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ORIGINAL ARTICLE

Isolation of a novel halothermophilic strain of the genus *Gracilibacillus* from Howz-e Sultan hypersaline lake in Iran

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ABSTRACT

Background and Objectives: Halothermophilic bacteria are adapted to high osmolarity and can grow in high saline environments and high temperatures. This study was aimed at the isolation of halothermophilic bacteria from Howz-e Sultan hypersaline lake in the central desert zone in Iran.

Materials and Methods: Samples were collected and after preparing dilutions, the samples were cultured on Molten haloid agar with different salt concentrations (5-35%), then the plates were incubated at 35-70ºC in both aerobic and anaerobic conditions. Biochemical characterizations, utilization of carbon sources, production of exoenzymes and antibiotic susceptibility were investigated. Taxonomic and phylogenetic analyses were performed using 16S rRNA gene sequences.

Results: One of the isolated bacteria was found to be Gram-positive, hyperhalophilic, thermophilic, endospore-forming, and was named as 1-9 h isolate. The bacterial cells were bacilli-shaped, which produced endospores at a subterminal position. This isolate was an aerobe and facultative anaerobe and grew between pH 5.0 and 10.0 (optimal growth at pH 7.0-7.5), at temperature between 15°C and 65°C (optimal growth at 40-45°C) and at salinity of 9-32% (w/v) NaCl, growing optimally at 18% (w/v) NaCl. On the basis of 16S rRNA gene sequence analysis, isolate 1-9 h belongs to the genus *Bacillus* within the phylum Firmicutes and showed the closest phylogenetic similarity to *Gracilibacillus* sp. IBP-V003 (99.0%).

Conclusion: Based on the results of its phenotypic and genotypic properties, strain 1-9 h represents a novel strain of the genus *Gracilibacillus*. It can be used in various fields of industry and biotechnology.

Keywords: Bacillaceae; Halobacteriales; Salinity; Extreme environments

INTRODUCTION

Genus *Gracilibacillus* instituted to accommodate species previously assigned to *Bacillus* but differing from core *Bacillus* taxa in sequence characteristics and physiology. The genus *Gracilibacillus* was labeled by researchers (1) to accommodate halophile, endospore-forming bacteria. At the time of writing, the genus includes seven species with validly published names: *Gracilibacillus halotolerans*, the type species, from surface mud of the Great Salt Lake (1, 2), *Gracilibacillus dipsosauri*, from a desert iguana (3), *Gracilibacillus orientalis* (4), *Gracilibacillus lacisalsi* (5) and *Gracilibacillus saliphilus* (6), from saline lakes, *Gracilibacillus boraciitolerans*, from soil (7) and *Gracilibacillus halophilus*, from a saline soil (8). '*Gracilibacillus quinghaiensis*' has been described from salt-lake sediment (9), but this name has not yet been validly published. These bacteria thrive in a maximum of 11-30% NaCl and at 50°C while, hyperhalophilic, with optimum growth at 20%

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(w/v) NaCl and a growing range of 7-32% (w/v) NaCl. Moderately thermophilic, with optimum growth at 40-50ºC and a growth temperature range of 28-60ºC (2, 10, 11).

Hypersaline habitats exist throughout the world in the form of saline soil and saline water. One of the more famous hypersaline habitats is the Howz-e Sultan hypersaline lake in the central desert zone of Iran. Many reports have proposed that halophile microorganisms can be multi-extremophile. Assessing the physiology of halophile microorganisms and their adaptation to the environment permits a better comprehension of the extremophile characteristics of the microorganisms. Halophiles have been applied in a number of biotechnological applications that making them a concerning and important option of the research subject in this revolution of biotechnology. In this study of the microbial diversity of the Howz-e Sultan hypersaline lake has been investigated. A *Gracilibacillus* strain was isolated from a saline soil sample collected. This strain is considered to represent a novel strain of the genus *Gracilibacillus.* In this paper, we describe its phenotypic and chemotaxonomic characteristics, including DNA–DNA relatedness and 16S rRNA gene sequence analyses.

MATERIALS AND METHODS

Isolation and selection of microorganism. Bacteria were isolated from soils and sediments of Howz-e Sultan hypersaline lake situated in the central desert zone in Iran, using an enrichment procedure. The enriched culture broth was serially diluted and spread on Molten Haloid (MH) medium agar plates (pH 7). Serial dilution was carried out using sterile saline 9g/l. Dilutions up to 10^{-7} were completed to perform the spread plate technique. After solidification, the plates were incubated at 35°C for 7-10 days. The colonies were purified by repeated transfers on solid culture media. The Molten Haloid (MH) medium containing [g/L]: yeast extract [10], protease peptone [5], glucose [1], NaCl [100], $MgCl_2.6H_2O$ [7], $MgSO_4.7H_2O$ [9.6], $CaCl_2.2H_2O$ [0.36], KCl [2], $NaHCO_3$ [0.06], NaBr [0.026], (Difco, Himedia & lio) (12, 13). After solidification, the petri dishes with samples were incubated for 7–10 days at 35°C and the number of grown colonies was counted.

Also for isolation of halothermophile organisms, growth at various NaCl concentrations was investigated in MH broth, with the addition of various concentrations of NaCl (0% to 35%) 0, 1, 2, 3, 4 and 6 M, at intervals of 0.5%. Growths at pH 4–10 (at intervals of 0.5 pH unit) and tempratures 15, 25, 30, 37, 40, 45, 50, 55, 60 and 65°C was investigated in MH broth, in shaker incubator (VISION, VS8480 SRN, South Korea) at 120 *rpm* (14, 15).

The growth of the halothermophilic strain of *Gracilibacillus* was performed using inoculum of 24-hour culture (1.5×10^{12} cfu/ml) from the agar plates into MH agar medium. Bacterial growth was measured with a spectrophotometer by OD at 660 nm (16- 18).

Phenotypic identification. The isolate was phenotypically characterized using a combination of phenotypic tests. Cell shape, size, and arrangement were examined on MH agar at 35°C after 7 days. The method with modification was used for Gram staining (19). Flagella were examined as described by Grossart et al. (20). Catalase and oxidase activity, motility, growth at different salt concentrations, anaerobic growth, indole production, the methyl red/Voges–Proskauer reaction, H_2S production, and nitrate reduction were determined as described by Barrow and Feltham (21). MH medium with 0.25% agar, 1% glucose, and bromocresol purple as the indicator were used for the oxidative/fermentative (OF) test. Hydrolysis of casein, gelatin, starch, Tween 80, urea, xylane, and DNA was determined as described by Namwong et al. (14). Acid production from carbohydrates was assessed in the medium explained by Tindall et al. (22). All tests were carried out in medium supplemented with 10% NaCl (except for the investigation of the effects of NaCl on growth) (23). The potency to utilize several compounds was tested in a medium defined by Carrasco et al. (4) supplemented with 10% NaCl. Carbohydrates were used at a final concentration of 0.5% (w/v). Whenever amino acids were consumed as substrates, the basic medium contained neither Potassium nitrate nor Ammonium phosphate. Growth under anaerobic conditions on MH agar plates with or without KNO_3 (1% w/v when present) was performed in a Gaspak anaerobic jar. Antibiotic susceptibility to discs of P, penicillin; S, streptomycin; T, oxytetracycline; CP, ciprofloxacin; CX, cloxacillin; E, erythromycin; AM, ampicillin; GM, gentamicin; NV, novobiocin; B, bacitracin; and C, chloramphenicol was controlled according to the conventional Reller et al. method (24). Inhibition zones were explicated according to the producer's manual (Oxoid) after that plates

were incubated at 30°C for 48 h (4, 9). It should be noted that all tests were repeated three times.

Confirmation of novel strain of *Gracilibacillus*. Identification of novel strain *Gracilibacillus* was verified by sequencing the 16S rRNA genes. This method was carried out as follows: DNA was extracted from the isolates by a standard kit (Roche-Germany). Then amplification of the 16S rRNA gene was performed by the PCR method and eventually the products were sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing (14, 25, 26).

DNA extraction and amplification of 16S rRNA gene. The purity of the extracted DNA was assessed based on the absorbance of the extracted DNA at 260 and 280 nm using a Biophotometer (Eppendorf-Germany) and then the purity was calculated based on absorbance ratio 260/280 nm. The separated DNA with proportion (260/280 nm) 1.9≤ communicating to 121 µg DNA/ ml was applied for the reinforcement and amplification of 16S rRNA by PCR. Amplification of 16S rRNA was performed using universal primers produced by TAG Kopenhagen (Denmark). The sequence of forwarding and reverse primers were 5'AGGAG GTGATCCAACCGCA-3' and 5'-AACTGGAGGAAGGTGGGGA-3', respectively.

Each reaction was performed in a total volume of 26.5 µl containing 14.5 µl of molecular biology grade water (Sigma Aldrich Company Ltd.), 2.5 µl of 10× PCR buffer (Cinnagen-Iran), 1 µl of each forward and reverse PCR primers, 1 µl of a 10 mM dNTPs (Cinnagen-Iran), 0.5 µl of Smart Taq polymerase (Cinnagen-Iran), 1 µl of 50 mM $MgCl_2$ (Cinnagen-Iran) and 5 µl of DNA template. PCR amplification conditions on an Eppendorf thermocycler were as follows: 95°C for 4 min, followed by 35 cycles of 95°C for 40 s, 56°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 5 min and storage at 4°C. All products of PCR gained in the previous process were run on a 1.5% (w/v) agarose gel with a 100 bp DNA ladder (Fermentas-Russia). Products of PCR were electrophoresed at 75 V for 20 min and then pieces of DNA were visualized using ethidium bromide and photographed by Uvidoc (Japan). Following the visualization of unmixed and pure DNA bands, the PCR products were sent to Macrogen in South Korea for DNA sequencing. The 16S rRNA arranged data for bioinformatics applications were exposed to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/) to identify each respective 16S rRNA gene amplicon. The phylogenetic tree was drawn based on the 16S rRNA gene sequence. The branching scheme was generated by the neighbor joining method (14, 25, 27-29).

RESULTS

Identification and characterization of *Gracilibacillus* strain (1-9 h). According to previous studies on Howz-e Sultan hypersaline lake in Qom and the isolation of 350 bacterial isolates, the initial naming of each isolate was done based on the sampled area number and the sample number prepared and the salinity of the isolates. In the case of isolate 1-9 h, number 1 indicates the sampling area of the lake and number 9 indicates a ninth of a strain isolated from that area, and the letter "h" indicates the salt-loving isolate means "Halophile".

Bacterial cells were Gram-positive rods, almost 0.25-0.35 µm wide and 3.5-4.5 µm long. Oviform endospores were generated in a terminal situation. This isolate was motile through peritrichous flagella. Colonies were circular, opaque, pasty and 0.2-0.5 mm in diameter. Cells were aerobes and facultative anaerobes. They utilized glucose by oxidative and non-oxidative mechanisms. Catalase, methyl red test, indole production, production of H_2S, nitrate reduction and hydrolysis of starch, Tween 80 and gelatin, utilization of citrate were positive. Oxidase, Voges–Proskauer reaction, hydrolysis of casein, urea, DNA, and xylene were negative. Phenotypic specifications were arranged in the species explanation and Table 1. Acid was produced from D-glucose, L-arabinose, sucrose, D-xylose and but not from maltose, D-fructose, D-mannitol, D-mannose, D-galactose, glycerol, ribose, lactose. This isolate was susceptible to chloramphenicol (30 µg), but was resistant to bacitracin (10 U), penicillin (10 µg), streptomycin (10 µg), oxytetracyclin (1 µg), ciprofloxacin (5 µg), cloxacillin (30 µg) and erythromycin (15 µg), ampicillin (10 µg), gentamicin (10 µg), novobiocin (30 µg).

Halophilic and thermophilic level for growth of *Gracilibacillus* strain (1-9 h). The growth pattern as determined by OD at 660 nm indicates that the culture was in 24 h of log-phase but predominantly it was in stationary phase for 48 h followed by a decline in the growth, as the viable cell density decreased

Table 1. Biochemical characterization of *Gracilibacillus* strain (1-9 h)

Biochemical Characterization									
Catalase	Oxidase	MR	VP	Acid & gas production from glucose	Indole	Sulfide production	Mutility	Citrate	Nitrate reduction
+	_	+	_	A+/g _	+	+	+	+	+
Utilization of carbon sources									
Amylase	Protease		DNase	Lipase	Xylanase		Urease		Gelatinase
+	_		_	+	_		_		+
Glucose aerobic	Glucose anaerobic		Arabinose	Maltose	Sucrose	Fructose	Xylose		Mannitol
+	+		+[w]	_	+	_	+		_

Enzyme activities in specific media with NaCl content of 2M
* A, Acid; g, gas; –, negative; W, weakly positive; +, Positive.

upon observing the culture density. The growth profile showed that this strain had a fast growth rate as it entered the log phase within 4 h of the lag phase. In general, bacterial growth was maximum until 48 h (Fig. 1). Growth of the strain occurred over a pH range of 5.0 to 10.0 (optimum pH 7.5) (Fig. 2), also growth was observed in the presence of 9- 32% (1.5-5 M) NaCl (optimum 15-21 %), but maximum growth was in 18% (3M) NaCl concentration (Fig. 3). It was able to grow in 15- 65°C and the optimum temperature was 40-45°C (Fig. 4), which indicates that the halothermophilic strain of *Gracilibacillus* could be presumed as hyperhalophilic and moderately thermophilic (16, 17).

16S rDNA sequencing. The isolate was identified according to the 16S rDNA genetic analysis. The sequence had 684 bp lengths and the guanine-cyto-

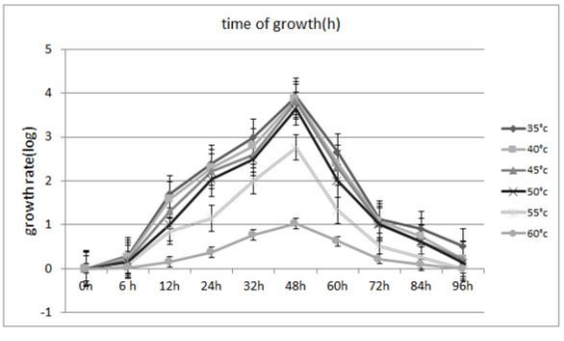

Fig. 1. Growth curve of *Gracilibacillus* strain (1-9 h) in MH broth medium

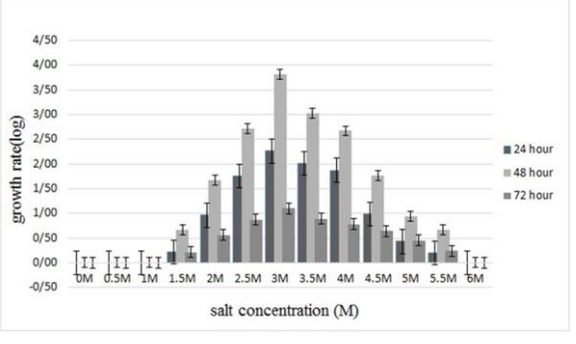

Fig. 3. Optimum salt concentration for growth of *Gracilibacillus* strain (1-9 h)

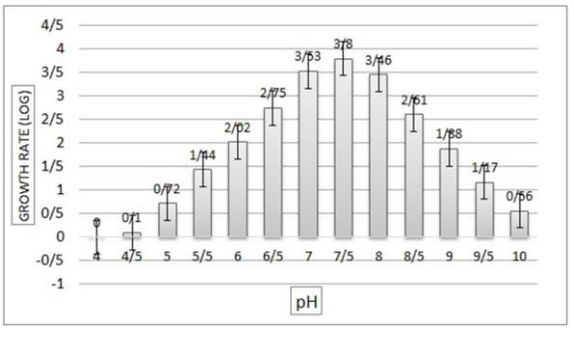

Fig. 2. Optimum pH for growth of *Gracilibacillus* strain (1-9 h)

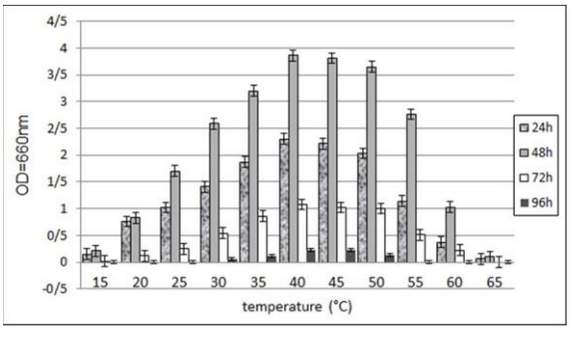

Fig. 4. Optimum temperature for growth of *Gracilibacillus* strain (1-9 h)

sine (GC) content of the sequence was found as 43.6 (mol %). BLAST analysis of the strains revealed that it had the closest match (99%) with *Gracilibacillus* sp. IBP-V003. The sequence was deposited in NCBI Gene Bank under accession numbers HM021766.1 (Table 2).

5AAATTAAATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGCAGCGAAGCGGCAACGCATTAGCAAATCCCATAAATCCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGTATGAAGCCGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAACACCCGAAGTCGGTGGGGTAACCTTTGGAGCCAGCCGCCGAAGGTGGGGCCAATGATTGGGGTGAAGTCGTAACAAGGTAACCCACTATTAGAGGTTCCTTTGTTCCCTAACTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAACAAAATACATTTATTTTCCTTGCTATGATGTATTTAAATTATTTCTGAATATTTTACTAAAAAGGGAATGTGAGCTCCACTTTCAAGC-3

DISCUSSION

Comparison of the 16S rRNA gene sequence of strain 1-9 h with those of other members of the family *Bacillaceae* indicated that it was placed in the genus *Gracilibacillus* and was closely related to *Gracilibacillus* sp. IBP-V003 (99.0% similarity), *Gracilibacillus* sp. IBP-VN3 (98.2%), *Gracilibacillus* sp. TP2-8 (98.0%), *Gracilibacillus* sp. JSM 078103 (98.0%), *Gracilibacillus* sp. BH235 (97.0%), *Gracilibacillus saliphilus* strain YIM 91119 (97.0%), *Gracilibacillus halotolerans* strain NN (96.0%), *Gracilibacillus halophilus* strain YIM C55.5(96.0%), *Gracilibacillus* sp. YIM C229(96.0%), *Gracilibacillus* sp. HVA-1(96.0%) and *Gracilibacillus* sp. YIM-kkny13 (96.0%). Phylogenetic trees based on 16S rRNA gene sequences are shown in Fig. 5, also there are differ-

Table 2. 16S rDNA genetic analysis

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HM021766.1	*Gracilibacillus* sp. IBP-V003 16S ribosomal RNA gene, partial sequence	684	684	67%	0.0	99%

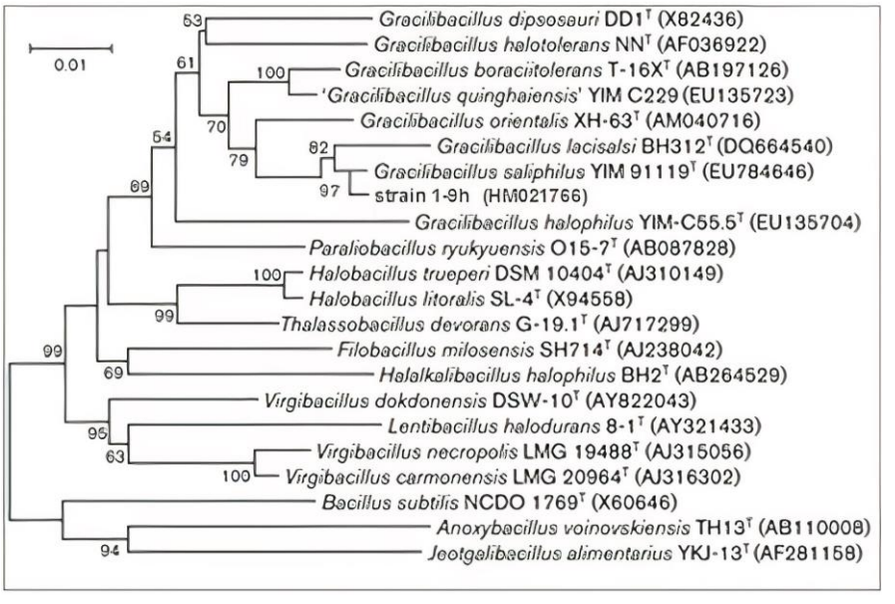

Fig. 5. Phylogenetic tree showing relationships between strain 1-9 h, *Gracilibacillus* species and related taxa based on 16S rRNA gene sequences. The branching plan was procreated by the neighbor-joining procedure. Bootstrap percentages ≥53% based on 1000 replications are shown at nodes. Bar, 0.01 substitutions per nucleotide position.

Table 3. Differential characteristics of 1-9 h and type strains of *Gracilibacillus* species.

Specifications	1	2	3	4	5	6	7	8	9
Shape of spore*	O	S	S	S	E	S	E	S	E
NaCl concentration for growth (%, w/v)									
Range	9–32	1–22	0.5–18	3–20	0.5–8	0–11	0–20	0–15	7–30
Optimum	15–21	10–15	5–7	10	1–3	0.5–3	0	3	15
Temperature for growth (°C)									
Range	15–65	4–45	15–50	15–45	4–45	11–37	6–50	28–50	28–60
Optimum	40-45	37	40	37	37	25–28	47	45	45–50
pH for growth									
Range	5–10	6–8	5.5–10	5–9	6–8.5	6–10	5–10	NI	6–9
Optimum	7.5	7	7.5–8	7	7–7.5	7.5–8.5	7.5	7.5	7
Reduction of Nitrate	+	+	+	−	+	−	+	+	+
Hydrolysis of:									
Gelatin	++	−	−	+	−	−	+	+	+
Starch	++	+	−	−	−	+	+	−	+
Urea	−	+	−	−	+	−	+	−	NI
Acid from:									
Glycerol	-	−	+	−	W	+	NI	−	W
Lactose	−	+	−	−	−	+	NI	+	−
Melezitose	-	−	−	−	−	+	NI	−	−
Carbon source utilization									
Glucose	+	+	+^{w}	+	-	+	+	+	-
Arabinose	W	+	−	−	NI	NI	NI	NI	NI
Maltose	−	+	−	−	NI	NI	NI	NI	NI
fructose	-	−	+	+	NI	NI	NI	NI	NI
xylose	+	-	−	+	NI	NI	NI	NI	NI
Mannitol	_	+	+	+	-	-	+	NI	NI
DNA G+C content (mol%)	43.6	40.1	39	37.1	40.9	35.8	38	39.4	42.3

*E, Ellipsoid; O, oviform; S, spheral.

Strains: 1, 1-9 h (data from this study); 2, *G. saliphilus* YIM 91119^{T} (6); 3, *G. lacisalsi* DSM 19029^{T} (5); 4, *G. orientalis* CCM 7326^{T} (4); 5, '*G. quinghaiensis*' DSM 17858 (9); 6, *G. boraciitolerans* JCM 21714^{T} (7); 7, *G. halotolerans* JCM 7302^{T} (1); 8, *G. dipsosauri* JCM 7303^{T} (1); 9, *G. halophilus* DSM 17856^{T} (8). Pieces of information were gained from the sources listed unless demonstrated. NI, no information available. −, negative; W, weakly positive; +, Positive;

ential phenotypic characteristics of 1-9 h with other type strains of *Gracilibacillus* species. To avoid repeating sentences and show the differences more clearly, these differences are categorized and accurately and comparatively listed in Table 3 (27-29).

In addition, strain 1-9h and its closest relative, *Gracilibacillus* sp. IBP-V003, showed differences in susceptibility to penicillin (10 μg), streptomycin (10 μg), oxytetracyclin (1 μg), ciprofloxacin (5 μg), cloxacillin (30 μg) and erythromycin(15 μg), ampicillin (10 μg), gentamicin (10 μg), novobiocin (30 μg), bacitracin (10 U), and resistance to chloramphenicol (30 μg) (this study).

CONCLUSION

Isolate 1-9 h was distinguished from *Gracilibacillus* species by differential phenotypic characteristics, such as spore shape, ranges of NaCl concentration, temperature and pH for growth, biochemical characteristics, acid production, and carbon source utilization, as shown in Table 3. On the basis of its phenotypic and genotypic properties, isolate 1-9 h represents a novel strain of the genus *Gracilibacillus.*

As mentioned above, the data from this study suggested that the investigated lake showed optimum conditions for hyperhalophile bacteria. On the other

hand, the high diversity of bacteria and phenotypic identification of the isolates illustrated that the strains could be a member of the genera viz., *Halobacillus*, *Halobacterium*, and *Halococcus*, as the major biota of ancient origin, that have been shown to be associated with ancient salt lake (Howz-e Sultan) samples. Our finding showed, the huge diversity of halophilic bacteria exists in Howz-e Sultan hypersaline lake hence, this area can be considered as a perfect region for investigation on halophilic bacteria.

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