Iranian Journal of Fisheries Sciences 14(1) 45-59 2015

Isolation and identification of halophilic bacteria from Urmia Lake in Iran

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Received: August 2013 Accepted: August 2014

Abstract

Halophiles are in all three domains of life: Archaea, Bacteria and Eucarya. Halophilic microorganisms in addition to form major part of life biodiversity can have many biotechnological applications. The objective of this research is isolation and identification of halophilic bacteria from Urmia Lake in Iran and the study of its bacterial biodiversity. After sampling of brines from Urmia Lake from 10 stations and depth of approximately 30-50 cm, in April 2011 and transfer to the laboratory in the sterile conditions, samples were enriched and cultured on defined media, and incubated. After appearance of colonies, selected strains were studied based on morphology, physiology and biochemical characteristics. For phylogenetic identification, their genomic DNA were extracted and amplified by PCR technique. Therefore their sequences were determined by genetic experiment based on 16S rRNA gene sequence and their similarity were analysed in GenBank of EzTaxon database. Finally the phylogenetic tree was constructed. Studied strains belonged to three genera: *Halomonas* 50% (including *H. andesensis* LC6(T) [12.5%], *H. gomseomensis* M12(T) [12.5%], *H. hydrothermalis* Slthf2(T) [12.5%], *H. boliviensis* LC1(T) [6.25%] and *H. janggokensis* M24(T) [6.25%]), *Salinivibrio* 25% (including *S.costicola*subsp. *alcaliphilus* DSM 16359(T) [18.75%] and *S. sharmensis* BAG(T) [6.25%]) and *Idiomarina* 25% (including *I .loihiensis* L2TR(T) [25%]).

Keywords: Halophilic bacteria, Species diversity, Isolation, Phylogenetic, DNA analysis, Urmia Lake

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Introduction

Microorganisms requiring extreme environments for growth are called extremophiles (MacElroy, 1974). Extremophilic microorganisms can thrive in extreme environments such as unusual levels of salt, pH, pressure, temperature, *etc*., and those which are adapted to live in hypersaline habitats are considered halophiles (Kumar and Gummadi, 2009). Halophiles are microorganisms that adapt to moderate and high salt concentrations. They are found in all three domains of life: Archaea, Bacteria and Eukarya. Halophilic bacteria grow over an extended range of salt concentrations (3–15% NaCl, w/v and above), unlike the truly halophilicarchaea whose growth is restricted to high saline environments (Litchfield, 2002). Halophiles represent valuable sources of various biomolecules which can offer potential applications for biocatalysis and biotransformation (Schiraldi and Rosa, 2002). Hypersaline environments are those with salt concentrations above that of sea water (3.3% total dissolved salts) (Oren, 2002a). Urmia Lake (located in the northwest of Iran) can be considered as one of the largest permanent hypersaline lakes in the world and resembles the Great Salt Lake in the western USA in many respects of morphology, chemistry and sediments (Kelts and Shahrabi, 1986).The predominance of the Na^+ and Cl^- ions illustrates the thalassohaline character of Urmia Lake (Sorgeloos, 1997). Therefore, Urmia Lake is an oligotrophic lake of thalassohaline origin (AzariTakami, 1993). Unfortunately, this lake has faced with drought problem in recent years. Fig. 1 shows the drought process of Urmia Lake during 1969-2011, based on satellite images (Pengra, 2012).

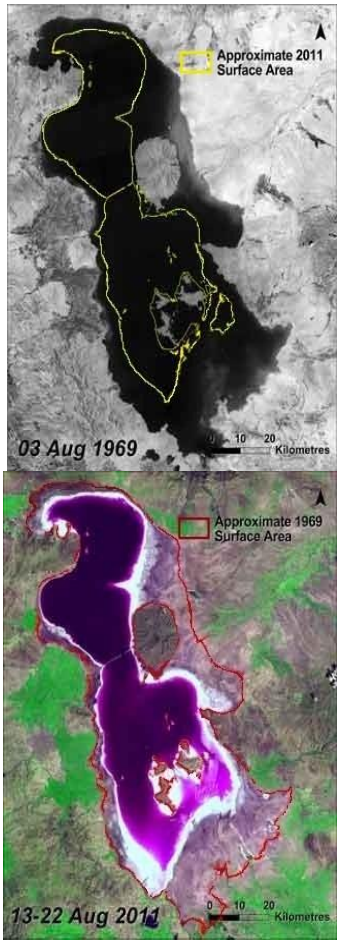


Figure 1: The drought process of Urmia Lake during 1969-2011

The aim of this research is the study of halophilic bacteria from Urmia Lake. These strains can form a small part of Urmia Lake biodiversity and be the base of wide applications in biotechnology too.

Materials and methods

Sampling

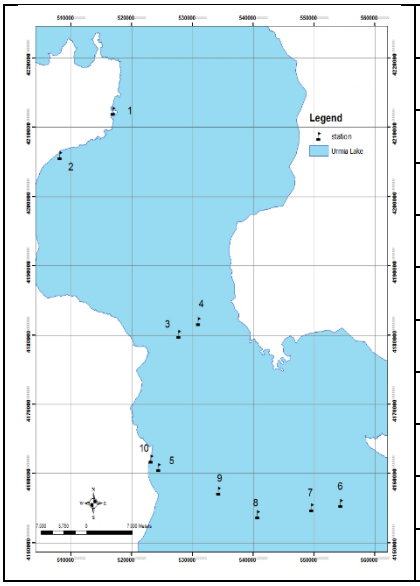
Urmia Lake in the northwestern corner of Iran is one of the largest permanent hypersaline lakes in the world. From among the regions with sampling ability, 10 stations were selected.

Water samples were collected in April 2011 from 10 stations (from depth of

approximately 30-50 cm) and transferred to the laboratory under sterile conditions.

Figs. 2 and 3 depict the images of sampling stations and their locations on the map.

Figure 2: The images of sampling stations, Urmia Lake, 2011
a: station No. 1; b: station No. 2; c: station No. 5; d: station No. 3



Stations No.	Geographic position
1	38S 0516914 4212350
2	38S 0508193 4205915
3	38S 0527776 4180091
4	38S 0530952 4181940
5	38S 0524422 4160858
6	38S 0554403 4155666
7	38S 0549579 4155046
8	38S 0540712 4154011
9	38S 0534303 4157435
10	38S 0523190 4162056

Figure 3: The locations of sampling stations on the map

Growth conditions and isolation

Samples were added to Nutrient Broth (NB; MERCK) and Alkaline Peptone Water (APW; MERCK) media. Samples were enriched by the methods of (Halako *et al*., 2005; Amoozegar *et al*., 2008) and incorporation of tow methods.

Enrichment in the Nutrient Broth was carried out in two forms:

1- First 10ml of water samples were centrifuged at 3000 rpm for 5 minute. After removing the top part of the sample, 1ml bottom sample of tube was added to 30ml Nutrient Broth (pH 7.2-7.4, supplemented with 5% (w/v) salt of lake).
2- Water samples without centrifuge were added to 30ml Nutrient Broth (pH 7.2-7.4, supplemented with 5% (w/v) salt of lake).
Then samples were incubated at 35˚C on an orbital shaking incubator at 150 rpm for 48-72 hours.

Enrichment in the Alkaline Peptone Water was carried out following on:
10ml of water samples were centrifuged at 3000 rpm for 5 minute. After removing the top part of the sample, 1ml bottom sample of tube was added to 9ml of Alkaline Peptone Water (pH 8.6±0.2, supplemented with 5% and 10% (w/v) salt of lake) and incubated at 37˚C for 24-48 hours. Therefore samples were cultured on Nutrient Agar (NA; MERCK) (Amoozegar *et al*., 2008) and MacConkey Agar w/0.15% Bile Salts, CV and NaCl (Mac A; HIMEDIA) media (This medium was selected for the isolation of Gram-negative strains), and incubated at 35-37 ˚C for 24-48 hours.

All of the media were contained with 5% and 10% (w/v) salt (the salt of lake). Repeated cultures were carried out to achieve pure cultures.

Phenotypic characteristics and phylogenetic analysis

Phenotypic characteristics including morphological, physiological and biochemical tests were determined for each strain.Colonial morphology was described by using standard microbiological criteria, such as pigmentation, form, colonial elevation and opacity. Cell morphology was examined by light microscopy. Gram staining was performed (Murray *et al*., 1994) and the result was confirmed by the KOH test (Baron and Finegold, 1990). Motility was analysed by the wet-mount method (Murray *et al*., 1994). Other phenotypic and biochemical characteristics were checked by using standard procedures and as recommended by Smibert and Krieg (1994).

Because of genotypic and phylogenetic studies, 16 isolates were selected for genetic experiment based on 16S rRNA gene sequence. First genomic DNA of the selected strains was extracted by IBRC genomic DNA extraction kit on following the manufacturer's recommended procedure and amplified by PCR technique. The 16S rRNA gene of the selected strains was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

The amplification program was done by initial denaturation at 94˚C for 3min followed by 30 cycles of denaturation at 94 ˚C for 1min, annealing at 57-58˚C for 1min, extension 72˚C for 1.5min, last extension 72˚C for 10min and hold time25˚C for 10sec.Then amplified PCR products were sent to Macrogen Co. (South Korea) for 16S rRNA gene sequencing. Data obtained from sequencing were edited by bioinformatic software of Chromas Pro. and saved in FASTA format. At last sequences similarity of these strains were analysed on comparison with registered strains in

GenBank of EzTaxon database (EzTaxon server 2.1) (Chun *et al*., 2007).After identification of closest strains to studied strains, data were aligned by Clustal_X software (Larkin *et al*., 2007).

Finally their phylogenetic tree was constructed by MEGA5 software and the neighbor-joining method (Saitou and Nei, 1987; Tamura *et al*., 2011).

Results

The average of temperature at sampling stations, pH and total salinity of samples were 13.7 ˚C, 7.02 and 32.4%.

Colonies were round with entire edges, convex, shiny to opaque or translucent. Isolated strains produced creamy colonies on Nutrient Agar and pink colonies on MacConkey Agar. All cells were Gram-negative, motile and rod-shaped which curved rods cells were seen between of them.The results of morphologic, physiologic and biochemical tests were listed in Table 1.Also Table 2 shows the comparison of 16S rRNA gene sequences in ExTaxon.

Table 1: Morphologic, physiologic and biochemical characteristics.

Tests		URM 1	URM 3	URM 5	URM 7
Isolation media	Centrifuge of sample	–	–	–	Centrifuge
	Enrichment medium	NB	NB	NB	NB
	Culture medium	NA	NA	NA	NA
	Salinity (w/v)	5%	5%	5%	5%
Colony pigmentation		Cream	Cream	Cream-Yellowish	Cream
Colony morphology		Round, Convex, Entire edges, Translucent	Round, Convex, Entire edges, Translucent	Round, Convex, Entire edges, Bright	Round, Convex, Entire edges, Opaque
Cell morphology		Short rod	Short rod	Short curved rod	Short curved rod
Gram staining		Gram-negative	Gram-negative	Gram-negative	Gram-negative
KOH		+	+	+	+
Motility		+	+	+	+
Oxidase		+	+	+	+
Catalase		+	+	+	+
O / F		+ / +	+ / +	+ / -	+ / -
S / I / M		- / - / +	- / - / +	- / - / +	- / - / +
Urease		+	+	+	+
Nitrate reduction		W	+	-	W
Simmons' Citrate		-	-	W	-
TSI		A/A	K/A	K/K	K/K
Methyl-red		-	-	+	-
Voges-Proskaeur		-	-	-	-
Gelatinase activity		-	-	+	+
Hydrolysis of Aesculin		-	-	-	-
Lysine decarboxylase		-	-	ND	-

Table 1 continued:				
Ornithine decarboxylase	-	-	ND	-
Aargininedihydrolase	+	-	ND	-
Tests	**URM 10**	**URM 11**	**URM 12**	**URM 14**
Isolation media: Centrifuge of sample	Centrifuge	Centrifuge	Centrifuge	Centrifuge
Isolation media: Enrichment medium	NB	APW	APW	APW
Isolation media: Culture medium	Mac A	NA	NA	Mac A
Isolation media: Salinity (w/v)	5%	5%	5%	5%
Colony pigmentation	Pink	Dark Cream	Cream	Pink
Colony morphology	Round, Convex, Entire edges, Opaque	Round, Convex, Entire edges, Shiny	Round, Convex, Entire edges, Shiny	Round, Convex, Entire edges, Shiny
Cell morphology	Short rod	Short rod	Short curved rod	Short curved rod
Gram staining	Gram-negative	Gram-negative	Gram-negative	Gram-negative
KOH	+	+	+	+
Motility	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
O / F	+ / -	+ / -	+ / -	+ / +
S / I / M	- / - / +	W / - / +	- / - / +	W / - / +
Urease	W	W	W	W
Nitrate reduction	-	-	-	+
Simmons' Citrate	+	-	W	-
TSI	A/A , H_2S	K/K	K/K	K/A , H_2S
Methyl-red	-	+	+	-
Voges-Proskaeur	-	-	-	+
Gelatinase activity	-	+	+	+
Hydrolysis of Aesculin	-	-	-	-
Lysine decarboxylase	-	+	ND	-
Ornithine decarboxylase	+	-	ND	-
Aargininedihydrolase	-	-	ND	-
Tests	**URM 16**	**URM 20**	**URM A**	**URM B**
Isolation media: Centrifuge of sample	Centrifuge	Centrifuge	Centrifuge	Centrifuge
Isolation media: Enrichment medium	APW	APW	APW	APW
Isolation media: Culture medium	NA	Mac A	NA	Mac A
Isolation media: Salinity (w/v)	10%	10%	10%	10%
Colony pigmentation	Dark cream	Pink	Cream	Dark pink
Colony morphology	Round, Convex, Entire edges, Opaque	Round, Convex, Entire edges, Opaque	Round, Convex, Entire edges, Shiny	Round, Convex, Entire edges, Translucent
Cell morphology	Rod	Rod	Short curved rod	Short curved rod

Table 1 continued:

Gram staining	Gram-negative	Gram-negative	Gram-negative	Gram-negative
KOH	+	+	+	+
Motility	+	+	+	+
Oxidase	-	-	+	+
Catalase	+	+	+	+
O / F	+ / -	+ / -	+ / -	+ / -
S / I / M	W / - / +	- / - / +	+ / - / +	- / - / +
Urease	-	-	W	-
Nitrate reduction	-	-	-	+
Simmons' Citrate	+	+	W	-
TSI	K/A	A/A	K/K	K/A
Methyl-red	+	+	+	-
Voges-Proskaeur	-	-	-	-
Gelatinase activity	-	-	+	+
Hydrolysis of Aesculin	+	+	-	+
Lysine decarboxylase	-	-	+	-
Ornithine decarboxylase	-	-	-	-
Aargininedihydrolase	+	-	-	-

	Tests	URM C	URM E	URM F	URM I
Isolation media	Centrifuge of sample	–	Centrifuge	–	Centrifuge
	Enrichment medium	NB	APW	NB	NB
	Culture medium	Mac A	Mac A	NA	NA
	Salinity (w/v)	5%	10%	5%	5%
Colony pigmentation		Pink	Light pink	Light cream	Cream
Colony morphology		Round, Convex, Entire edges, Opaque	Round, Convex, Entire edges, Opaque	Round, Convex, Entire edges, Opaque	Round, Convex, Entire edges, Opaque
Cell morphology		Rod	Rod	Rod	Short curved rod
Gram staining		Gram-negative	Gram-negative	Gram-negative	Gram-negative
KOH		+	+	+	+
Motility		+	+	+	+
Oxidase		+	-	+	+
Catalase		+	+	+	+
O / F		+ / -	+ / -	+ / -	+ / -
S / I / M		- / - / +	- / - / +	- / - / +	- / - / +
Urease		W	W	+	W
Nitrate reduction		+	W	+	+
Simmons' Citrate		+	-	+	-
TSI		A/A , H_2S	K/A	K/A	A/A
Methyl-red		+	+	-	+
Voges-Proskaeur		-	-	-	-
Gelatinase activity		-	-	-	+
Hydrolysis of Aesculin		-	-	-	+
Lysine decarboxylase		-	-	-	-

Table 1 continued:				
Ornithine decarboxylase	-	-	-	-
Aargininedihydrolase	-	-	-	-

NB: Nutrient Broth; NA: Nutrient Agar; APW: Alkaline Peptone Water; Mac A: MacConkey Agar; W: Weak

Table 2: The comparison of 16S rRNA gene sequences in ExTaxon.

Strain	**Closest strain in GenBank (EzTaxon)**	**Accession**	**Similarity**
	H.hydrothermalis Slthf2(T)	AF212218	97.7
URM 1	*H.alkaliphila* 18bAG (T)	AJ640133	97.6
	H.venusta DSM 4743(T)	AJ306894	97.5
	H. hydrothermalis Slthf2(T)	AF212218	98.3
URM 3	*H.alkaliphila* 18bAG(T)	AJ640133	98.2
	H.venusta DSM 4743(T)	AJ306894	98.1
	I.loihiensis L2TR(T)	AE017340	98.9
URM 5	*I.abyssalis* KMM 227(T)	AF052740	98.5
	I.ramblicola R22(T)	AY526862	98.5
	*S.costicola*subsp. *alcaliphilus*DSM 16359(T)	AJ640132	95.8
URM 7	*S.sharmensis* BAG(T)	AM279734	95.6
	S.costicola subsp. *costicola* NCIMB 701(T)	X95527	95.3
	H.andesensis LC6(T)	EF622233	97.4
URM 10	*H.venusta* DSM 4743(T)	AJ306894	97.3
	H.alkaliphila 18bAG(T)	AJ640133	97.1
	I.loihiensis L2TR(T)	AE017340	99.5
URM 11	*I.abyssalis* KMM 227(T)	AF052740	99.1
	I.ramblicola R22(T)	AY526862	99
	I.loihiensis L2TR(T)	AE017340	99.6
URM 12	*I.ramblicola* R22(T)	AY526862	99.3
	I.abyssalis KMM 227(T)	AF052740	99.2
	S.sharmensis BAG(T)	AM279734	92.1
URM 14	*S.costicola* subsp. *alcaliphilus* DSM 16359(T)	AJ640132	92
	S.costicola subsp. *costicola* NCIMB 701(T)	X95527	91.4
	H.gomseomensis M12(T)	AM229314	99.4
URM 16	*H.arcis* AJ282(T)	EF144147	97.6
	H.subterranea ZG16(T)	EF144148	97.5
	H.gomseomensis M12(T)	AM229314	99.5
URM 20	*H.arcis* AJ282(T)	EF144147	98
	H.subterranea ZG16(T)	EF144148	97.4
	I.loihiensis L2TR(T)	AE017340	99.4
URM A	*I.abyssalis* KMM 227(T)	AF052740	99
	I.ramblicola R22(T)	AY526862	98.9
	*S.costicola*subsp. *alcaliphilus*DSM 16359(T)	AJ640132	99.3
URM B	*S.costicola* subsp. *costicola* NCIMB 701(T)	X95527	98.6
	S.costicola subsp. *vallismortis* DSM 8285(T)	AF057016	97.4
	H.boliviensis LC1(T)	AY245449	98
URM C	*H.neptunia* Eplume1(T)	AF212202	97.9
	H.variabilis DSM 3051(T)	AJ306893	97.5
	H.janggokensis M24(T)	AM229315	99.9
URM E	*H.subterranea* ZG16(T)	EF144148	99.7
	H.arcis AJ282(T)	EF144147	98.5
	H.andesensis LC6(T)	EF622233	94.3
URM F	*H. venusta* DSM 4743(T)	AJ306894	94.2
	H. hydrothermalis Slthf2(T)	AF212218	94.2
	*S.costicola*subsp.*alcaliphilus*DSM 16359(T)	AJ640132	99.7
URM I	*S.costicola* subsp. *costicola* NCIMB 701(T)	X95527	98.9
	S.sharmensis BAG(T)	AM279734	97.8

From total strains, 50%, 25% and 25% of strains belonged to members of the genera

Halomonas, *Salinivibrio* and *Idiomarina*, respectively.

The genus *Halomonas* [50%] contained *H.andesensis* LC6(T) [12.5%], *H.gomseomensis* M12(T) [12.5%], *H.hydrothermalis* Slthf2(T) [12.5%], *H.boliviensis* LC1(T) [6.25%] and *H.janggokensis* M24(T) [6.25%]). The genus *Salinivibrio* [25%] included *S.costicola* subsp. *alcaliphilus* DSM [18.75%] and *S.sharmensis* BAG(T) [6.25%]. The identified genus *Idiomarina* [25%] only belonged to *I.loihiensis* L2TR(T).

The study of 16S rRNA gene similarities showed more than 99% similarity for 50% of strains, 98.7–99% similarity for 6.25% of strains and less than 98.7% similarity for 43.75% of strains.

The analysis of 16S rRNA gene similarity along with phenotypic characteristics suggested that some of these strains can be representatives of new species in Urmia Lake.Furthermore, phylogenetic relationships of the studied strains are shown by phylogenetic tree in Fig.4.

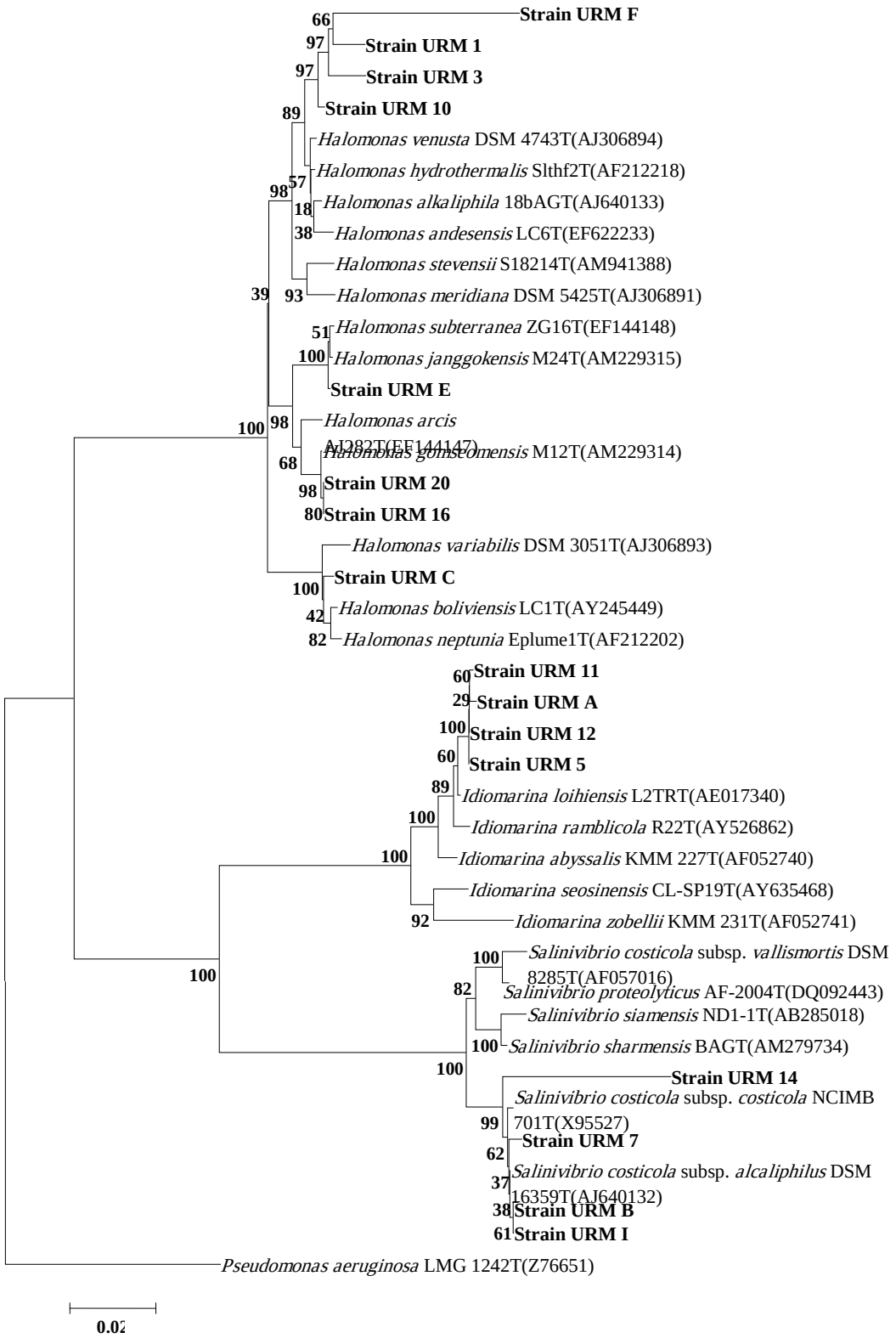


Figure 4: Neighbour-joining tree based on 16S rRNA gene sequences (Bootstraps were done using 100 replications and *Pseudomonas aeruginosa* LMG 1242T(Z76651) was used as an outgroup).

Discussion

Increased attention has been given in the last few years to moderately halophilic bacteria. Several studies have been conducted on their ecology, taxonomy and phylogeny as well as their biotechnological applications (Ramos-Cormenzana, 1991; Ventosa *et al*., 1998a, b; Sanchez-Porro *et al*., 2003).

In this study, the moderately halophilic Gram-negative bacteria members of the genera *Halomonas, Salinivibrio* and *Idiomarina* were isolated from Urmia Lake and their phenotypic and genotypic characteristics were studied. Most environmental isolates able to produce hydrolytic enzymes belonged to the Gram-negative genera *Salinivibrio* and *Halomonas*, two genera widely distributed in hypersaline environments (Ventosa, 1988; Ventosa, *et al*. 1998b).*Halomonas* is the largest genus in the family *Halomonadaceae*, with more than 50 recognized species until 2010 (Guzman *et al*., 2010). This genus can tolerate or require a high salt concentration for growth (Ventosa *et al*., 1998b). In addition, in our research, most of the reported strains belonged to the genus *Halomonas*. We isolated members of *Halomonas* from water samples of Urmia Lake but *Halomonas* have been isolated from water, soil, and seafood and depth-water samples from saline environments in the world (Oren, 2002b). Also, second abundance within the identified genera in the present research belonged to the strains of the genus *Salinivibrio*. Members of this genus are moderately halophilic bacteria distributed in salted meats, brines and several hypersaline environments (Rao *et al*., 1998).

In similar studies, *salinivibrio* was not isolated from Urmai Lake. Such as the results from ZununiVahed *et al*. (2011), strains of the genera *Halomonas* and *Idiomarina* were isolated but there wasnot any strain of *salinivibrio*.Maybe the present study is the first report of *salinivibrio* from Urmia Lake.Moreover, we isolated *Idiomarina loihiensis* sp. from surface water of Urmia Lake (depth of 30-50 cm), while the first isolation and identification of this species was from sediment at a depth of 11000 m in the Mariana Trench (Donachie *et al*., 2003).

The said strains were only part of the microbial communities in Urmia Lake. The aim of this investigation was isolation and identification of halophilic bacteria from Urmia Lakethat it led to identification of new strains from this lake.However, the presentation of new or unusual isolates, require a polyphasic approach.There were polyextremophile microorganisms within isolated strains too. The extremophiles that could tolerate more than one factor of harsh conditions are called polyextremophiles. They can withstand a variety of stresses (Chela-Flores, 2013).

S.costicola subsp. *alcaliphilus* and *S.sharmensis* are haloalkaliphilic bacteria (Romano *et al*., 2005, 2011).

H.boliviensis is a halophilic, psychrophilic, alkalitolerant bacterium and *H.hydrothermalis* is a psychrotolerant halophile too (Kaye *et al*., 2004; Quillaguaman *et al*., 2004).Besides, the genus *Idiomarina* has been proposed aspsychrotolerant heterotrophic halophilic

as well (Ivanova *et al*., 2000).The 16S rRNAgene sequence similarity value has played an important role in delineating novel taxa and in the identification of isolates. Stackebrandt and Goebel (1994) suggested that a 16S rRNA gene sequence similarity of 97% should become the boundary for delineation of prokaryotic species, which has been well accepted among microbiologists.

More recently, Stackebrandt and Ebers (2006) proposed a more relaxed cut-off value of 98.7–99%, after inspection of a large amount of recently published data. Even though this new proposal requires further validation and discussion, it is evident that high quality of sequencing should be the prerequisite to the use of lower similarity cut-off values for the identification of prokaryotes.

Thus, the studied strains can be novel members of halophilic bacteria by supplemental studies such as DNA-DNA hybridization, G+C content and more chemotaxonomic and phenotypic studies.
In addition, this article shows that Urmia Lake and its bacterial stores can have wide biotechnology applications.

Acknowledgment

This study was supported by Iranian Biological Resource Center and General Office Department of Environment West Azarbaijan Province. The authors are gratefulfor the valuable help of these organizations.

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