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Diversity of Culturable Halophilic Archaea and Bacteria from Chott Tinsilt and El Malah Salt-Lake in Algeria

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Abstract: At the limits of life, hyper-saline aquatic ecosystems; Chott and Sebkha are a model of choice of extreme environments, housing a halophilic microflora that had to adapt to these conditions. In Algeria, these ecosystems are poorly studied. However, our study was carried out on the waters of Chott Tinsilt and Sebkha El Malah. The study of this microflora revealed the presence of a significant morphological, physiological and metabolic diversity. The molecular study allowed us to access to a phylogenetic affiliation including an Archean Species (ATS1) and 7 bacterial species (A1, A2, A3, A4, B1, B4, B5). The results showed that these isolates were related to the genera *Haloferax* (for the strain ATS1) and *Halomonas* (strains A1, A2 and A4), *Staphylococcus* (strain A3), *Salinivibrio* (strain B1), *Planococcus* (strain B4) and *Halobacillus* (strain B5). Most isolates produced hydrolases at high salt concentrations. The Production yields obtained are very promising for applications in the biotechnology and industrial microbiology.

Keywords: Chott, Halophiles, 16S rRNA, Phylogenic Analysis, MEGA6.06

Introduction

Saline and hypersaline environments constitute the largest ecosystems on the planet (Ghosh *et al*., 2019). During recent years, the microbial diversity of such environments has been widely explored (Borsodi *et al*., 2013). Halophiles can be found in a wide range of hypersaline environments that are widely distributed in various geographic areas of the earth, such as marine salterns, salt lakes and saline soils (Amoozegar *et al*., 2019).

These microorganisms can be found in the three domains of life, whose Archaea, Bacteria and Eukarya are distinguished by their requirement of salinity conditions for growth (Amoozegar *et al*., 2019). They may be classified into three main classes according to the quantity of their salt (NaCl) requirements: slight halophiles which require 0.2 to 0.85 M, Moderate halophiles, which require 0.85 to 3.4 M and extreme halphiles, which need 3.4 to 5.1 M of NaCl. Halotolerant microorganisms are those that can grow in the presence and absence of high concentrations of salt (Singh *et al*., 2019).

Due to their ability to adapt in hostile conditions, halophiles present specific characteristics, with biotechnological and industrial interests such as, the capacity to produce hydrolytic enzymes with stable and optimal activities under harsh conditions of temperature, salt concentration and pH; an extreme state which leads to the denaturation and aggregation of most proteins (DasSarma and DasSarma, 2015).

Many halophiles are capable of secreting extracellular hydrolytic enzymes such as amylases, cellulases, xylanases, proteases and lipases (Govender *et al*., 2009; Enache and Kamekura, 2010; Delgado-García *et al*., 2015). These enzymes are able to catalyze the hydrolytic reactions under high salt concentrations and they are called halophilic hydrolases (Delgado-García *et al*., 2012; Liu *et al*., 2019).

Halophiles form a group of extremophiles relatively few explored compared to thermophiles, alkalophiles and

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acidophiles, although they have been used traditionally for the production of salt and even for the preservation of foodstuffs, few products derived from halophiles (for example, beta-carotene from *Dunaliella* and ectoine from moderately halophilic bacteria) have been able to enter the world market (Oren, 2010). However, no haloarchean enzyme has reached the level of production and commercial distribution to date (Das *et al*., 2019).

In addition, Algeria has several wetlands and hypersaline lakes, with a specific typology and ecology, of which 50 are classified as sites of international importance as Ramsar sites (Aliat *et al*., 2016). However, all aspects related to the microbiota composition (diversity and bioactivity) are few studied and remain unidentified (Hacene *et al*., 2004; Kebbouche-Gana *et al*., 2009; Boutaiba *et al*., 2011; Kharroub *et al*., 2014; Quadri *et al*., 2016; Menasria *et al*., 2018; 2019).

In this report, we tried to discuss the diversity of the halophilic bacterial communities living in two Sebkha located in the semi-arid zones of north-eastern Algeria by describing their phenotypic characteristics and phylogenetic affiliation, as well as their potential production of hydrolytic enzymes and their applications in various biotechnological processes.

Materials and Methods

Sampling, Isolation and Cultivation of Halophilic Bacteria and Archaea

Water samples from two sites (chotts and sebkha) located in the Hauts Plateaux of Northeastern Algeria, were collected aseptically in sterile plastic bottles and were directly cultivated. The samples were collected from Chott Tinsilt (Biskra) and Sebkha EL Malah (Medea) in the northeastern of the Algerian Sahara.

In order to isolate halophilic archaeal and bacterial strains, 10 mL of diluted water sample was added in 100 mL of the modified Standard Halophilic (SH) medium (Oren *et al*., 1995) containing (in g/L): $MgCl_2 \cdot 6H_2O$, 20; K_2SO_4, 5; $CaCl_2$, 0.1; yeast extract (Difco), 1; starch, 2 and supplemented with different total salt concentrations (5-30%). The pH was adjusted to 7.2 before autoclaving. For solid medium, 20 g/L Bacto-agar (Difco) was added. The plates were incubated for 7-15 days at 40°C. After six weeks of incubation, pure cultures were obtained and stored on solid SH medium with appropriate total salt concentration at 4°C.

Phenotypic Characterization of the Isolated Strains

Colony morphology was observed under optimal growth conditions on solid SH medium after incubation at 40°C for 7 days. Gram staining of cells was performed as described by Dussault (1955) for Archean isolates and by the Burke method (Murray *et al*., 1994) for bacterial isolates. Growth and optimal conditions were determined at different temperatures (4, 25, 30, 37, 40, 55 and 60°C), pH (4.0, 5.0, 6.0, 8.0, 9, 0, 10.0 and 12.0) and in various salt concentrations (0-30%) on SH agar for 7-15 days of incubation. The growth was carried out by spreading 0.2 mL of a culture suspension of each strain on the surface of the respective media.

Molecular Identification by 16S rRNA Sequencing and Phylogenetic Analysis

Genomic DNA Extraction and PCR Amplification

Extraction of genomic DNA from the isolated strains is carried out during their exponential growth phase using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The 16S rRNA gene was amplified with the bacterial primers pair Fd1 (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3') and Rd1 (5'-AAG -GAG-GTG-ATC-CAG-CC-3') (Aguilera *et al*., 2009) and archaeal primers pair 21F (5'-TTCCGGTTGA TCCTGCCGGA-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Kebbouche-Gana *et al*., 2009). The PCR mixture is composed of 0.5 µL of each primer (50 mM); 10 µL of Biogenic LOADTM Taq polymerase (1X); 0.5 µL of template DNA. The mixture is completed with 38.5 µL of sterile water. The Taq used is a ready prepared PCR reaction mixture containing the following components: Taq polymerase, dNTP, PCR buffer, MgCl2 and deposition buffer. PCR amplification consisted of an initial denaturation at 94°C for 5 min, followed by 30 denaturation cycles at 94°C for 30 s, annealing at 52°C for 45 s, extension at 72°C for 2 min and a final polymerization step of 72°C for 10 min.

Sequencing and Phylogenetic Analysis

PCR products were determined and sequencing was performed by using the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The obtained sequence was compared with sequences available in the public sequence databases using the BLAST search program (http://www.ncbi.nlm.nih.gov). The multiple sequence alignment was performed by means of the ClustalW program. Phylogenetic and molecular evolutionary genetic analyses were performed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06 (Tamura *et al*., 2013). Evolutionary distance matrix was generated and phylogenetic tree was constructed using the neighbor-joining method. The topologies were evaluated by bootstrap sampling expressed as percentage of 1000 replicates.

Screening of Hydrolytic Exo-Enzymes

To get a qualitative detection of producing extracellular hydrolyses, different enzymatic agar plate assays were performed using a drop spot technique. The standard methods referenced for each enzyme were modified to make suitable condition for growth which corresponds to each isolate as described for amylase (Montalvo-Rodriguez *et al*., 1998), gelatinase (Frazier, 1926), esterase (Sierra, 1957), lipase (Kouker and Jaeger, 1987), cellulase (Teather and Wood, 1982), protease (Gonzalez *et al*., 1978), xylanase (Ghio *et al*., 2012), chitinase (Roberts and Selitrennikoff, 1988) and tannase (Aissam, 2003). After incubation of each isolate for 3 to 10 days, the results were expressed qualitatively as Levels of Enzymatic Activities (LEA) (High activity, medium activity, low activity and no activity) (Latorre *et al*., 2016) using the formula LEA = diameter of the zone of clearance/the diameter of colony in millimeters.

Results and Discussion

Isolation and Phylogenetic Characterization of the Halophilic Isolates

In recent years, halophilic prokaryotes have been widely explored and have been found in a wide range of saline environments. For their biotechnological applications, extreme and moderate halophilic bacteria have received high attention (Edbeib *et al*., 2016; Margesin and Schinner 2001; Oren, 2002). However, culture-based methods remain an essential approach to obtaining new microbes that offer application of new potentials and an understanding of their ecophysiological and environmental functions (Menasria *et al*., 2018). Throughout this work, we have characterized seven moderate halophiles and one extreme halophilic isolate. The isolates were named (ATS1, A1, A2, A3, A4, B1, B4 and B5). The color of colonies ranged from pale-pink to red-orange. The cells were Gram-negative polymorphic, short rods or coccus. All stained Gram negative and were catalase- and oxidase positive.

All the isolates were halophilic, had a salt concentration for growth at least 5% (w/v) NaCl and could tolerate salt concentration up to 20-25% (w/v) NaCl. The isolates grew best between 30 and 45°C and pH of 6 to 8, with the exception of some isolates showed a growth at 50°C and pH up to 9. Overall, the isolates showing a large range of growth temperature extend from 25°C to 50°C (Fig. 1). The microbial community of hypersaline environments is dominated by well-adapted halophilic microorganisms which in many cases are polyextremophiles with the ability to grow optimally not only at high salt concentrations but also at high or low pH values and temperatures (Bowers *et al*., 2009; Mesbah and Wiegel, 2012).

Sequencing of the 16S rRNA genes and phylogenetic analysis of the 8 selected strains allowed the identification of 6 genera: *Haloferax, Halomonas, Staphylococcus, Salinivibrio, Halobacillus* and *Planococcus* (Fig. 2 and Table 1). The isolates revealed high degree of similarity, with the closest described species in terms of colony, cells morphologies and physiological characteristics.

The results presented in Table 1 indicate the description of six different genera with the dominance of *Halomonas* (3 strains) followed by *Halobacillus* (2 strains) and *Staphylococcus, Salinivibrio, Planococcus, Haloferax* (1 strain each). *Haloferax* specie was isolated from Sebkha Al Malah. The strain ATS1 has been identified as a member of the family *Halobacteriaceae*. The complete 16S rRNA gene sequence of the strain ATS1 was 1490 bp (Genbank accession number: MF353936) and showed 98% significant sequence similarity to that of the *Haloferax mediterranei* strain ATCC 33500T. This species was found in Sebkha Ezzemoul (Ain M'Lila) (Kharroub, 2007), Sidi Ameur (Bousaâda) (Quadri *et al*., 2016) as well as in Sebkha Medghacen and Sebkha El Hamiet (Menasria *et al*., 2018).

Three strains (A1, A2 and A4) were found to be phylogenetically attached to members of the genus *Halomonas* with at least 96% similarity. These strains were isolated from different sites: Chott El Beïdha, Chott Tinsilt, Chott Kralla, Sebkha Djendli, Sebkha El Hamiet, Sebkha Medghacen and Sebkha Oumache in Algerian Saline Ecosystems (Sebkha and Chott) located in arid and semi-arid ecoclimate zones (Northeastern Algeria) (Menasria *et al*., 2019).

Whereas, the sequence of the A3 strain showed a low level of homology with those of reference strains close to the genus *Staphylococcus arlettae* strain 3m-3 (JX188021) with 95% homology. This test alone can justify the classification of this strain in a new species. The 16S sequence of the rDNA of the B1 strain has 96% homology to that of *Salinivibrio costicola* strain cepa 6 (X95531) and 94% to that of *Salinivibrio proteolyticus* strain M1SED9 (JX501730) (Table 1). These are halophilic bacteria commonly found in hypersaline aquatic habitats and in salty foods. This genus includes five species and two subspecies (Galisteo *et al*., 2019).

While the strain B3 and B5 were 99 and 98% homology respectively, to that of *Halobacillus karajensis* isolate HK-03 (HG931925), *Halobacillus dabanensis* isolate HD-02 (HG931924) and *Halobacillus trueperi* isolate HT-01 (HG931926). These species had also been isolated from saline soil (Iran) (Amoozegar *et al*., 2003), salt lakes in Xinjiang, China (Liu *et al*., 2005) and the Great Salt Lake Utah (USA) (Spring *et al*., 1996).

The strain B4 is affiliated to the species *Planococcus rifietoensis* strain SAL-15 (HE573181) and *Planococcus*

citreus strain NBRC 15849 (NR_113814) with a homology of 96%. This species has also been isolated from saline soils in Pakistan (Rajput *et al*., 2013) and salt ponds in Korea (Yoon *et al*., 2010).

Screening for Halophilic-Active Hydrolytic Enzymes

The enzymatic production of halophilic microorganisms is poorly exploited commercially. One of the advantages of these enzymes is its ability to catalyze reactions under extreme conditions, mainly at high salt concentrations, but also at high or low pH values and temperatures (Enache and Kamekura 2010; Mesbah and Wiegel, 2012). These properties allow them to be used in industrial processes using rigorous physico-chemical conditions (Setati, 2009; Oren, 2010). These enzymes are called halozymes, enzymes that exhibit tolerant or salt-dependent catalytic activity (Gupta *et al*., 2016).

Table 1: Phylogenetic affiliation of the bacterial and Archean strains studied

Isolates	Genus	Related species	Similarity%	Accession number
Al	*Halomonas*	*Halomonas venusta*	96	AY553074
		Halomonas janggokensis	96	NR_04248
		Halomonas variabilis	96	AM945682
		Halomonas subterranean	96	NR_04411
A2	*Halomonas*	*Halomonas venusta*	97	AY553074
		Halomonas variabilis	97	AY505527
		Halomonas janggokensis	97	KM488473
A3	*Staphylococc*	*Staphylococc arlettae*	95	JX188021
A4	*Halomonas*	*Halomonas aquamarina*	97	EU624431
		Halomonas venusta	96	KF933652
B1	*Salinivbrio*	*Salinivbrio costicola*	96	X95531
		Salinivibrio proteolyticus	94	JX501730
B3	*Halobacillus*	*Halobacillus karajensis*	99	HG931925
		Halobacillus dabanensis	99	HG931924
		Halobacillus trueperi	99	HG931926
B4	*Planococcus*	*Planococcus rifietoensis*	96	HE573181
		Planococcus citreus	96	NR_11381
B5	*Halobacillus*	*Halobacillus karajensis*	98	HG931925
		Halobacillus dabanensis	98	HG931924
		Halobacillus trueperi	98	HG931926
ATS1	*Haloferax*	*Haloferax mediterranei*	98	MF353936

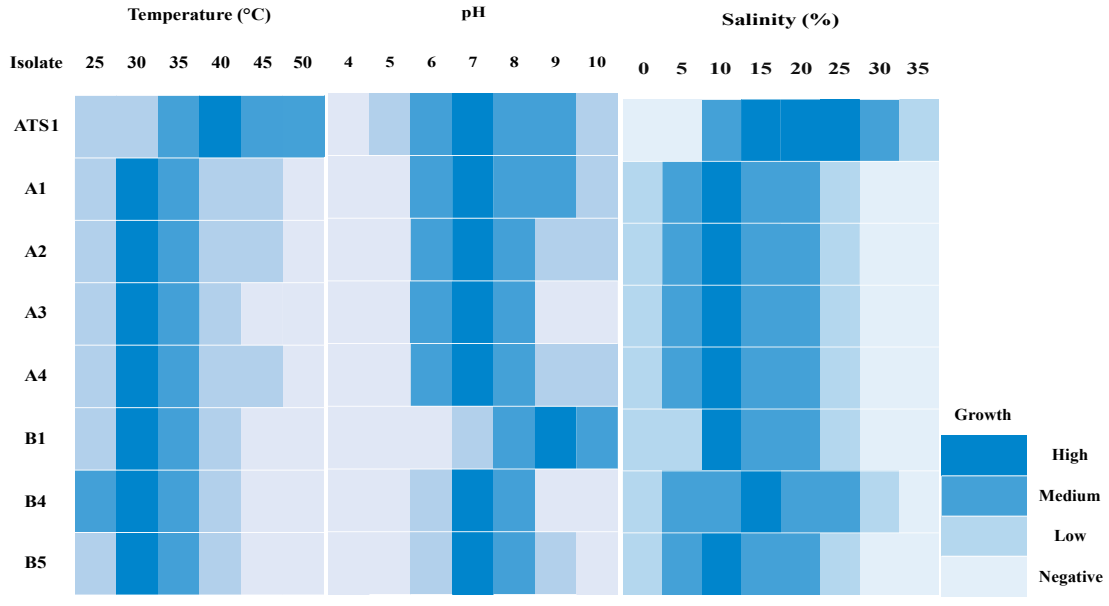

Fig. 1: Heat map plots of physiological features of halophilic isolates

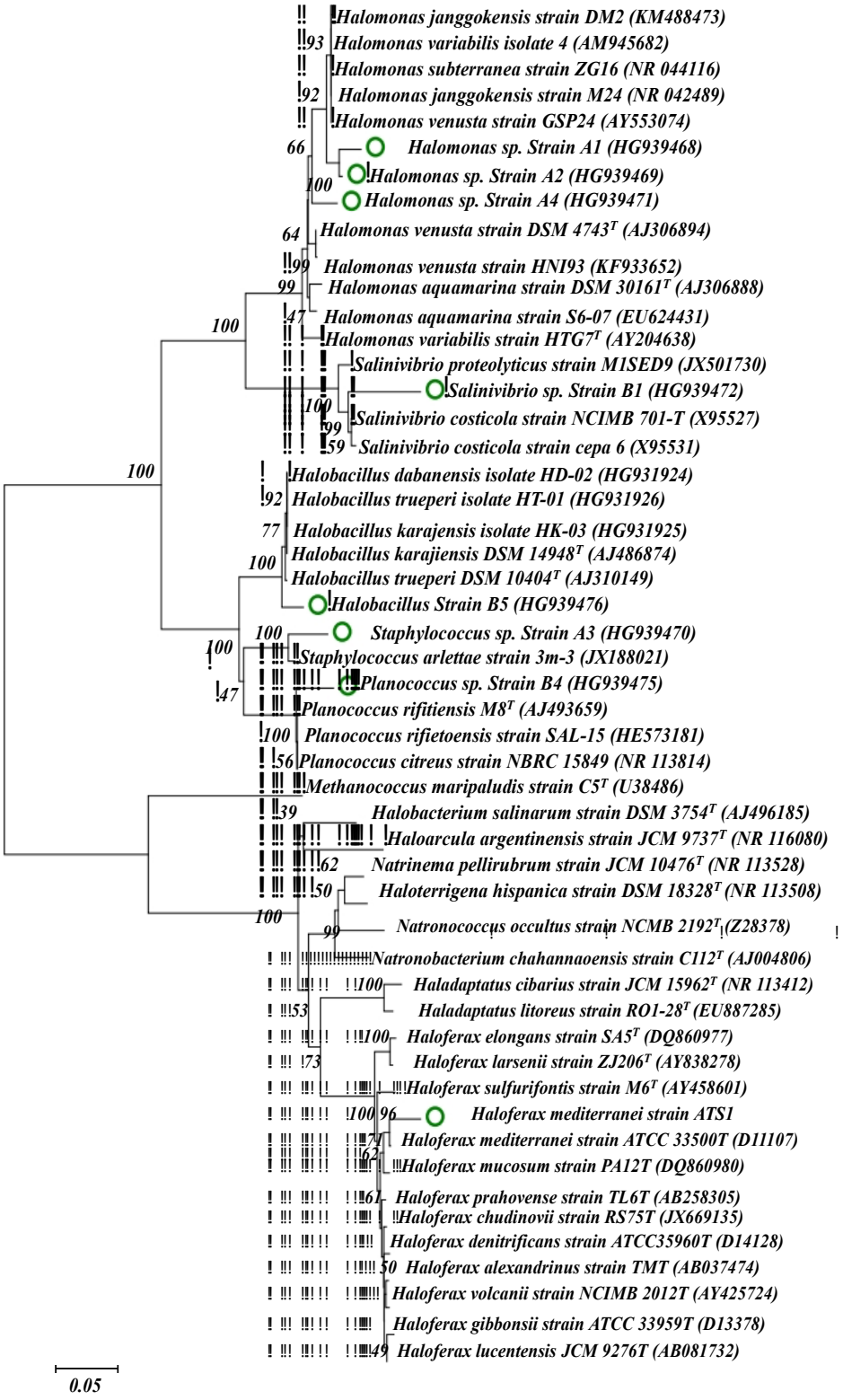

Fig. 2: Dendrogram indicating the position of strains A1, A2, A3, A4, B1, B4, B5 and ATS1 (green circle) based on their 16S rDNA sequences by MEGA6.06 software. The numbers above the branches refer to bootstrap values. *Methanococcus maripaludis* strain C5T (U38486) was used as the out group. The scale bar indicates a substitution of 5 nucleotides for each 10 nucleotides sequence. The sequence access numbers publicly available in Genbank are shown in parentheses.

All the eight isolates were tested for extracellular hydrolase producing activity by plate assay on starch, Carboxymethylcellulose (CMC), tween, olive oil, skim milk, xylane, tannic acid, colloidal chitin and gelatin agar plates for Amylase (Amy), Cellulose (CMC), Esterase (Est), Lipase (Lip), Protease (Prot), Xylanase (Xyl), Tannase (Tan), Chitinase (Chit) and Gelatinase (Gel) respectively.

Screening for extracellular halophilic enzymes showed that the majority of isolates produced at least four hydrolytic enzymes, respectively (Fig. 3). Lipases, esterases, amylases and proteases constitute the most abundant enzymes (produced in the majority of isolated halophilic strains). Followed by gelatinase and tannase in terms of activity. Many isolates showed several hydrolytic activities combined, mainly strain ATS1 affiliated to *Haloferax mediterranei*.

Sánchez-Porro *et al*. (2003) showed the abundance of five hydrolases including amylase, protease, lipase, DNase and pullulanase in a moderate halophilic community isolated from water and Spanish saline sediments, with an abundance of activity amylolytic. Though, Moreno *et al*. (2009) studied the diversity of extreme halophiles producing lipases, proteases, amylases and nucleases in crystallization ponds located in Southern Spain, at a rate of 70% of the total of isolates, while no DNAase activity has been detected. In a similar study, Cojoc *et al*. (2009) have elucidated the extracellular hydrolytic activities of halophilic bacteria, with lipolytic and proteolytic activities predominant for the isolated strains.

The same results were obtained by Makhdoumi Kakhki *et al*. (2011) reporting the capacity of halophilic archaea from hypersaline lake Aran-Bidgol (central desert region in Iran), to produce different types of hydrolases, concluding that *Halorubrum* and *Haloarcula* were the most dominant Haloarchean genera with a high enzyme production rate comprising amylase respectively, DNase and lipase. However, Kharroub *et al*. (2014) report the capacity of halophilic archaea from Algerian Sebkha to produce amylolytic enzymes with a high prevalence. Furthermore, Gupta *et al*. (2016) found that the five bacterial isolates halophilic produce one or more haloenzymes such as amylase, protease, lipase and glutaminase.

Menasria *et al*. (2019) report that the enzymes amylase, esterase, gelatinase and nuclease activities are classified first in the group of hydrolases. While Menasria *et al*. (2018), report that the esterase and inulinase activities have the highest activities. Probably because biotopes represent an important reservoir of organic matter which can stimulate the metabolic activity of isolated halophilic archaea. The results of this present work as well as those of Boutaiba *et al*. (2006) also demonstrate that Archean halophilic isolates can produce lipase with a high concentration of NaCl.

According to these results, a great diversity was displayed concerning their biotechnological aptitudes to produce metabolites of interest, particularly the ATS1 strain which displayed the best potential for the production of lipase, protease and tannase among all the halophilic strains studied. The results obtained confirm the great capacity of the isolated strains to synthesize enzymatic activities. These biomolecules are essential metabolites that allow bacteria to catabolize nutrients in the environment to ensure their lives. In addition, these natural capacities are used for the benefit of biotechnology in various fields (Zhao *et al*., 2010).

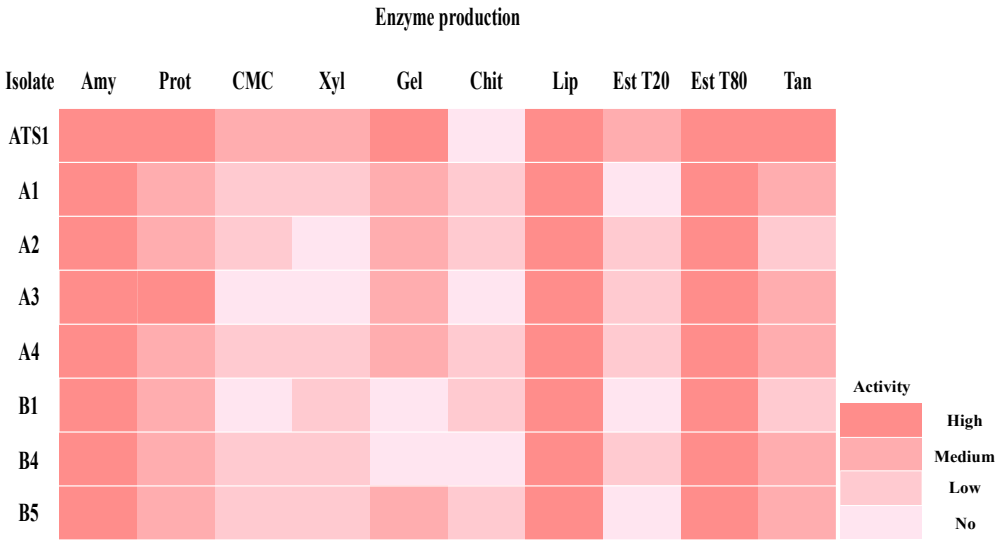

Fig. 3: Heat map plots of enzymatic activities of halophilic isolates

Conclusion

This work studies the biodiversity of halophilic isolates from the Algerian Saline wetlands. The data have shown that different sebkha ecosystems of Tinsilt and Al Malah are good sources of isolation of extreme and moderate halophiles by the culture dependent approach producing extracellular hydrolytic enzymes of biotechnological importance. However, further studies are necessary to better understand the microbial ecology, their distribution and their functional diversity in such hypersaline environments.

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Author's Contributions

Akmoussi-Toumi Siham, Khemili-Talbi Souad, Kebbouche-Gana Salima, Najjari Afef: Conceived and designed the study, provided supervision of analyzed data and wrote the paper.

Akmoussi-Toumi Siham, Lenchi-Izouine Nesrine, Khelfaoui Mohamed Amine and Mokhtari Wafa: Performed experiments and Analyzed data.

Sayah Amna, Bouarab Ghania, Ferrioune Imen: Contributed reagents/materials/analysis tools.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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