J Genet Resour2020;6(1): 1-11 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2020.17847.1170*

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Culturable Diversity and Enzyme Production Survey of Halophilic Prokaryotes from a Solar Saltern on the Shore of the Oman Sea

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ARTICLE INFO

Article history:
Received 04 December 2019
Accepted 07 January 2020
Available online 20 January 2020

Keywords:
Diversity
Hydrolytic enzymes
Solar salterns
Halophiles

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p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

The prokaryotic residents of the Tis solar saltern in the southeast of Iran on the shore of Oman Sea were investigated by the culture-dependent methods. Sequencing of the PCR-amplified fragments of *16S rRNA* genes revealed that bacterial populations were related to *Actinobacteria*, *Bacteroidetes*, *Balneolaeota*, *Firmicutes*, and *Proteobacteria*. They were phylogenetically identified as members of *Bacillus* (35%), *Aliifodinibius* (15%), *Longibacter* (10%), *Halomonas* (10%), *Arthrobacter* (5%), *Luteimonas* (5%), *Ornithinibacillus* (5%), *Rhodovibrio* (5%), *Staphylococcus* (5%), and *Tamilnaduibacter* (5%). All archaeal isolates were belonged to the order *Halobacteriales* in the following genera: *Haloferax* (33%), *Haloarcula* (27%), *Halogeometricum* (11%), *Halococcus* (5%), *Halomicroarcula* (5%), *Halorubrum* (5%), *Halostagnicola* (5%), and *Natronoarchaeum* (5%). Semi-quantitative evaluation of six hydrolytic enzymes, including amylase, cellulase, lipase, pectinase, protease, and urease among these strains, revealed that urease (47%) and amylase (41%) had the highest production frequency. The average production rates were observed for lipase (25%) and protease (30%), while the pectinase (12%) and cellulase (4%) productions were rare among these halophiles. The most potent bacterial/archaeal strains for the enzymes production were as: *Longibacter/Natronoarchaeum* (amylase), *Bacillus/ non archaeum* (cellulase), *Tamilnaduibacter/ Haloferax* (lipase), *Bacillus/ Haloferax* (pectinase), *Bacillus/ Haloferax* (protease), and *Staphylococcus/ Halococcus* (urease). This first report about the prokaryote populations of the solar salterns in Iran demonstrated its high microbial diversity and potentials for the production of industrially interesting enzymes.

Please cite this paper as: Hashemzehi A, Makhdoumi A, Asoodeh A. 2020. Culturable Diversity and Enzyme Production Survey of Halophilic Prokaryotes from a Solar Saltern on the Shore of the Oman Sea. *J Genet Resour* 6 (1): 1-11 doi: *10.22080/jgr.2020.17847.1170*

Introduction

Saline environments are usually referred to as the ecosystems that the concentrations of salts are equal or higher than those find in marines (3.0% w/v total salts) (Oren, 2015). Halophiles are a group of microorganisms that are the main prokaryotic residents in saline environments. They divided into moderate and extreme halophiles based on the amount of salt that supports their best growth. While moderate halophiles are members of the bacterial domain and growth optimally below 15% (w/v) salts, extreme halophiles almost belong to the *Archaea* and growth well up to 30% (w/v) salts (Kushner & Kamekura, 1998). Living in these extreme environments required some special cellular characteristics to encounter various stressful conditions. Accordingly, halophilic microorganisms harbor some specific features (enzymes and other cell components), which can be used in various industrial and environmental applications not founded in non-halophilic (micro)organisms (Yin *et al*., 2015). Exploring the prokaryotes from the non-considered environments may result in the isolation of new

halophiles with the probable potentials in biotechnology.

Solar slatterns are artificial saline environments where seawater is trapped in serial ponds. The salinity increased gradually by the effects of temperature and salt produced at the last pond named crystallizer pond (Oren, 2015). The crystallizer ponds are artificially extreme environments, and their salinity reaches up to 30-35% (10 times more than marines). They defined as thalassohaline hyper-saline environments as their salt composition respect the seawater, *i.e.* sodium and chloride are the dominant cations and anions (DasSarma & Arora, 2002). While the brine shrimps (*Artemia* spp.) and chlorophyll-containing phototrophic eukaryote (*Dunaliella*) live at the lower salinities (middle ponds), halophilic bacteria and archaea are the main biotas in crystallizer ponds (Anton *et al*., 2000). There are several hypersaline environments in Iran, and their microbial populations were investigated by either culture dependents or independent approaches. The major well-studied Iranian saline lakes were as Aran-bidgol salt lakes, Bakhtegan Lake, Gomishan wetland, Howz Soltan salt lake, Incheh Borun wetland, Maharloo salt lake, Tashk lake and Urmia lake (Safarpour *et al*., 2018). All of these saline lakes are natural systems, which means they have geogenic sources. Despite the progress in the study of microbial diversity of natural saline lakes in Iran, the anthropogenic (artificial) saline lakes (solar salterns) were neglected, and there is not any report regarding the microbial diversity of saltern ponds in Iran. In this study, for the first time, we sampled a solar pond on the shore of the Oman Sea, and its aerobic prokaryotic population was determined by the culture-dependent method. The potentials of these halophiles for the production of some biotechnologically important enzymes were also investigated.

Materials and Methods

Isolation of samples

This solar saltern located at the Makran coast, 5km away from Chabahar city (25°22′ 01’’; °45′ N, 60°36′ 46′’ E,) was sampled in October 2017 (Fig. 1). The brine and soil samples of the last pond (crystallizer pond) were collected in the sterile plastic containers and kept in the dark at environmental temperature until analyzed in the laboratory. The anions and cations concentrations of salt samples were analyzed by titration and atomic absorption methods, respectively (Saad *et al*. 1998). Resident prokaryotes were isolated under aerobic conditions on different growth media. The 20% and 10% Salt Water medium (Nercessian *et al*., 2015) prepared from the 25% (w/v) salt water solution (g/l): NaCl 195, $MgSO_4.7H_2O$ 49.5, $MgCl_2.6H_2O$ 16.2, KCl 5.0, $CaCl_2.2H_2O$ 0.94, $NaHCO_3$ 0.25, and NaBr 0.625. The salt water was supplemented by (g/l) peptone 10.0, yeast extract 2.0, and agar 15.0; pH 7.2. The Solar Salt medium consisted of (g/l): 200 (20%) and 100 (10%) solar salt of crystallizer pond, peptone 10.0, yeast extract 2.0 and agar 15.0; pH 7.2. All samples were serially diluted and inoculated to the mentioned media, and plates were incubated aerobically at 37 °C for eight weeks. The pure isolates were obtained after successive cultivation. All isolates were cultured in SW (Salt Water) broth medium supplemented by 0-25% (w/v) NaCl with 5% intervals to determine their relation to salt.

A **B** **C**

Fig. 1. Location map of the Tis solar saltern and sampling pond used in the diversity study. A=Solar ponds located in Sistan and Baluchestan province; B= Aerial image of salt ponds; C= crystallizer pond.

The growth was determined by measuring the absorbance at 600 nm. They were classified as halotolerant when salt-free medium supports their growth. The strains were determined as moderate and extreme halophiles when the optimal growth was achieved in the medium contained below and above 15% (w/v) NaCl, respectively (Kushner & Kamekura, 1998). The preliminary microbiological tests (Gram staining, colony, and microscopic morphology, oxidase, and catalase) were performed based on the standard protocols (Smibert & Krieg, 1994).

Amplification of *16S rRNA* genes

The genomic DNA of the extremely halophilic archaea was extracted after the simply lysed the cells by adding distilled water. Convention phenol-chloroform approach was used for the precipitation of proteins and other cellular components. DNA precipitation was achieved by adding cold ethanol. Precipitated DNA was washed with 70% ethanol, then the ethanol was removed, and 200 µl distilled water was added and then stored at -20 °C until use (Burns *et al*., 2004). Bacterial genomic DNA was extracted by the Genomic-DNA extraction kit (Cinagen, Iran), according to the manufacturer's recommended procedure. The *16S rRNA* genes were amplified using either 27F foreward Bacteria-specific primer (5'-AGAGTTTGATCATGGCTCAG-3') (Lane *et al*., 1985) and 21F Archaea-specific primer (5'-TTCCGGTTGATCCTGCCGGA-3') (DeLong, 1992) in combination with the universal 1492R reverse primer 5'-GGTTACCTTGTTACGACTT-3' (Lane *et al*., 1985). PCR conditions were used: 94 °C for 2 min, followed by 30 cycles of 94 °C for 60 s, 55 °C for 60 s (51 °C for archaeal strain) and 72 °C for 60 s, with final 7 min extension at 72 °C. The sequencing was carried out based on the Sanger method at the sequencing service of Macrogen (Seoul, South Korea). The related sequences were obtained from GenBank (www.ncbi.nlm.nih.org) using BLASTN and through the Ez-Biocloud server (Yoon *et al*., 2017). Phylogenetic analysis was performed using the software package MEGA version 7 using the multiple alignments generated by MUSCLE webserver (Kumar *et al*., 2016). The isolates were grouped as an operation taxonomic unit (OTU) where their *16S rRNA* genes have 97% or more similarity.

Extracellular hydrolytic enzyme activity

The semi-quantitative screening was performed to determine the ability of the halophilic strains for the production of some hydrolytic enzymes, including amylase, cellulase, lipase, pectinase, protease, and urease. The moderate and extreme halophiles were inoculated in the 10% and 20% SW broth medium and kept at 40 ºC for 24 and 72 hours, respectively. The culture supernatant was removed by centrifugation at 10000 rpm for 15 min, and the cell pellets were suspended in sterile 10% and 20% salt water (10^8 CFU/ml) for the moderate and extreme halophiles, respectively. A total of 20 µl of each prokaryotic suspension was inoculated in the different screening agar plats and incubated for 3 and 7 days at 40 ºC for moderate and extreme halophiles, respectively. The semi-quantitative measurement of the enzyme production was determined based on the ratio of the enzyme activity zone (halo zone) to the colony diameter (Cody, 1989). We defined the strains as weak, moderate, and strong enzyme producers when their EC ratio (Enzyme zone to Colony size) ratio was <2, 2<ratio<3, and >3, respectively.

Extracellular amylase

To determination of amylase activity, starch (30 g/l) was added to the 10% and 20% SW solid medium. After the growth period, plates were flooded by gram staining Lugol's iodine solution. The clear zone around the colonies was assumed as the amylase activity (Amoozegar *et al*., 2003).

Extracellular cellulase

Extracellular cellulase activity of the moderate and extreme halophilic strains was determined in the 10% and 20% solid SW medium supplemented by 0.5% (w/v) carboxy methyl cellulose. The clear zone around the colonies after the addition of (0.1 w/v) Congo red solution indicated the cellulase activity (Ghose, 1987).

Extracellular lipase

The production of lipase by the moderate and extreme halophile was evaluated in the 10% and 20% SW solid medium supplemented by 10 (g/l) olive oil, respectively. White sediment around

the colonies showed the lipolytic activity of the strains (Samad *et al*., 1989).

Extracellular pectinase

Pectinase enzyme was determined by inoculation of the moderate and extreme halophiles in the 10% and 20% SW medium contains 5(g/l) pectin substrate. The presence of the clear zone around the colonies follows the flooded by Lugol's iodine solution indicated the pectin hydrolyzing activity (Samad *et al*., 1989).

Extracellular protease

The basal skim milk (20 g/l) was added to the 10% and 20% SW solid medium to identify the moderate and extreme halophile protease producing strains, respectively. The clear zone around the colonies after the incubation periods indicated the proteolytic ability (Amoozegar *et al.*, 2008).

Extracellular urease

To detect the urease activity of the moderate and extreme halophiles, 10% and 20% SW solid medium was supplemented by 2.0% (w/v) filter-sterilized urea as a substrate and phenol red (0.012 (w/v)) as the pH indicator. The appearance of the pink-red color around the colonies indicated the hydrolysis of the urea (Natarajan *et al*., 1995).

Data Analysis

All experiments were performed at least three times. Statistical analysis independent *t-test* was performed by SPSS software ver. 16 software (IBM Co) to determine the significance of the difference between archaeal and bacterial enzyme production rate (p-value less than 0.05). One-way analysis of variance was performed to evaluate no significance difference between EC ratio for each strain/enzyme in at least three independent replicates ($p>0.05$).

Results

In this study, the diversity of the cultivable aerobic prokaryotes from Tis solar saltern in the southeast of Iran was determined (Fig. 1). The water of the Oman Sea was trapped in shallow ponds where the salinity was increased by evaporation and salt harvested from the last crystallizer pond. Total salt concentrations and pH of the brine sample were determined by pH-conductivity meter and was equal to 27% (v/w) and pH 6.8. The results of chemical analysis of the ions revealed that monovalent cations and anions, i.e. sodium and chloride were dominant, while divalent ions like sulfate and magnesium were present in smaller concentrations (Table. 1).

Table 1. Chemical characterization of Saltern salt

Parameter	Ion Concentration (g/l)
Na^+	56.9
Mg^{2+}	13.5
Ca^{2+}	0.24
K^+	11.6
Cl^-	191.5
SO_4^{2-}	23.9
HCO_3^-	0.85
CO_3^{2-}	0.15
pH	6.8
Temperature(º C)	40.3
Lon Lt	N 25° 22′ E 60° 36′

The composition of salts, as well as its marine sources, confirmed that this saline environment is a thalassohaline system. Among inland natural salt lakes in Iran, some like Aran-bidgol salt lake was also identified as thalassohaline. We will compare the prokaryotic populations of these artificial and natural Iranian thalassohaline lakes in follow. We also compared the microbial population with the other solar saltern around the world. Two different isolation medium, i.e. SW and SS, represented a similar isolation rate with the mean of 5×10^6 CFU/ml. A total of 476 aerobic isolates were obtained from 10%, and 20% salt-containing medium and 100 strains were selected for further analysis after trimming possible redundant based on the preliminary microbiological tests. A total of 55, 34, and 11 isolates were identified as extreme, moderate, and halotolerant strains based on the amounts of salts that support their (best) growth. Extreme halophiles were more isolated from 20% SW and SS medium and moderate strains were more isolated from 10% SW and SS medium.

The sequences of the 16S rRNA gene of 48 strains were properly obtained and deposited in the GenBank database under accession numbers MN750215- MN750252. All of the 20 analyzed moderate halophile strains were identified as bacteria in the following phyla: *Actinobacteria Bacteroidetes*, *Balneolaeota*, *Firmicutes*, and *Proteobacteria* (Fig. 2). Bacterial isolates

clustered into 10 OTUs (Table 2) and were phylogenetically identified as members of *Bacillus* (7 strains), *Aliifodinibius* (3 strain), *Longibacter* (2 strain), *Halomonas* (2 strain), *Arthrobacter* (1 strain), *Luteimonas* (1 strain), *Ornithinibacillus* (1 strain), *Rhodovibrio* (1 strain), *Staphylococcus* (1 strain), and *Tamilnaduibacter* (1 strain). The large numbers of the obtained bacteria belonged to the *Bacillus* genus, and all of them were obtained from the soil sample. Regarding the brine sample, the abundance of *Aliifodinibius*, *Longibacter*, and *Halomonas* were similar to those reported from other salterns in South Korea (Lim et al., 2004), China (Xia *et al*., 2016), and turkey (Çınar & Mutlu, 2016). In comparison to the Iranian inland thalossohalin salt lake, Aran-bidgole, the isolated bacteria were also similar except that we could not obtain *Salinibacter* strains (Makhdoumi-kakhki *et al*., 2012).

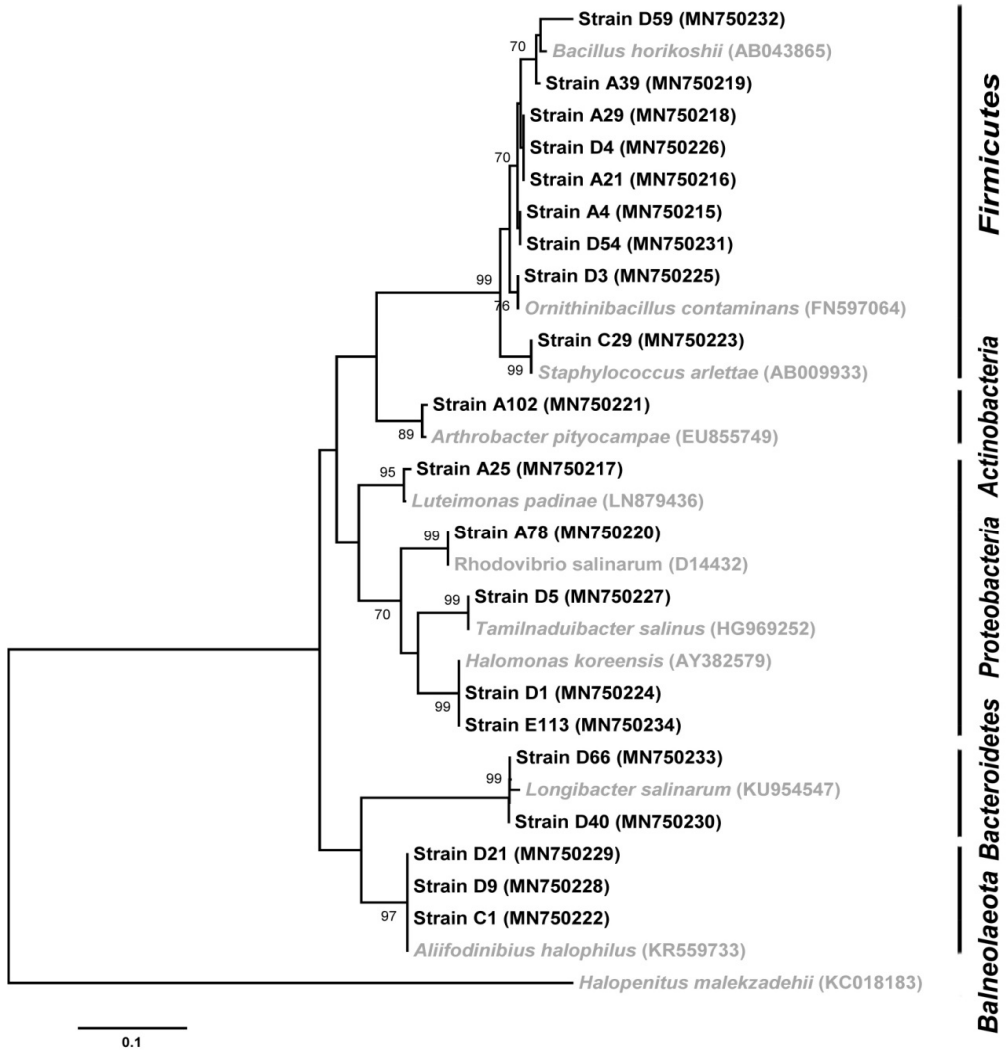

Fig. 2. Phylogenetic inferences based on 16S rRNA gene sequences from isolates belonging to the *Bacteria* domain. The tree is based on the neighbor-joining analysis. The sequence of *Halopenitus malekzadehii* was used as the outgroup.

Table 2. Comparison of isolates sequences obtained from Tis saltern pond with those available in Ezbiocloud (Chun *et al.* 2017).

Bacteria				Archaea			
OTU-97 %	**Strains**	**Species**	**% SIM***	**OTU-97 %**	**Strains**	**Species**	**% SIM***
1	A4	*Bacillus coreaensis*	98.2	1	E106	*Haloferax prahovense*	99.6
	A21	*Bacillus atrophaeus*	99.9		F28	*Haloferax mediterranei*	99.9
	A29	*Bacillus pakistanensis*	98.3		F139	*Haloferax larsenii*	99.9
	A39	*Bacillus horikoshii*	94.8		F161	*Haloferax larsenii*	99.9
	D4	*Bacillus subtilis*	99.8		E33	*Haloferax sulfurifontis*	98.5
	D54	*Bacillus oceanisediminis*	99.4		F144	*Haloferax mediterranei*	98.9
	D59	*Bacillus litoralis*	98.7	2	F126	*Haloarcula hispanica*	99.2
2	C1	*Aliifodinibius halophilus*	100		F17	*Haloarcula hispanica*	98.7
	D9	*Aliifodinibius halophilus*	99.6		F45	*Haloarcula hispanica*	98.5
	D21	*Aliifodinibius halophilus*	99.8		F67	*Haloarcula tradensis*	98.9
3	D40	*Longibacter salinarum*	98.2		F69	*Haloarcula tradensis*	96.8
	D66	*Longibacter salinarum*	99.8	3	E118	*Halogeometricum borinquense*	99.9
4	E113	*Halomonas koreensis*	99.6		E59	*Halogeometricum borinquense*	99.9
	D1	*Halomonas koreensis*	98.5	4	F104	*Halococcus saccharolyticus*	99.4
5	A102	*Arthrobacter pityocampae*	98.0	5	F101	*Halomicroarcula salina*	97.5
6	A25	*Luteimonas padinae*	98.7	6	F59	*Halorubrum xinjiangense*	99.6
7	D3	*Ornithinibacillus contaminans*	97.1	7	F153	*Halostagnicola kamekurae*	98.5
8	A78	*Rhodovibrio salinarum*	94.9	8	F61	*Natronoarchaeum philippinense*	99.7
9	C29	*Staphylococcus arlettae*	99.8				
10	D5	*Tamilnaduibacter salinus*	99.7				

* **% Similarity**

All of the 18 extreme halophiles were belonged to the archaea and clustered into eight OTUs (Fig. 3). They were phylogenetically identified as members of *Haloferax* (6 strains), *Haloarcula* (5 strains), *Halogeometricum* (two strains), *Halococcus* (one strain), *Halomicroarcula* (one strain), *Halorubrum* (1 strain), *Halostagnicola* (one strain), and *Natronoarchaeum* (one strain) (Table 2). The results are compatible with the archaeal diversity of other solar salterns in Peru (Maturrano et al., 2006), Slovenia (Pašić *et al*., 2005), and Australia (Oh *et al*., 2010), where the *Haloferax* and *Haloarcula* were the most common taxa. Comparison the archaeal diversity between this artificial salt system and Aran-bidgole salt lake revealed the significant differences; while the main isolated halophilic archaea from the natural saline lake were *Halorubrum* (>55%) only the small parts of archaeal strains from Tis solar saltern were belong to this genus (5%). Interestingly, some isolated species from the Tis solar saltern like *Haloferax, Halostagnicola,* and *Halococcus*, were not isolated from the inland thalasohaline lakes in Iran (Amoozegae & Mehrshad, 2013). The results showed this new artificial saline system, harbor microbial populations (especially in the archaea domain) that were not reported from other previously studied natural saline systems in Iran.

The ability of halophile strains for the production of six hydrolytic enzymes, including amylase, cellulase, lipase, pectinase, protease, and urease, were determined by a semi-qualitative approach (Fig. 4). Besides the intrinsic salt stability of halophilic enzymes, their stability over the harsh industrial conditions like the presence of an organic solvent, high pH, and temperature offer their potentials in biotechnological processes. The production of pectinase, protease and urease were not significantly different between halophilic bacteria and archaea obtained from Tis solar saltern ($p>0.05$). However, the halophilic bacteria were most potent to produce lipase and cellulase, while the amylase production was more common for the archaeal strains ($p<0.05$) (Fig. 5). The frequency of enzymes production among all obtained strains was presented in Fig. 6. A. The high enzyme production rates were observed for urease, amylase, protease, and lipase, while relatively low rates of pectinase and cellulase production were observed. The potency of these hydrolytic enzymes was determined based on the mentioned EC ratio and categorized to weak (Fig. 6 B), moderate (Fig. 6 C) and strong (Fig. 6 C). This classification determined that the halophilic strains are good producers for the potent amylase. They produced cellulase and urease with moderate activity, and finally, the lipase, pectinase and protease produced by these strains mostly showed weak activity.

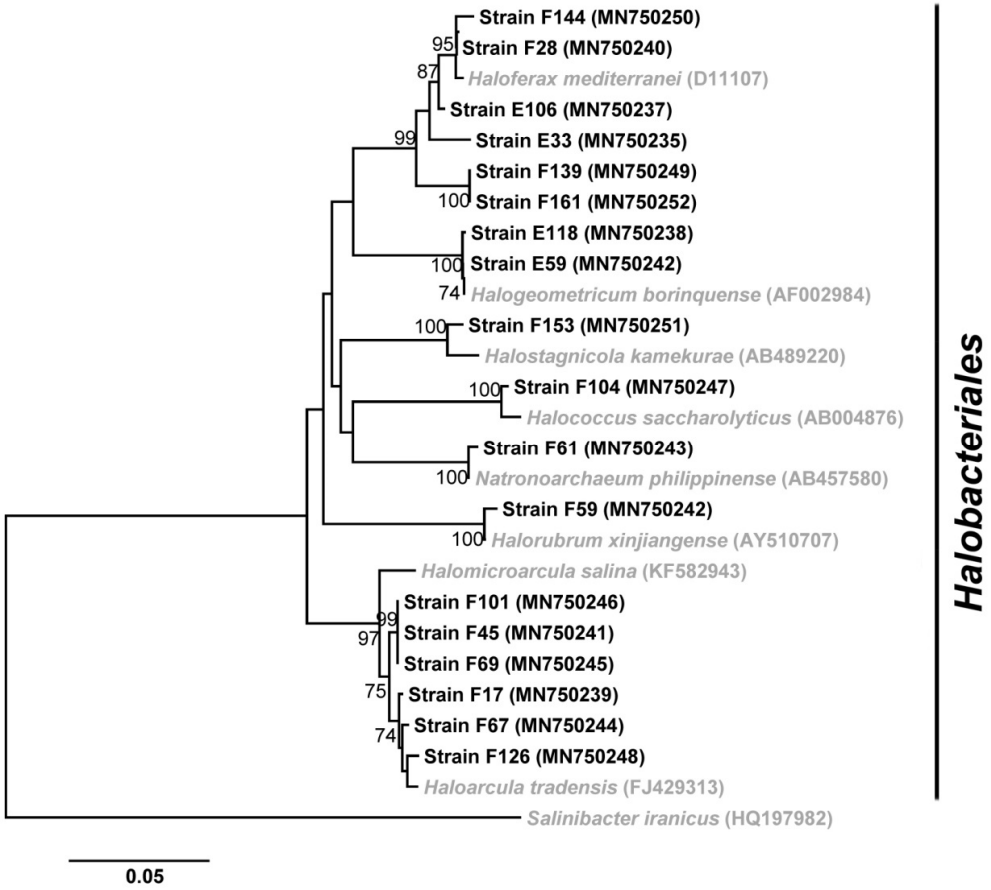

Fig. 3. Phylogenetic inferences based on 16S rRNA gene sequences from isolates belonging to the Archaea domain. The tree is based on the neighbor-joining analysis. The sequence of *Salinibacter iranicus* used as the outgroup.

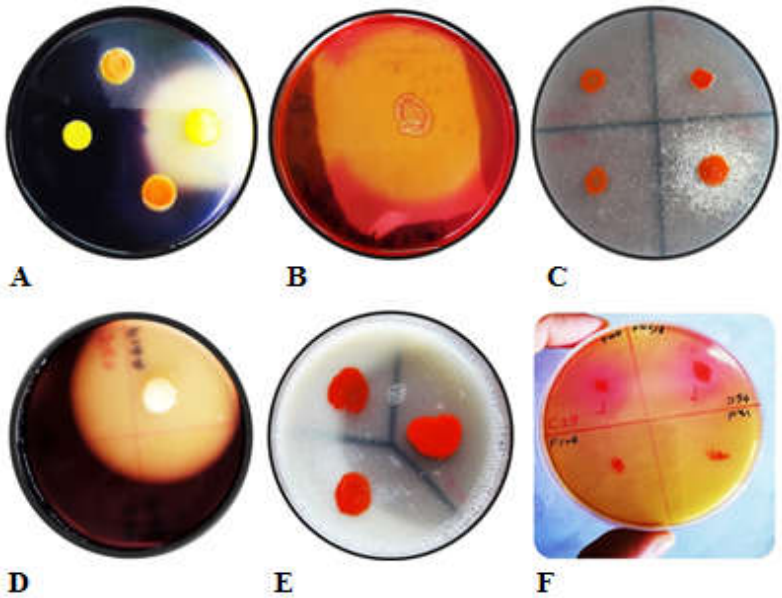

Fig. 4. Assay of various enzyme activity on plates with solid SW medium supplemented with correspond substrates: A= Amylase; B= Cellulose; C= Lipase; D= Pectinase; E= Protease; F= Urease.

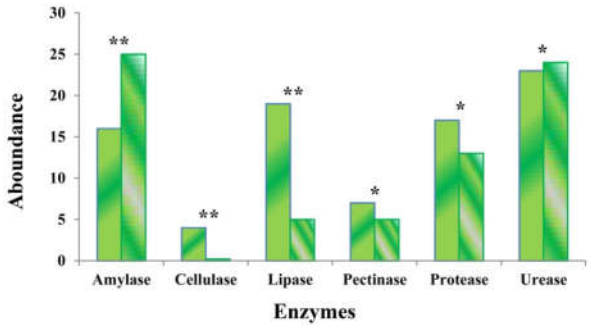

Fig. 5. Enzyme production frequency among bacteria (left columns) and archaea (right columns) obtained from Tis solar saltern:*= not significance (p>0.05), **= significance (p<0.05).

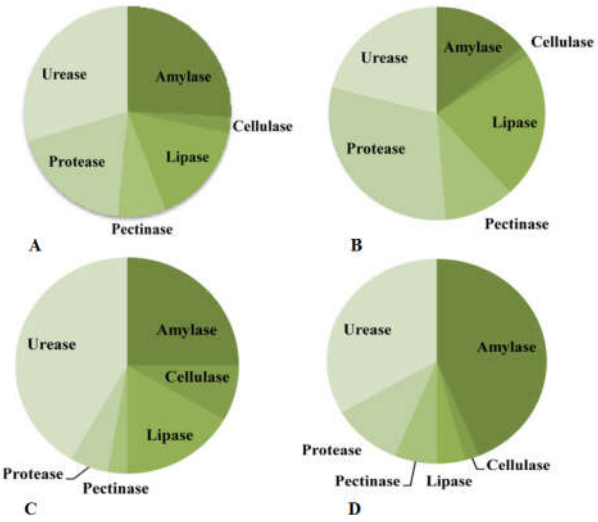

Fig. 6. Enzyme production frequency among halophilic strains obtained from Tis solar saltern. A: the overall enzyme production rate; B, C, and D: rate of enzymes with the weak, medium, and strong activities, respectively.

Amylase production was observed among 41 strains. A majority of these strains were strong or moderate enzyme producers (21 and 9, respectively). The highest level of amylase productions observed by the strains F61 (*Natronoarchaeum*) and D40 (*Longibacter*), where their EC ratio were equals to 11.25 and 5.5, respectively. Amylase is a common hydrolytic enzyme from halophilic prokaryotes. Incompatible with our results, the various studies showed a high amylase activity among the halophiles obtained from saline lakes (Sánchez-Porro *et al*., 2003; Dang *et al*., 2009; and Moreno *et al*., 2013).

The cellulase production rate was low in this study, and only four strains could hydrolysis cellulosic substrate. Lignocellulose substances are one of the most abundant raw materials that can be used in the production of various fermented products such as bioethanol, enzymes, organic acids, etc. However, lignocellulosic materials should be hydrolyzed into simple sugars to be used by appropriate microbes. Cellulases are usually used for the conversion of lignocellulose into simple sugars. In the saccharification process, the raw materials were pretreatment by Ionic liquids to disrupt the lignin and waxy materials. Hence, it is necessary to produce cellulase enzymes that are stable in the presence of salt (Gunny *et al*., 2015). Among the strains obtained from Tis solar saltern, the most potent strain was D59 with the EC equals to 2.5 and was identified as *Bacillus* sp.

A total of 25 strains could produce lipase, and most of them (19 strains) were bacteria. Most of these halophilic strains weakly produce lipase (17 strains), while the six and two strains produced moderate or high levels of the enzyme, respectively. The most potent bacterium and archaeum were strain D5 (*Tamilnaduibacter*) and E106 (*Haloferax*) with EC as 2.54 and 3.50, respectively. Similar to our results, lipase producers strains *Tamilnaduibacter* and *Haloferax* were isolated from the salt pan (Krishnamurthi et al., 2015) and saltern pond (Rathod *et al*., 2016).

Pectinase activity was not determined among strains isolated from saline environments in several studies (Makhdoumi-kakkhi *et al*., 2011; Biswas and Paul, 2013). However, 12 strains of isolated prokaryotes from Tis solar saltern showed pectinase activity. Of them, eight strains produce a low level of the enzyme. One strain produces moderate and remained three strains were the strong producers of pectinase. The highest pectinase activity in bacterial isolates was detected in strain D59 (EC 3.67), which identified as *Bacillus*. The most potent archaeal strain for pectin hydrolyzing activity, strain F28 (EPR 4.27), was a member of *Haloferax*, where its pectinolytic activity was previously determined (Menasria *et al*., 2018).

The urease activity was evaluated in the 47 halophilic strains. A total of 16, 15, and 16 strains were identified as weak, moderate, and strong urease producers. The most potent ureolytic strains were as C29 (*Staphylococcus*) and F104 (*Halococcus*) with EPR equals to 3.75 and 4.28, respectively. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Recently it received much attention due to its impacts on environmental health. In a process named bioconsolidation, released ammonium groups from urea hydrolysis induce the precipitation of calcite, which can be used as a cement to bind small particles in the soil.

Conclusion

The prokaryotic diversity of Tis solar saltern was determined by the aerobic culture-dependent approach. The pond showed high prokaryotic diversity from various bacterial and archaeal groups, including *Actinobacteria*, *Bacteroidetes*, *Balneolaeota*, *Firmicutes*, *Proteobacteria* (*Bacteria*) and *Halobacteria* (*Archaea*) in more than 18 different taxa. Some isolates like *Halococcus* and *Halostagnicola* were not isolated in the previously studied natural thalassohaline lake in Iran. The strains could produce remarkable amounts of hydrolytic enzymes. The most common and potent enzymes were amylase and urease while the cellulase and pectinase were relatively rare. This first report about the culturable aerobic microbial populations of solar salterns in Iran showed these environments are exciting sources for obtaining the halophiles with the potentials in biotechnology.

Acknowledgment

This work was supported by a grant from Ferdowsi University of Mashhad (41314/3).

Conflicts of interest

The authors have no conflict of interest to declare.

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