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Chapter 1

Kinetics of Halophilic Enzymes

Luis Alberto Cira-Chávez, Joseph Guevara-Luna, Marisela Yadira Soto-Padilla, Brenda Román-Ponce, María Soledad Vásquez-Murrieta and María Isabel Estrada-Alvarado

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Abstract

Hypersaline environments are those with salt concentrations 9–10 times higher (30–35% of NaCl) than sea water (3.5% of NaCl). At high concentrations of soluble salts, cytoplasm—mainly of bacteria and archaea—is exposed to high ionic strength and achieves osmotic equilibrium by maintaining a cytoplasmic salt concentration similar to that of the surrounding media. Halophilic enzymes are extremozymes produced by halophilic microorganisms; they have similar characteristics to regular enzymes but different properties, mainly structural. Among these properties is a high requirement of salt for biological functions. Furthermore, the discovery of enzymes capable of degrading biopolymers offer a new perspective in the treatment of residues from oil deposits, under typically high conditions of salt and temperature, while giving valuable information on heterotrophic processes in saline environments.

Keywords: halotolerants, halophiles, *salt-in*, synthesis intracellular compounds, extremonzymes

1. Introduction

Extreme environments involve a wide range of extreme conditions (pH, temperature, pressure, light intensity, oxygen, nutrient conditions, heavy metals, and salinity). Hypersaline environments are those with salt concentrations 9–10 times higher (30–35% of NaCl) than sea water (3.5% of NaCl). These sites are widely distributed around the world and can harbor microorganisms from three different life domains (archaea, bacteria, and eukaryota); together, these microorganisms are known as halophiles, which survive or even thrive in saline environments [1].

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2. Classification of halophiles and halophile environments

Nowadays, several classifications of halophiles have been suggested; the classification proposed by Ollivier et al. [2] considers those microorganisms capable of growing in salt concentrations ≥150 g L^{-1} (15% w/v, 2.5 M) as halophiles. Another classification considers the optimum growth salinity as follows: mild halophiles (1–6%, w/v NaCl), moderate halophiles (7–15%) and extreme halophiles (15–30%) [3]. On the other hand, Ventosa and Arahal [4] defines halophiles as organisms that have an optimal growth above 3% salt concentration; if the optimal growth occurs between 3 and 15% salt, they are regarded as moderate halophiles; and when it occurs above 15% and up to halite saturation (34%), they are regarded as extreme halophiles. In addition, DasSarma and DasSarma [5] described halophiles as those organisms that thrive from sea salinity (~0.6 M) up to saturation salinity (>5 M NaCl). However, the most complete and widely used classification scheme was proposed by Kushner and Kamekura [6], in which halophilic microorganisms are separated into six groups based on their salt requirement and tolerance (**Table 1**): non-halophiles are those that have optimal growth in culture media containing less than 0.2 M NaCl; slight halophiles (marine bacteria) grow best in media with 0.2–0.5 M NaCl; moderate halophiles grow best with 0.5–2.5 M NaCl; borderline extreme halophile that growth best at 2.5–4.0 M; extreme halophiles show optimal growth in culture media containing NaCl concentration between 4 and 5.9 M; and finally halo-tolerant microorganisms, which are non-halophiles that can tolerate high salt concentrations but do not require salt to survive; any microorganism viable at 2.5 M of NaCl is considered extremely halotolerant. Archaea and bacteria are the most widely distributed organisms in hypersaline environments [7], especially in those in which salinities exceed 1.5 M (about 10%). In recent years, halophilic organisms are mainly isolated from saline environments, such as salt lakes, marine solar salterns, saline soils, and marine sediments (see **Table 1**). However, halophile bacteria have also been isolated from some non-common places, for example, textile effluents, halophytes, mine tailings as well as processed foods (**Table 1**).

2.1. Hypersaline environments

Hypersaline environments are extreme habitats with limited microbial diversity as result of high salt concentrations and other environmental factors. Nowadays, most environmental studies have been carried out on aquatic habitats, such as saline lakes and solar salterns used for the production of salt for commercial purposes [8]. Nevertheless, halophilic bacteria can be found in other habitats including saline soils, salted foods and other products, hides, and deep-sea brine pools [7, 9–11]. Depending on whether they originated or not from seawater, hypersaline environments are classified as thalassohaline and athalassohaline, respectively.

2.1.1. Thalassohaline environments

The thalassohaline environments are saline environments of marine origin, which contain the following ions: Cl^{-1}, Na^{+}, Mg^{2+}, SO_4^{2-}, K^{+}, Ca^{2+}, Br^{-}, HCO_3^{-}, and F^{-} [4]. Some examples of thalassohaline as explained as follows.

Category	Salt tolerance (M)	Example	Isolation site	References
Non-halophile	<0.2	*Vibrio palustris* EAdo9^T and *Vibrio spartinae* SMJ221^T	Salt-marsh plants	[106]
Slight halophile	0.2–0.5	*Paracoccus* sp. GSM2	Textile mill effluent	[107]
		Bacillus sp. NY6	Saline wastewater	[108]
		Zunongwangia endophytica CPA58^T	Tissues of the halophyte *Halimione portulacoides*	[109]
Moderate halophile	0.5–2.5	*Salinispora arenicola* CNH-643^T and *Salinispora tropica* CNB-440^T	Marine sediments	[110]
		Salinispora pacifica CNR-114^T	Marine sediments	[111]
		Martelella endophytica YC6887^T	Root of *Rosa rugosa*	[112]
		Streptomyces halophyticola KLBMP 1284^T	Stems of *Tamarix chinensis*	[113]
		Labrenzia suaedae YC6927^T	Root of *Sauceda maritime*	[114]
		Kocuria arsenatis CM1E1^T	*Prosopis laevigata*	[115]
		Proteus sp. NA6	Textile effluent drain	[116]
		Candidatus *Desulfonatronobulbus propionicus*	Hypersaline soda lakes	[117]
		Novosphingobium pokkalii L3E4^T	Rhizosphere of saline-tolerant pokkali rice	[118]
		Marinobacter aquaticus M6-53^T	Marine saltern located in Huelva, Spain	[119]
		Agrobacterium salinitolerans YIC 5082^T	Root nodules of *Sesbania cannabina* grown in a high-salt and alkaline environment	[120]
		Salinicola tamaricis F01^T	Leaves of *Tamarix chinensis*	[121]
		Salinirubellus salinus ZS-35-S2^T	Marine solar saltern	[122]
Borderline extreme halophile	2.5–4.0	*Aliifodinibius halophilus* 2W32^T	Marine solar saltern	[123]
		Desulfosalsimonas propionicica PropA^T	Hypersaline sediment of the Great Salt Lake	[124]
		Salinibacter iranicus CB7^T and *Salinibacter luteus* DGO^T	Aran-Bidgol salt lake, Iran	[125]
		Halanaerobium sehlinense 1Sehel^T	Sediments of the hypersaline lake Sehline Sebkha	[126]
		Sporohalobacter salinus CEJFT1B^T	Under the salt crust of El-Jerid hypersaline lake in southern Tunisia	[127]
		Lentibacillus kimchii K9^T	Korean fermented food (kimchi)	[128]
		Marinobacter salexigens HJR7^T	Marine sediment	[129]
		Gracilimonas halophila WDS2C40^T	Marine solar saltern	[130]
		Salinifilum proteinilyticum Miq-12^T	Wetland in Iran	[131]
		Natronospira proteinivora Bsker1^T	Marine solar saltern	[132]

Category	Salt tolerance (M)	Example	Isolation site	References
Extreme halophile	4–5.9	*Salinibacter ruber* M31^T	Saltern crystallizer ponds in Alicante and Mallorca, Spain	[12]
		Limimonas halophila IA16^T	Mud of the hypersaline Lake Aran-Bidgol, Iran	[133]
		Desulfonatronobacter acetoxydans APT3	Hypersaline soda lake	[47]
Halo-tolerant	A non-halophile that tolerant salt; if it is viable 2.5 M, in is considered extremely halotolerant	*Brevibacterium salitolerans* TRM 415^T	Sediment from a salt lake	[134]
		Kineococcus endophytica KLMMP 1274^T	Halophytic plant (*Limonium sinense*)	[135]
		Anditalea andensis ANESC-S^T	Alkali-saline soil	[136]
		Brevibacterium jeotgali SJ5-8^T	Traditional Korean fermented seafood	[137]
		Salimicrobium sp. LY19	Saline soil	[138]
		Brevibacterium metallicus NM3E2^T	Edge of mine tailings	[139]
		Bacillus subtilis BLK-1.5	Salt mines	[76]
		Halomonas nigrificans MB G8645^T	Acid curd cheese called Quargel	[140]

Table 1. Classification of bacteria based on their salinity tolerance according with criteria proposed by Kushner and Kamekura [6].

2.1.1.1. Solar salterns

These sites have a similar composition to seawater and they are used for salt production by evaporation. They generally consist of several ponds interconnected to form the so-called multipond system. Seawater is pumped or allowed to flow into the first ponds, and as a consequence of solar evaporation, the concentration of salts increases slightly and the water is moved to the next ponds, where it will concentrate further. Finally, in the last pond (called crystallizer), common salt is precipitated [4]. Many studies have focused on the isolation of bacteria harbored in hypersaline environments, identifying the following major groups: Bacteroidetes [12], Firmicutes [13–15], ϒ-Proteobacteria [13, 16–17], and ϒ-Proteobacteria being the most abundant.

2.1.1.2. Soils

Saline soils are those with an electrical conductivity (EC) higher than 4 dS mL^{-1}, approximately 40 mM NaCl [18]. Nowadays, salinized areas are increasing at rate of 10% annually for various reasons, including low precipitation, high surface evaporation, weathering of native

rocks, irrigation with saline water, and poor cultural species practices [19]. Several studies on hypersaline soils mainly isolated moderate halophiles and non-halophilic bacteria affiliated with different genera of the following taxonomic groups: Firmicutes [20–22], actinobacteria [23, 24], and proteobacteria [25, 26].

2.1.1.3. Great Salt Lake

In 1957, a rock-filled railroad causeway was completed across the lake, dividing it into a northern and a southern basin. The northern arm presented high salinity (33%), while the southern arm separated by a semipermeable rock causeway contains a moderate concentration of salt (12%) [27]. A number of studies on the isolation of bacteria from the sediment from Great Salt Lake have been carried out [28, 29].

2.1.2. Athalassohaline environments

These are environments that do not have a marine origin and their ionic proportions are quite different from that of the dissolved salts in seawater [30]. They reflect the composition of the surrounding geology, topography, and climate conditions, often particularly influenced by the dissolution of mineral deposits [31].

2.1.2.1. Dead Sea

The Dead Sea, which is actually an inland lake, is famous for being so saline that people can float with ease on its surface. The site is composed mainly of divalent ions like Mg^{2+} [32]. The Dead Sea is a hypersaline lake with 34% salinity, and its name is due to the lack of any living macroscopic creatures. The lake consists of a deeper northern basin and a shallow southern basin, which has been recently dried up and used for commercial mineral production [33]. The water level is dependent on the balance between amount of freshwater inflow and evaporation [32]. The Jordan River is the main source of freshwater inflow, in addition to several water springs and the complex system of underwater springs, which has been recently discovered [34]. Metagenomic studies demonstrated the presence of Halobacterium-like sequences and Mg^{2+} transport-related proteins, suggesting a potential adaptation to the high magnesium concentration by Dead Sea halophiles [35]. In addition, metagenomic sequence analysis and amino acid profiling also demonstrated the presence of halophiles never previously isolated or sequenced in the Dead Sea [35, 36]. It was recently discovered that the Dead Sea harbors some bacteria with biotechnological properties, such as *Bacillus persicus* 24-DSM, which showed antimicrobial activity [33].

2.1.2.2. Soda lakes

Athalassohaline alkaline salt lakes (or soda lakes), rich in NaCl, $NaHCO_3$, and Na_2CO_3, are usually formed by dissolution of rocks that are low in magnesium and calcium, which would

otherwise cause carbonate to precipitate [37, 38]. The most studied lakes are those in the East African Rift Valley, continental Russia, and the USA. In addition to being able to tolerate high pH values and elevated salinities, microbes inhabiting soda lakes have to cope with low availability of NH_4^+, caused by weak dissociation of ammonia at high pH. An accumulation of stressful but volatile NH_3 may occur in enclosed alkaline-saline systems such as sea ice or locally in soda lakes [32]. Several studies about bacteria isolation from soda lakes from different continents are available [39–47].

Halophilic microorganisms have several biotechnological applications, such as β-carotene production of fermented foods. In recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers, and valuable compounds from halophiles may present advantages for the development of biotechnological production processes.

2.2. Biology and adaptation of halophilic bacteria

The first chemical stress encountered during the evolution of life on earth may have been salt stress. Thus, from the beginning, organisms must have evolved strategies and effective mechanisms for the stabilization of protoplasmic structures and ion regulation [48]. At high concentrations of soluble salts, cytoplasm—mainly of bacteria and archaea—is exposed to high ionic strength and achieves osmotic equilibrium by maintaining a cytoplasmic salt concentration similar to that of the surrounding media. This can affect microbes via two primary mechanisms: osmotic effect and specific ion effects. Soluble salts increase the osmotic potential (more negative) of the soil water, drawing water out of cells which may kill microbes and roots through plasmolysis [49, 50].

To thrive in the hypersaline environment, halophiles have two main adaptation mechanisms to prevent NaCl from diffusing into the cells. The first mechanism is accumulation of inorganic ions (mainly KCl) for balancing osmotic pressure. This mechanism is mainly utilized by aerobic and extremely halophilic archaea and some anaerobic halophilic bacteria [32, 49, 51]. In contrast, most halophilic bacteria accumulate water soluble organic compounds of low molecular weight, which are referred to as compatible solutes or osmolytes, to maintain low intracellular salt concentration [52–54].

2.2.1. Salt-in mechanisms

As mentioned above, microorganisms that grow optimally in the presence of extremely high salinities (up to 5 M NaCl), accumulate intracellular potassium and chloride ions in concentrations higher than the external NaCl concentration to maintain a turgor pressure. This so-called "salt-in" strategy is observed in *Halobacteriales* (archaea) and *Halanaerobiales* (anaerobic halophilic bacteria) [55, 56]. The mechanism ("salt-in" to balance "salt-out") requires far-reaching adaptations of the entire intracellular machinery, as all enzymes and functions in the cytoplasm have to be functional in the presence of molar concentrations of KCl [57]. A characteristic feature of halophilic proteins from microorganisms that accumulate KCl for osmotic balance is their highly acidic nature, with a great excess of acidic amino acids (glutamate and

aspartate) over basic amino acids (lysine and arginine). Such proteins are highly negatively charged compared to their non-halophilic equivalents. In addition, halophilic proteins generally have a low content of hydrophobic amino acids [58, 59].

2.2.2. Synthesis of intracellular compounds

As explained above, microorganisms have the ability to adapt to or tolerate stress caused by salinity by accumulating osmolytes, also known as compatible solutes. The compatible solute strategy is broadly known in domain archaea, bacteria, as well as eukarya. Organisms accumulate organic solutes by uptake from the environment or *de novo* synthesis of organic compounds, such as sugars and polyols, amino acids and their derivatives, and other compatible solutes for protection against salinity stress [60–62].

Organic solutes act as stabilizers for biological structures and allow the cells to adapt not only to salts but also to heat, desiccation, cold, or even freezing conditions [63]. Many halophilic bacteria accumulate ectoine or hydroxyectoine as the predominant compatible solutes. Other intracellular compatible solutes include amino acids, glycine betaine and other compounds accumulated in small amounts [54].

Mei et al. [64] describe the physiology of a *Natrinema* sp. strain J7–2, an extremely halophilic archaea isolated from a salt mine in China, under salt stress conditions (15, 25, and 30% NaCl). This strain showed the highest growth rate at 25–30% of NaCl, while at 15% cells were more fragile. Furthermore, the glycerolipid and amino acidic metabolism showed a significant difference in cellular transcripts levels, perhaps playing a role in membrane production/alteration or in accumulation of specific amino acids (glutamate family—Glu, Arg and Pro; aspartate family—Asp; and aromatic amino acids—Phe and Trp), especially Glu and Asp as carbon substrates and energy resources or compatible solutes.

The most common inorganic solutes used as osmolytes by salinity tolerant microbes are potassium cations, while proline and glycine betaine are the main organic osmolytes [65]. However, the synthesis of these compounds requires high amounts of energy [50, 66]. Given these high energetic requirements, there are few reports of halophilic microorganisms that can produce compatible solutes to mitigate the stress by variable concentrations of salts. The capacity of two halophilic strains is noteworthy: *Planococcus* sp. VITP21 and *Bacillus* sp. VITP4, which are capable of *de novo* synthesis of two rarely occurring diamino acids, Nε-acetyl α-lysine and Nδ-acetyl ornithine, respectively; besides the well-known ectoine and proline [67] as simple diamino acidic molecules to tolerate salt stress.

2.3. Production of extremozymes

Halophilic enzymes are extremozymes produced by halophilic microorganisms; they have similar characteristics to regular enzymes but different properties, mainly structural. Among these properties is a high requirement of salt for biological functions. In recent years, different studies have focused on the detection of halophiles in saline environments in order to isolate and characterize new enzymatic activities. This resulted in several halophile hydrolases being

described, including amylases, lipases, and proteases. Furthermore, the discovery of enzymes capable of degrading biopolymers offer a new perspective in the treatment of residues from oil deposits, under typically high conditions of salt and temperature, while giving valuable information on heterotrophic processes in saline environments.

2.3.1. Extremozymes-producing halophiles

Nowadays, investigation on the production of extremozymes from different bacterial genus and halophilic archaea has intensified. This interest is due to their capacity to efficiently catalyze a process and show optimal activities at different salt concentrations. Halophiles are the most probable source of extremozymes, since they are also capable of tolerating alkaline pH and high temperatures, as reported by several authors [68–79].

Most of the evaluation studies on the enzymatic capacities of halophiles begin with the isolation of these microorganisms from environments considered extreme due to specific characteristics such as high salt concentrations, high pH values, and extreme temperature conditions. Sánchez-Porro et al. [80] report the isolation of moderately halophile strains from water and salterns in different areas of southern Spain: Almería (Cabo de Gata), Cádiz (San Vicente and San Fernando), and Huelva (Isla Bacuta, Río Tinto and Isla Cristina). Isolates have been identified as members of the genera *Salinivibrio, Bacillus, Salibacillus, Halomonas, Chromohalobacter, Salinicoccus*, and *Marinococcus* and they showed amylase, protease, lipase, and DNase activities.

In 2007, Vidyasagar et al. [68] isolated the extreme halophile *Chromohalobacter* sp. from solar lanterns, and subsequently produced and partially purified a halo-thermophile protease extracellular enzyme. *Chromohalobacter* sp. required a 4 M concentration of NaCl for optimal growth and protease secretion, and no growth was observed under 1 M NaCl. The initial pH of the medium for growth and enzyme production was in the interval of 7.0–8.0, with an optimum value of 7.2. Halophile *Salinivibrio* sp. isolated from Bakhtegan Lake in southern Iran also produced an extracellular protease [81].

Rohban et al. [82] studied extremophiles in Howz Soltan, a hypersaline lake located in central Iran. The organisms successfully isolated produced a wide variety of extracellular enzymes, where 84.4% had lipase activity, 76.6% amylase, 43.2% protease, 41.1% inulinase, 39.8% xylanase, 29.4% cellulase, 14.2% DNase, and 12.1% pectinase. Halophile strains were identified as members of the following genera: *Salicola, Halovibrio, Halomonas, Oceanobacillus, Thalassobacillus, Halobacillus, Virgibacillus, Gracilibacillus, Salinicoccus*, and *Piscibacillus*. Most of the lipase and DNase producers belonged to the *Gracilibacillus* and *Halomonas* genera, respectively, while most of the organisms capable of producing hydrolytic enzymes (amylase, protease, cellulase, and inulinase) were part of Gram-positive genera, such as *Gracilibacillus*, *Thalassobacillus, Virgibacillus*, and *Halobacillus*.

In 2011, Perez et al. [83] reported the isolation and purification of a lipase obtained from the *Marinobacter lipolyticus* SM19 halophile, isolated from a saline habitat in southern Spain. The properties of this enzyme are of great potential for the food industry. Li and Yu [84] isolated the halophile strain LY9, which has amylolytic properties, from soil samples obtained in Yuncheng, China. The strain LY9 was identified as a member of the halobacillus genus and it was discovered that the production of amylase secreted for this strain depended on the salinity

of the growth medium. The maximum production of amylase was observed in the presence of 10% KCl or 10% NaCl. Maltose was the main product from hydrolysis of soluble starch, pointing out to β-amylase activity.

In agreement with previous studies from 2003, 2007, and 2009, Shahbazi and Karbalaei-Heidari [85] reported the capacity of *Salinivibrio* sp. to produce extracellular low molecular weight proteases, and Jayachandra et al. [86] reported the isolation and identification of extracellular activity of hydrolytic enzymes from bacteria in the *Salinicoccus* sp. genus. Strain JAS4 was isolated from the Arabal soil in the west coast of Karnataka, India. These bacteria showed great potential to produce extracellular enzymes such as amylase, protease, inulinase, and gelatinase. Also in 2012, Kumar et al. [70] isolated halophiles from different saline environments in India, by means of morphological, biochemical, and 16S rRNA analyses. The authors identified the genera *Marinobacter, Virgibacillus, Halobacillus, Geomicrobium, Chromohalobacter, Oceanobacillus, Bacillus, Halomonas*, and *Staphylococcus* as having hydrolase activities of industrial relevance, pointing out the presence of amylases, lipases, and proteases. A new genus of marine bacteria is included among halophiles that are capable of producing extracellular hydrolytic enzymes, according to the research by Ardakani et al. [69], who isolated extracellular hydrolytic enzymes from the water and sediments of the Persian Gulf, in that site the isolation of bacteria that produced enzymes belong to the *Pseudoalteromonas* genera, and the activities include amylase, protease, and lipase.

During 2013, studies were made on enzyme-producing halophilic archaea capable of synthesizing two new alcohol-dehydrogenases, amylase and a thermostable halo-alkaliphile α-amylase; the producing organisms were identified as *Haloferax volcanii, Natrialba aegyptiaca* and *Halorubrum xinjiangense*, respectively. Hagaggi et al. [87] reported the isolation of the extremely halophilic archaea *Natrialba aegyptiaca*, from a salty soil near Aswan in Egypt. This organism is capable of producing an extracellular halophilic amylase that digests raw starch; therefore the enzyme may be used to efficiently process different vegetable sources. Moshfegh et al. [71] isolated a thermostable halo-alkaliphile α-amylase from an archaea located in the salty water of the Urmia Lake, which lies in northeast Iran. The producing organism was identified as *Halorubrum xinjiangense*, based on its morphological, biochemical, and molecular properties. Nigam et al. (2013) [72] tested the alkaline proteases produced by halophilic bacteria isolated from the Sambar Lake in Rajasthan for keratolytic activity. Moreno et al. [88] have shown that some microorganisms from hypersaline environments in Spain are able to produce hydrolytic enzymes; these have been related to the genera *Salinivibrio, Halomonas, Chromohalobacter, Bacillus-Salibacillus, Salinicoccus, Marinococcus, Halorubrum, Haloarcula, Halobacterium, Salicola, Salinibacter*, and *Pseudomonas*.

The bacteria isolated from a saline lake in Iran produced lipases, these bacteria belonging to the genera *Salicola, Halovibrio, Halomonas, Oceanobacillus, Thalassobacillus, Halobacillus, Virgibacillus, Gracilibacillus, Salinicoccus*, and *Piscibacillus*. On the other hand, sediments of deep waters in China have been found to contain amylase-producing organisms from the genera *Alcanivorax, Bacillus, Cobetia, Halomonas, Methylarcula, Micrococcus, Myroides, Paracoccus, Planococcus, Pseudomonas, Psychrobacter, Sporosarcina, Sufflavibacter*, and *Wangia*. In a desert in Chile, enzymes with DNase activity were related to the genera *Bacillus, Halobacillus, Pseudomonas, Halomonas*, and *Staphylococcus* [89].

A halo-alkaliphile, thermostable extracellular protease was reported by Selim et al. [74] produced by *Natronolimnobius innermongolicus* WN18 (HQ658997), an organism that belongs to the genus *Natronolimnobius* and was isolated from the sodium lake of An-Natrun, Egypt. Another halophile studied in the same year was the marine bacterium *Zunongwangia profunda,* due to its production of a new α-amylase resistant to low temperatures and tolerant to high concentrations of NaCl (4 M) [90].

Gupta et al. [75] reported a halo-alkaliphile isolated from a soil sample collected from the Sambhar Lake in Rajasthan, northern India, which produced an extracellular alkaline protease; the results of the analysis of gene 16S rRNA showed a 98% match with *Halobiforma* sp. Del Campo et al. [91] and Kumar and Khare [92] used fermentation in a solid medium to produce an esterase from halophilic archaea (*Natronococcus* sp. TC6, *Halobacterium* sp. NRC-1, and *Haloarcula marismortui*). These authors also optimized the production and nano-immobilization of *Marinobacter* sp. for an efficient hydrolysis of starch.

The *Kocuria* is mentioned as an example of a genus capable of producing extracellular amylases [77]. On the other hand, archaea *Halobacterium* sp. was isolated from samples of fermented fish and was considered a strong source of halophilic protease [93]; the bacterium *Bacillus licheniformis* isolated from sea water and sediments in Alexandria Eastern Harbor, Egypt, together with *Bacillus subtilis* isolated from the salt mines in Karak, Pakistan, were found to be producers of extracellular amylases and proteases, respectively [76, 94].

Dumorné et al. [95] stated that that the halophiles *Acinetobacter, Haloferax, Halobacterium, Halorhabdus, Marinococcus, Micrococcus, Natronococcus, Bacillus, Halobacillus*, and *Halothermothrix* produce extremozymes such as xylanases, amylases, proteases, and lipases. Halophile bacterium *Idiomarina* produces two extracellular proteases and was isolated in Badab-Sourt, Irán [79]. The same year, Hosseini et al. [96] described the isolation of bacteria capable of nitrite reduction that belonged to five different genera: *Bacillus, Halobacillus, Idiomarina, Oceanobacillus,* and *Virgibacillus,* in a paper regarding denitrifying halophile bacteria. Isolates capable of producing nitrate reductase were found among the genera *Halobacillus* and *Halomonas*. Another study on soils was carried out by Bhatt et al. [78], who isolated halo-alkaliphile bacteria from the saline desert soil in Little Rann of Kutch, India. Phylogenetic analysis indicated that isolates belong to phylum Firmicutes, which comprises lower G + C Gram-positive bacteria of different genera. Most of the halophilic isolates produced proteases (30% of isolates), followed by cellulases (24% isolates), CMCases (24% of isolates), and amylases (20% of isolates).

2.4. Physicochemical parameters and kinetic properties of extremozymes from halophilic microorganisms

Halophilic enzymes have specific mechanisms for solubility at high salt concentrations, such as a highly negative superficial charge given by carboxylic groups that depend on high salt concentrations to remain soluble. Halophilic archaea are known to secrete active proteases at high concentrations of NaCl (4 M), and to accumulate high concentrations of KCl in their cytoplasm in order to face osmotic stress, while maintaining the conformation of their proteins. The study made by Akolkar and Desai [97] suggests that proteases from haloarchaea may be active and stable in the presence of osmolytes different from NaCl/KCl at different degrees, as shown by the kinetics and thermodynamic analyses of casein hydrolysis produced by *Halobacterium* sp., in the presence of a compatible solute (sodium glutamate). In 2012, Zhang et al. [98] demonstrated

that NaCl may improve the thermal stability of enzymes, and the presence of NaCl or KCl increases enzymatic activity 10-fold, approximately; this agrees with other investigations that demonstrate that enzyme activity depends on the concentration of NaCl or KCl, as well as on the substrate, pH, and presence of ions. Moshfegh et al. [71] demonstrated that the concentration of NaCl 4 M or KCl 4.5 M determines the maximum activity of halo-alkaliphile α-amylase produced by archaea *Halorubrum xinjiangense*, besides improving its thermal stability. In a similar manner, Selim et al. [74] showed that the protease activity of *Natronolimnobius innermongolicus* depends on high concentrations of salt to remain active and stable. Proteases purified by Faghihi et al. [79] increased their activity in the presence of metallic ions such as Mn^{2+} and Cu^{2+}, while decreasing activity when exposed to Hg^{2+} and Fe^{2+}. Both proteases were strongly inhibited by SDS, while DDT, EDTA, and 2-mercaptoethanol may stimulate their activity.

The affinity of an enzyme to hydrolyze a substrate is determined by the Michaelis constant (K_m), which is the concentration of substrate at which the reaction velocity is half the maximum velocity (V_{max}). V_{max} is the maximum velocity when the system is saturated with substrate. The value of *Km* is a measure of the enzyme-substrate affinity, and at the moment there are very few determinations made on extremozymes from halophilic organisms. **Table 2** shows the values of kinetic constants for extremozymes currently available.

Nowadays, recombinant DNA techniques and genetic engineering are used to obtain customized extremozymes to be used for specific purposes, greatly improving their catalytic ability, as demonstrated by Kui et al. [99] with the expression of genes from extremozyme β-1,4-xylanase, which was cloned from *Nesterenkonia xinjiangensis* and expressed in *Escherichia coli*. This enzyme was thermostable, retaining more than 80% of the initial activity after incubation at 60°C for 1 h, and more than 40% activity at 90°C for 15 min. In the same way, Qin et al. [90] cloned a novel gene that codifies a new α-amylase, which is active at low temperatures and tolerant to salt (AmyZ), from the marine bacterium *Zunongwangia profunda*, this protein was also expressed in *Escherichia coli*. It was observed that AmyZ is one of the few α-amylases that tolerate both low temperatures and high salinity, which makes it a potential candidate for research in basic and applied biology.

2.5. Halophile extremozyme applications

As mentioned above, halophiles are good sources of several extremozymes, and among them hydrolases have been the most studied, mainly amylases, proteases, lipases, xylanases, cellulases, and DNases. Some extremozymes from halophiles exhibit extraordinary biochemical properties, which show the potential for industrial applications. It has been demonstrated that extremozymes derived from halophiles are able to function under harsh conditions and remain

Microorganism	Extremonzyme	km (mg/mL)	V_{max}	Reference
Nesterenkonia xinjiangensis	Xilanasa	16.08	45.66 μmol/min-mg	[99]
Bacillus sp.	Celulasa	3.18		[98]
Halorubrum xinjiangense	α-amilasa	3.8	12.4 U/mg	[71]
Aspergillus gracilis	α-amilasa	6.33	8.36 U/mg	[87]
Kocuria sp.	Amilasa	3.0	90.09 U/ml	[77]

Table 2. Kinetics parameters of extreme-enzymes produced by halophiles microorganims.

stable and active with different properties than conventional enzymes, offering opportunities in several applications such as environmental bioremediation, food processing, and residual water treatment. Recent research points out the application of halophilic extremozymes in the production of biofuels. Since several halophiles are also alkaliphiles, their enzymes are of interest for the textile and detergent industries, and some have been explored as raw materials in the production of commercial enzymes, particularly proteases and amylases [5, 100–105].

3. Conclusions

Halophile microorganisms have the ability to adapt to or tolerate stress caused by salinity by accumulating osmolytes. Halophilic microorganisms have several biotechnological applications, in recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers, and valuable compounds from halophiles may present advantages for the development of biotechnological production processes. Halophiles are the most probable source of extremozymes, since them also capable of tolerating alkaline pH and high temperatures.

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Author details

Luis Alberto Cira-Chávez[1], Joseph Guevara-Luna[2], Marisela Yadira Soto-Padilla[3], Brenda Román-Ponce[2,4], María Soledad Vásquez-Murrieta[2] and María Isabel Estrada-Alvarado[1]*

*Address all correspondence to: maria.estrada@itson.edu.mx

1 Departamento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora, Sonora, Mexico

2 Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Prol. de Carpio y Plan de Ayala, Ciudad de Mexico, Mexico

3 Instituto de Ingeniería y Tecnología, Universidad Autónoma de Ciudad Juárez, Mexico

4 Departamento de Microbiología y Genética, Edificio Departamental, Salamanca, Spain

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