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Isolation and characterization of denitrifying halophilic bacteria from Bahr Al-Milh Salt Lake, Karbala, Iraq

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ABSTRACT

Nitrogen fixation is considered as a significant biological process, which is important in agricultural and environmental implications. Herein denitrifying bacteria from Bahr Al-Milh Salt Lake, Karbala, Iraq, was reported using molecular and phenotypical characteristics. Samples were collected from eastern parts of the Bahr Al-Milh. The strains were grown in different saline concentrations of nutrient broth (2.5–22.5%). Nitrate and nitrite reduction activities were assessed for all the isolates. Molecular analysis was performed by BLAST alignment and MEGA7 software. The 16S rRNA sequences of newly found strains were submitted in the GenBank database. 218 strains were isolated, 76.6% of which were nitrate reductase and 25.5% nitrate-nitrite reductase (NiR) positive strains. 68% slightly and 32% moderately halophilic bacteria were found. Isolates with nitrite reduction activity belonged to five genera including *Bacillus*, *Halobacillus*, *Idiomarina, Oceanobacillus,* and *Virgibacillus*. The isolates with the ability of producing nitrate-NiR consisted of bacteria in genera *Halobacillus* and *Halomonas*. Apart from industrial and biotechnological applications, the present information might be useful to fertilize the saline soil for agricultural aims. The isolated strains could be considered as a source of halotolerant enzymes in agriculture and environmental implications in hypersaline areas. To the best of our knowledge, this is the first microbiological study on halophilic bacteria from Bahr Al-Milh Salt Lake.

1. INTRODUCTION

One of the significant characteristics of some prokaryotes is their ability to live under extreme conditions (pH value, pressure, salinity, temperature, oxygen, and starving conditions) [1]. Not many microorganisms are able to tolerate large amount of salt, but a group of salt-loving bacteria have been successfully adjusted to the high concentrations of NaCl, therefore, they are called halophilic microorganisms. They have a large diversity found in three domains of *Archaea, Bacteria, and Eukarya*. They mostly require above 10–15% of salt concentration for survival [2]. Hypersaline environments have a universal distribution represented mainly in aquatic areas such as coastal, deep-sea locations, salt lakes, and also saline alluvial soils [3]. Halophilic bacteria have evolved in a way they can resist rigid situations which their counterparts fail. To stand unusual saline amount, halophilic organisms have developed internal mechanisms to balance the osmotic stress of the environment and to overcome the high salinity complications leading to their functionality in such circumstances [4]. A notable example of this salt-dependent feature is the disintegration taking place in the cell envelope and consequently inactivation of membrane-bound enzymes on lowering the NaCl concentration. Peculiar physiology of these bacteria involving adaptation to hypersaline environments has led to the investigation of halophilic enzymes [5].

Nitrogen fixation is considered as one of the significant biological processes in soil, which is influenced by environmental factors such as pH, temperature, and oxygen. [6]. Regarding the importance of N-cycle in agriculture and environmental implications, nitrogen metabolism of bacteria has been in the spotlight of recent years' studies [7]. In the past, the primary driving of food crops was to maximize productivity and enhance the yield potential. Nowadays, the requisite is to provide sustainability along with productivity in the long-term; therefore, it will be mandatory to reload the reserves of nutrients that are lost or removed from the soil. As for nitrogen (N), it can be inserted into agricultural systems in the form of artificial N-fertilizers or obtained from atmospheric N_2 fixed by biological processes [8]. Soil salinity might have an effect on fertility by disrupting the activity of nitrogen turnover [6]. Majority of bacteria use nitrate as an inorganic source of nitrogen [9,10]. One of the strategies evolved in microorganisms living in such extreme environments is the developed enzymes capable of functioning even in salinity zones. Whenever there is a high amount of salt, and consequently the oxygen concentration is decreased below

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normal, nitrate plays the role of electron receptor instead of oxygen in dissimilatory and assimilatory metabolism. The microorganism performing this action is called denitrifier. Nitrate reductase (NR) and nitrite reductase (NiR) are the key enzymes involved in the stepwise reduction of nitrate to nitrite and the following nitrite to ammonia [11].

Bahr Al-Milh (Lake Razazah) is one of the artificial Salt Lakes located 10 km west of Karbala, Iraq (32°00' – 33°15' N and 42°00'–43°45' E). While the salt concentration of the lake has been rising due to rainfall shortage, temperature increase, etc., Bahr Al-Milh is considered as an extreme ecosystem with potential halophilic bacteria sources [12]. A number of eukaryotic microorganisms from Bahr Al-Milh Salt Lake were defined in previous studies [13,14]. However, to our knowledge, no report has been published to describe the prokaryotic diversity of the lake so far. The present study relied on culture methods. Nitrate-nitrite reducing halophilic bacteria was isolated and characterized using physiological, morphological, and molecular techniques.

2. MATERIALS AND METHODS

2.1. Sampling and Preparations

Fifteen water and soil samples were collected from different locations from eastern parts of the Bahr Al-Milh in February 2014. Samples were put into labeled sterile plastic tubes and bags, then transported to microbiology laboratory in <24 h. The temperature of the area was estimated between 20°C and 25°C. The strains were cultured in different saline concentration of nutrient broth - 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 15.0%, 17.5%, 20%, and 22.5% - containing artificial seawater (ASW) with the following composition: 175 g NaCl, 20 g $MgCl_2.6H_2O$, 5 g K_2SO_4, and 0.1 g $CaCl_2$, and 2 mL filtered saline lake water as the source of trace elements. Final volume was then reached to 1 L with distilled water. The pH 7–8.5 was provided with the addition of 1 N sodium hydroxide.

The culture media were incubated at 37°C for a week. 1 mL of each sample was transferred to nutrient agar medium with the same saline concentrations where they incubated for another 1 week. The number of colonies formed was counted at day 7 following by 2-week further incubation. Morphology of the cells was observed by light microscopy (model BH2; Olympus). Gram staining and KOH lysis tests were performed according to previous reports [15,16].

2.2. Enzymatic Activities

To assess nitrate and nitrite reduction activities, the isolates were cultured in tubes of 1% peptone water with 0.1% potassium nitrate and 0.03% sodium nitrite, respectively. Durham tubes were placed into test tubes to allow an assessment of gas production in the media due to nitrite reduction. Test tubes were sealed with airtight screw tops, incubated for 5 days at 37°C. A solution containing 8% sulfanilic acid and 0.5% alpha-naphthylamine in acetic acid was used to determine nitrate reduction to nitrite after forming a red color. The positive result of nitrite reduction was considered as gas production in the inverted Durham tube. Appearance of red color after addition of zinc dust indicates nitrite reduction to nongaseous products [17]. Catalase production was determined by adding a drop of 3% (v/v) H_2O_2 and observing its hydrolysis and the consequent gas formation [18]. The oxidase activity was detected according to Kovacs [19]. Determination of utilization of glucose and sucrose as a sole source of carbon as well as acid production from them was measured as recommended by Ventosa *et al*. [20]. Nitrate and NiR producers were selected for further molecular analysis.

2.3. Molecular Analysis

The DNA was extracted (bioron sample preparation Kit-Korea) and purified. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) run with two universal bacterial primers: 8F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'GGTTACCTTGTTACGACTT-m3') [21]. PCR solution contained 5 µl template DNA, 17 µl dH_2O, 1 µl primers, 1 µl dNTP, 0.75 µl $MgCl_2$ (50 mM), 25 µl PCR amplification buffer (1X), and 2 U Taq DNA polymerase. Amplification was carried out with Techne TC-3000X thermal cycler (UK) as follow: Initial denaturation at 95°C for 5 min, followed by 30 1-min cycles at 95°C, 30 s at 58°C, 1 min at 72°C, and 10 min at 72°C for the final extension [22]. After electrophoresing [Figure 1], PCR products - single 1400 bp of DNA fragments - were then purified using Gene All Gel Extraction Kit (Korea). Sequencing of 16S rRNA was performed by Macrogen Biotechnology Company (Korea) using an automated sequencer.

The newly sequenced 16S rRNA was aligned with BLAST and ribosomal database project and compared with alike bacterial sequences available in National Center for Biotechnology Information. Drawing phylogenetic trees were completed using MEGA7 software [23]; neighbor-joining [24], maximum likelihood [25], and maximum-parsimony [15] algorithms were applied. Bootstrap analyses based on 1000 replications determined the confidence values of the branches. 16S rRNA sequences of newly found strains were submitted in the GenBank database [Table 1].

3. RESULTS AND DISCUSSION

A total of 218 strains were isolated from enrichment cultures, 76.6% of which was determined to be only NR positive, and 25.5% found to produce both nitrate and NiR. Optimum pH ranged 7.0–7.5 and optimum temperature was determined to be around 37°C. Based on the definition of halophiles, no extremely halophilic bacteria were detected, and the frequency of slightly and moderately halophilic isolates was found to be 68% and 32%, respectively [20]. The optimum salinity to favor growth was 10% ASW. They mostly consist of Gram-positive bacteria (73.8%, $n = 161$) followed by just about one-fourth of Gram-negative bacteria (26.2%, $n = 57$). From morphological aspect, they included 57.8% ($n = 126$) rods or short rods and 42.2% ($n = 92$) cocci appearing in singles, pairs or short chains.

According to the results, almost half of the bacteria, which were capable of producing only NR were Gram-positive (53.1%). Same frequency is obtained for nitrate- NiR positive bacteria (50.0%). Of the bacteria with the ability of producing NR, 12 isolates were randomly selected for further molecular analysis. Four bacteria with the ability to produce gas through nitrite reduction were selected accidentally for supplemental molecular tests as well. Table 2 shows the characterization

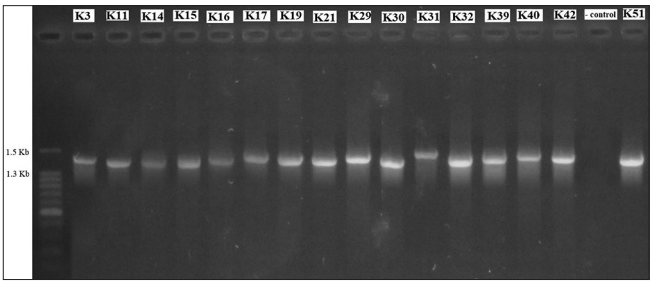

Figure 1: Agarose gel electrophoresis of the polymerase chain reaction products.

Table 1: The accession numbers of the isolates.

Strains	Accession number	Strains	Accession number
Bacillus sp. K3	KT59792	*Halobacillus* sp. K15	KT991680
Bacillus sp. K14	KT597928	*Halobacillus* sp. K16	KT597929
Bacillus sp. K21	KT200230	*Halobacillus* sp. K17	KT353098
Bacillus sp. K32	KT223787	*Halobacillus* sp. K51	KT223788
Virgibacillus sp. K11	KR347118	*Halomonas* sp. K29	KT353099
Virgibacillus sp. K19	KT597930	*Halomonas* sp. K40	KT597931
Oceanobacillus sp. K30	KT281118	*Halomonas* sp. K42	KR909223
Oceanobacillus sp. K31	KT281119	*Idiomarina* sp. K39	KT200229

Table 2: Phenotypic characteristics of nitrate and NiR positive isolates.

Strains	Gram	Oxidase	Catalase	Nitrate reduction	Nitrite reduction	Acid production from		Carbon source utilization	
						Sucrose	Glucose	Sucrose	Glucose
Bacillus sp. K3	+	+	+	+	−	+	+	+	+
Bacillus sp. K14	+	+	−	+	−	+	−	+	−
Bacillus sp. K21	+	−	+	+	−	−	−	−	−
Bacillus sp. K32	+	+	+	+	−	+	+	+	+
Virgibacillus sp. K11	+	+	+	+	−	−	−	−	−
Virgibacillus sp. K19	+	+	+	+	−	−	+	−	+
Oceanobacillus sp. K30	+	+	+	+	−	+	+	+	+
Oceanobacillus sp. K31	+	−	+	+	−	−	−	−	−
Halobacillus sp. K15	+	+	+	+	−	−	−	−	−
Halobacillus sp. K16	+	+	+	+	−	+	−	+	−
Halobacillus sp. K17	+	−	+	+	−	+	−	+	−
Halobacillus sp. K51	+	+	+	+	+	−	−	−	−
Halomonas sp. K29	−	−	+	+	+	−	−	−	−
Halomonas sp. K40	−	+	+	+	+	−	+	−	+
Halomonas sp. K42	−	+	+	+	+	−	−	−	−
Idiomarina sp. K39	−	+	+	+	−	−	+	−	+

NiR: nitrite reductase.

of the very strains. Catalase activity was reported for 93.7% of them, and 75.0% were positive for oxidase activity. The ability to produce acid from glucose and sucrose were observed in 37.5% and 37.5% of isolates, respectively. Similar results were obtained from using the mentioned carbohydrates as a sole source of carbon.

Based on the morphological and molecular (sequences of genes encoding for 16S rRNA) analysis, taxonomic groups of isolates were specified. Using bootstrap values, the isolates that were able to produce NR without nitrite reduction activity belonged to five different genera including *Bacillus*, *Halobacillus*, *Idiomarina*, *Oceanobacillus,* and *Virgibacillus*. The isolates with the ability of producing nitrate- NiR consisted of bacteria in genera *Halobacillus* and *Halomonas*. Figure 2 presents the phylogenetic trees of isolates. The 16S rRNA partial gene sequences of strains have been deposited in the GenBank database.

Recent studies have seen a surge in investigating the diversity of halophilic microorganisms in extreme environments including salt lakes [26]. Both microbiological and molecular studies have showed the presence of a great number of halophilic microorganisms in these saline environments [27-28]. Bahr Al-Milh Salt Lake on the west of city Karbala, Iraq, was made with the aim of water supply to this religious and strategic city but its saline bed and excessive water evaporation created a saline ecosystem. During recent years, apart from high salinity, drastic physico-chemical changes, extreme temperature variations, and dryness have made it a target for microbiology studies. In the present work, both molecular and microbiological tests revealed the presence of halophilic bacteria in a wide range of diversity in the Bahr Al-Milh Salt Lake. More than one-third of isolates were able to produce N-cycle key enzymes, which indicated the potential of further studies about agricultural, industrial and biotechnological applications of this lake's microorganisms.

The BLAST results showed that the isolated bacteria were mostly belonging to genera *Bacillus*, *Halobacillus*, *Halomonas, Idiomarina*, *Oceanobacillus,* and *Virgibacillus*. The results concur with the study of Rohban *et al*. that reported *Salicola* as the most dominant genus in Howz Soltan Lake, Qom, Iran [29]. In addition, *Bacillus*, *Halobacillus*, *Lysinibacillus*, *Oceanobacillus*, and *Virgibacillus* were among the genera found in extreme environments of India [30]. The most common bacteria from different saline environments in South Spain were assigned to genus *Salinivibrio* [5]. This is in agreement with the earlier study on halophilic bacteria from Alborz oil field, Iran, which showed 50% of isolates, belonged to genera *Halomonas* [31].

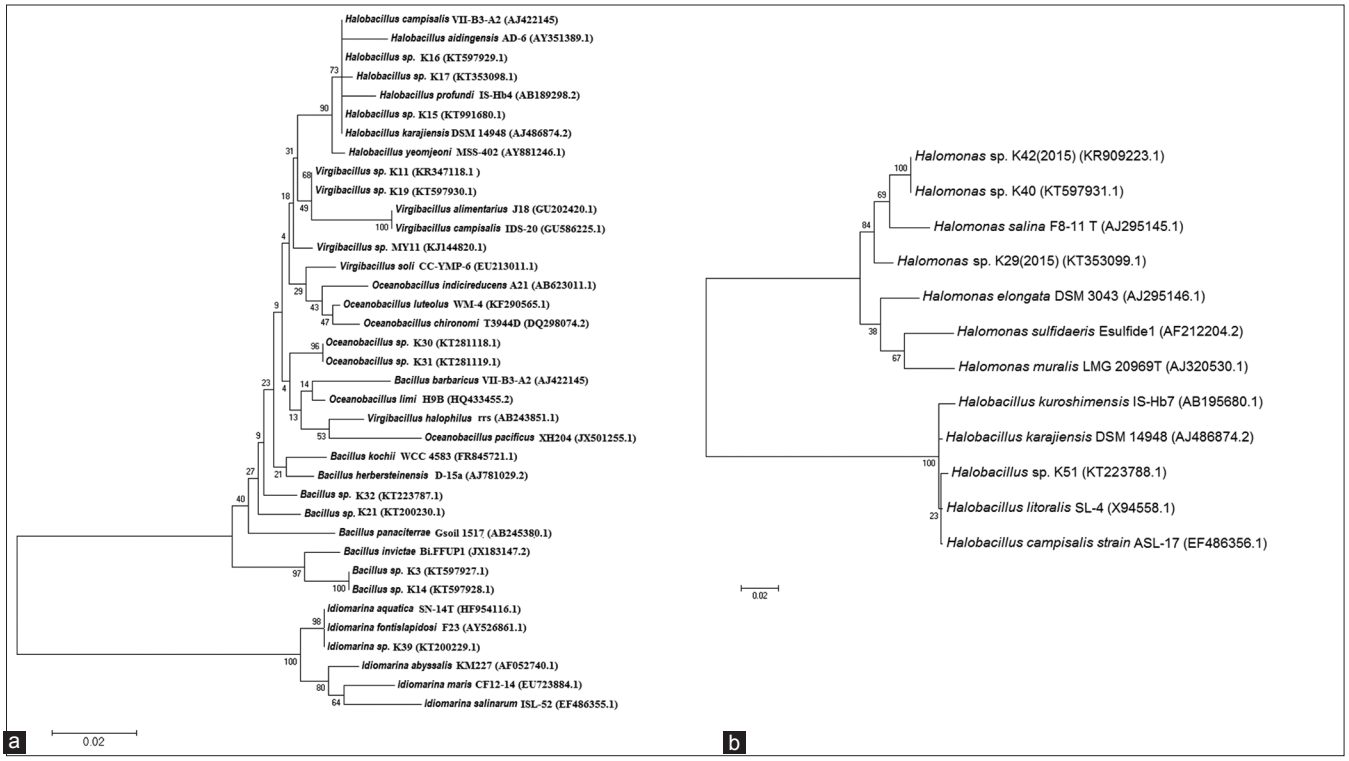

Figure 2: (a) Phylogenetic tree of the halophilic isolates with the ability of producing nitrate reductase, (b) phylogenetic tree of the halophilic isolates which are able to produce both nitrate and nitrite reductase. The trees were constructed according to the neighbor-joining method showing the position of isolates based on the partial 16S rDNA sequence comparison. The numbers of branches indicated bootstrap values and the accession numbers of the reference strains are presented in brackets.

Although species of the genus *Salinivibrio* can be found in low salt concentration, in this study any member of this genus was not identified [29]. The study of microbial diversity of Qinghai Lake, China, revealed *Actinobacteria* and *Acidobacteria*/*Holophaga* as the dominant Gram-positive bacteria while they did not find among the present isolates [32]. The member of genus *Salicola* was isolated from a saline spring in Mahdasht, Alborz province, Iran [33].

Totally, the number of denitrifying bacteria is fewer than that of other heterotrophs in different ecosystems; as a result, isolating or identifying of these strains seems to be more difficult than other ones. According to Li *et al*. [34], aerobic denitrifying bacteria can be isolated from activated sludge sampled from sewage disposal. A number of studies examined denitrifying bacteria for usage in the development of a bioprocess that could potentially restore the function of ion-exchanges agents. For instance, a moderately haloalkalophilic denitrifying bacterium belongs to genus *Halomonas* was isolated from a series of saline-alkaline lakes located in Grant County, Washington State [35]. Study of a member of *Pseudomonas* genus, isolated from a sample of crude oil, Pembina oilfield, Canada, showed a significant increase of Fe(II) during short incubation in association with increased production and accumulation of nitrite [36]. Apart from the above-mentioned application of denitrifying microorganisms, these bacteria can be applied for treating wastewaters containing NH_4^+ or NO_3 –at high concentrations and those carrying the very ions [37].

4. CONCLUSION

The present information might be useful for industrial microbiologists, biotechnologists, and others who are interested in fertilizing saline soil for agricultural purposes. In addition, the isolated strains could be considered as a source of halotolerant enzymes in agriculture and environmental implications in hypersaline areas.

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