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Article *in* Microbiology · July 2013

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EXPERIMENTAL ARTICLES

Isolation and Identification of Moderately Halophilic Bacteria Producing Hydrolytic Enzymes from the Largest Hypersaline Playa in Iran[1]

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Received August 14, 2012

Abstract—Iran has many hypersaline environments, both the permanent and seasonal ones. One of the seasonal hypersaline lakes in the central desert zone is Aran-Bidgol Lake in which microbial diversity has not been characterized, thus the potential usage of this microbial community in biotechnology remained unknown. In this study, screening the halophilic hydrolytic enzyme-producing bacteria from different areas of this lake led to isolation of 61 gram-positive and 22 gram-negative moderately halophilic bacteria. These bacterial isolates were shown to produce a wide variety of hydrolytic enzymes including DNase, inulinase, amylase, lipase, pectinase, protease, chitinase, pullulanase, cellulase, and xylanase. The most common enzymes were DNase and inulinase in gram-positive bacteria, lipase in gram-negative bacteria, and pullulanase and cellulase in gram-positive cocci. Interestingly, combined hydrolytic activates were observed in some isolates. According to their phenotypic characteristics and comparative partial 16S rRNA sequence analysis, the moderately halophilic strains belonged to the genera *Halobacillus*, *Thalassobacillus*, *Bacillus*, *Salinicoccus*, *Idiomarina*, *Salicola*, and *Halomonas*.

Keywords: moderate halophiles, hydrolytic enzymes, hypersaline lake

DOI: 10.1134/S0026261713040176

Hypersaline lakes fall into two categories according to their origin: thalassohaline and athalassohaline. Thalassohaline lakes are those that originated by evaporation of seawater and have an ionic composition reflecting that of seawater, with Na^+ as the dominant cation and Cl^- as the dominant anion. Athalassohaline environments are natural brines that have ionic compositions different from that of seawater. Hypersaline environments harbor a considerable diversity of extremely halophilic archaea as well as halophilic and halotolerant bacteria. There are several hypersaline lakes in Iran in both categories, for which the microbial population remains unidentified.

Aran-Bidgol Lake, in the centre of Iran, is a hypersaline lake and the largest playa in Iran. On satellite images, the lake (area ~647 km^2) looks like a triangle headed to the north (Fig. 1). The shores of the lake are covered with salt deposits formed by accumulation of flood and surface water throughout the centuries. The depth of salt layers varies between 5 and 54 meters; they are separated from each other by clay layers. The surroundings of the lake are extremely marshy and the marches are much larger at the west of the lake. In most seasons, the lake is dry and saturated with salt. Most salts in this lake are NaCl, Na_2SO_4, $MgCl_2$, $MgSO_4$ and traces of carbonate ions, so it may be considered a thalassohaline lake [1].

Moderately halophilic bacteria are a group of halophilic microorganisms growing optimally in salt concentrations ranging from 5 to 15% [2]. They constitute a heterogeneous group of microorganisms including species belonging to various genera that possess such features as rapid growth, low nutritional demands, and the ability to utilize a variety of compounds as the sole carbon and energy source.

The potential importance of moderately halophilic bacteria in various biotechnological processes such as production of fermented foods, compatible solutes, biodegradable plastics, and treatment of saline wastewaters, is evident [3].

One of the commercial applications of these microorganisms is production of extracellular hydrolytic enzymes which are active and stable at high salt concentrations and also often alkalin and thermotolerant These organisms are able to produce a variety of hydrolytic enzymes with optimal activities in wide

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Table 1. Sampling conditions, sampling sites, and distribution of halophilic isolates in Aran-Bidgol Lake

Sampling site	Geographical location	pH	*T*, °C	Sample type	Total number of isolates
Site I South East	H: 808 m N: 34.31265 E: 51.7667	7	32	Saline soil	10
				Brine	4
				Saline mud	5
				Salt sediments	5
Site II South west	H: 809 m N: 34.58166 E: 51.76385	6.7	34	Saline soil	3
				Brine	1
				Saline mud	5
				Salt sediments	0
Site III West	H: 805 m N: 34.50729 E: 51.72439	7.2	29	Saline soil	0
				Brine	1
				Saline mud	5
				Salt sediments	16
Site IV East	H: 804 m N: 34.50106 E: 51.76385	7.1	30	Saline soil	3
				Brine	4
				Saline mud	2
				Salt sediments	9
Site V North	H: 799 m N: 34.64378 E: 51.83838	7.5	30	Saline soil	0
				Brine	2
				Saline mud	0
				Salt sediments	8

range of salinity such as amylase, lipase and protease [4–9].

In this study, we attempted to determine the diversity of ten hydrolytic enzymes, including amylase, cellulase, chitinase, DNase, inulinase, lipase, pectinase, protease, pullulanase, and xylanase in halophilic bacterial strains, which will provide information about their potential applications in biotechnological processes.

MATERIALS AND METHODS

Sample collection and growth conditions. The samples were collected during dry seasons (July–November, 2007) from five sites representing different saline environments consisting of brine, multicolor solar salt, saline soil, and saline mud. Table 1 and Fig. 1 show the map of Aran-Bidgol Lake and the sampling locations.

The temperature and pH on the sampling sites were between 30–38°C and 7.0–7.8, respectively. Samples were collected in sterile plastic containers and were cultured not later than 18 h after collection. All samples were cultured in saline nutrient agar with a final concentration of 10% sea salt containing (g/L): NaCl, 81; $MgSO_4 \cdot 7H_2O$, 9.7; $MgCl_2 \cdot 6H_2O$, 7.0; $CaCl_2$, 3.6; KCl, 2.0; $NaHCO_3$, 0.06; NaBr, 0.026, and supplemented with 5% yeast extract, the pH was adjusted to 7.3 with Tris-base buffer before autoclaving. Samples were incubated at 34°C for up to 2 weeks depending on the growth rate of the isolates. After primary isolation and purification, the strains were stored in sealed plates at 4°C for some months. Liquid nitrogen storage was used for long-time preservation.

Screening for extracellular hydrolytic activities. For qualitative detection of enzyme production, the relevant enzymatic assays were performed on agar plates (except for the pullulanase activity assay, which was performed in liquid medium). Standard methods were modified to make suitable conditions for growth of moderately halophilic bacteria. The pH of all media was adjusted on 7.2–7.4 with Tris-base buffer. The different assay media used are described below.

Determination of extracellular hydrolytic enzymes activity. Amylolytic activity on plates was determined qualitatively by the method described by Amoozegar et al. [5], using starch agar medium (Merck, Germany) containing 10% total salts. After incubation at 34°C for one week, the plates were flooded with 0.3% I_2–0.6% KI solution; a clear zone around the colony indicated hydrolysis of starch.

Cellulase activity of the cultures was screened on solid medium containing (g/L): carboxymethylcellulose (CMC), 5; $NaNO_3$, 1; K_2HPO_4, 2; KCl, 1; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 0.5; glucose, 1; agar, 17. After incubation at 34°C for 7 days, the plates were flooded with 0.1% Congo red solution. The clear zones around the colonies were the sign of cellulase activity [10].

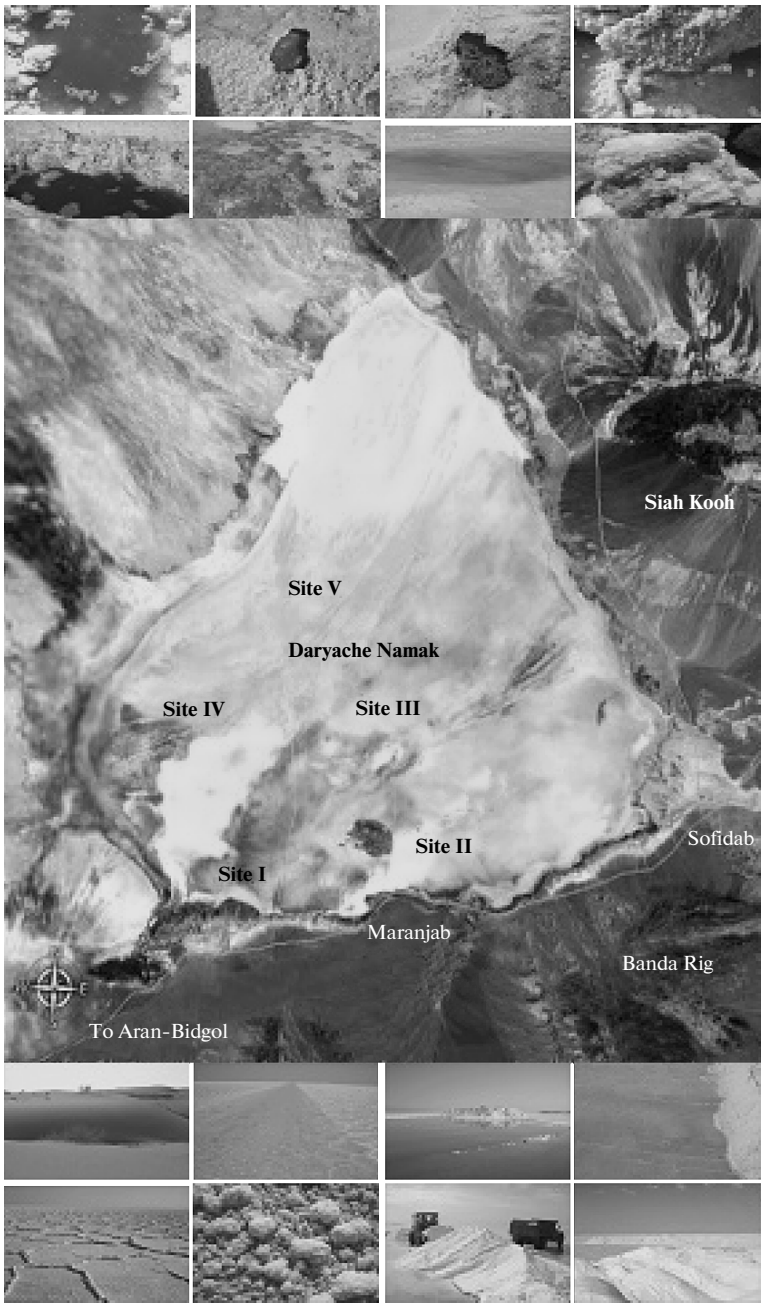

Fig. 1. The map of Aran-Bidgol Lake and the locations of sampling.

Chitinase activity was detected using the modified medium presented by Shaikh et al. [11]. Colloidal chitin (1 g/L) was added to 10% saline nutrient agar. After incubation at 34°C for 7 days, the clear zones around the colonies indicated chitinase activity.

DNase activity of the strains was determined using 42 g/L of DNase test agar medium (Merck), supplemented with 10% total salt and 0.008 g/L toluidine blue. After incubation at 34°C for 4 days, the plates were flooded with 1 N HCl solution. Clear halos around the colonies showed DNase activity [12].

Production of inulinase by halophilic strains was detected in the medium containing (g/L): inulin, 2; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 3; agar, 20; supplemented with an appropriate concentration of salt. Since inulin was used as the sole source of carbon in this medium, bacterial growth after 4 days of incubation at 34°C indicated the presence of inulinase activity [13].

To observe lipase production, the strains were cultured on nutrient agar plates containing olive oil (2.5%), Victoria blue (4 mg/dL) and 10% total salt

Table 2. Taxonomic identification of the 83 environmental isolates able to produce different hydrolytic enzymes

Genus	Amylase	Protease	Lipase	DNase	Inulinase	Xylanase	Cellulase	Pullulanase	Pectinase	Chitinase	Total
Halobacillus	20	13	22	28	22	1	3	7	15	14	154
Thalassobacillus	5	4	4	6	5	5	1	2	1	1	34
Salinicoccus	2	0	0	1	1	0	4	0	1	1	10
Marinococcus	0	1	0	0	1	0	0	0	0	0	2
Nesterenkonia	0	0	0	0	1	0	1	0	0	0	2
Staphylococcus	0	0	0	1	0	1	0	0	0	0	2
Halomonas	2	1	2	1	6	2	2	2	3	2	23
Salicola	1	0	8	2	0	0	0	1	1	0	13
Idiomarina	0	0	1	1	0	0	0	0	0	0	2
Non-identified	2	1	0	0	4	1	0	4	2	2	16
Total	32	20	37	40	40	10	11	16	23	20	249

with an initial pH of 7.2–7.4. The plates were incubated at 34°C for 4 days and the colonies with blue color zones were identified as lipase producing strains [14, 15].

The presence of pectinolytic activity was determined on plates with the medium containing the following (g/L): pectin, 10; $(NH_4)_2SO_4$, 1.4; K_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.02%; nutrient solution, 1 g/L ($FeSO_4 \cdot 7H_2O$, 0.005 g/L; $MnSO_4 \cdot H_2O$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4; $CaCl_2 \cdot 2H_2O$, 0.1; agar, 20 and 10% total salt. After incubation at 34°C for 7 days, the plates were flooded with 0.3% I_2–0.6% KI solution. Clear zones around the colonies showed pectinolytic activity [16].

Proteolytic activity of the cultures was screened in skim milk agar containing 10% skim milk, 2% agar, supplemented with 10% total salt. Clear zones around the growth after 3 days were taken as an evidence of proteolytic activity [7].

To detect pullulanase activity, the strains were cultured in saline liquid medium containing the following (g/L): yeast extract, 1; pullulan, 5; and appropriate concentration of salts and incubated for 72 h.

Clearness of the medium after addition of 97% ethanol indicated that the strains produced pullulanase, since the interaction between pullulan and ethanol leads to formation of white precipitate of the non-degraded pullulan [17].

Xylanase activity was detected by using a saline medium containing the following (g/L): xylan, 10; yeast extract, 2; peptone, 5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; agar, 20. After incubation at 34°C for 7 days, the plates were flooded with 0.1% Congo red solution. The clear zones around the colonies indicated qualitative xylanase activity [18].

Phenotypic and genotypic identification of the isolates. Morphological and physiological characteristics of the isolates studied on nutrient agar or nutrient broth plus 10% NaCl. Gram reaction, motility, colony shape and color, catalase, urease and oxidase activities, nitrate reduction, Tween 80 hydrolysis, Voges–Proskauer and methyl red reactions were checked as recommended by Simbert and Krieg [19]. Acid production from carbohydrates and carbon and nitrogen sources utilization were determined according to Ventosa et al. [20]. Susceptibility of the strains to antibiotics was determined on Muller-Hinton agar containing 10% NaCl.

To determine the optimal temperature and pH for growth of strains, broth cultures were incubated at temperatures of 5–55°C at intervals of 5°C and at pH values of 5–11 at intervals of 0.5 pH units. Growth at different salt concentrations (0, 2.5, 5, 7.5, 10, 15, 20, 25, and 30%) was tested on nutrient broth at pH 7.5. Growth was monitored by optical density at OD_{600} using the spectroscopic method (model UV-160 A; Shimadzu, Japan).

Isolates with growth optima at 3–15% NaCl were considered as moderately halophilic strains [2].

Some strains which exhibited potential for production of extracellular hydrolytic enzymes were chosen for detailed investigation. The genomic DNA of these strains was extracted by DNA extraction kit (Roch, Germany) according to the manufacturer's recommendations and the 16S rRNA gene was amplified using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTA-CCTTGTTACGACTT-3'). A PCR cycler (SensoQuest) was used for amplification. Amplification reaction mixture contained 1.25 μL of each primer, dNTP (10 mM) 0.5 μL, PCR buffer 2.5 μL, $MgCl_2$ (50 mM) 0.75 μL (BIONEER, South

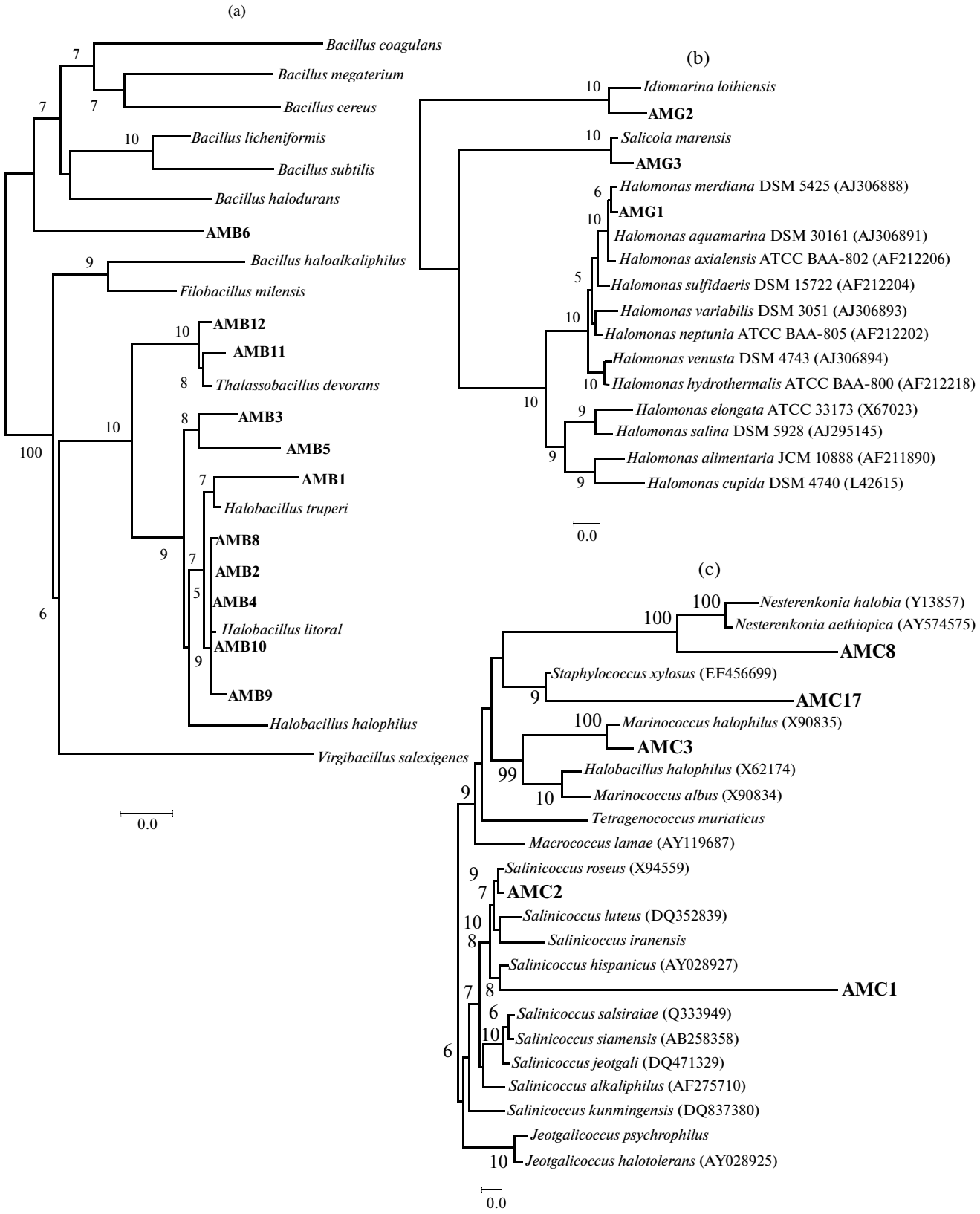

Fig. 2. Phylogenetic tree showing the position of the halophilic strains isolated from Aran-Bidgol Lake, based on comparison of the partial 16S rRNA gene sequences. (a), relation of selected gram-positive rods (AMB1, AMB2, AMB3, AMB4, AMB5, AMB6, AMB7, AMB8, AMB9, AMB10, AMB11, AMB12) to other bacilli and related genera. (b), relation of selected gram-negative strains (AMG1, AMG2, AMG3) to other rod-shaped gram-negative bacteria. (c), relation of selected gram-positive cocci (AMC1, AMC2, AMC3, AMC8, AMC17) to other gram-positive cocci.

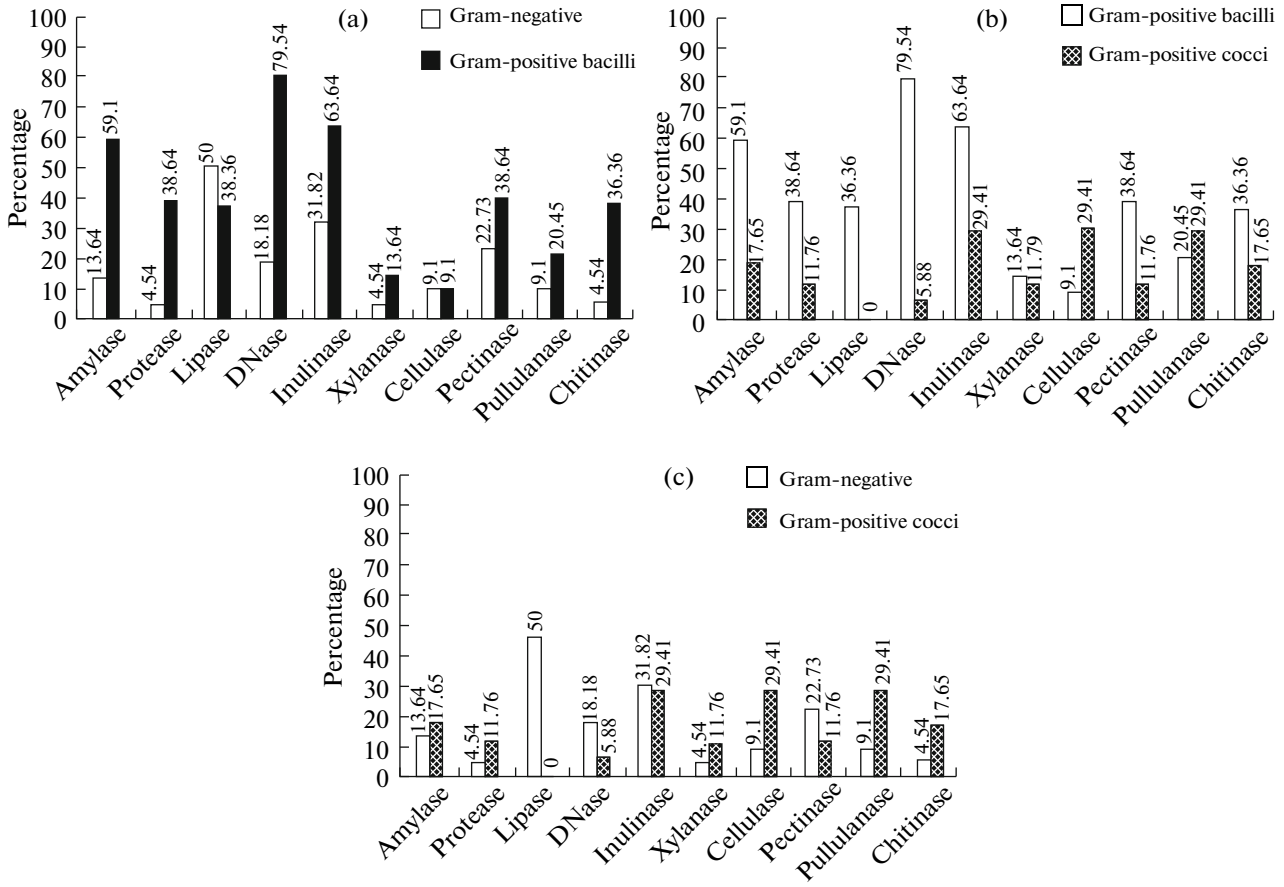

Fig. 3. Comparative diagram of the percentage of isolates with hydrolytic activity. (a) Comparative c for gram-negative and gram-positive rods. (b) Comparative diagram for gram-positive bacilli and gram-positive cocci. (c) Comparative diagram for gram-negative and gram-positive cocci.

Korea), template DNA 1 μL, DMSO 1.25 μL, smartaq DNA polymerase (Cinnagen, Iran) 0.5 μL and deionized H_2O 16 μL, in a final volume of 25 μL.

The following conditions were used for amplification of the 16S rRNA gene: 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 55°C for 1 min and 72°C for 1.5 min, with final 10 min extension at 72°C. Then the PCR products were checked on agarose gel by ethidium bromide staining and were purified using PCR purification kit (Bioneer, South Korea). The purified PCR products were sequenced in both directions using an automated sequencer by Microgene Company (South Korea). The phylogenetic relationship of the isolates was determined by comparing the sequencing data with the related 16S rRNA gene sequences in the GenBank database of the National Center for Biotechnology Information, via BLAST search.

Phylogenetic analysis was performed using the software packages PHYLIP [21] and MEGA version 5 [22], after multiple alignment of data available from public databases by CLUSTAL_X [23]. Pairwise evolutionary distances were computed using the correction method [24] and clustering was performed using the neighbor-joining method [25]. Bootstrap analysis was used to evaluate the tree topology by means of 1000 alternative trees [26].

RESULTS AND DISCUSSION

Isolation of halophilic isolates from Aran-Bidgol Lake. A total of 83 moderately halophilic bacterial strains were obtained from saline soil, saline mud, brine and multicolor solar salt of Aran-Bidgol Lake, among them there were 44 gram-positive rods, 24 gram-negative rods and 17 gram-positive cocci.

Table 1 shows the sampling conditions, sites-and distribution of halophilic isolates from Aran-Bidgol Lake.

Characterization of selective strains. Based on their ability to produce more hydrolytic enzymes, 20 strains selected from total 83 isolates. According to phenotypic characteristics and comparison of partial 16S rRNA gene sequences, the isolates were identified as the members of the following genera: *Halobacillus, Bacillus, Thalassobacilus, Salinicoccus, Nesterenkonia, Marinococcus, Staphylococcus, Halomonas, Idio-*

marina, and *Salicola*. The tree showing the phylogenetic position of the isolates is shown in Fig. 2.

The taxonomic position of 83 environmental isolates able to produce different hydrolytic enzymes determined using their 16S rRNA gene sequences, is shown in Table 2.

Hydrolytic activity of halophilic isolates. The ability to produce ten different hydrolytic enzymes was tested qualitatively among the 83 isolates. A total of 40, 40, 32, 27, 24, 20, 20, 16, 11, and 9 strains were able to produce DNase, inulinase, amylase, lipase, pectinase, protease, chitinase, pullulanase, cellulose and xylanase, respectively. Combined hydrolytic activity was also detected in some strains (Table 2).

The number of hydrolytic activities was higher for gram-positive moderately halophilic rods than for gram-negative rods and gram-positive cocci (Fig. 3).

The gram-positive isolates showed mainly amylolytic, proteolytic, inulinolytic, nucleolytic, pectinolytic, chitinase, xylanolytic and pullulanase activities, while gram-negative rods and gram-positive cocci showed mainly lipolytic and cellulolytic activity, respectively.

In similar studies, Sanchez-Porro et al. [27] showed that hydrolytic enzymes including amylase, protease, lipase, DNase, pullulanase were the most abundant enzymes produced by moderately halophilic bacteria from salterus in Spain. Also, Zavaleta et al. [28] showed that amylase, lipase, and protease were important hydrolytic enzymes produced by halophilic bacteria isolated from Pilluana brines, Peru. Moreno et al. [29] investigated the diversity of extreme halophiies, producers of lipase, protease, amylase and nuclease, in hypersaline ecosystems in Southern Spain. Cojoc et al. [30] showed that six enzymes (amylase, gelatinase, protease, lipase, cellulase, and xylanase) were the most abundant hydrolytic enzymes produced by bacterial isolates from a subterranean rock salt crystal in Slani Prahova area. Govender et al. [31] investigated isolation of hydrolase-producing bacteria (xylanase, cellulase, mannanase) from Sun Pan solar saltern in Botswana. Rohban et al. [32] screened the halophilic bacteria from Howz Sultan Lake (Iran) which produced 9 extracellular hydrolases.

In this study compared with previous studies, two points are noteworthy: first, frequency occurrence of 10 hydrolytic extracellular enzymes produced by moderately halophilic bacteria of Aran-Bidgol Lake was shown. Second, ecological studies on saline environment in Aran-Bidgol Lake led to isolation and identification a large number of moderately halophilic bacteria capable of producing a mixture of mentioned enzymes. These strains may be valuable for biotechnological purposes.

The strains producing xylanase and cellulase isolated were less frequent than producers of other investigated enzymes. Unlike Sanchez-Porro et al. [27], in this study we isolated no xylan-degrading halophilic bacteria, strains producing xylanase were isolated in this study, most of them belonging (five isolates) to filamentous gram-positive spore-forming bacillus *Thalassobacillus*. Low number of xylanase-producing strains is likely due to the low amount of this polymer in saline environments, so these strains are not common in these habitats. Isolated DNase- and inulinase-producing strains were most common compared to other hydrolytic enzymes studied.

All groups of bacterial isolates were capable of producing DNase, however, most DNase-producing strains belonged to genus *Halobacillus*, while in the study by Sanchez-Porro et al. [27], DNase production was not observed in gram-positive cocci and 28 DNase-producing strains belonged to *Bacillus* and *Halobacillus*. Inulinase is an enzyme which was not previously reported in halophiles; 40 isolates were capable of producing this enzyme. Of these, 22 inulinase-producing strains belonged to *Halobacillus* and were able to grow on inulin as the sole carbon source, which is justifiable according to the high abilities of these bacteria to utilize different compounds in their environment.

Lipase is a kind of enzymes which was more frequently produced by gram-negative strains than by gram-positive bacteria. Such finding is similar to the results of Sanchez-Porro et al. [27], while in this study gram-negative strains capable of producing lipase belonged to the genus *Salicola,* mandatory halophilic *Gammaproteobacteria*. *Salicola* sp. strain IC10 was originally identified as a lipase producer by Moreno et al. [29]. Characteristics of this strain were similar to those of our isolate.

Genotypic grouping of strains. Most gram-positive environmental isolates producing hydrolytic enzymes belonged to the genus *Halobacillus* which is accordance with the results of Sanchez-Porro et al. [27] and Rohban et al. [32]. Most gram-negative isolates belonged to the genera *Halomonas* and *Salicola*. Moreover, strains from the genera *Thalassobacilus, Nesterenkonia, Staphylococcus*, and *Idiomarina* have been isolated in this screening, while no strain of these genera was isolated in the study by Sanchez-Porro on different saline environments of southern Spain. Unlike the study of Rohban et al. on the saline environment of Howz Soltan Lake, we isolated no *Piscibacillus* or *Oceanobacillus*. Also, Sanchez-Porro et al. isolated several strains from the genus *Salinivibrio* in their studies, while we isolated no strains of this genus. Higher concentration of salts in Aran-Bidgol and Howz Soltan lakes may be an acceptable explanation, since these bacteria have optimum growth at 2.5 to 10% NaCl and are able to grow up to 17% NaCl, while concentration of salts in these salt lakes is much higher and reaches up to 30%.

Strains of the genus *Chromohalobacter* were isolated by Sanchez-Porro in their screenings which have not been found in this study. A small number of our

gram-positive and gram-negative strains producing hydrolytic enzymes belonged to the genera *Thalassobacilus* and *Idiomarina,* respectively. The remaining strains did not belong to any of the above genera according to 16S rRNA sequence, phylogeny and phenotypic traits.

Generally, the genus *Halobacillus* and the genera *Halomonas* and *Salicola* were dominant among gram-positive and gram-negative bacteria, respectively. *Bacillus* is known as a producer of extracellular hydrolytic enzymes and species belonging to this genus are used to produce industrial enzymes for most industrial process.

Production of amylase, protease, lipase, DNase, inulinase, xylanase, cellulase, pectinase, plunanase, and chitinase was observed in all three groups (gram-positive bacilli, gram-negative bacteria, and gram-positive cocci). Only lipase production was not observed in gram-positive cocci.

Taxonomic identification of 83 environmental isolates from Aran-Bidgol Lake with were capable of producing different hydrolytic enzymes revealed that 20 amylase, 13 protease, 22 DNase, 28 inulinase, 15 pectinase and 14 chitinase bacteria producers belonged'to the genus *Halobacillus*; 5 xylanase producers belonged to the genus *Thalassobacillus*, 8 lipase producers belonged to the genus *Salicola* and 4 cellulase producers belonged to the genus *Salinicoccus*, respectively. These findings demonstrate the importance of *Halobacillus* and *Thalassobacillus* in producing hydrolytic enzymes.

Some strains were able to produce a mixture of hydrolytic enzymes including one strain capable of producing eight enzymes, three strains capable of producing seven enzymes, seven strains capable of producing six enzymes, five strains capable of producing five enzymes, nine strains capable of producing four enzymes, twelve strains capable of producing, three enzymes, and fourteen strains capable of producing two enzymes. Sanchez-Porro et al. [27] reported only four strains with five hydrolytic activities. Moreno et al. [29] also identified three strains with hydrolytic activity of amylase and lipase and one strain with hydrolytic activity of protease and lipase. Unlike their work, a strain without any enzymatic activity has not been observed in this study.

Strains with several hydrolytic activities are considered valuable for biotechnological targets. Generally, the genus *Halobacillus* is capable of producing more combined enzymesthan the genus *Halomonas*. Ecological studies in saline environment of Aran-Bidgol Lake led to isolation of a wide variety of relatively halophilic bacteria that possess a potential for hydrolysis of polymers such as xylan, pullulan, pectin and carboxymethylcellulose.

ACKNOWLEDGMENTS

This study was supported and conducted by Tehran University. The authors would like to thank their colleagues from the Extremophiles Lab group at Tehran University.

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