Original article

Isolation and characterization of *Halobacterium salinarum* from saline lakes in Iran

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Abstract

Introduction and objective: Extremely halophilic Archaea belonging to the order *Halobacteriales* had been isolated from various hyper saline environments such as the Dead Sea, the Great Salt Lake, Sabkhas Lake, and natural or artificial Salterns. The main aim of this study was to isolate and characterize *Halobacterium salinarum* strains from samples of saline soils and lake water collected from four distinct regions of Iran.

Materials and methods: Saline soil samples and lake water samples were collected from different zones of Iran including a high salinity lake in Qom and Fars and also Seawater of Oromiea Lake and Persian Gulf. For isolation of *H. salinarum* from collected samples, *Halobacterium salinarum* medium were used. Isolates were identified by biochemical tests such as motility and morphological features, then identification confirmed by molecular characterization. Optimum NaCl requirement, pH and temperature were determined.

Results: The obtained results showed two isolates were motile, rod, Gram-negative, oxidase and catalase positive, with red pigmentation which grew in the presence of 25% NaCl. Isolates could hydrolyze casein and gelatin. The result of molecular characterization showed that two strains were *H. salinarum*. Basic local alignment search tool (BLAST) analysis showed 99% homology between these strains and *H. salinarum* strain R1.

Conclusion: In this investigation we succeeded to isolate and identify *H. salinarum* in Iran saline ecosystems. There is a large diversity among halophilic bacteria in Iran ecosystems and isolation of *H. salinarum* from saline lakes will provide better information about halophilic bacteria in Iran ecosystems.

Keywords: *Halobacterium salinarum*; Halophilic bacteria; Archaea; Saline; Saline Lake

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Introduction

It is well known that extremely halophilic bacteria are commonly found in brine with a high concentration of sodium chloride, marine and rock salt, dehydrated kitchen salt, and even in purified salt. The peculiar properties of these bacteria concerning their needs to NaCl for optimal growth, poor use of carbohydrates, and requirement for proteins from dead organisms present in salt led to the assumption of the presence of a well developed enzymatic complex responsible for the amino acid metabolism [1].

Extremely halophilic Archaea belonging to the order *Halobacteriales* had been isolated from various hyper saline environments such as the Dead Sea, the Great Salt Lake, Sabkhas Lake, and natural or artificial Salterns [2]. The *Halobacterium* genus is the best khown genus of *Halobacteriaceae* family. *H. salinarum* is the type genus of this family that has a light driven proton pump in the purple memberane [3]. Three *Halobacterium* species were isolated from saline environment, these species include: *H. salinarum, H. halobium* and *H. cutirubrum* [4].

Halophilic Archaea has a number of useful applications and potential new applications in biotechnological processes are being investigated. They produce products such as bacteriorhodopsin for information processing and ATP generation; novel extracellular polysaccharides; exoenzymes such as amylase, cellulase, xylanase, lipase, and proteases; poly-β-hydroxyalkanoate for biodegradable plastic production; and a protein from *H. salinarum* significant for cancer research [5].

In some extremely halophilic Archaea there was a retinal-binding membrane protein with seven membrane-spanning segments is as Bacteriorhodopsin (BR). The function of this protein is light driven proton pump. This protein can be applied in optical memory, artificial retina, photon switch and so on. Extremely halophilic Archaea with economic importance continues to be isolated from different saline habitats in the world and new taxa in the *Halobacteriales* are still being described [6]. Hypersaline environments are present in Iran. The main aim of this study was to isolate and characterize *H. salinarum* strains from samples of saline soil and water samples collected from four distinct regions of Iran.

Materials and methods

Collection of samples

Saline soil samples and lake water samples were collected from different zones of Iran including a high salinity lake in Qom and Fars and also Seawater of Oromiea Lake and Persian Gulf. Saline soil and lake water samples were collected in sterile mason jars, wrapped in aluminum foil, placed on ice and immediately transported to the laboratory for further analysis.

Growth media and culture condition

Halobacterium Salinarum (HS) medium were used for isolation of *Halobacterium* from collected samples. The medium contained (gl^{-1}): NaCl, 250; KCl, 2; $MgSO_4$, 20; Tri-Na-Citrate, 3; $FeSO_4.7H_20$, 0.02; Casamino acids, 5; $MnCl_2.4H_20$; 0.026 and yeast extract, 5 and for solidification, 20 gl^{-1} agar was added. The pH was adjusted to seven before autoclaving.

Each sample was inoculated into a 250ml conical flask with HS medium (250ml) for enrichment. Flasks were incubated for nine days in a shaker (150rpm) at 37°C and then 10ml of the culture was transferred to a new flask with 250ml HS medium. After three subcultures, 100µl were spread on HS agar medium and incubated at 37°C for nine days. After the

incubation period, different colonies were picked and purified on HS agar [7].

Identification of isolates

Morphological and biochemical tests

Isolates were examined for motility and morphological features in wet mounts. Gram staining was carried out by using acetic acid fixed samples, as described by Dussault [8]. Colony appearance and pigmentation were determined on HS medium agar after growth for nine days. Nitrate reduction was tested in liquid HS medium supplemented with 0.5% $NaNO_3$. Formation of gaseous products from nitrate was detected by the presence of gas bubbles in Durham tubes. Anaerobic growth in the presence of L-arginine was tested according to Oren and Litchfield [7].

Production of acids from sugars was examined in HS medium supplemented with 0.5% (w/v) of sugars tested [9]. Indole production was determined by adding Kovac's reagent to the HS broth supplemented with 1% (w/v) tryptone. To determine starch hydrolysis, the strains were streaked onto HS agar plates with 1% (w/v) soluble starch and the plates were flooded with iodine solution after growth was obtained. Catalase was detected by adding a 1% (v/v) H_2O_2 solution to colonies on HS agar. The presence of oxidase was determined with tetramethyl *p*-phenylendiamine-HCl. Tween 80 and gelatin hydrolysis was tested as recommended by Gutierrez and Gonzales [10].

HS agar with 0.5% sodium thiosulfate was used to test the production of H_2S. Casein hydrolysis was determined by observing the formation of clear zones around colonies on agar medium with 0.15% (w/v) skim milk powder. Appropriate positive, (*H. salinarum* NRC 5812 purchase from Japan bacterial collection) and negative controls (H_2O) were run for all of the above tests and they were carried out at least three times [11].

Molecular identification

For genomic DNA extraction, cells were disrupted in a bead beater, and the released DNA was isolated and purified with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v). The 16S rDNA sequence primers, designed according to Ozcan *et al.* [12] were rDNA22F (5′-ATTCCGGTTGATCCTGC-3′ (positions 6-22) and rDNA1521R (5′ AGGAGGTGAT-CCAGCCGCAG-3′ (positions 1540-1521). The *Escherichia coil* 16S rRNA sequence was used as the position reference.

The PCR thermal cycling conditions were as follows: Initial denaturation at 94°C for 10mins followed by: 25 cycles of 94°C for 1min, 55°C for 1min and 72°C for 2mins. Final extension for 10mins at 72°C. PCR products (200bp) were electrophoresis on a 2% horizontal agarose gel (Sigma USA). Gels were stained in a solution of ethidium bromide and visualized with a UVP UV transilluminator (UVP Inc., San Gabriel, Calif). The desired PCR products (200bp) were extracted from gel using the gel extraction kit (Fermentas, Germany). The sequences of amplified product were determined by automated dideoxynucl-eotide methods with the ABI Prism Big Dye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer) on an ABI Prism 377XL DNA sequencer.

Growth curve and purple membrane synthesis of H. salinarum strain HM-2

Strain HM-2 was grown on HS medium with 25% NaCl to obtain the growth curve. Growth was recorded daily as absorbance in 600nm. Synthesis of purple membrane was assayed during growth by measuring the absorbance at 570nm.

Determination of optimum NaCl requirement, pH and temperature

Salt requirement and tolerance were examined in HS broth in which NaCl concentration was varied (13,15,17,19,23, 26,29). Flask were inoculated with the isolate *H. salinarum* and incubated on shaker (150rpm) for nine days at 30°C [13]. Turbidity (OD600nm) was recorded every day. The pH tolerance was tested in 25% of NaCl medium (HS) adjusted to pH values of 4.5, 5.5, 6.5, 7.5 and 8.5. Optimum temperature for growth was assayed by the growth at 25, 30 and 37°C [14].

Results

Isolation of strains from saline ecosystems in Iran

From different saline soil and water samples grown on HS agar, ten colonies were selected according to colony shape, size, consistency and pigmentation: four from Qom saline Lake (HQ1, HQ2, HQ3 and HQ4), three from Oromiea Lake (HO1, HO2 and HO3), and three from Fars Lake (HM1, HM2 and HM3). None of the Persian Gulf samples did not grows in saline HS medium. They were isolated in pure culture, and the isolated strains were subjected to a range of phenotypic tests. All isolates were motile; Gram-negative, rod, oxidase and catalase positive and grew in 25% NaCl. These properties, together with the colony pigmentation and the requirement for high salt concentrations, suggested that all were members of the family *Halobacteriaceae*.

Physiological and biochemical characteristics

From preliminary screening tests two isolates (HQ-2 and HM-2) with red pigmentation were selected for further studies. Since other isolates can not grow in high concentration of NaCl (250g/l), they were ignored in the future study. Two isolates (HQ-2 and HM-2) were catalase and oxidase positive, motile and have red pigmentation. Both isolates were able to hydrolyse of casein and gelatin. Neither of the isolates were abled hydrolyzing starch. Tween 80 was hydrolyzed only by HM-2 strain. A summary of the biochemical properties of these strains has been presented in table 1 showing that these two isolates belong to *Halobacterium* genus.

Molecular identification

DNA was extracted from two strains (HQ-2 and HM-2) and was quantified spectrophotometrically at 260nm and 280nm. The purity of DNA obtained by the equation of 260/280. PCR performed with 16S rDNA archeal primers. The sequences of design primers are as follow: HS-F 5′-AAGGTGGTGCACGAATAAG-G-3′ and HS-R 5′-ACGTACCCCTCAGGTCTTCC-3′. The PCR product were extracted from gel and sequenced. Both isolates yielded PCR products of amplified 16S rDNA gene derived sequences with archaeal specific primers.

The PCR products (200bp) were extracted from the gels and sequenced. The results of sequencing showed that both strain are belong to *H. salinarum*. The derived sequences were basic local alignment search tool (BLAST) and after BLAST the results show that this sequence has 99% homology with *H. salinarum* strain R1. Table 2 shows the sequencing results.

Growth properties of H. salinarum strain HM-2 and HQ-2

Figure 1 illustrates the growth curve and purple membrane synthesis of HM-2 and HQ-2 strain. As shown, strain HM-2 and HQ-2 reached maximum growth after six days (OD600=0.8) and then entered the stationary phase. Synthesis of purple membrane followed the same pattern and

maximum production was observed at the end of the logarithmic phase.

Optimum NaCl requirement, pH and temperature of HM-2 and HQ-2 strain

Figure 2 shows the effect of different concentrations of NaCl on growth of HM-2 strain. As shown, 22% NaCl was the best concentration for the growth of HM-2 strain. Figure 3 illustrates that the optimal pH for the growth of HM-2 strain was 7. This figure showed that the natural pH=7 is better for the growth of this strain. Figure 4 shows the effect of three temperatures on the growth of HM-2 strain. As shown, 37°C was the best temperature for the growth of HM-2 strain.

Table 1: Biochemical characteristic of isolated strains

Biochemical tests	HQ-2 Strain	HM-2 Strain
Morphology	Rod	Rod
Gram staining	Negative	Negative
Motility	+	+
Catalase	+	+
Oxidase	+	+
Reduction of nitrate to nitrite	-	-
Formation of gas from nitrate	-	-
Anaerobic growth on argenine	+	+
Acid production from glucose	-	-
Indole production from tryptophan	-	+
Starch hydrolysis	-	-
Tween 80 hydrolysis	-	+
Gelatin hydrolysis	+	+
Casein hydrolysis	+	+
Pigmentation	Light Red	Brick Red

Table 2: Sequence and high homology of isolated strains

Strain	Sequence of 16S rDNA (200 bp)	Maximum identity
HM-2	AAGGTGGTGCACGAATAAGGACTGGGCAAGACCGGTGCCAGC CGCCGCGGTAATACCGGCAGTCCGAGTGATGGCCGATCTTATT GGGCCTAAAGCGTCCGTAGCTGGCTGAACAAGTCCGTTGGGA AATCTGTCCGCTTAACGGGCAGGCGTCCAGCGGAAACTGTTCA GCTTGGGACCGGAAGACCTGAGGGGTACGT	*H. salinarum* strain R1 99%
HQ-2	AAGGTGGTGCACGAATAAGGACTGGGCAAGACCGGTGCCAGC CGCCGCGGTAATACCGGCAGTCCGAGTGATGGCCGATCTTATT GGGCCTAAAGCGTCCGTAGCTGGCTGAACAAGTCCGTTGGGA AATCTGTCCGCTTAACGGGCAGGCGTCCAGCGGAAACTGTTCA GCTTGGGACCGGAAGACCTGAGGGGTACGT	*H. salinarum* strain R1 99%

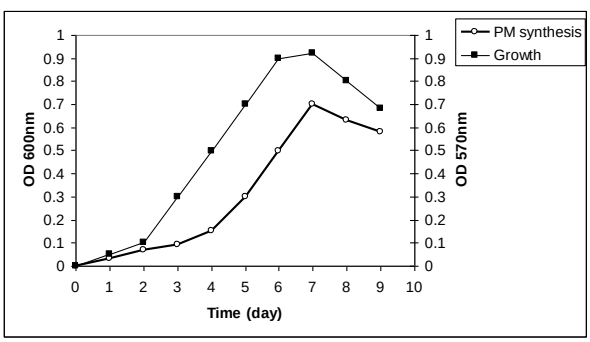

Fig. 1: Growth curve and purple membrane synthesis of *H. salinarum* strain HM-2

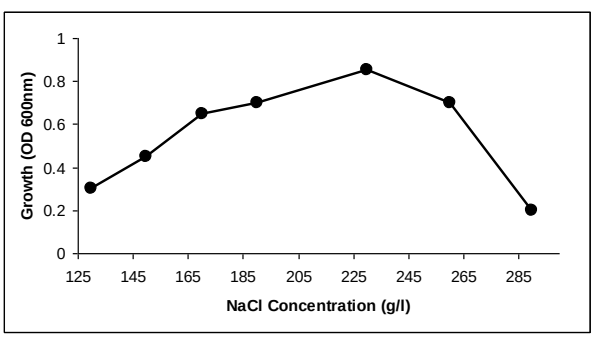

Fig. 2: Effect of NaCl concentration on growth of *H. salinarum* strain HM-2

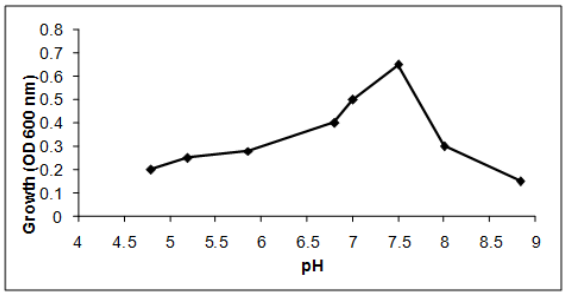

Fig. 3: Optimal pH for growth of *H. salinarum* strain HM-2

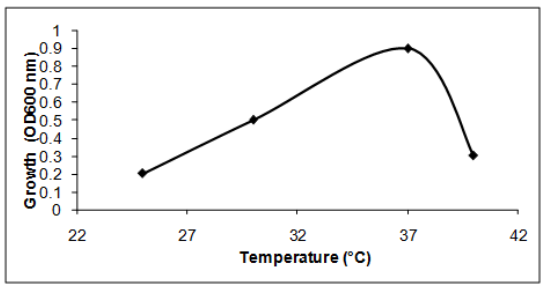

Fig. 4: Optimal temperature for growth of *H. salinarum* strain HM-2

Discussion

In the current study, 10 Archeal strains were isolated from four distinct parts of Iran. All isolates examined belong to the Archeal domain, family *Halobacteriaceae*. That *Halobacterium* is extremely halophilic is by the following evidences: nature of the culture, red color pigmentation and biochemical tests. The genus *Halobacterium* is usually isolated from different ecosystems such as saline lakes [15], Dead Sea [16], halite brine inclusion [2] and Saltern Ponds [17]. Mormile *et al.* [2] isolated *Halobacterium* from halite crystals in Death Valley at California.

They use PCR analysis with haloarchaea-specific 16S rDNA primers to identifying this genus. They concluded that this genus is *H. salinarum.* The genus *Halobacterium* is not known to be dominate in the Archeal community of salt lakes. We recovered two strains from Qom saline Lake (HQ-2) and Fars Lake (HM-2) that can be classified within the genus *Halobacterium* on the basis of their phenotypic characteristics.

Ozcan *et al.* [12] were characterized ninety-five extremely halophilic strains isolated from six distinct saline regions of Turkey by biochemical test. They suggest that molecular method is better than biochemical test for identification of halophilic bacteria. Analysis of 16S rDNA sequence of these strains showed that both isolated strains were *H. salinarum.* However, they exhibited some difference in biochemical properties such as Tween 80 hydrolysis and Indole production from tryptophan. Also these physiological varieties can be useful in biotechnological application. These applications can be production of bacteriorhodopsin with diverse physicochemical properties in different strains.

Synthesis of purple membrane (PM) parallel to bacterial growth demonstrates that PM is a primary product which was also shown by other researchers [18,19]. Zeng *et al.* [13] studied the effect of NaCl concentrations on growth of *H. salinarum*. They found that 25% NaCl was the best concentration for growth of *H. salinarum*. In this study, 22% NaCl concentration yielded maximum growth of our isolates.

Conclusion

In this investigation we succeeded to isolate and identify *H. salinarum* from Iran saline ecosystems. This is the first report on isolation of *H. salinarum* from Iran ecosystemsecosystem.

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