environment [62].

TABLE IV. SALT CONCENTRATIONS AND pH FOR OPTIMAL ENZYME ACTIVITIES OF *H. MARISMORTUI*

Enzyme	Salt concentration (M)	Activity (%)	pH	Ref
MDH (W)	1.25 NaCl	100	7.1	[64]
CPx (W)	0.5-0.7 NaCl	85	7.0	[42]
	1.0-2.0 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	100		
EST (W)	0.5 NaCl	100	7.5	[18]
	3.5 NaCl	90		
EST (R)	2.0 NaCl	90	8.5	[44]
	3.0 KCl	100		
NR (W)	2.0 NaCl	100	7.0	[47]
SRS (R)	1.5 KCl	100	7.5	[50]
	3.5-4.5 NaCl	100		
ACS (R)	0.5-3.0 NaCl	14	7.5	[53]
	1.25 KCl	100		
ADH* (R)	3.0 KCl	90	8.0	[54]
	3.0 KCl	70	9.0	
	2.0 KCl	100	10.0	
	2.0 KCl	70	8.0	
	2.0 KCl	80	7.0	
	2.0 KCl	100	6.0	
	4.0 KCl	50	5.0	
XDH (R)	1.5 NaCl	100	8.3	[55]
	1.5 KCl	100		
	0.1 MgCl <sub>2</sub>	100		
APH (W)	1.5 NaCl	50	9.0	[49]
	0.0-3.0 KCl	32		
	0.0034 CaCl <sub>2</sub>	100		

W and R are wild and recombinant enzymes, respectively. MDH is Malate dehydrogenase; CPx Catalase-peroxidase; EST esterase; NR Nitrate reductase; SRS Seryl-tRNA synthetases; ACS AMP-forming acetyl-CoA synthetase; ADH alcohol dehydrogenase; XDH D-Xylose Dehydrogenase; APH Alkaline phosphatase. \*ADH performs two characteristic reactions: oxidation in alkaline pH and reduction in acid pH.

The required salt concentrations for the folding of *H. marismortui* malate dehydrogenase (HmaMDH) and esterase (HmaEST) were studied. HmaMDH showed a large increase of transition energy from 0.15 to 0.85 M NaCl, using activation energy analysis. It was suggested that the HmaMDH structural conformation changed due to the increase in the activation energy [63]. The HmaEST secondary structure emerged in a range of 0.25 to 0.5 M KCl, as revealed by far-UV circular dichroism [44].

The concentration and type of salt required for optimal catalytic activities is an inherent feature for each enzyme. Moreover, halophilic enzymes show a marked dependence on the salt concentration for activity and stability at different pH and incubation temperatures (Table V).

Some comparative studies of activities and stabilities of wild and recombinant enzymes at different types and concentrations of salts have been carried out. For instance, the wild HmaMDH retained complete stability at 2 M NaCl and 1.5M KCl, while the recombinant HmaMDH retained complete stability at 3M NaCl and 2 M KCl, after 24 h of incubation at 24°C and pH 8 [64, 65] (Table V). As shown in Table V, concentrations and types of salts are different for optimal activity and stability of wild enzyme and its respective recombinant. These differences could be attributed to divergences in glycosylation patterns, as reported for *Hfx. volcanii* [66]. Finally, it is worth noting that *H. marismortui* enzymes require different salt concentrations, suggesting a relation between the salt requirements and their location in the cell (cytoplasm, ribosomes, membrane).

TABLE V. OPTIMAL CONDITIONS OF pH, INCUBATION TEMPERATURE AND SALT CONCENTRATION FOR STABILITY OF *H. MARISMORTUI* ENZYMES.

Enzyme	Time (h)	pH	T (°C)	Salt conc. (M)	Residual activity (%)	Ref
MDH (W)	9	7.3	30	3.5 NaCl	100	[63]
	9	7.3	30	3.5 KCl	100	
	9	7.3	30	2.5 NH <sub>4</sub> Cl	100	
	9	7.3	30	1.5 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	90	
	9	7.3	30	1.5 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	90	
MDH (W)	24	8.0	20	2.0 NaCl	100	[64]
MDH (R)	24	8.0	20	3.0 NaCl	100	
MDH (R)	24	6.0	25	1.5 NaCl	100	[67]
	24	7.0	25	1.5 NaCl	100	
	24	8.0	25	2.0 NaCl	100	
	24	9.0	25	2.0 NaCl	100	
	24	6.0	25	0.9 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	100	
	24	7.0	25	0.9 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	100	
	24	8.0	25	1.2 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	100	
	24	9.0	25	1.2 (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	100	
CPx (W)	24	8.0	25	1.5 KCl	100	[65]
CPx (W)	24	8.0	25	1.0 KF	100	
CPx (R)	24	8.0	25	2.0 KCl	100	[65]
	24	8.0	25	1.0 KF	100	

Enzyme	Time (h)	pH	T (°C)	Salt conc. (M)	Residual activity (%)	Ref
LipC (R)	1	8.5	45	3.5 NaCl	95	[45]
	1	8.5	45	3.0 KCl	100	
SRS (R)	24	7.5	37	1.25 KCl	100	[50]
	24	7.5	37	1.5-4.5 NaCl	100	
	24	7.5	37	0.5-1.5 MgCl <sub>2</sub>	100	
APH (W)	24	9.0	20	3.5 NaCl	80	[49]
	24	9.0	20	3.5 M NaCl	80	
	24	9.0	20	3.5 M NaCl	75	

W and R are wild and recombinant enzymes, respectively. **MDH** is Malate dehydrogenase; **CPx** Catalase-peroxidase; **LipC** esterase; **SRS** Seryl-tRNA synthetase; **APH** Alkaline phosphatase.

## XII. CONCLUSIONS

The use of halophiles products in the industry is expanding. For instance, purple membrane proteins (rhodopsin) are used for photochemical applications [68] and halophilic living cells are used for environmental bioremediation (treatment of saline wastewaters) [5]. Additionally, the issuance of polymer production patents increases annually. Due to the need for more environmentally friendly technology, halophilic archaea are considered as a potential source of enzymes, catalyzing reactions with reduced water activity, including nonaqueous solvents.

Difficulties in the study of *Haloarcula marismortui* are common for other halophilic archaea. The main problem in archaeal cultures is the low biomass production and consequently, the low concentration of cell components (including enzymes). Moreover, halophilic microorganisms require special equipment for culturing, supporting high salt concentrations and supplying the required oxygen levels. Different approaches have been proposed to resolve these problems like recombinant DNA techniques, modification of culture conditions and bioreactor design to improve biomass and enzymes production.

The expression of enzymes in nonarchaeal hosts is often accompanied by a lack of enzymatic activity, caused by the formation of inactive inclusion bodies and the intrinsic difficulties to disaggregate them and recover the active enzyme.

Currently, halophilic microorganisms have been considered as suitable hosts for recombinant protein production (i.e. *Haloferax volcanii*) to avoid inclusion bodies [69–71]; however, it still remains difficult to achieve high cellular growth and product yield. On the other hand, in most cases, the modification of culture conditions and the bioreactor design has been insufficient to reach a high

concentration of biomass and enzymes. However, in some cases such as productions of lipid pigments and PHA polymers, these approaches have been successfully applied.

Studies of *H. marismortui* enzymes have been performed by using cells cultures or molecular biology techniques. However, the majority of studies on *H. marismortui* enzymes were conducted using molecular biology techniques, revealing itself as the best way to produce enzymes from extreme halophilic microorganisms. The hosts and vectors used to obtain the recombinant enzymes from *H. marismortui* are summarized in Table VI.

TABLE VI. HOST AND VECTORS USED FOR EXPRESSION OF RECOMBINANT ENZYMES OF *HALOARCUA MARISMORTUI*.

Enzyme	Vector	Strain host	Enzyme active	Ref
MDH	pET11a	<i>E. coli</i> K 71/18 <i>E. coli</i> K12 HMS174, <i>E. coli</i> BL21(DE3)LysS	Yes	[36]
	pET11a	<i>E. coli</i> BL21 (DE3)	Yes	[72]
NRs	pT7 Blue T, pNR1, pNR2	<i>E. coli</i> NarG	No	[46]
CPx	Lambda Zap, R408, pBlue-script, pvc40, pBk 72	XLI- blue HRF strain	NR	[73]
	pUC119, pHK, pWL102, pWLHK, pWLHKM2 44A	<i>Hfx. volcanii</i> , <i>Hfx. denitrificans</i> JCM884 <sup>T</sup>	Yes	[58]
SRS	pUC18, pET, pCR <sup>TM</sup> II	<i>E. coli</i> DH5a, <i>E. coli</i> HMS174, <i>E. coli</i> GI724, <i>E. coli</i> B HMS174 (DE3)	No	[50]
ACD	pET17b	<i>E. coli</i> BL21 (DE3)-RIL	Yes	[52]
EST	pET14b	<i>E. coli</i> BL21(DE3)pLysS	Yes	[44]
	pET28a	<i>E. coli</i> BL21 (DE3)	Yes	[45]
ADH	pSTBlue-1, pRV1, pTA963	<i>E. coli</i> NovaBlue, <i>E. coli</i> XL10-Gold <i>Hfx. volcanii</i> (DS70), <i>Hfx. volcanii</i> (H1209)	Yes	[54]
	pET17b	<i>E. coli</i> BL21	Yes	[55]

**MDH** Malate dehydrogenase; **NRs** Nitrate reductase; **CPx** Catalase-peroxidase; **SRS** Seryl-tRNA synthetase; **ACS** ADP-forming acetyl-CoA synthetase; **EST** esterase; **ADH**

alcohol dehydrogenase; **XDH** D-Xylose Dehydrogenase. NR Non reported.

Post-translational modification (N-glycosylation) is common in Archaea as a strategy adopted by extremophiles to cope with the physically challenging environments where they proliferate [74]. The *in situ* folding and post-translational modification of recombinant enzymes in *Haloferax volcanii* host might confer similar properties to those found in *H. marismortui* wild enzymes. Protein post-translation modifications in *Hfx. volcanii* include permanent or temporary covalent attachment of one or more biomolecules, such as sugars, lipids, signal peptide cleavage and sanylation (a process that resembles ubiquitylation, it is thought to be linked to proteasome-mediated degradation) [75]. Differences of catalytic activity and stability between wild and recombinant haloarchaeal enzymes have been widely documented. Probably, using *Haloferax volcanii* as an enzyme producer host, these differences could be minimized.

TABLE VII. PURIFICATION PROCEDURES FOR *H. MARISMORTUI* ENZYMES.

Enzyme	Purification steps	Final Yield %	Ref
MDH	1 Acetone, 2 Ammonium sulfate, 3 DEAE cellulose, 4 Electrophoresis, 5 Electrophoresis.	0.5	[35]
	1 Sonication, 2 Ammonium sulfate precipitation, 3 Sepharose 4B chromatography, 4 DEAE-cellulose chromatography, 5 Gel filtration Sephadex G-100 chromatography, 6 Hydroxylapatite chromatography, 7 Affinity chromatography NAD <sup>+</sup> -Sepharose.	48	[63]
	1 Ammonium sulfate precipitation, 2 Sepharose 4B chromatography, 3 Hydroxylapatite Bio Gel HT chromatography.		[36]
	Sepharose Mono Q chromatography.		[77]
	1 cell disruption, 2 Sefarose 4B chromatography, 3 fractionation on DE52 cellulose chromatography, 4 concentrated and dialysed, 5 hydroxyapatite column chromatography, 6 buffer exchanged (by Centricon system with a membrane of 30-kDa).		[67]
	1 Ammonium sulfate precipitation, 2 Sepharose 4B chromatography, 3 Hydroxylapatite Bio Gel HT chromatography.		[78]
	1 Membrane fraction, 2 Protein solubilization, 3 DEAE-Toyopearl chromatography, 4 Gel-filtration Sephacryl S-300 chromatography.	44.8	[46, 79]

Enzyme	Purification steps	Final Yield %	Ref
CuNiR	1 Soluble fraction from lysed cell, 2 Two butyl-Toyopearl chromatographies, 3 Sepharose CL-6B chromatography, 4 Gel filtration Sephacryl S300 chromatography, 5 Octyl sepharose chromatography, 6 Hydroxyapatite chromatography.	17.8	[47]
GDH	1 Ammonium sulfate precipitation, 2 Sepharose 6B chromatography, 3 DEAE cellulose chromatography, 4 affinity chromatography.	58	[48]
APH	1 CsCl density gradient ultracentrifugation, 2 Gel filtration Sepharose 6B chromatography.		[49]
CPx	1 sepharose 4B chromatography, 2 DEAE cellulose chromatography, 3 Dialysis, 4 Hidroxyapatite chromatography, 5 Gel filtration chromatography.	55	[42]
CPx	1 DNase treatment and ultracentrifugation, 2 butyl-Toyopearl 650 M chromatography, 3 Sephacryl S-300 chromatography, 4 Octyl Sepharose chromatography, 5 Gel-filtration chromatography.		[80]
SRS	1 cell disruption, 2 DEAE Sepharose CL 6B chromatography, 3 Utrogel AcA (Sepracor) chromatography, 4 Hydroxyapatite HA Utrogel (Sepracor) chromatography.	8.2	[50]
ACD / ACS	1 Cell-free extract, 2 Heat precipitations, 3 Q-sepharose chromatography, 4 Superdex 200 chromatography, 5 Phenyl-Sepharose chromatography.	6	[52]
		28	[53]
EST	1 Cell-free extract, 2 Heat precipitations, 3 Q-sepharose chromatography, 4 Superdex 200 chromatography, 5 Phenyl-Sepharose chromatography.	70	[44]
	1 cell disruption, 2 Affinity chromatography (IMAC), 3 Hydrophobic interaction chromatography, 4 proteolytic cleavage	38.5	[45]
ADH	1 IMAC with Ni <sup>2+</sup> affinity chromatography.		[54]
XDH	1 cell disruption 2 Sepharose CL 4B chromatography, 3 Phenyl sepharose chromatography, 4 Superdex chromatography.	10	[55]
	1 Inclusion bodies refolding, 2 Superdex chromatography, 3 Resource Phenyl chromatography.		



**MDH** Malate dehydrogenase; **NRs** Nitrate and Nitrite reductases; **GDH** Glutamate dehydrogenase; **FD** 2Fe-ferredoxin; **APH** Alkaline phosphatase; **CPx** Catalase-peroxidase; **SRS** Seryl-tRNA synthetase; **ACD** ADP-forming acetyl-CoA synthetase; **ACS** AMPforming acetyl-CoA synthetase; **EST** esterase; **ADH** alcohol dehydrogenase; **XDH** D-Xylose dehydrogenase.

New replicative vectors have been developed using origins of DNA replication taken from indigenous haloarchaeal plasmids [76]. Additionally, *Halobacterium* sp and vectors have been successfully employed as an expression system for archaeal enzymes [76].

Finally, the purification methods and yields of *H. marismortui* enzymes are summarized in Table VII. Several purification steps have been performed for that purpose, which generally consist of cell disruption, precipitation with solvents or ammonium sulfate and chromatographic procedures (ionic, hydrophobic, affinity and gel filtration). Nevertheless, purification yields of enzymes are generally poor; therefore, new improved procedures should be implemented.

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