

Full Length Research Paper

Isolation and characterization of extremely halotolerant *Bacillus* species from Dead Sea black mud and determination of their antimicrobial and hydrolytic activities

Maher Obeidat

Department of Biotechnology, Faculty of Agricultural Technology, Maher Obeidat, Al-Balqa Applied University, Al-Salt 19117, Jordan.

Received 29 May, 2017; Accepted 2 August, 2017

This is the first study that investigated the isolation of extremely halotolerant *Bacillus* species from Dead Sea black mud. Nine isolates obtained from black mud were considered to be extremely halotolerant *Bacillus* based on morphological, physiological, and biochemical properties. Most of their colonies were white to light yellow and circular to irregular. All isolates were Gram-positive rod-shaped endospore-forming bacteria, facultative anaerobes, oxidase negative, catalase positive, mesophilic, extremely halotolerant, reacted positively for tryptophan deaminase and Voges-Proskauer, hydrolyzed gelatin and aesculin, and assimilated potassium gluconate. Most of the isolates were found to hydrolyze *o*-nitrophenyl-beta-D-galactoside (ONPG) and *p*-nitrophenyl-β-D-galactopyranoside (PNPG) as well as arginine, and assimilate D-mannose, N-acetylglucosamine, D-maltose, and malic acid. All isolates were considered to be nitrate reducers, six of them were nitrite producers and three were N₂ producers, suggesting that they may play an important role in nitrification-denitrification processes and in the nitrogen cycle in soil. Based on 16S rRNA gene sequence analysis, the isolates were found to share very high identities (97-99%) with their closest phylogenetic relative and they were assigned to eight *Bacillus* species (*B. oceanisediminis*, *B. subtilis*, *B. firmus*, *B. paralicheniformis*, *B. methylotrophicus*, *B. amyloliquefaciens*, *B. sonorensis*, and *B. malikii*). Interestingly, several enzymatic activities were detected from nonhemolytic isolates DSM2 and DSM7 that were identified as *B. paralicheniformis*. It was found that only DSM2 isolate produced promising antimicrobial activities. Its aqueous extract showed the highest significant antifungal activity. Whereas, *n*-butanol and methanol extracts showed significant antibacterial and antifungal activities against human skin pathogens and against other frequent human pathogens.

Key words: Halotolerant, *Bacillus*, nitrification, antimicrobial, hydrolytic.

INTRODUCTION

Halophilic and halotolerant microorganisms that inhabit hypersaline environments can be found in all three domains of life and they exhibit different metabolic

pathways. Halophilic microorganisms are classified into mild (require at least 1% NaCl), moderate, and extreme halophiles (require up to 30% NaCl) (Madigan and

Martinko, 2006). Whereas, halotolerant microorganisms possess the ability to grow in the absence of NaCl and also in the presence of high concentrations of NaCl. The halotolerant microorganisms that do not require salt for growth but grow well above 2.5 M salt (that is, above 15% NaCl) are considered extremely halotolerant (Kushner, 1978). Halophilic microorganisms maintain cell structure and function in hypersaline environments by osmoregulation which has been performed by synthesis of compatible solutes strategy, which have been used in industry, and salt-in strategy (Boone and Garrity, 2001; Madigan and Martinko, 2006; Oren, 2006). They play an important role in production of hydrolytic enzymes (Oren, 2006). Halotolerant bacteria are used in numerous industrial processes such as production of salty foods and in maintenance of soil health in saline environment (Vreeland, 1993).

The Dead Sea, in Jordan, is the second largest hypersaline lake in the world after the Great Salt Lake in the western United States. The Dead Sea is the lowest place on earth (Gavrieli et al., 1999). Therefore, it is unique by its high salt concentration especially magnesium, high barometric pressure, high partial oxygen pressure, unique UV radiation that found to be in the range of that reported at high altitudes such as the Alps and the Andes, low humidity, and rarity of rain (Avriel et al., 2011). Furthermore, the Dead Sea is unique in its microbiological environment. The Dead Sea pH is close to neutral (pH 6.1) and from which a large number of novel members of halophilic microorganisms have been provided including extremely halophilic archaea such as *Haloarcula marismortui*, new species of halophilic bacteria such as *Chromohalobacter marismortui*, novel halophilic fungal species such as *Gymnascella marismortui* (Oren, 2010). The Dead Sea was also the natural habitat for the green algae of the genus *Dunaliella* (Oren, 2010).

Dead Sea is rich in highly mineralized and sulfide-rich black mud. Black mudpacks that are abundantly distributed along the shore of the Dead Sea attract patients worldwide, who seek a cure for several skin diseases and rheumatic disorders (Abels et al., 1995; Abels and Kipnis, 1998; Oumeish, 1996; Halevy and Sukenik, 1998; AbdelFattah and Schultz-Makuch, 2004; Portugal-Cohen et al., 2015). Dead Sea black mud minerals have the potential to serve as skincare actives because they affected the expression of various genes that contribute to skin elasticity (Portugal-Cohen et al., 2015). The black mud deposits in three regions where the runoff streams flow into the Dead Sea; including, Jordan Valley, Jordanian Moab Mountains, and Judean Mountains (Rudel, 1993). The mineral mud is also extensively used as an ingredient in cosmetic preparations

(Ma'or et al., 1996). It was reported (Abdel-Fattah, 1997) that the level of trace elements in the Dead Sea mud was less than those in any other sea mud and its major component is carbonate (40%) and less than 1% organic matter.

The purpose of the current study was to isolate and characterize halotolerant *Bacillus* from Dead Sea black mud. The hydrolytic and antimicrobial activities as well as the biochemical properties of the screened isolates were also determined. To our knowledge, this is the first study that investigated the presence of extremely halotolerant *Bacillus* in the black mud of the Dead Sea and examined their enzymatic and antimicrobial activities since they have not been previously reported.

MATERIALS AND METHODS

Collection of samples

Thirty black mud samples were collected from three regions along the shore of the Dead Sea, Jordan (Dead Sea; north, DSN; middle, DSM, and south, DSS). Ten mud samples collected from each region in 500 mL sterile glass containers from 30-40 cm below the surface, 50-100 m away from the sea shoreline and away from human activities.

Isolation of bacteria

An equal volume of sterile distilled water was added to each mud sample and mixed well to get a homogenous mixture. Then, one milliliter from each mixture was serially diluted (10 folds) and 100 μ L aliquots were plated by spreading on tryptone soy agar (TSA) plates supplemented with 10% (w/v) NaCl and incubated under aerobic conditions for 72 h at 30°C. The different developing colonies were selected and purified by subculturing on TSA medium, and then, were stored in tryptone soy broth (TSB) containing 20% glycerol at -80°C until usage.

Phenotypic and physiological characterization of the isolates

Colony and cell morphology as well as Gram staining and endospore staining were performed for each isolate according to the standard protocols (Holt et al., 1994). Catalase and oxidase activities were investigated for each isolate. Growth temperature and pH as well as anaerobic growth were also determined. The effect of NaCl on the growth of isolated colonies was also examined.

To determine the growth temperature and pH, the isolates were tested at temperatures in the range 25 to 60°C at 2.5 unit interval and pH in the range 4.0 to 12.0 at 0.5 unit interval in nutrient broth (NB) medium. The growth of isolates was determined after 48 h of incubation by McFarland standards. The effect of NaCl concentration on the growth of isolates was considered by incubating the isolates at 37°C for 48 h in 10 mL NB medium containing 0.0 to 30% (w/v) NaCl at 2.5% interval.

The hemolytic activity of the isolates was tested on blood agar medium containing 5% (w/v) fresh human erythrocytes, by inoculating 50 μ L of each prepared extract into each well (5 mm

i.d.) prepared on the blood agar plates. The type of hemolysis was determined after incubation of plates at 37°C for 48 h (Carillo et al., 1996).

On the other hand, the biochemical properties of the isolates were recognized based on API-20E and API-20NE systems (BioMerieux, USA). In addition, several enzymatic activities of the isolates were determined by API-ZYM system (BioMerieux, USA) and the enzymatic activity of amylase and caseinase were determined for isolates according to Harley and Prescott (2002).

Molecular characterization of the isolates

Genomic DNA extraction

The bacterial isolates as well as the reference strains *B. cereus* ATCC 14579 and *E. coli* ATCC 8739 were inoculated into 20 mL of Luria Bertani (LB) broth and incubated overnight at 37°C with shaking at 150 rpm. Cultures were centrifuged at 14000 rpm for 5 min. Cell pellets were washed three times, then used for DNA isolation using Wizard Genomic DNA purification kit (Promega, USA, part no. A1120) according to the manufacturer's instructions. The extracted genomic DNA was electrophoresized in 1% (w/v) agarose gel and photographed by UV Transillumination (Perez-Roth et al., 2001).

PCR amplification of the 16S rRNA gene

The 16S rRNA gene of the isolates and the reference strains was amplified by adding 1 μ L of cell culture to a thermocycler microtube containing 5 μ L of 5X *Taq* buffer, 0.5 μ L of each 10 μ M Fd1 and Rd1 primers, 3 μ L of 25 mM MgCl₂, 0.5 μ L of 25 mM dNTPs, 0.25 μ L of *Taq* polymerase (5U μ L⁻¹), and 38 μ L of sterilized distilled water. Universal primers Fd1 and Rd1 (Fd1, 5'-AGAGTTTGTATCCTGGCTCAG-3' and Rd1, 5'-AAGGAGGTGATCCAGCC-3') were used to obtain a PCR product of ~1.5 kb corresponding to base positions 8-1542 based on *E. coli* numbering of the 16S rRNA gene (Winker and Woese, 1991). PCR program used was an initial denaturation for 1 min at 95°C and the samples subjected to 30 cycles for 20 s at 95°C, 30 s at 55°C, and 1 min and 30 s at 72°C. This was followed by a final elongation step for 5 min at 72°C. The PCR products were analyzed on 1% (w/v) agarose gels.

Sequencing and phylogenetic analysis

The sequences of the 16S rRNA gene from PCR products of the isolates and the reference strains were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequences were compared with those contained within GenBank (Benson et al., 1999) by using a basic local alignment tool (BLAST) search (Altschul et al., 1990). The most closely related 16S rRNA gene sequences to the isolates of this study were retrieved from the database. Retrieved sequences were then aligned and the phylogenetic tree was constructed by the use of DNAMAN 5.2.9 sequence analysis software. The obtained sequences were also submitted to GenBank to provide an accession number for each sequence. The reference strains *B. cereus* ATCC 14579 and *E. coli* ATCC 8739 were used as in-group and out-group bacteria, respectively.

Antimicrobial activity

Preparation of extracts

Bacterial cultures were grown in 500 mL NB for two weeks and they

were centrifuged at 14,000 rpm for 10 min. The supernatant was extracted with equal volume of different solvent types (*n*-butanol, methanol, ethanol, acetone, and water) for two weeks at room temperature with shaking at 150 rpm. The extracts were then filtered through 0.45 μ m membrane syringe filter. The filtrate was evaporated at 40°C in water bath. After evaporation, the remained residues were resuspended in phosphate buffer saline (PBS) to achieve a concentration of 200 mg/mL concentration and used for screening of antimicrobial activity.

Test microorganisms

In order to examine the antibacterial and antifungal activities of the prepared extracts from black mud isolates, 11 reference bacteria; including, *Staphylococcus aureus* ATCC 25923, Methicillin resistant *S. aureus* ATCC 95047 (MRSA), *Streptococcus pyogenes* ATCC 8668, *Salmonella typhimurium* ATCC 14028, *E. coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27253, *Klebsiella pneumoniae* ATCC 7700, *Klebsiella oxytoca* ATCC 13182, *Enterobacter aerogenes* ATCC 35029, *Proteus mirabilis* ATCC 12453, and *Proteus vulgaris* ATCC 33420 and two reference fungi (*Aspergillus brasiliensis* ATCC 16404 and *Candida albicans* ATCC 10231) were used.

Multidrug resistance of test microorganisms to some standard antibiotics

Seven standard antibiotics (Ampicillin 10 μ g, Chloramphenicol 30 μ g, Erythromycin 15 μ g, Nalidixic acid 30 μ g, Penicillin G (10 units), Streptomycin 10 μ g, and Vancomycin 30 μ g) were tested for multidrug-resistance against test bacteria, and two standard antibiotics (Cycloheximide 250 μ g, Nystatin 10 μ g) were used to investigate the resistance of test fungi. Aliquots of 50 μ L from each test bacterium were swabbed uniformly on nutrient agar (NA) medium and thereafter one disk from each standard antibiotic was placed on NA medium surface and incubated at 37°C for 24 h. For test fungi, the same procedure was accomplished but using potato dextrose agar (PDA) medium and incubation at 28°C for 48 h. The antimicrobial activities were determined by measuring the diameter of generated inhibition zones.

Preparation of inoculums

For antimicrobial activity, reference bacteria and fungi were cultured in NB at 37°C for 24 h and Sabouraud dextrose broth (SDB) at 28°C for 48 h, respectively. The cultures were adjusted to achieve 2 \times 10⁶ CFU/mL for bacteria and 2 \times 10⁵ spore/mL for fungi. The antimicrobial activities were performed by using agar-well diffusion method (Perez et al., 1990). Aliquots of 50 μ L from each test microorganism were swabbed uniformly on NA medium for bacteria and on PDA medium for fungi, and allowed to dry for 5 min. Sterile cork borer (6 mm diameter) was used to make wells in the seeded agar. Then, 50 μ L aliquot from each prepared extract was added into each well and allowed to stand on the bench for 1 h for proper diffusion and after that incubated at 37°C for 24 h for test bacteria and at 28°C for 48 h for test fungi. The antimicrobial activities were determined by measuring the diameter of formed inhibition zones. Negative controls using 50 μ L PBS were also run in the same manner and parallel to the treatments. These studies were performed in triplicates and all data were expressed as the mean \pm standard deviation (SD). For statistical evaluation of data for generated inhibition zones, one-way ANOVA (Tukey's studentized range) was applied and significant differences were considered significant at *P* < 0.05.

Table 1. Phenotypic and growth characteristics of extremely halotolerant bacteria screened from black mud of the Dead Sea.

Isolate	DSN1	DSN2	DSN3	DSM2	DSM3	DSM5	DSM7	DSS3	DSS8
Colony shape	Rigid irregular	Rigid irregular	Rigid irregular	Rigid irregular	Circular	Rigid irregular	Circular	Circular	Mucoid irregular
Pigmentation	White	White	Creamy	White	White	White	Translucent	Light yellow	Paige
Cell shape	Single rods	Coccobacilli	Single rods	Single rods	Single rods	Chain rods	Single rods	Chain rods	Chain rods
Sporulation	+	+	+	+	+	+	+	+	+
Gram stain	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+
Growth range									
NaCl (% (w/v))	0-25	0-20	0-20	0-20	0-30	0-25	0-20	0-20	0-20
pH ^a (Optimum)	4.5-9(6.8)	5-9(6.5)	5-9(6.5)	6-9(6.8)	6-9(6.8)	5-11(6.3)	6.5-9(7.0)	4.5-9(6)	5-9(6.3)
Temperature (°C)	25-45(38)	25-45(37)	25-40(36)	25-45(37)	25-45(38)	25-45(38)	25-45(36)	25-45(37)	25-50(37)

^aGrowth of isolates at different pH measured at 37°C.

RESULTS

Phenotypic characterization

Sixty-two aerobic bacterial isolates were harvested from the collected black mud samples with low colony forming unit (CFU) count (18,520-24,733 CFU/g) and most of them (47 isolates) have *Bacillus* characteristics (data are not shown). Based on morphological, physiological, and biochemical properties, only nine isolates which obtained from the Dead Sea black mud met the criteria of extremely halotolerant *Bacillus* (Tables 1 and 2). These isolates were considered extremely halotolerant *Bacillus* and different based on colonial morphology, Gram staining, cell shape, endospore formation, catalase test, and NaCl requirements for growth (Table 1). Most of the developed colonies were white to light yellow circular to irregular on TSA (Table 1). All isolates comprised Gram-positive rod-shaped endospore-forming microorganisms.

Physiological characterization

All isolates grew aerobically and anaerobically, thus the isolates were considered facultative anaerobes (Table 1). All isolates reacted negatively with oxidase and positively with catalase. As shown in Table 1, all isolates were capable to grow at temperature ranging from 25 to 45°C (optimal growth at temperature 36-37°C) except isolate DSN3 grow up to 40°C, at pH ranging from 5 to 9 (optimum pH for growth is between 6.3 to 7), and able to grow in the range of 0-20% salt concentration. Two isolates (DSN1 and DSM5) were found tolerant to 25% (w/v) NaCl and one isolate (DSM3) tolerated up to 30% (w/v) NaCl.

Biochemical characterization

The isolates were tested by different biochemical tests including API-20E and API-20NE (Table 2).

For API-20E test, it was found that all isolates were positive for tryptophan deaminase, Voges-Proskauer, and gelatinase activity. Ortho-Nitrophenyl- β -galactoside (ONPG) hydrolysis and arginine dihydrolase were found to be positive for all isolates except isolate DSM2 and isolate DSN2, respectively. It was observed that only one isolate (DSM2) utilizes citrate, one isolate (DSM7) ferments D-glucose, and one isolate (DSS3) ferments D-mannitol. Whereas, fermentation or oxidation of inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose was negative for all isolates. All isolates reacted negatively to lysine decarboxylase, ornithine decarboxylase, H₂S production, urease, and for indol production. Isolates DSN2, DSM2, DSM5, DSM7, DSS3, and DSS8 were found to be nitrite producers. Whereas, the remaining isolates (DSN1, DSN3, and DSM3) were dinitrogen gas producers. For API-20NE (Table 2), it was observed that all isolates were nitrate reducers but they were unable to reduce nitrite, aesculin

Table 2. Biochemical identification of extremely halotolerant bacteria isolated from Dead Sea black mud.

Isolate	DSN1	DSN2	DSN3	DSM2	DSM3	DSM5	DSM7	DSS3	DSS8
API-20E									
ONPG hydrolysis	+	+	+	-	+	+	+	+	+
Arginine dihydrolase	+	-	+	+	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	+	-	-	-	-	-
H ₂ S production	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-
Tryptophan deaminase	+	+	+	+	+	+	+	+	+
Indol production	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+	+	+	+
Gelatinase activity	+	+	+	+	+	+	+	+	+
Fermentation/oxidation									
D-glucose	-	-	-	-	-	-	+	-	-
D-mannitol	-	-	-	-	-	-	-	+	-
Inositol	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-
D-sucrose	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-
NO ₂ production	-	+	-	+	-	+	+	+	+
N ₂ production	+	-	+	-	+	-	-	-	-
API-20NE									
NO ₃ reduction	+	+	+	+	+	+	+	+	+
NO ₂ reduction	-	-	-	-	-	-	-	-	-
Hydrolysis of									
Aesculin	+	+	+	+	+	+	+	+	+
PNPG	+	+	+	-	+	+	+	+	+
Assimilation of									
D-mannose	+	+	+	-	+	+	+	+	+
N-acetylglucosamine	+	+	+	-	+	+	+	+	+
D-maltose	+	+	+	-	+	+	+	+	+
Potassium gluconate	+	+	+	+	+	+	+	+	+
Capric acid	-	-	-	-	-	-	-	+	-
Adipic acid	-	-	-	-	-	-	-	-	-
Malic acid	+	+	+	+	+	+	+	-	+
Phenylacetic acid	-	-	-	-	-	-	-	-	-

hydrolyzers, and potassium gluconate assimilators. Except isolate DSM2, all isolates were found to hydrolyze p-nitrophenyl-β-D-galactopyranoside (PNPG), and assimilate D-mannose, N-acetylglucosamine, and D-maltose. All isolates, except isolate DSS3, were positive for malic acid assimilation and only one isolate was positive for capric acid assimilation. None of the isolates assimilates adipic acid and phenylacetic acid.

Molecular characterization

For further identification of the screened isolates from Dead Sea black mud, genomic DNA was extracted from the isolates and amplified by PCR, and then 16S rRNA gene sequences were analyzed. The 16S rDNA of the isolates was amplified with Fd1 and Rd1 primers. The amplified genomic DNA of the isolates and the reference

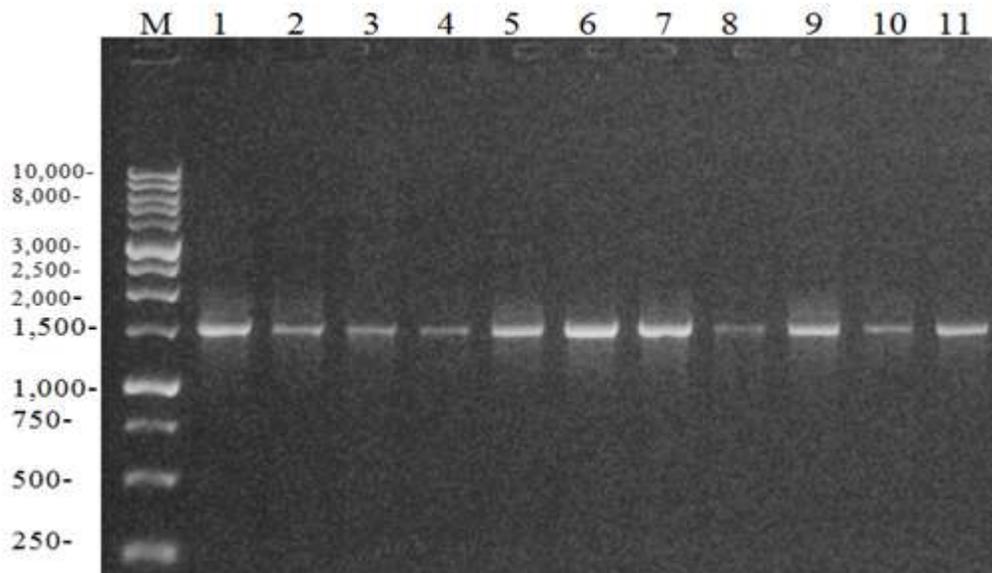


Figure 1. Agarose gel (1%) electrophoresis of PCR amplification of 16S rRNA gene fragments with oligonucleotide forward primer Fd1 and reverse primer Rd1 of nine isolates and two reference strains. Lanes 1-11: Isolates, DSN1, DSN2, DSN3, DSM2, DSM3, DSM5, DSM7, DSS3, DSS8, and the reference strains *B. cereus* ATCC 14579 and *E. coli* ATCC 8739, respectively. Lane M: 1 kb DNA ladder marker (Genedirex, USA).

strains produced PCR band with about 1500 bp in size (Figure 1). The obtained 16S rRNA gene sequences were aligned by BLAST alignment of GenBank sequences. Moreover, the sequences were submitted to the GenBank database and the accession numbers were kindly provided for the submitted sequences (Table 3). Based on BLAST alignment of GenBank sequences to 16S rDNA sequences, all isolates were allocated to the genus *Bacillus* with very high identities ranged from 97 to 99% (Table 3). The reference strain showed 99% similarity to the same species level of its closest phylogenetic relative *B. cereus* ATCC 14579 (Accession no. NR074540). Three isolates (DSN2, DSN3, and DSM5) had 97% sequence identity and the highest GC content. The remaining six isolates showed 99% sequence identity to the closely related phylogenetic *Bacillus* species. In addition, the aligned sequences showed high alignment scores to the closest phylogenetic relative and showed 100% query coverage, 0% gaps, and significant e-values, which equal zero. Based on the sequences alignment, it was clearly observed that the halotolerant *Bacillus* isolates were highly related to eight *Bacillus* species (*oceanisediminis*, *subtilis*, *firmus*, *paralicheniformis*, *methylophilicus*, *amyloliquefaciens*, *sonorensis* and *malikii*).

Based on the obtained sequences, a phylogenetic tree was constructed (Figure 2). The phylogenetic analysis of the 16S rRNA gene sequences reflected the affiliation of all extremely halotolerant isolates with the genus *Bacillus*, evidencing high bootstrap values at nodes (99-100%), and appeared closely related to the reference strain *B.*

cereus ATCC 14579 with high bootstrap value (97%) (Figure 2). The 16S rRNA gene sequence of the extremely halotolerant isolates were clustered into two subclusters; subcluster-I (99% bootstrap confidence value at the node) that includes six isolates (DSN2, DSM2, DSM3, DSM5, DSM7, and DSS3) and subcluster-II which groups three isolates (DSN1, DSN3, and DSS8) together with 100% bootstrap confidence value at the node.

Hemolytic and hydrolytic activities

The black mud *Bacillus* isolates were tested for their hemolytic activity against human erythrocytes (Table 4). It was found that five *Bacillus* isolates (DSN1, DSN2, DSN3, DSM3 and DSS8) exhibited hemolysis against human erythrocytes but they did not display any of the examined hydrolytic activities including amylase, caseinase, and API-ZYM hydrolysaes (Table 4). However, the remaining non-hemolytic isolates (DSM2, DSM5, DSM7 and DSS3) showed various enzymatic activities. Isolates DSM5 and DSS3 produced alkaline phosphatase only. Isolate DSM7 produced eight enzymatic activities including amylase, caseinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and α -chymotrypsin. Whereas, the black mud halotolerant *Bacillus* isolate DSM2 was considered to display several hydrolytic activities which reacted positively for 17 enzymes; including, amylase, caseinase, alkaline

Table 3. The comparison of the 16S rRNA gene sequences of nine extremely halotolerant *Bacillus* isolates harvested from black mud with the 16S rRNA gene sequences in the GenBank.

Isolate ^a	Sequence				
	GenBank accession no. ^b	No. of nucleotides ^c (GC%)	Closest phylogenetic relative ^d	Score ^e	% identity ^f
DSN1	KY848801	1074 (50.6)	<i>Bacillus oceanisediminis</i> (HE801980)	1943	99
DSN2	KY848802	1113 (59.8)	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> strain BGSC 3A28 (NR104873)	1890	97
DSN3	KY848803	1086 (50.8)	<i>Bacillus firmus</i> strain NBRC 15306 (NR 112635)	1842	97
DSM2	KY848804	1116 (50.5)	<i>Bacillus paralicheniformis</i> strain KJ-16 (NR137421)	2050	99
DSM3	KY848805	1306 (49.7)	<i>Bacillus methylotrophicus</i> strain CBMB205 (NR116240)	2399	99
DSM5	KY848806	1133 (53.7)	<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> strain FZB42 (NR075005)	1921	97
DSM7	KY848807	1115 (49.9)	<i>B. paralicheniformis</i> strain KJ-16 (NR137421)	1991	99
DSS3	KY848808	1142 (50.8)	<i>Bacillus sonorensis</i> strain NBRC 101234 (NR113993)	2074	99
DSS8	KY848809	1119 (50.6)	<i>Bacillus malikii</i> strain NCCP-662 (NR146005)	2043	99
<i>Bacillus cereus</i> ATCC 14579	KY848810	947 (52.2)	<i>B. cereus</i> ATCC 14579 (NR074540)	1927	99
<i>Escherichia coli</i> ATCC 8739	KY848811	1291 (51.9)	<i>E. coli</i> strain NBRC 102203 (NR114042)	2316	99

^a*B. cereus* ATCC 14579 was used as reference strain and *E. coli* ATCC 8739 was used as out-group. ^bThe accession number for each sequence was provided from GenBank database. ^cThe number of 16S rRNA gene nucleotides used for the alignment. ^dGenBank accession number was provided between parentheses. ^eThe matching score with the closest phylogenetic relative has 0.0 e-value, 0% gaps, and 100% query coverage. ^fThe percentage identity with the 16S rRNA gene sequence of the closest phylogenetic relative of bacteria.

phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-B1-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, and α -glucosidase. However, the hydrolytic activities of β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, and β -fucosidase were not detected in all isolates.

Antimicrobial activity

Antibacterial and antifungal activities of the extremely halotolerant *Bacillus* isolated from black mud of the Dead Sea were performed against test bacteria and fungi that exhibited multidrug

resistance (Table 5), by preparing bacterial extracts using organic solvents (*n*-butanol, methanol, ethanol, and acetone) and aqueous solvent (water). Unfortunately, it was found that only one isolate (DSM2) exhibited different arrays of antimicrobial activity (Table 5). Aqueous and acetone extracts of DSM2 isolate exhibited no antibacterial activity but produced antifungal activity. Aqueous extract showed the highest significant antifungal activity against test fungi (*A. brasiliensis* ATCC 16404 and *C. albicans* ATCC 10231). Interestingly, both *n*-butanol and methanol extracts of DSM2 isolate were exhibited significant antibacterial and antifungal activities against all test microorganisms investigated in this study. On the other hand, it was observed that ethanol extract of DSM2 exhibited narrower ranges of antibacterial activity against test

bacteria, except *Klebsiella* and *Proteus*, and it did not show inhibitory effects against any test fungus.

DISCUSSION

A little research efforts have focused on isolation of halophilic and halotolerant microorganisms from Dead Sea black mud and no previous study demonstrated the isolation of extremely halotolerant *Bacillus* from black mud. Therefore, this is the first study that investigated the isolation and characterization of extremely halotolerant *Bacillus* from Dead Sea black mud. Furthermore, this is the first study that examined the hydrolytic and the antimicrobial activities of extremely halotolerant *Bacillus* isolated from black mud of

Table 5. Antimicrobial activity of extremely halotolerant *Bacillus* isolates DSM2 against multidrug resistant bacteria and fungi.

Test microorganism ^a	Antibiotic resistance ^b	Inhibition Zone ^c (mm)				
		<i>n</i> -Butanol	Methanol	Ethanol	Acetone	Water
<i>Staphylococcus aureus</i> ATCC 25923	A, E, P, V	18.7±1.2 ^c	18.3±1.5 ^c	12.3±0.6 ^b	0 ^a	0 ^a
MRSA ATCC 95047 ^a	A, P	13.3±2.1 ^c	15.0±1.7 ^c	10.7±1.2 ^b	0 ^a	0 ^a
<i>Streptococcus pyogenes</i> ATCC 8668	A, P, V	19.7±1.6 ^c	20.3±3.1 ^c	13.0±3.5 ^b	0 ^a	0 ^a
<i>Pseudomonas aeruginosa</i> ATCC 27253	A, C, E, P, S	13.3±0.6 ^c	14.0±2.6 ^c	10.3±0.6 ^b	0 ^a	0 ^a
<i>Escherichia coli</i> ATCC 8739	A, E, V	19.3±3.0 ^c	17.3±0.6 ^c	12.7±1.6 ^b	0 ^a	0 ^a
<i>Klebsiella oxytoca</i> ATCC 13182	C, E, N, P, S, V	16.0±1.0 ^b	15.7±1.2 ^b	0 ^a	0 ^a	0 ^a
<i>Klebsiella pneumoniae</i> ATCC 7700	A, P, V	13.7±0.6 ^b	17.3±1.5 ^c	0 ^a	0 ^a	0 ^a
<i>Enterobacter aerogenes</i> ATCC 35029	A, E, P, V	17.3±3.1 ^c	16.7±2.5 ^c	11.3±2.5 ^b	0 ^a	0 ^a
<i>Proteus mirabilis</i> ATCC 12453	A, P	13.7±1.6 ^b	13.3±3.0 ^b	0 ^a	0 ^a	0 ^a
<i>Proteus vulgaris</i> ATCC 33420	A, P, V	16.0±1.7 ^b	14.7±1.5 ^b	0 ^a	0 ^a	0 ^a
<i>Salmonella typhimurium</i> ATCC 14028	A, N, P, V	20.3±2.5 ^c	21.3±1.6 ^c	9.7±1.5 ^b	0 ^a	0 ^a
<i>Candida albicans</i> ATCC 10231	NY	15.7±1.2 ^c	17.7±1.5 ^c	0 ^a	11.7±1.2 ^b	21.3±1.5 ^d
<i>Aspergillus brasiliensis</i> ATCC 16404	CY, NY	19.3±1.5 ^c	18.3±2.5 ^c	0 ^a	9.0±2.0 ^b	23.3±0.6 ^d

^aMRSA is methicillin resistant *S. aureus*. ^bA: Ampicillin 10 µg, C: Chloramphenicol 30 µg, E: Erythromycin 15 µg, N: Nalidixic acid 30 µg; P: Penicillin G (10 units), S: Streptomycin 10 µg; V: Vancomycin 30 µg; CY: Cycloheximide 250 µg; NY: Nystatin 10 µg. CY and NY activities were not determined for bacteria. The resistance for A, P, and S when inhibition zone (IZ) ≤ 11 mm; for C when IZ ≤ 12 mm, for E, N, and V when IZ ≤ 13 mm; and for CY and NY when IZ ≤ 8 mm. ^cThe inhibition ratio was represented as means ± SD. Means ± SD within column followed by the same letter are not significantly different (Tukey's studentized range test: α = 0.05).

the Dead Sea.

In the current study, it was observed that black mud contained low bacterial colony count and dominated by *Bacillus*. This is consistent with Ma'or et al. (2006) findings who reported that most bacteria in black mud of the Dead Sea was endospore-forming bacteria. Based on the phenotypic, physiological, biochemical, and phylogenetic characteristics, nine different bacterial isolates were considered as extremely halotolerant *Bacillus*. All black mud *Bacillus* isolates were Gram-positive, rod-shaped, endospore-forming, facultative anaerobes, and grew at 25 to 45°C, at pH 5 to 9, and at 0 to 20% (w/v) NaCl. Based on those phenotypic and physiological properties, the isolates appeared to be highly closed to extremely halotolerant bacteria of the genus *Bacillus*. Based on biochemical tests, all isolates were found to be positive for tryptophan deaminase, Voges-Proskauer, gelatinase activity, aesculin hydrolysis, and potassium gluconate assimilation. Most of the isolates were found to be positive for arginine dihydrolase, ONPG and PNPG hydrolyses, and for assimilation of D-mannose, N-acetylglucosamine, D-maltose, and malic acid. In terms of phenotypic, physiological, and biochemical characters, the identification of those isolates as *Bacillus* was in agreement with previous studies (Holt et al., 1994; Koneman et al., 1997; Fritze, 2004).

Interestingly, it was found that all isolates were nitrate reducers that are capable to reduce nitrate (NO₃) to nitrite (NO₂) but they were unable to reduce nitrite to dinitrogen gas (N₂). On the other hand, six of the isolates were able to produce nitrite and the remaining three isolates were

able to produce N₂ from nitrate. Therefore, the obtained isolates in the current study can perform intermediates of nitrification under aerobic conditions and denitrification under anaerobic conditions or low molecular oxygen levels. This result suggests that extremely halotolerant *Bacillus* isolated from the Dead Sea black mud played an important role in nitrification-denitrification processes and in the nitrogen cycle in soil. It was reported previously (Hall, 1986; Zumft, 1997; Zhang et al., 2012) that facultative anaerobic bacteria including *Bacillus* can perform both nitrification and denitrification. Zhang et al. (2012) reported that *B. methylotrophicus* is an efficient heterotrophic nitrification-aerobic denitrification bacteria, this is in agreement with the results of this study. Denitrification is commonly used in wastewater treatment and to prevent ground water pollution with nitrate due to over use of chemical fertilizers in agriculture (Mulvaney et al., 1997; Foglar et al., 2005). Therefore, extremely halotolerant *Bacillus* isolated from the Dead Sea black mud and possibly the black mud itself can be used in treatment processes of wastewater, in protection of groundwater from nitrate pollution, and in maintenance of soil health.

The phylogenetic analysis of the 16S rRNA gene sequences reflected the affiliation of extremely halotolerant isolates with the genus *Bacillus* and clustered together with the reference strain *B. cereus* ATCC 14579 (Figure 2). All isolates shared very high identities (97-99%) with their closest phylogenetic relative. In addition, the reference strain (*B. cereus* ATCC 14579) as well as the out-group strain (*E. coli* ATCC 8739) showed 99% identities to the same species level.

Furthermore, it was revealed that sequences with identities greater than 85, 95 and 97% are assigned to the same phylum, same genus, and same species, respectively (Schloss and Handelsman, 2005). However, most published libraries are restricted to 97 to 99% identity, thus sequence identity equals to or greater than 97% is assigned to the same species level (Stackebrandt and Goebel, 1994). Therefore, in this study, the standard 97% sequence identity with the closely related *Bacillus* was used to assign *Bacillus* isolates to the same species level. Based on this, extremely halotolerant *Bacillus* isolates screened from Dead Sea black mud were assigned to eight *Bacillus* species (Table 3). The identification of the isolated *Bacillus* species in this study as halotolerant *B. oceanisediminis*, *B. subtilis*, *B. firmus*, *B. paralicheniformis*, *B. methylotrophicus*, *B. sonorensis*, and *B. malikii* was in agreement with previous studies (Garabito et al., 1998; Palmisano et al., 2001; Roongsawang et al., 2002; Berrada et al., 2012; Zhang et al., 2012; Abbas et al., 2015; Dunlap et al., 2015) which demonstrated that those *Bacillus* species can tolerate increased salt concentrations. Whereas, the remained *B. amyloliquefaciens* was not previously defined as a halotolerant bacterium but Zar et al. (2013) demonstrated that this bacterium has the ability to produce halotolerant enzymes. The result of sequence analysis obtained in this study is in agreement with Romanovskaya et al. (2014) who isolated three *Bacillus* strains from black mud of the Dead Sea and found them closely related to *B. licheniformis* and *B. subtilis*.

The results presented in this study indicated that only nonhemolytic bacilli produced enzymatic activities (Table 3). Two *Bacillus* isolates DSM2 and DSM7, which have been defined according to 16SrRNA as *B. paralicheniformis* (accession numbers KY848804 and KY848807, respectively), were found to produce some economically important industrial enzymes such as amylase, lipase and several proteases. Interestingly, 17 enzymatic activities were detected from DSM2; including proteolytic enzymes such as trypsin, saccharolytic/amylolytic enzymes such as amylase, lipolytic enzymes such as lipase, and nucleolytic enzymes such as alkaline phosphatase. The positive results on several enzymes activity are indication of potential applications of such bacterial hydrolyases in biotechnology. Therefore, those two isolates could receive considerable attention due to the production of industrially important enzymes that could be used in food industry, bioremediation, and biosynthesis.

In the purpose of screening antimicrobial activities of the isolates, surprisingly all extremely halotolerant *Bacillus* isolates were found to exhibit neither antibacterial activity nor antifungal activity except DSM2 isolate (assigned to *B. paralicheniformis*). It was clearly observed that aqueous extract of DSM2 showed the highest significant antifungal activity against *A. brasiliensis* and *C. albicans*. Extracts prepared by *n*-

butanol and methanol showed significant antibacterial and antifungal activities against multidrug resistant human skin pathogens (*S. aureus*, MRSA, *S. pyogenes*, *P. aeruginosa*, and *C. albicans*) and against other frequent human pathogens (*S. typhimurium*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *E. aerogenes*, *P. mirabilis*, *P. vulgaris*, and *A. brasiliensis*) that exhibited resistance for at least two antibiotics. Ethanol extracts exhibited antibacterial activity against some test bacteria but they did not show inhibitory effects against test fungi. Ma'or et al. (2006) screened the antimicrobial activity of the Dead Sea black mud but he did not examine the antimicrobial activities of bacteria naturally occurred in the black mud. It was demonstrated that Dead Sea black mud exhibited slight inhibitory effect against the skin pathogen bacterium *Propionibacterium acnes* and the skin pathogen fungus *C. albicans* but the black mud did not show antibacterial effect against *E. coli* and *S. aureus* (Ma'or et al., 2006). Therefore, this is the first study that evaluated the antibacterial and antifungal activities of bacteria isolated from Dead Sea black mud, in particular extremely halotolerant *Bacillus*. Based on the antimicrobial activity result achieved through this study, the treatment of the Dead Sea black mud by extracting solvents before use in therapy to get better effect against pathogenic microorganisms especially skin pathogens needs to be evaluated by further studies.

Syed and Chinthala (2015) found that three *Bacillus* species (*B. licheniformis*, *B. cereus*, and *B. subtilis*) had significant levels of heavy metal detoxification. On the other hand, Momani et al. (2009) revealed that heavy metals content in the black mud of Dead Sea of Jordan was less than their contents in other types of mud. This might be due to detoxification of heavy metals by halotolerant *Bacillus* that dominate black mud as described in this study. Moreover, Abbas et al. (2015) reported that *B. malikii* is heavy metal tolerant, suggesting that extremely halotolerant *Bacillus* in black mud may play a role in lowering heavy metal content in black mud by detoxification processes. This needs further experimental studies.

The results of this study demonstrated that extremely halotolerant *Bacillus* isolated from Dead Sea black mud could be used in several industrial applications such as wastewater treatment, groundwater protection, food industry, and enzyme industry. In addition, the byproducts of DSM2 isolate can be used for pharmaceutical and medicinal purposes for instance treatment of bacterial infections, in particular multidrug resistant bacteria such as methicillin-resistant staphylococcus aureus (MRSA), skin and soft tissues therapies, and other potential medical applications.

CONFLICT OF INTERESTS

The authors has not declared any conflict of interests.

ACKNOWLEDGEMENTS

The author is grateful to "the Scientific Research Support Fund / Ministry of Higher Education of Jordan, grant no. M-Ph/2/14/2008" for financial support. The author also extends his thanks to Mr. Ismail Otri for technical assistance.

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