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Phylogenetic and Biochemical Characterization of a New Halo-Thermotolerant, Biofilm-Forming *Bacillus* from Saline Lake of Iran

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Abstract

In this study, five halotolerant *Bacillus* isolates from Aran-Bidgol Saline Lake in Iran were identified from saline environments. Screening of the bacteria led to the identification of a unique halo-thermotolerant *Bacillus*. On the basis of genetic and phenotypic data, this isolate was closely related to *Bacillus licheniformis*. But isolated *Bacillus* can be distinguished from *B. licheniformis* by salt tolerance, 16S rDNA sequence and some different physicochemical properties. Thus, suggested that the isolate was not the known *Bacillus*. Optical density analysis indicated strong biofilm formation for this strain. Also this isolate exhibited average tolerance to 1–25 mM concentrations of zinc and was sensitive to all concentrations of nickel. In biosurfactant production assay, this *Bacillus* exhibited the high activity for semi-quantitative oil displacement test (3.14 ± 0.02 cm^2) and evaluated positive for drop-collapse test and hemolytic activity. Moreover, amylase, protease and DNase enzymes produced in presence of 10–20% salt of medium. Therefore, identified *Bacillus* could supply potential microbial materials for bioremediation purposes and biotechnological applications.

Key words: biofilm formation, halo-thermotolerant *Bacillus*, phylogenetic analysis, Saline Lake

Introduction

The properties of saline and hypersaline habitats on earth are reflected in the great diversity within the microbial communities adapted to life under the prevailing conditions (Oren, 2002a). The aspects that attracted the attention of researchers were mainly those related to their physiological adaptation to highly saline concentrations and their ecology (Ghozlan *et al*., 2006; Ventosa *et al*., 1998a; 1998b). Recently, increasing interest, in microorganisms from hypersaline environments led to the discovery of several new bacterial species and genera (Yildiz *et al*., 2011). Besides their important role in the ecology of hypersaline environments, these prokaryotes, could be used in a multitude of potential applications in various fields of biotechnology (Coronado *et al*., 2000). They are a good quality source for compatible solutes that can be used as salt antagonists, stabilizers of biomolecules and whole cells, or stress-protective agents (Margesin and Schinner, 2001). Other useful bio-substances are exoenzymes, such as new isomerases and hydrolases that are active and stable at high salt concentrations. In addition, biopolymers such as biosurfactants and exopolysaccharides are of interest in enhanced oil recovery processes, degradation of industrial residues and toxic chemicals that can pollute hypersaline habitats has also been claimed (Coronado *et al*., 2000; Margesin and Schinner, 2001; Ventosa *et al*., 1998b).

On the other hand, in their natural environment, some halophilic bacteria occur in microbial aggregates as biofilm communities. It seems that the biofilm structure allows the attachment to various substrates and the

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survival of cells by their interactions with ions such as heavy metals (Davey and Otoole, 2000; Maugeri *et al.*, 2010; Poli *et al.*, 2010). Thus, detoxifying ability of these microorganisms can be manipulated for bioremediation of heavy metals in wastewater systems (Kamika and Momba, 2011). Therefore extreme environments can offer novel microbial biodiversity that produces varied and promising useful bio-substances for biotechnological applications (Llamas *et al.*, 2010; Mata *et al.*, 2006; Nichols *et al.*, 2005). Aran-Bidgol Saline Lake in the central part of Iran is a hypersaline environment that similarly to other hypersaline ecosystems is subjected to drastic physicochemical conditions including high salinity, high radiation and strong changes in temperatures and dryness which make it an applicable study target for microbiologists.

In this study, we report the determination of phylogenetic properties, phenotypic features, physiological and biochemical characteristics of a new halo-thermotolerant *Bacillus* from the Aran-Bidgol Saline Lake of Iran with a practical perspective on biotechnology. Also, important properties of this isolate such as biofilm formation ability, biosurfactant production, extracellular hydrolytic activates, nickel and zinc resistance were evaluated.

Experimental

Material and Methods

Physicochemical analysis of the samples. Samples for isolation of bacteria were collected 10 cm below the water surface of the lake in June 2011. Ion content of the water samples were measured according to standard methods (Lenore *et al.*, 1989). Na^+, K^+ and Ca^{2+} were quantified by flame spectrophotometer (Genway, Uk), Mg^{2+} was quantified by atomic absorption spectrophotometer (Analytik Jena, Germany). PH and temperature were determined *in situ*.

Enrichment, bacterial isolation and culture conditions. Enrichment procedures were performed in medium including brine sample enriched with 2.5 g/l yeast extract (Difco) and 5 g/l tryptone (Difco). One hundred ml of each enriched medium was placed into 250 ml flasks. Cultures were incubated at 35°C and in an orbital shaker, at 150 rpm, during 3–7 days. After 4 days of incubation, the enrichment culture were spread on a saline nutrient agar plates, with a final concentration of 10% sea salt, containing (per liter): NaCl, 250 g; $MgCl_2\ 6H_2O$, 13 g; $MgSO_4\ 7H_2O$, 20 g; KCl, 4 g; $CaCl_2\ 2H_2O$, 1 g; $NaHCO_3$, 0.2 g for moderately halophilic bacteria and 20% (w/v) for extremely halophilic microorganisms (Rohban *et al.*, 2009), supplemented with 2.5% yeast extract and 5% tryptone, solidified with 10–12 g/l agar. Different colonies were picked and passaged several times to obtain pure cultures. Microbial cultures were stored at −80°C in the isolation medium supplemented with 10% glycerol.

Morphological, physiological and biochemical tests. All assay media for characteristics of the isolates were supplemented with 10% and 20% sea salt. Physiological and biochemical tests were performed as recommended by Smibert and Krieg (1994). Hydrolytic enzymes activities of isolates were screened qualitatively according to Rohban *et al.* (2009). Cultures were tested in triplicate and compared with negative and positive control. For assays of salt tolerance, cultures were incubated in nutrient broth containing 0, 2, 5, 7 and 10% (w/v) NaCl. Duplicate culture tubes containing 6 ml medium were inoculated with a loopful of 24 h culture grown in nutrient broth at 30°C. The inoculated tubes were incubated at 37°C and monitored for growth at 2 day.

16S rDNA analysis. The genomic DNA of isolates was extracted by QIAamp DNA Mini Kit (Qiagene, Germany) according to the manufacturer's instructions. The 16S rDNA gene was amplified by using primers FD1 (5'-CGAATTCGTCGACAACAGAGTTTGATC-CTGGCTCAG-3') and RP1 (5'-CCCGGGATC CAA-GCTTACGGTTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991). PCR was performed in a thermal cycler (Bioneer, South Korea). The reaction mix included smarTaq DNA polymerase (Cinnagen, Iran) 0.25 µl, 0.5 µl of each primer, dNTP (10 mM) 0.5 µl, PCR buffer (10X) 2.5 µl, $MgCl_2$ (50 mM) 0.75 µl, template DNA 2 µl, and dH_2O 18 µl, in a final volume of 25 µl. The PCR amplification was performed using the following program: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 61°C for 30 s, and extension at 72°C for 2 min with final 10 min extension at 72°C. PCR product was analyzed on 1% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized under ultra violet transillumination (Syngene InGenius, US). PCR products were sequenced using an automated sequencer (ABI system, 3730XL) by Macrogen Company in Korea. The phylogenetic relationship of the isolates were determined by comparing with the related sequences in the GenBank database by advanced BLAST searches from National Center for Biotechnology Information. Among the isolated strains, SL1 isolate, was selected for further identification based on its high salt and thermo tolerance, hydrolytic activity, biochemical properties and 16S rDNA data

Phylogenetic tree of SL1 isolate. Phylogenetic tree analysis was performed by using the software package MEGA5 version (Tamura *et al.*, 2011) after obtaining multiple alignments of data available from public databases using CLUSTAL W (Thompson *et al.*, 1994). Pair

wise evolutionary distances were computed using the correction method and clustering was performed using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 2,000 replicates (Zharkikh and Li, 1995).

Biofilm formation of SL1 isolate. Quantitative analysis of biofilm production was performed as described by Seno *et al*. (2005), with some modifications. Briefly, the isolate was grown in tryptic soy broth (TSB) with 10% and 20% (w/v) sea salt. From each culture, 20 µl samples and 180 µl of TSB were dispensed in the wells of sterile 96-well flat-bottomed microtiter plate (BD Biosciences) and incubated at 35°C for 48 h. The control wells contained only TSB medium. After 48 h, wells were washed three times with distilled water, dried and fixed for 30 min at 80°C. Adhered cells were stained with 0.5% crystal violet solution for 30 min. The stain was washed out with distilled water. In order to quantify adhered cells, 220 µl of ethanol-acetic acid (95: 5, vol/vol) was added to each well for 15 min. Optical density (OD) of eluted stain was measured at 590 nm by using a microtiter plate reader (Awareness technology INC, U.S). Each assay was performed triplicate. As a control uninoculated medium was used. The strain with $A_{590} < 0.5$, and $A_{590} \geq 0.5$ were defined as weak and strong biofilm formers, respectively.

Biosurfactant production of SL1 isolate. Examination of biosurfactant production and surface tension was performed by Drop-collapse, Oil displacement and hemolytic activity tests (Bodour *et al*., 2003; Morikawa *et al*., 1993). Briefly, the isolate was cultured in broth medium and incubated at 36°C for 3 days. Cell suspensions were centrifuged (10,000 g) and the cell-free supernatant was used for analysis. Drop-collapse test was performed in the polystyrene lid of a 96-microwell plate. Cultures were tested in triplicate. The broth medium alone was a negative drop-collapse control. In oil displacement test, 15 µl of crude oil were added on the surface of distilled water (40 µl) in a Petri dish. Then, 10 µl of the medium supernatant were quietly put on the center of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 second. Also, the hemolytic activity of SL1 was screened on blood agar plates containing 5% (v/v) human blood.

Effects of nickel (Ni^{+}) and zinc (Zn^{+2}) on SL1 growth. Toxicity of nickel ($NiSO_4$) and zinc ($ZnCl_2$) determined by using methods of Hassen *et al.* (1998). Different concentrations of each metal prepared. The ranges of concentrations for heavy metals were 0, 1, 3, 5, 10, 25, 50, 100, 125 and 150 mM. Ion solutions were prepared in tubes with a final volume of 10 ml of nutrient broth (Merck, Germany). A metal-deficient Medium inoculated with the 200 µl of microorganism. A metal-supplemented media without the bacteria (abiotic control) were used as negative controls. After 24 h incubation, bacterial growth was measured (OD 600 nm) (CE2021, U.K). Each assay was performed triplicate.

Nucleotide sequence accession numbers. The 16S rDNA sequences have been submitted to GenBank with accession numbers: strain SL1 (JQ996502)

Results

Physicochemical analysis of the water samples. Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} content of samples were 37500 ppm, 4500 ppm, 2400 ppm and 23741 ppm, respectively. The results showed that the water samples from the studied lake were saturated with Na^{+} and contained high levels of Mg^{+2}. The pH of samples was between 7.13–7.49 and average of temperature at the sampling sites was 42°C.

Phenotypic characterizations of the isolates. The analysis of the isolates could be further identified them as part of a major *Firmicutes* group. The *Firmicutes* group related to the *Bacillaceae* family was represented by five strains. The results of phenotypic characterizations of isolates are shown in Table I. Among the isolates, SL1 grew well in wide range (up to 10%) of NaCl concentrations and 20–55°C of temperature. Optimum growth in wide range of salt indicated that the NaCl is not required for growth of SL1; therefore the isolate was regarded as a halotolerant *Bacillus*. Also this isolate produced three important hydrolytic enzymes in presence of 10% and 20% sea salt.

Phylogenetic analysis. Phylogenetic analysis based on 16S rDNA gene sequence comparisons revealed that the isolates SL2 and SL4 resembled to *Bacillus safensis* by 98% and 99% respectively. There were a 99% similarity between the isolate SL3 and *Bacillus pumilus* and 99.5% between the SL5 and *Bacillus sonorensis*. Also, the isolate SL1 fell within the branch encompassing members of the genus *Bacillus* and was related to *Bacillus licheniformis* with 97.0% 16S rDNA gene sequence similarity (Fig. 1). But SL1 isolate can be distinguished from *B. licheniformis* by physicochemical properties, 16S rDNA sequence and phylogenetic tree. Thus, suggested that the strain was not the known *Bacillus* and was tentatively named as *Bacillus* sp. SL1. This *Bacillus* considered as a unique microorganism for further study.

Biofilm formation and biosurfactant production of SL1 isolate. In Biofilm formation assay, optical density (OD) of eluted stain in 590 nm was 1.25 ± 0.31. This result indicated that the SL1 isolate have a strong ($OD_{590} \geq 0.5$) biofilm formation in present of 10% and 20% sea salt concentration. In semi-quantitative test for biosurfactant production the SL1 exhibited the high activity for oil displacement test toward Crude

Table I
Phenotypic characterizations and biochemical properties of isolates

Phenotypic Characterizations	SL1	SL2	SL3	SL4	SL5
Gram reaction	+	+	+	+	+
Spore formation	+	+	+	+	+
Mucoid Colony	+	–	–	+	–
Motility	–	+	–	+	+
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Voges-Proskauer test	+	+	+	+	+
Acid from					
D-Glucose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Gas from glucose	–	–	–	–	–
Utilization of Citrat	–	–	–	–	–
Formation of					
Indole	–	–	–	–	–
H_2S	–	–	–	–	–
NaCl required for growth	–	–	–	–	–
Hydrolytic activity					
Amylase	+	–	–	–	–
Protease	+	+	+	+	+
DNase	+	–	+	–	+
Hemolytic Activity					
α	+	–	–	–	–
β	–	+	+	+	–
Growth in NaCl					
2%	+	+	+	+	+
5%	+	+	+	+	–
7%	+	+	+	+	–
10%	+	Nd*	–	Nd*	–
12%	+	–	–	–	–
Growth at					
5°C	–	–	–	–	–
10°C	–	+	+	+	–
30°C	+	+	+	+	+
40°C	+	+	+	+	+
50°C	+	+	–	+	+
55°C	+	–	–	–	+
65°C	–	–	–	–	–
Optimum pH	7.0	7.0	7.0	7.0	7.0

* Non detectable

Oil ($3.14 \pm 0.02\ cm^2$) and clear haloes on blood agar plate. Also, this strain evaluated positive for drop-collapse test and emulsified crude oil in broth medium within 48 h of cultivation.

Zinc and nickel resistance of SL1 isolate. The ability of the SL1 to tolerate zinc and nickel was tested by tube method. The high concentrations of zinc in broth medium were effective on bacterial growth (Fig. 2). This strain exhibited average degree of tolerance to 1–25 mM concentrations of zinc (OD_{600}nm 0.480–0.141) and could live in medium with more than 25 mM zinc. On the other hand, nickel inhibited the growth of strain at very low concentrations. The lower optical density values revealed that the bacterial growth was affected due to the presence of metal in the growth medium (Fig. 2).

Discussion

Recent decades have seen a flow in studies on extreme environments including hypersaline ecosystems (Demergasso *et al.*, 2004; Oren, 2002b). The hypersaline Lake Aran-Bidgol is located at an altitude of 800 m in an area with an arid to semiarid continental climate. It was formed by the deposition of halite sediments from an ancient sea in different geological periods (Makhdoumi-Kakhki *et al.*, 2011). According to the results of physicochemical analysis, sodium and magnesium concentrations of water samples were a high level, similarly to thalassohaline systems content. In addition, several interfering factors such as season, temperature, moisture and depth of sampling site could affect the ion concentrations of lake.

New halophilic *Bacillus* species from Aran-Bidgol saline Lake have been previously described. Recently *Bacillus iranensis* isolated from saline mud of this lake by Bagheri *et al.* (2012). In the present study, among the isolated strains, SL1 isolate was selected for further identification for its high halo-thermo tolerance and ability to produce most important industrial enzymes in present of 10% and 20% sea salt. Since most industrial procedure are performed under specific physicochemical conditions which may not be definitively adjusted to the optimal points needed for the activity of the existing enzymes; therefore, it would be of great importance to have enzymes that exhibit best possible activities at various ranges of salt concentration, pH and temperature. It is interesting to note that combined hydrolytic activity was detected in many halophilic strains (Rohban *et al.*, 2009). Thus, these organisms are an excellent source of such enzymes that may be active at extreme conditions (Gomes and Steiner, 2004).

As determined by phylogenetic analysis, the SL1 isolate was closely related to the *Bacillus licheniformis*. But, according to the 16S rDNA sequencing and some physicochemical properties, SL1 was different from *B. licheniformis* that previously described. *B. licheniformis* is a Gram-positive, endospores forming and industrial organism that can be isolated from soils

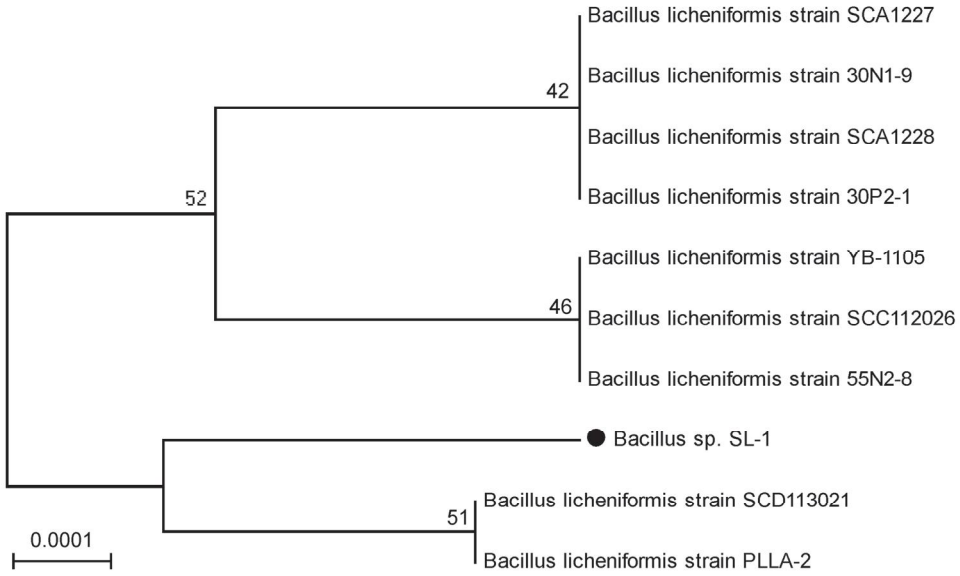

Fig. 1. Phylogenetic tree based on 16S rDNA sequences, showing the relationship of the isolate SL1 to other members of the genus *Bacillus*.

Numbers at nodes are percentage bootstrap values based on 2,000 replications. Bar 0.0001 substitutions per nucleotide position

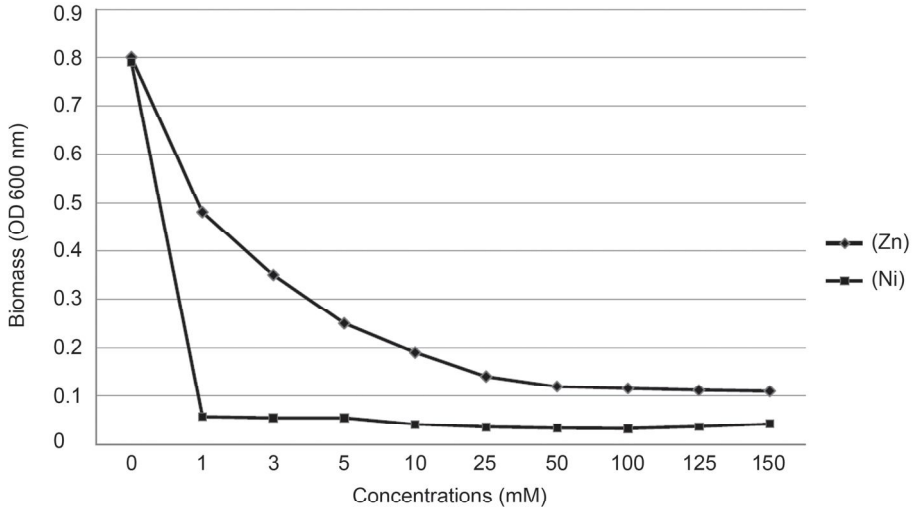

Fig. 2. Effects of different concentrations of zinc and nickel on growth of *Bacillus* sp. SL1

and plant material all over the world (Veith *et al.*, 2004). Moreover, there are reports that this organism is olated from marine environments frequently (Ettoumi *et al.*, 2009). But, to our knowledge, there is no report of *B. licheniformis* isolation from hypersalin Lakes, so far. Thus we tentatively named the SL1 isolate as *Bacillus* sp. SL1.

According to the results, SL1 isolate produced a strong biofilm in present of 10% and 20% sea salt. Also, this isolate exhibited tolerance to 1 mM to 25 mM concentration of zinc metal. A possible explanation for this ability is that SL1 isolate is protected in the environment conditions by using the biofilm formation. Based on previous investigations, the biofilm matrix has the potential to prevent diffusion of certain antimicrobial agents and heavy metal adsorption, thus restricting dispersion of compounds from the surrounding environment into the biofilm (Davey and Otoole, 2000; Maugeri *et al.*, 2002; Tourney *et al.*, 2009). Microbial biofilms contain bioorganic metal-complexing functional groups; thereby play an important function in metal cycling in contaminated environments. The results of Toner *et al.* (2005) study confirmed the importance of phosphoryl functional groups in zinc absorption by a bacterial biofilm. Also, there is a report of halophilic, thermotolerant *B. licheniformis* (B3-15), isolated from marine hot spring at Vulcano Island that was highly resistant to zinc (Maugeri *et al.,* 2002).

On the other hand, the growth of SL1 isolate was inhibited at very low concentration of nickel. In contrast of our study, Kamika and Momba (2011) found a *B. licheniformis*-ATTC12759 could tolerate nickel at concentrations ranging between 1and 2 mM. These observations of *Bacillus* sp. SL1 are in disagreement with previous reports of *B. licheniformis* so far and mostly confirmed that these different probably could be to special properties of various strains of *Bacillus* isolated from unusual environment.

In this study, the *Bacillus* sp. SL1 evaluated as a good biosurfactant producer similar to *B. licheniformis* strains that isolated from diverse locations (Maugeri *et al.*, 2002; Yakimov *et al.*, 1995). These microorganisms and their compounds can be used to enhance oil recovery, clean oil storage tanks, increase flow though pipelines reduce the heavy oil viscosity and stabilize fuel water-oil emulsions (Safary *et al.*, 2010). Thus probably this microorganism can play a significant role in the bioremediation and treatment of industrial wastewater.

In conclusion on the basis of results, the present study could represent a new halo-thermotolerant *Bacillus* for remarkable amylase, protease, DNase, biosurfactant and exopolysaccharides in order to usage in bioremediation, biocatalysts production, medical and pharmaceutical industrial.

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