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Review

Halophilic microorganism resources and their applications in industrial and environmental biotechnology

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Abstract: Hypersaline environments are extreme habitats on the planet and have a diverse microbial population formed by halophilic microorganisms. They are considered to be actual or potential sources for discovery bioactive compounds, compatible solutes including novel and/or extraordinarily enzymes. To date, a number of bioactive compounds for the use in various fields of biotechnology which show assorted biological activities ranging from antioxidant, sunscreen and antibiotic actions have been reported. In addition, some halophilic microorganisms are capable of producing massive amounts of compatible solutes that are useful as stabilizers for biomolecules or stress-protective agents. The present review will impart knowledge and discuss on (i) potential biotechnological applications of bioactive compounds, compatible solutes and some novel hydrolytic enzymes; (ii) recent efforts on discovery and utilization of halophiles for biotechnological interest; (iii) future perspective of aforementioned points.

Keywords: algae; bioactive compound; compatible solute; cyanobacteria; halophile; hypersaline

Abbreviations:
MAA: mycosporine-like amino acid;
RNS: reactive nitrogen species;
ROS: reactive oxygen species.

1. Introduction

Halophilic microorganisms comprise a heterogeneous group of microorganisms and require salts for optimal growth. They have been isolated from diverse salinity environments, varying from natural brines, hypersaline lakes to saturation salinities. Diverse halophilic population includes Archaea, Bacteria, and Eukarya [1–3]. Most commonly observed halophiles are Archaea and Bacteria such as *Halobacterium*, *Halomonas* and *Salinibacter* [1]. The halophilic Eukarya such as *Dunaliella salina* is one of nature's richest sources of carotenoids known to date [4]. Halophiles are able to thrive in hypersaline (~ 0.6 M) up to saturation salinity (>5 M NaCl) environments [5]. They have evolved several different molecular and cellular mechanisms to respond to the salt-stress condition. A primarily used 'salt-in strategy' can be achieved by raising the salt concentration in the cytoplasm, and their enzymes tolerate or require high-salt concentration which is well-recognized in halophilic Archaea and Bacteria [2,5]. The well-known mechanism is the so-called 'salt-out strategy' or 'organic-osmolyte mechanism' allowing an osmotic adaptation by excluding salts and/or synthesizing *de novo* compatible solutes [5,6]. This strategy is also used in Archaea and Bacteria. In addition, molecular basis of protein halotolerance and adaptation of halophilic enzymes to high salinity is by increasing a substantial number of protein charges and increased hydrophobicity [7–9].

Over the past few decades, halophiles have been considered for biotechnological applications. Diverse response mechanisms of halophiles under high-salinity conditions cause the production of various valuable biomolecules. It has been recognized that halophiles are also major sources of stable enzymes that function in very high salinity, an extreme condition that results in denaturation and aggregation of most conventional proteins [7,8]. For instance, the α-amylase isolated from *Haloarcula* sp. functions optimally at 4.3 M salt at 50°C, and is stable in solvents benzene, toluene and chloroform [10,11]. Furthermore, halophiles are also considered to be potential sources for discovery of bioactive compounds, compatible solutes, unique enzymes including other potential biotechnological uses. Currently only two industrial processes that employ halophilic microorganisms, namely carotenoid production by halophilic alga *Dunaliella* and ectoine production by halophilic bacterium *Halomonas elongate* are realized [2]. There are a number of bioactive compounds that show assorted biological activities ranging from antioxidant, sunscreen, and antibiotics actions derived from halophiles [4,12,13]. Various studies show that some halophiles are capable of synthesizing massive amounts of compatible solutes such as glycine betaine and ectoine [5,6]. These small organic molecules are useful as stabilizers of biomolecules or stress-protective agents. Thus, the halophiles have a broad biotechnological applications ranging from agriculture to biomedical (Table 1). In this review (i) discovery and biotechnological potential of bioactive compounds having antioxidant and sunscreen actions are highlighted; (ii) special emphasize is placed on compatible solutes and some extraordinarily hydrolytic enzymes; (iii) recent efforts on utilization of halophilic enzymes are discussed; and (iv) future perspective in biotechnology is mentioned.

2. Antioxidant Pigments

It was evident that extreme halophilic Archaea particularly in the families Haloferacaceae and Halobacterium (e.g. *Halobacterium salinarum*, *Halobaculum gomorrense*, *Haloferax mediterranei* and *Haloarcula marismortui*) inhabit the extremely halophilic environments such as Great Salt Lake and the Dead Sea in which salt concentrations are extremely high [1,2,14]. They overcome and

survive the challenge of these environments by adopting ‘salt-in strategy’, allowing their cells accumulate intercellular KCl equal to NaCl in surrounding medium. Their enzymes generally tolerate or require 4–5 M salt [5]. In addition to these adaptabilities, the extremely halophilic Archaea are also capable of producing extraordinary red pigments known as carotenoid compounds to combat the high salt and intense UV radiation [15]. It was shown that these colored pigments have strong antioxidant, immune boosting activities and likely protecting premature ageing [4,16,17].

Although many halophilic Archaeaproduce carotenoids, the halophilic Eukarya *Dunaliella salina* is the only halophilic organism used in industry for the production of carotenoids [4]. Carotenoids responsible for the red coloration in *Dunaliella salina* are found in algal chloroplasts, whereas those in halophilic Archaeaare dispersed throughout the cell membrane. Although there is a much greater abundance of *Dunaliella salina*, the red pigments on the cell membrane of Archaea are more visibly accessible. Therefore, *Dunaliella salina* has a much less overall impact to coloration. *Dunaliella salina* contains the bioactive compounds with strong antioxidant properties comprise of β-carotene, lycopene, phytoene, derivatives of bacterioruberin, and salinixanthin [4].

Carotenoids are hydrophobic compounds and generally consisting of a C40 hydrocarbon backbone. However, the majority of carotenoid derived from Archeae is the C50 carotenoid such as α-bacterioruberin, which is found in all archaeal strains [18]. All carotenoids possess a long conjugated chain of double bond and a near bilateral symmetry around the central double bond, as common chemical features [19]. This chain may be terminated by rings and can be complemented with oxygen-containing functional groups. Based on chemical structure and the oxygen presence, carotenoids can be classified into two different groups. The first group is carotenes (or carotenoid hydrocarbons), composed of only carbon and hydrogen. Examples of carotene molecules are lycopene and β-carotene. The second group is xanthophylls (or oxygenated carotenoids) in which their structures are oxygenated and may contain carbonyl, epoxy, hydroxyl, methoxy or carboxylic acid functional groups [19]. Lutein, canthaxanthin, zeaxanthin, violaxanthin, capsorubin and astaxanthin are known as xanthophyll carotenoids [20]. The most relevant biological functions of carotenoids are associated with their antioxidant properties, which directly emerge from their molecular structure. For instance, xanthophyll carotenoids in particular are free radical scavengers of both Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). It was shown that asthaxanthin and canthaxantin are better antioxidants and scavengers of free radicals than β-carotene [4,18,20].

Carotenoids are colorful natural products responsible from yellow, orange, red, to purple colors. They are extensively used as dyes and functional ingredients in food products including cosmetics [4]. Since carotenoids possess strong antioxidant, immune boosting activities and likely protecting premature ageing, they are also widely applied in pharmaceutical and medical fields such as antitumor and heart disease prevention agents [4,17]. Carotenoids are also used as enhancers of *in vitro* antibody production [21]. To date, carotenoids can be obtained from a wide variety of organisms from bacteria to higher plants [4,17,22,23], but the production using extremely halophilic microorganisms are of particular interest. Production of carotenoids has been carried out using halophilic alga *Dunaliella salina*. The first pilot plant for production of the carotenoid: β-carotene was established in the 1960s in USSR [24]. Commercial-scale production of the carotenoids in open ponds was also carried out in Australia and Israel [25]. Various technological processes such as *in situ* extraction process and so-called milking process have been developed [26]. In addition, continuous production in a turbidostat without *in situ* extraction was also developed [27]. They have unique features for carotenoids production. For instance, the extremely high-salt tolerance of halophiles prevents contamination by other organisms thus enables the cultivation under non-sterile

conditions. In addition, extraction and purification of carotenoids are quite simple by direct lysis under hypoosmotic condition. Although this feature is advantageous, care should be taken to remove the salt from products during the actual industrial process. Increased carotenoid production has been established using sea-water cultivation and genetic manipulation, including the feasibility of downstream processes of the cells [28,29]. Taken together, carotenoid production from halophiles has potential advantage for biotechnological aspect.

3. Mycosporine-like Amino Acids

In the past few decades, one major concern was the increase in the solar UV radiation reaching to the Earth's surface due to industrially released chemicals. Marine organisms are a group having direct impact from detrimental effects on the solar UV radiation. Diverse mechanisms for UV protection have been reported at molecular to behavioral levels [30–32]. Accumulating evidence has shown that the synthesized secondary metabolite compounds such as scytonemins and mycosporine-like amino acids (MAAs) and non-enzymatic antioxidants (i.e., carotenoids) are crucial for scavenging ROS generated due to UV exposure [30]. The specific metabolites that are capable of absorbing/screening UV are called UV-sunscreen compounds.

Among various sunscreen compounds, MAAs are of particular interest because of their potent, stable and UV absorption maxima around 310–360 nm, and highly photoprotective properties such as high molar coefficients (ε = 28, 100–50,000/M/cm) [30,33]. MAAs mainly show absorption in the UVB region, thus they are excellent UVB absorbers known to date. Chemical structures of MAAs are low-molecular-weight and water-soluble molecules containing a substituted cyclohexenone or an imino cyclohexene ring. The parent core structure of most MAAs is 4-deoxygadusol [30]. With the attachments of different amino acids at positions C1 and C3 into core structure 4-deoxygadusol, over 20 MAAs were discovered. Recently, glycosylated MAAs were reported [34,35].

Biosynthesis and accumulation of MAAs were mostly reported in microorganisms that thrive in hypersaline conditions such as marine cyanobacteria and eukaryotic algae [36–38]. These organisms have evolved the capacity to synthesize and accumulate MAAs upon the adaptation from a detrimental effect of solar UV radiation. It was shown that the MAAs protect the cell by absorbing UV radiation and dissipating the energy as heat without generating ROS [30]. Even various MAAs were discovered, the major MAAs found in marine microorganisms are shinorine, porphyra-334, and mycosporine-glycine [39]. It was evident that a community of unicellular cyanobacteria in a hypersaline environment in Dead Sea contains a large amount of MAAs such as mycosporine-2-glycine and euhalothece-362 [39–41]. It was shown that halophilic cyanobacterial community inhabiting a gypsum crust accumulated large concentration of MAAs (at least 98 mM) [41]. High accumulation level is one of merits using halophilic strains that would be exploited in a large amount for industrial applications.

MAAs is one of the valuable molecules that are the basis for important photoprotective constituents. These compounds are therefore promising candidates for use in cosmetic and pharmaceutical applications. They also would be biotechnologically exploited in diverse ways. It was shown that the shinorine, porphyra-334 and mycosporine-glycine can protect the human fibroblast cells from UV-induced cell death [42]. These three MAAs further showed anti-inflammation activities in response to UV radiation, suggesting the potential use in anti-skin aging [43]. Methanolic extract of porphyra-334 was evaluated its UVA absorption properties compared with two commercial sunscreens such as NIVEA and BOOTS, showing significant UVA screening properties that may be used as a potential natural source for UVA protective sunscreens [44]. Besides

photoprotective constituents, some MAAs act as antioxidants to prevent cellular damage resulting from UV-induced ROS production [42,43]. It was found that mycosporine glycine extract exhibited strong antioxidant activity in the 2,2-diphenyl-1-picryhydrazyl (DPPH) assay. Porphyra-334 and shinorine also had moderate antioxidant activities [42]. Recently, the glycosylated products of MAAs found in *Nostoc commune* were also shown to have scavenging activity *in vitro* [34,35]. It should be noted that mycosporine-glycine having strong antioxidant property is one of the major MAAs found in marine cyanobacteria [39]. In fact, a precursor (or core structure) of most MAAs, *i.e.* 4-deoxygadusol, exhibited strong antioxidant properties and its retro-biosynthesis through bacterial conversion of algal MAAs has been performed for commercial applications [45,46]. To date, over 20 MAAs have been discovered, but only few reports have shown their antioxidant properties. This remains to be further clarified. Several lines evidence have shown that MAAs have multiple roles as a UV sunscreen and antioxidant relevant. Due to these properties of MAAs, their application in cosmetics for preserving and protecting purposes are developing rapidly. Thus, applications of MAAs in industrial and medicinal fields would be a useful approach. Exploration of novel MAAs from halophilic microorganisms can be suitable candidates for the development of natural UV-sunscreen. Moreover, biosynthesis of MAAs has attracted considerable interest for metabolic engineering.

4. Compatible Solutes

Compatible solutes are a group of low-molecular mass organic compounds that act as osmolytes to help organisms survive under various stress conditions. Metabolic accumulation of compatible solute is often regarded as the second line of defense. This strategy is one of the foremost tolerance mechanism adopted by halophiles [5,6]. Compatible solutes can be accumulated inside the stressed cells via transportation and/or *de novo* biosynthesis at high amounts without adversely affecting the cellular metabolism. Previously, it was believed that compatible solutes can function to mimic the Hofmeister stabilizing ions by being preferentially excluded from the protein surface and its immediate hydration sphere, and which stabilizes folded protein structures, promote subunit assembly and tend to promote salting-out [47]. Another theory, the osmophobic effect was proposed. It appears that compatible solutes are selected as molecules which exhibit the unfavorable interaction with the peptide backbone. Because the peptide backbone is highly interacted to compatible solute in the denatured state, thus this effect preferentially raised the free energy of denatured state, shifting the equilibrium to promote the folded configuration. This is a thermodynamic force in nature that complements the well-recognized hydrophobic interaction, hydrogen bonding, electrostatic and dispersion forces drive protein folding [48]. There are variety of compatible solutes, including amino acids, sugars, polyols and their derivatives, and nitrogen-containing compounds [49,50]. Halophilic bacteria and Archaea generally synthesize *de novo* nitrogen-containing compounds as their major compatible solutes. The most predominant solutes found in moderate halophiles are the amino acid-derivatives glycine-betaine and ectoine [5,6].

The types of compatible solutes accumulated inside halophilic strains can determine the degree of halotolerance. Low salt-tolerant strains (refers to the maximum tolerance up to 0.7 M NaCl) generally accumulate simple sugars such as sucrose and/or trehalose [51,52]. Sucrose accumulation in low salt-tolerant strains occurs as a cellular response to salt stress, with a high level of intracellular concentration, in molar range. Thus, sucrose serves as a compatible solute in response to salt stress. Besides this function, these sugars could play crucial roles as a signaling molecule and involved in glycogen synthesis as well as insoluble polysaccharide synthesis [53]. The moderately salt-tolerant

strains (refers to the maximum tolerance up to 1.8 M NaCl) also accumulate glucosylglycerol [54,55]. The important role of glucosylglycerol in protection of cells against salt stress has been shown to be due to the sustainability of cells division [56].

The last group refers to the highly salt-tolerant strains with salinity tolerance capacity up to 3 M NaCl, and which accumulate ectoine and the quaternary ammonium compounds such as glycine betaine and glutamate betaine [5,6,57]. Additionally, compatible solute namely trimethylamine-N-oxide was identified from the hypersaline stromatolite-associated cyanobacteria [58]. It has been shown that a high salt-tolerant strain (i.e., *Aphanothece halophytica*) accumulates high amount of glycine betaine under salt-stress condition [6]. This halotolerant cyanobacterium was originally isolated from the Dead Sea. *A. halophytica* possesses a biosynthetic pathway for glycine betaine via three step methylation of glycine catalyzed by two methyltransferases[6]. Glycine betaine was firstly identified biosynthetic pathway via glycine methylation in *Methanophilus*. By using Nuclear Magnetic Resonance analysis, it has been shown glycine betaine was produced from glycine in this microorganism, but no information of corresponding genes is available [59]. In addition to *de novo* biosynthesis, it has been shown that the accumulated glycine betaine can also occur via the transporter [60].

Halophiles are actual sources for producing compatible solutes of biotechnological interest at high concentrations. Compatible solutes ectoine and hydroxyectoine have many existing and potential applications. Ectoine and its hydroxyl derivative, hydroxyectoine, are of particular interest as stabilizers for proteins and protectors of membranes from desiccation [61]. Thus, these natural compounds were applied in cosmetics such as skin care products [62,63]. Hydroxyectoine can stabilize immunotoxins *in vitro*, and was found to lessen the side effects of immunotoxins in an animal model when ectoinewas co-administered [64]. It may thus have application as an excipient for cancer therapeutics.

For industrial application, production of ectoine is promising. It has shown that *Halomonas elongata* was employed to produce ectoine via the so-called milking process. Achievement of high cell density, i.e. over 40 g DW/L using *H. elongata* was reported. Milking process was carried out by using high-cell density cultures and then subjected to hypoosmotic condition. Consequently, ectoine was released into medium. Using this process, approximately 80% of the cytoplasmic ectoine was released to the culture medium. Finally, recovery, purification and crystallization of ectoine were conducted [65]. Furthermore, mutant strains ectoine-excreting and -degradation were developed. These mutants allow high production of ectoine and would be very useful in future application [66–67].

5. Novel Hydrolytic Enzymes

Halophilic microorganisms have been perceived as potential source of enzymes for various biotechnological applications. It has been shown that many enzymes derived from halophiles can function under harsh chemical and physical conditions. Being intrinsically stable and active under harsh conditions with different properties to those of conventional enzymes, halophilic enzymes therefore offer important opportunities in many applications, such as environmental bioremediation, food industry, and waste-water treatment. Screening and exploration of enzymes that can perform optimal activities at various ranges of extreme conditions are of great interest.

To date, potential use of various enzymes (i.e., hydrolases, lipases, esterases, proteases, nucleases etc.) derived from halophiles in industry have been reported [68–70]. Hydrolases constitute a class of enzymes widely distributed in nature. Several halophilic hydrolases have been described,

including amylases, lipases and proteases, and then used for biotechnological applications. It has been shown that α-amylase isolated from *Haloarcula* sp. functions efficiently at 4.3 M salt at 50°C, and is stable in benzene, toluene and chloroform [10,11]. Nanoimmobilization of α-amylase derived from moderately halophilic *Marinobacter* sp. EMB8 was carried out. Optimization of various parameters resulted in 96% immobilization efficiency and its activity retained after five rounds of repeated used. This is one of examples showing the feasibility of applications of halophilic enzymes in industry.

Some halophilic enzymes exhibit extraordinary biochemical properties. It was shown that the lipolytic enzyme LipBL, produced by the moderately halophilic bacterium *Marinobacter lipolyticus* catalyses the activity over a wide range of pHs and temperatures. The immobilized LipBL is capable of catalyzing regio- and enantioselective reactions. These reactions thus showed an excellent behavior in the production of free polyunsaturated fatty acids (PUFAs) and have advantages over other conventional lipases [71]. Being enantioselective biocatalyst, this lipolytic enzyme would be useful for production of fine chemicals. The resistant of lipase derived from the moderately halophilic *Salinivibrio* under high temperature was reported. This halophilic lipase is still active at 50°C [72].

Another class of hydrolytic enzymes with potential for various biotechnological uses is protease. It constitutes a large number of members, involved in many industries and currently the majority of worldwide enzyme sales. It was shown that the protease derived from moderately halophilic bacterium *Halobacillus karajensis* strain MA-2 conducted maximum enzyme activity at pH extremely alkaline values ranging from 8.0–10.0, with 55% and 50% activity remaining at pH 6 and 11, respectively [73]. This property presents a potential for biotechnological applications when alkaline conditions are required.

Different screening programs have been conducted to explore enzymatic activities derived from halophilic microorganisms [74,75]. In addition, genomics and metagenomics approaches are also attractive to explore halophilic enzymes [76,77]. Apart from these approaches, metabolic engineering for directing the overexpression of halophilic enzymes and bioengineering pathways in extreme halophiles would be interesting for biotechnological applications.

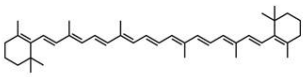
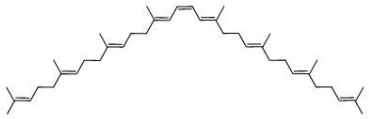
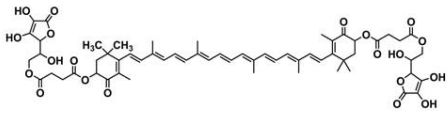
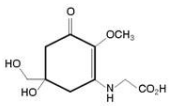
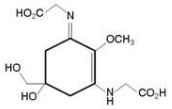
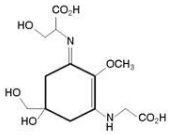
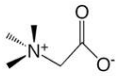
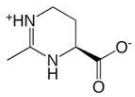
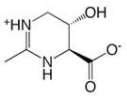
6. Conclusion and Future Perspectives

The biotechnological potentials of halophiles as natural source of unique biomolecules that exhibit distinct biological activities varying from antioxidants, sunscreens, compatible solutes and hydrolytic enzymes are described. These biomolecules are valuable and show commercial potential for food, pharmaceutical, biomedical, industrial and environment. The availability of these halophilic biomolecules and their advantages in production can be optimized to produce sustainable yields at industrial scale. The recent availability of various complete genome sequences of halophiles together with advances in omics technologies would further provide new opportunities for exploration, discovery and identification of unique properties and/or novel biomolecules derived from halophiles in the future.

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

Table 1. Structures of selected antioxidants, mycosporine-like amino acids and compatible solutes derived from halophilic microorganisms and biotechnological potentials.

Category	Chemical structures	Example compounds	Relevant function and biolotechnological potentials	Halophilic sources	References
Carotenoids		β-carotene	Antioxidant Food and supplement Colorant	*Dunaliella salina*	[4, 16, 19]
		Phytoene	Antioxidant Cancer preventing agents	*Dunaliella salina*	[4, 16, 17, 19]
		α-bacterioruberin	Antioxidant Control membrane rigidity	Haloarcheae	[18]
Mycosporine-like amino acids		Mycosporine-glycine	UV-Screening Antioxidant	Coral Zooxanthellae	[31] [78]
		Mycosporine-2-glycine	UV-Screening Osmoprotectant	*Aphanothece halophytica* *Euhalothece* sp.	[12] [40]
		Shinorine	UV-Screening Osmoprotectant	*Antarctic diatoms* *Gymnodnium catenatum.*	[79] [80]
Compatible solutes		Glycine betaine	Osmoprotectant Stress protectant	*Aphanothece halophytica*	[6]
		Ectoine	Osmoprotectant Cosmetics Skin care products	*Halomonas elongata* *Ectothiorhodospira halochloris*	[1, 5, 62, 63]
		Hydroxyectoine	Osmoprotectant Stabilize immunotoxin	*Halomonas elongata* *Ectothiorhodospira halochloris*	[1, 5, 62, 64]

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