

Isolation and identification of halophilic and halotolerant bacteria from the sediments of the Qeshm Island mangrove forest

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ABSTRACT

Due to specific environmental and ecological conditions, mangrove forests are known as marine transitional zones between sea and land, and, as such, they host organisms with high ecological plasticity. The mangrove forests of Qeshm Island (Iran) are relatively pristine habitats and represent an ideal target for investigating patterns of either aquatic or benthic biodiversity. To provide insights on microbial diversity in this area, nineteen halophilic and halotolerant bacteria were isolated from the sediments in 2017 during low tide. The extracted bacterial strains were studied morphologically by streaking, initial observation of colonies and bacterial staining, and characterized using a battery of biochemical tests including KOH, MR, VP, urease, TSI, S/I/M, Mac, LIA, ODC, ADH, oxidase, catalase, and tryptophan deaminase. The optimum growth of halophilic bacteria was observed in salt concentrations from 5 to 20% NaCl, whereas the extreme halophilic Gram-positive strain grew in salt concentration of up to 30% NaCl. Molecular analyses were also carried out on four halophilic strains and one extreme halophilic gram-positive bacteria. Phylogenetic taxonomy analysis, after 16S rDNA gene Sanger sequencing, revealed that the halophilic bacteria were closely related to the strain types of the genus *Bacillus* including *Bacillus licheniformis*, *Bacillus velezensis*, *Bacillus Paralicheniformis* and *Bacillus* sp. with 99% bootstrap value. The extreme halophilic strain was associated to strains of *Planococcus plaktortidis* with 100% bootstrap value.

INTRODUCTION

Mangrove forests are located in intertidal and transitional zones between aquatic and terrestrial environments (Lee *et al.*, 2006) with special ecological conditions (Thu and Populus, 2007) and occur in up to 70% of tropical and subtropical coastlines worldwide. They are important for their ecological values, highly production (Ghosh *et al.*, 2010), economic activities - such as fisheries and aquaculture - and biogeochemical cycles (Thu and Populus, 2007; Zahed *et al.*, 2010). They provide breeding and feeding areas for many macro- and micro-organisms.

The microbial community living in the sediments of mangrove forests are exposed to several spatially and temporally variable ecological, biogeographical and anthropogenic factors, including abundance of organic and inorganic matter, food web structure, nutrient cycling and pollution (Ghosh *et al.*, 2010). Since mangrove ecosystems endure periodic tidal flooding, environmental factors, such as nutrient availability and salinity, are highly variable, which make mangroves a unique ecosystem with specific characteristics (Holguin *et al.*, 2006).

Qeshm Island is the largest island in the Persian Gulf (Salehipour Milani and Jafar Beglu, 2012), and its mangrove forests, covering about 67.5 km² and located within the northwest estuaries of the island, in Khoor-e-khooran and sandy islands apposite villages of Tabl and Laft to Gooran (55° 63'E - 26° 75'N and 55° 80'E - 26° 93'N), are protected areas, international wetlands and biosphere reserves (Zahed *et al.*, 2010). They are also the

largest area of mangrove forests in the northern part of the Persian Gulf (Khosravi, 1992).

The Persian Gulf is experiencing stressful conditions due to high seasonal variations in temperature (<10°C in winter and >30°C in summer) (Khosravi, 1992; Zehzad and Majnounian, 1997). Annual rainfall in Iranian mangrove forests is typically <200 mm, evaporation exceeds rainfall (Joekar and Razmjoo, 1995) and, thus, salinity is high (38 to 50) (Zahed *et al.*, 2010; Salehipour Milani and Jafar Beglu, 2012). As a consequence, life in these environments shows a high tolerance to salinity and heat stress (Yan and Sinica, 2007).

The hypersaline environments which are derived from the sea could be divided into four groups according to the salinity range and organism origin: i) 6-7 to 10% salinity includes originally marine various biota, ii) 10-14% salinity includes usually halotolerant, halophilic or adapted biota, iii) 14-30% salinity includes both extremely and moderately halophilic bacteria, iv) more than 30% salinity contains only bacterial community living on the organic matter (Por, 1980). According to the microorganism's ranges of salinity tolerance, they are categorized into three groups: slightly, moderately and extremely halophiles (Ventosa *et al.*, 2006; Zhang *et al.*, 2014). Halophilic bacteria can be present in almost all hypersaline environments including marine or terrestrial, anaerobic or aerobic, polar and tropical, or acidic to alkaline environments (Javor, 2012).

Microorganisms from extreme environments are able to enhance oil recovery and bioremediation (Lima *et al.*, 2011), and produce important secondary compounds with potential to be used in pharmaceutical industries,

agriculture, etc. (Janek *et al.*, 2010). Isolated strains including *Bacillus* sp. from more tough environments, such as marine and salty sediments can produce surfactin products (Kiran *et al.*, 2010; Donio *et al.*, 2013). As an example, *Bacillus subtilis* is able to produce a cyclic lipopeptide surfactin, which is known as one of the most powerful surfactants (Kiran *et al.*, 2010). This product is active at extreme salinity, temperatures and pH levels (Pacwa-Plociniczak *et al.*, 2011). Other biological products, such as biosurfactants and antibiotics, are also produced by microorganisms living in extreme environments (Abdel-Mawgoud *et al.*, 2008). Although the molecular evolution of stability and life of macromolecules has not been fully revealed yet (Vasavada *et al.*, 2006), the survival and proliferation of microorganisms in extreme environmental conditions could depend on their ability to produce biologically active compounds (Valentine 2007), which in turn, could reasonably have a pharmaceutical importance. As an example, a pharmacologically important biosurfactant isolated from a halophilic *Bacillus* sp. BS3 at extreme environment of solar salt work has anticancer activity in breast cancer and antiviral activity in shrimps' white spot syndrome (Donio *et al.*, 2013). Also, halophilic bacteria are suitable choices for bioremediation of hypersaline habitats (Zhang *et al.*, 2014).

Many studies have indicated that due to the high diversity of bacterial species, evaluation of bacterial diversity using culture-dependent methods may lead to inaccurate information. Therefore, over the past two decades, many researchers have turned from culture-dependent approaches to molecular (16S rRNA-dependent) approaches. Although this change of method has led to the discovery of many new species of microbes, bacterial cultivation, wherever possible and with exclusion of viable but non-culturable (VBNC) bacteria (Li *et al.*, 2014), remains the best method to investigate their physiology under variable ecological conditions (Yeon *et al.*, 2005).

Culture-dependent methods can be technologically enhanced by molecular techniques and molecular analysis. Also, PCR-based methods following DNA technologies could be complementary for cultivation-dependent studies in order to discover the biodiversity or genetic differences of bacterial communities (Yoon *et al.*, 2003; Fry, 2004; Yeon *et al.*, 2005).

Studies on bacteria communities of soils and sediments have been conducted worldwide. In mangrove sediments, among the others, studies have been carried out on the diversity and biotechnology potential of bacteria in Brazil (Dias *et al.*, 2009), isolation of *Bacillus swezeyi* sp. nov. and *Bacillus haynesii* sp. nov. from Negev Desert of Isreal (Dunlap *et al.*, 2017), bacterial biodiversity in Sundarban, India (Ghosh *et al.*, 2010), recovery of novel bacteria diversity in China (Liang *et al.*, 2007), and so on.

To provide insights on microbial diversity in

mangrove sediments, we have isolated and characterized, for the first time ever, some bacterial strains from the sediments of the Qeshm mangrove forests in Iran.

METHODS

Sampling

Sediment sampling was carried out with sterile pestles in September 2017 from the northwestern part of the mangrove forests in Qeshm Island, at Soheili jetty (26° 78' N, 55° 76' E) (Fig. 1) during the maximum low tide from three zones of upper intertidal - near tree coverage (A); intertidal (B); and under tidal zones - near the sea water (C). The sediment samples were collected in sterile glass jars, put into ice powder and then transported to the laboratory in 4°C refrigerator for further analyses.

Bacteria cultivation, purification, and staining

Sediment samples from the three zones (A, B and C) were initially dissolved in 0.85% NaCl solutions. Different dilutions of 10⁻¹, 10⁻² and 10⁻³ were then prepared and cultured in Nutrient Agar (NA) at 30°C for 24 and 48 hours. Then, the grown colonies were isolated on new NA cultures for subsequent biochemical tests, staining, and molecular studies. NaCl tolerance of strains and their growth were tested. More specifically, for assays of salt tolerance, Nutrient Broth (NB) medium was supplemented with different sodium chloride concentrations of 5%, 10%, 15%, 20%, 25% and 30% (w/v). The isolated strains in different NaCl concentrations were incubated at 30°C and monitored for growth after 24 and 48 hours. The Gram reaction of bacteria was determined by Gram staining set (Qatran Shimi, Iran). Gram staining is a useful method for detecting the structure of bacterial cell wall. According to the wall structure of bacterial cell, after staining, they show a purple appearance in Gram-Positive bacteria or red in Gram Negative ones. Staining was followed in accordance with manufacturer's protocol. Growth and common biochemical tests for identification of microorganisms were performed as follows: KOH test (potassium hydroxide test) with 3% KOH solution was carried out for confirming the gram staining process. Catalase test was conducted for finding out whether the strains had catalase enzyme for decomposition of H₂O₂; MR (Methyl Red)/VP (Voges-Proskauer) test was used to distinguish and identify the general forms of bacteria and identify the final product of bacterial fermentation pathway. Urease test was done in urease broth medium culture to determine which strain produced the urease enzyme that hydrolyzes urea to ammonia and carbon dioxide. Sugar fermentation test was used in differential TSI or Triple Sugar Iron Agar culture medium containing three types of sugar (lactose, sucrose

and glucose). This test was carried out to differentiate the microorganisms according to their ability in reduction of sulfur and fermentation of carbohydrates. Sulfur reduction, indole production ability, and motility of strains were tested through culturing in SIM (sulfur, indole, and motility) medium to differentiate the strains.

Culturing in MacConkey agar was carried out for detection of gram-negative bacteria. The decarboxylation tests show the microorganism's ability in removing the carboxyl group from an amino acid, and are useful for the differentiation of *Enterobacteriaceae*. The decarboxylation test for Lysine amino acid was carried out in lysine iron agar (LIA). Ornithine decarboxylation test was conducted in ornithine decarboxylase agar (ODC). Arginine dihydrolase test was done with culturing the bacteria in arginine dihydrolase broth (ADH) medium to detect the bacteria producing arginine dihydrolase enzyme that decarboxylates arginine. Oxidase test was carried out to identify the organisms producing cytochrome oxidase enzyme. Indole test was carried out by culturing strains in tryptophan broth. This test shows the ability of bacteria in converting tryptophan into indole.

DNA extraction, PCR conditions, and molecular identification

The bacteria were cultured in 10 cc tryptophan broth and yeast extract with 10% salinity for proliferation. DNA was extracted by Genomic DNA Extraction Kit from Gram-Positive Bacteria (Gene Transfer Pioneers, Iran) and suspended in 30 ml elution buffer. Extracted DNA quality was evaluated by 1.5% agarose gel electrophoresis using Tris-borate-EDTA as the buffer and RedSafe™ (iNtRON, South Korea) for gel staining.

The extracted DNA was used as a template for amplification and sequencing. The universal bacterial primers, 27F (Forward: 5'-AGA GTT TGA TCM TGG CTC AG-3') (Lane, 1991) and 1525R (Reverse: AAG GAG GTG WTC CAR CC) (Lane, 1991) were used for amplification of 16S rDNA genes by Polymerase Chain Reaction. PCR reactions were carried out in 25 μ L of master mix, with 2.5 μ L of 10X PCR buffer (BIORON, Germany), 1.25 μ L of 100 mM MgCl₂ (BIORON, Germany), 1 μ L of 25 mM dNTPs (GeneAll, Korea), 1 μ L of each 10 pM forward and reverse primer (Pishgam, Iran), 5 U Taq DNA polymerase, and about 15 ng μ L⁻¹ of template.

The 16S rDNA was amplified using a DNA thermal

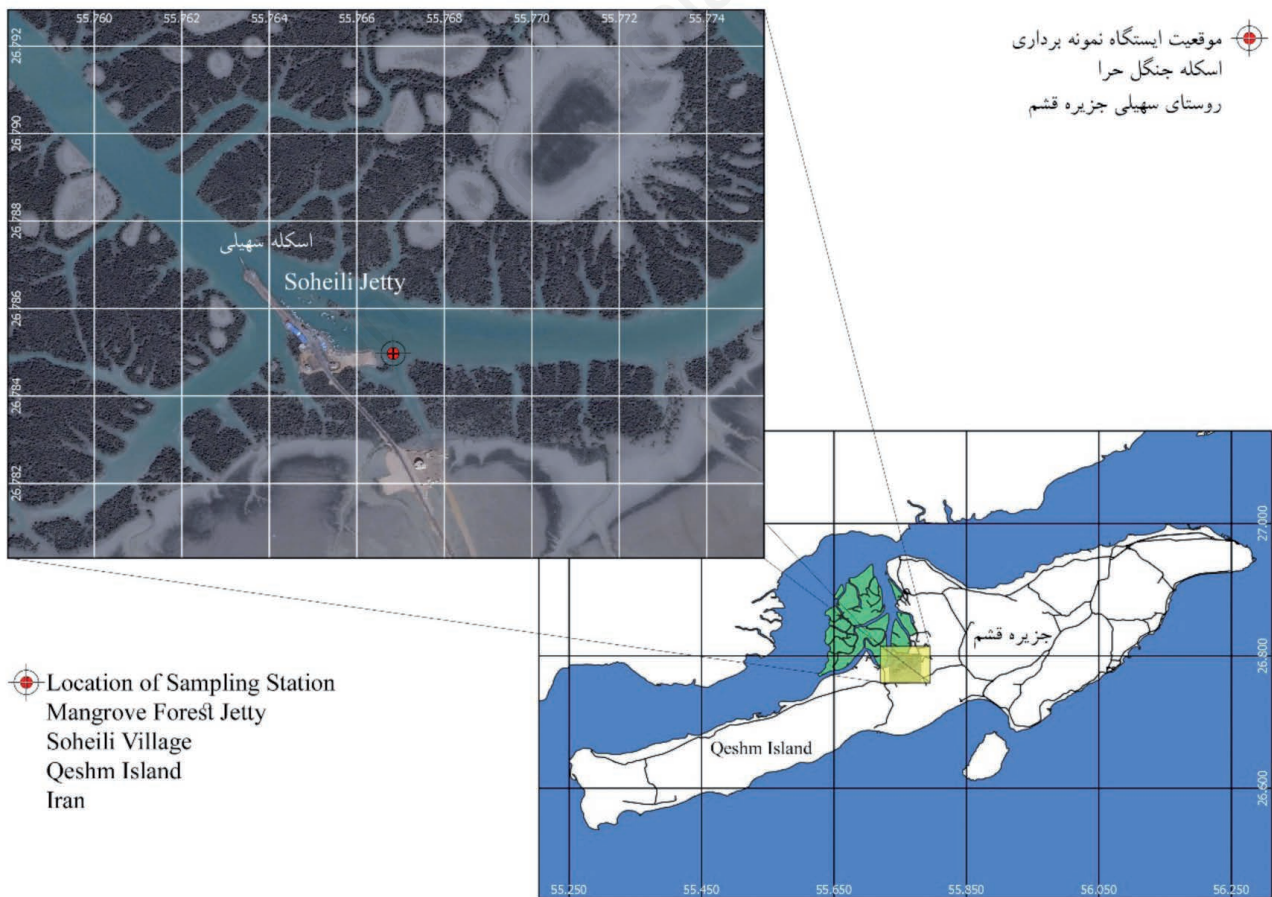


Fig. 1. Map of sampling site on Mangrove forest, Soheili jetty, the Northwestern part of Qeshm Island, Iran. Credit: Eghbal Zobeiri.

Molecular identification of the 16S rDNA gene sequences includes 12 strains of *Bacillus* sp. *Bacillus licheniformis* and *Bacillus paralicheniformis* in Clade 1 (Cl1), 6 strains of *Bacillus velezensis* in Clade 2 (Cl2) and 9 strains of *Planococcus plakortidis* and *Planococcus maritimus* in Clade 3 (Cl3) along with one outgroup. The analysis in this study showed that strains in the same clade are sister groups and clade 1 and 2 are supported as monophyletic groups with a bootstrap value of 100%. The blast results of Cl1 showed that PG-1-MS is associated with the species of *Bacillus licheniformis* strains of this clade with 99.90% (KJ526876.1, KJ526860.1, and EF472268.1) and 99.79% (MG980062.1) identities. In the same clade, the strain PG-3-MS has high identity with other strains of *Bacillus paralicheniformis* (100% identity with strain MH538129.1 and 99.93% identity with strains CP023666.1 and MF321832.1). Meanwhile, MH538129.1 and CP023666.1 have lower identity (99.79%) with *Bacillus licheniformis*. In addition, PG-4-MS was identified as *Bacillus* sp., and had 100% identity with other *Bacillus* sp. strains (MG814028.1 and MG814024.1) in the blast result, while the identity of PG-4-MS with *Bacillus paralicheniformis* is 99.93%. Another bootstrap value of 100% shows that clade 3 is paraphyletic for the previous monophyletic groups. The Clade 1 contains strains 1, 3 and 4 of the recorded mangrove sediment bacteria. Clade 2 has all *Bacillus velezensis* strains including our strain 2. Clade 3, as a paraphyletic group in the phylogenetic tree, includes the species *Planococcus plakortidis*, which corresponds to our strain 5. Strains PG-1-MS, PG-2-MS, PG-3-MS, and PG-4-MS belong to genus *Bacillus*, whereas PG-5-MS belongs to genus *Planococcus*. About 967 bp for PG-1-MS (Accession Number: MH847742.1) and 1442 bp for PG-3-MS (Accession Number: MH842119.1) were analyzed in the final alignment.

PG-1-MS and PG-3-MS were closely related to *Bacillus licheniformis* and *Bacillus paralicheniformis*, respectively (99% bootstrap value). The PG-4-MS (Accession Number: MH842120.1) was related to strains PG-1-MS and PG-3-MS and genus *Bacillus* with 99% bootstrap value, but other tests and biochemical analyses showed different results from other identified species mentioned in Tab. 1. The Neighbor-Joining phylogenetic tree (Fig. 2) revealed that strain PG-2-MS (Accession Number: MH838019.1) belongs to species *Bacillus velezensis* and is related to the other strains of species *Bacillus velezensis* with 99% bootstrap value. Blast results also showed that PG-2-MS has 100% identity with other strains of *Bacillus velezensis* (MF662480.1, MF662477.1, MF662476.1, MF662472.1, and MF662470.1) presented in clade 2. The last strain PG-5-MS (Accession Number: MH842121.1) is associated with other species of *Planococcus plakortidis* with 100% bootstrap value in phylogenetic taxonomy analysis.

Blast results revealed that PG-5-MS has more than 95.52% (MG705879.1, and MG705828.1) and 99.60% (MH384429.1, CP016539.2, NR_109414.1) identity with identity with *Planococcus plakortidis*. Although these blast results showed close relation between PG-5-MS and *Planococcus maritimus* (MG705829.1) with 99.52% identity, biochemical characteristics showed more similarity of PG-5-MS to *P. plakortidis*. Thus, the highest bootstrap values among our strains and other species of genus *Bacillus* and *Planococcus* were 99% (*Bacillus* sp., *Bacillus licheniformis*, *Bacillus paralicheniformis*, *Bacillus velezensis*) and 100% (*Planococcus plakortidis*), respectively.

Strains PG-1-MS, PG-2-MS, PG-3-MS, and PG-4-MS showed common features with genus *Bacillus* as they were all Gram-positive, rod-shaped, endospore-forming, motile and aerobic. PG-1-MS and PG-2-MS grew in up to 10% salinity in NB, but not in higher NaCl concentrations. PG-3-MS and PG-4-MS tolerated more salinity and grew in enriched NB with 20% NaCl. The growth condition in NB enriched by NaCl, interestingly, showed that PG-5-MS is an extreme halophilic strain that grew in 30% NaCl concentration.

Strain PG-1-MS belongs to aerobic, endospore-forming motile rod bacteria that produced yellow glossy round shaped with regular margin colonies in the NA. This halotolerant strain was molecularly identified as a strain of *Bacillus licheniformis* (more than 99.79% identity with other strains of *Bacillus licheniformis* in clade 1; Accession Number: MH837742.1). Strain PG-2-MS, molecularly identified as a strain of *Bacillus velezensis* (100% identity with other strains of *Bacillus velezensis* in clade 2 and 100% bootstrap value; Accession Number: MH838019.1), was aerobic *Diplococcus* bacteria with creamy white flat, viscose and irregular-margin colonies in the NA. Strain PG-3-MS, recorded as a strain of *Bacillus paralicheniformis* (more than 99.93% identity with other strains of *Bacillus paralicheniformis* in clade 1; Accession Number: MH842119.1), was an aerobic bacteria that formed convex, multilayered mounds of flower-like or amorphous, slime, approximately colorless (very light creamy-white) colonies with 2-3 mm in diameter in the NA culture. Strain PG-4-MS, identified as a strain of *Bacillus* sp. (99.93% identity with other strains of *Bacillus* sp. in clade 1; Accession Number: MH842120.1), formed spreading edged mucoid transparent light pinkish colonies on NA culture. Strain PG-5-MS, the strain of *Planococcus plakortidis* (more than 99.5% blast identity and 100% bootstrap value; Accession Number: MH842121.1), was gram-positive, non-spore forming, slow-growing, aerobic coccoid bacteria, and produced colonies with approximately 1 mm in diameter after about 72 hours at 30°C. The colonies were smooth, glossy, circular, small and round-shaped with regular-margin in orange on NA culture.

DISCUSSION AND CONCLUSIONS

Mean seawater salinity is ca. 35, and many microorganisms which live at this salinity can endure slightly higher salinities, whereas only halotolerant and halophilic species can grow in extremely hypersaline environments (Javor, 2012). Although, in comparison to other habitats, hypersaline environments possess low prokaryotic diversity (Guixa-Boixareu *et al.*, 1996), they are home to a high diversity of halotolerant bacteria and extremely halophilic bacteria (Yoon *et al.*, 2003). The microorganisms that tolerate very high salinity values show different metabolic features adapted to such extreme conditions (Zhang *et al.*, 2014).

The genus *Bacillus* consists of various species with some general characteristics: rod-shaped, and chain-forming gram-positive bacilli which can grow anaerobically or aerobically (Thwaite and Atkins, 2012), the majority of them being motile and having lateral

flagella (Stoica and Ionut, 2017). Their function in biochemical tests, growth features and even the phenotype of sporangia can influence their classification, and they can cope with unfavorable growth conditions due to their endospores (Zeigler and Perkins, 2015). Members of the genus *Bacillus* possess variable features and can live in different environments, or endure environmental variations of oxygen availability, humidity, temperature and nutrient accessibility (Nicholson, 2002). For example, different species of *Bacillus* might be aerobic or anaerobic, Gram-positive or Gram-variable and live in all environments from dust, soil and water (La Jeon *et al.* 2012) to skin and human gut (Mandell *et al.*, 2010). *Bacillus anthracis* and *Bacillus cereus* are human pathogens (Farrar, 1963). *Bacillus cereus* populations are endosymbionts in the gut of cockroach species *Blaberus giganteus* and have nutritional advantage for these insects (Feinberg *et al.*, 1999). It is reported that the species *Bacillus infernus* has been found nearly 3 km below the surface of earth (Boon *et al.*, 1995). Some even

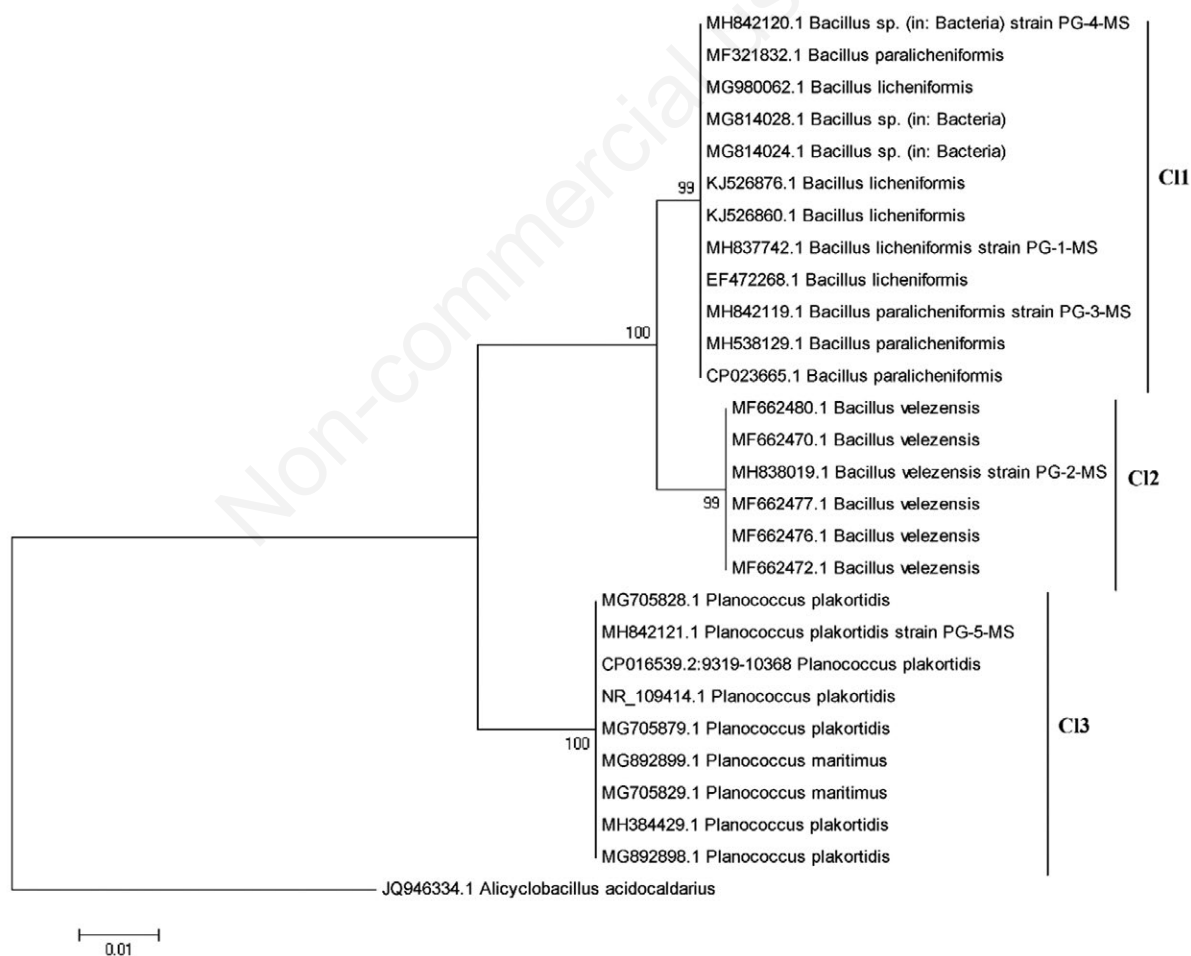


Fig. 2. Neighbor-joining phylogenetic tree for 27 strains, including Qeshm Mangrove Sediment species (PG-1-MS to PG-5-MS), based on 16S rDNA sequences. The numbers beside the branches are bootstrap values with 1000 replications. *Alicyclobacillus acidocaldarius* is selected as the outgroup.

could be found in extreme salty ecosystems such as solar salt works and condenser water with 155 ppt of salinity (Donio *et al.*, 2013). Any species might include different strains, which are sympatric (Palmisano *et al.*, 2001), so it is obvious that different strains of a species do not show the same features. Although there are strains of bacteria which are not potentially halophilic, with the passage of time, they can tolerate salinity as they adapt to their environment (Zhang *et al.*, 2014). The species of genus *Bacillus* have been increasingly important in applied microbiology and biochemical studies such as production of natural products, antibiotics, surfactants and enzymes (Ruiz-Garcia *et al.*, 2005; Jasim *et al.*, 2016). They have been studied also as probiotics in fish food and used as feeding supplement in aquaculture (Martínez Cruz, 2012; Ramesh *et al.*, 2015; Meidong *et al.*, 2017) and livestock (Kaewtapee *et al.*, 2017) for improving performance and health in animals (Meng *et al.*, 2010). Especially, *Bacillus licheniformis* and *Bacillus subtilis* have been used as pig supplementary diet (Larsen *et al.*, 2014; Jørgensen *et al.*, 2016). Different species of genus *Bacillus* could be found in various habitats from nature and plants (Arnesen *et al.*, 2008; Jasim *et al.*, 2016), and in intestinal tract of insects and mammals (Arnesen *et al.*, 2008) to soil (Palmisano *et al.*, 2001; Dunlap *et al.*, 2017) and water (Arjmand *et al.*, 2016). *Bacillus* enfolds about 70 species (Thwaite and Atkins, 2012) four of which have been recently presented as surfactant-producing bacteria, including *Bacillus licheniformis* (Weigmann, 1898) and *Bacillus velezensis* (Ruiz-Garcia *et al.*, 2005) isolated from Velez River in southern Spain for the first time, *Bacillus paralicheniformis* (Dunlap *et al.*, 2015) that have been isolated from fermented soybean paste for the first time, and *Bacillus* sp. which have been studied in the present paper.

As it was mentioned before, according to the continuously increasing number of identified bacteria with high similarity to each other, recognition via classical identification methods - such as analysis of physiological and morphological characteristics, the composition of cell walls and biochemical features - is very difficult (Fan *et al.*, 2017). There might be some other features other than morphology and biochemistry characteristics which make microorganisms distinguished from each other such as DNA sequences, fatty acid composition and even resistance to genetic transformation; these kinds of differences might exist even among the species of the same genus (Bacon and Hinton, 2011). Thus, there might exist some biochemical features which vary among species of a genus or strains of a species. The genus *Planococcus* was identified more than one century ago by Migula (1894), and was later modified by Nakagawa *et al.* (1996) (Migula, 1894; Nakagawa *et al.*, 1996; Kaur *et al.*, 2012). Some species of this genus are alkaliphilic and thermotolerant. A novel strain of species *Planococcus plakortidis* was isolated from a marine

sponge, *Plakortis simplex* (Kaur *et al.*, 2012) and received the authority by Kaur *et al.* (2012).

Planococcus plakortidis isolated from a genus of marine sponge showed positive activity for both catalase and oxidase (Kaur *et al.*, 2012) while *Planococcus* sp. MC01 (99.3% nucleotide identity with *Planococcus plakortidis*) (Ma *et al.*, 2013) and *Planococcus maritimus* (Yoon *et al.*, 2003) were found to be Catalase-Positive and Oxidase-Negative. Additionally, in our study, *Planococcus plakortidis* PG-5-MS had negative and positive activity for catalase and oxidase, respectively.

Our phylogenetic taxonomy analysis using 16S rDNA through NJ phylogenetic tree showed that clade C11 includes *Bacillus licheniformis*, *Bacillus paralicheniformis*, *Bacillus* sp. and their similar strains in a species, clade C12 consisted of *Bacillus velezensis* and similar strains and clade C13 possess *Planococcus plakortidis* and its strains (Fig. 2). The affinity of molecularly identified strains in trees showed 99% or more bootstrap value with other strains.

Bacillus licheniformis is a halophilic species, and different studies have shown that the presence of NaCl in the medium has better effects on bacterial growth and the production of bacterial products. There was positive growth on the medium enriched by 7% NaCl (v/w), and the grown bacteria successfully produced tannin acyl hydrolase (Mondal and Pati, 2000). NaCl concentration in the modified medium of a strain of *Bacillus licheniformis* isolated from saline soil had positive effect on γ -PGA production resulting in the most volumetric yield of this product (13.86 g l⁻¹) as the medium was enriched by 8% NaCl; the strain had positive growth in 12% concentration of NaCl as well (Wei *et al.*, 2010). Interestingly, the phylogenetic analysis has also revealed that two strains of genus *Bacillus* (*Bacillus swezeyi* and *Bacillus haynesii*) had a close relationship with the clade that included *Bacillus licheniformis* and its members, and they showed tolerance to up 12% NaCl (w/v) (Dunlap *et al.*, 2017). Shivaji *et al.* (2006) showed that *Bacillus licheniformis* forms white irregular, fried-egg-like colonies on NA, tolerated up to 11.6% NaCl (v/w) but not 17.4% and 23.4% concentrations of NaCl (v/w).

Bacillus velezensis showed growth capability at 12% w/v NaCl concentration and a temperature range of 15-45 °C; also, grew on TSA medium while producing rough white-creamy colonies with irregular edges (Ruiz-Garcia *et al.*, 2005), which was not far from our observations on this strain grown in NA. *Bacillus velezensis* was reported as a bacterium which acts as a plant growth promoting strain (Fan *et al.*, 2017). Presence of strain *Bacillus velezensis* in the collected sediments close to the plants in our research further proves the role of this strain in rhizosphere colonization. Dunlap *et al.* (2015) recorded a strain of *Bacillus paralicheniformis* as absolute anaerobic

gram-positive, motile strain that can form creamy mucoid, semitransparent colonies with 3-4 mm in diameter on R2A agar that can tolerate NaCl concentrations up to 10% (w/v) (Dunlap *et al.*, 2015).

Planococcus plakortidis was identified as a gram-positive coccoid strain for the first time in 2012 from the Bay of Bengal (Kaur *et al.* 2012). *Planococcus* sp. has been reported as a halotolerant and alkaliphilic slow-growing bacterium with orange-red colonies and 2-3 mm in diameter that grew after two days while the optimal temperature was 30 °C; in this study, the growth occurred at from 2% to 17% (w/v) NaCl (Ma *et al.*, 2013). The strains of *Planococcus maritimus* with 100% bootstrap value in Cl3 with *Planococcus plakortidis* and its strains in our study showed high affinity. *Planococcus maritimus* was isolated from seawater of the tidal zone in Korea for the first time. The biochemical characteristics of this species, however, showed either similarities (Gram-positive, cocci, motile, positive growth in 0% NaCl, negative urease) or dissimilarities (positive catalase, negative oxidase, tolerant to different salinities) (Yoon *et al.*, 2003) to the putative *Planococcus plakortidis* strain isolated in our study. Positive growth in 15-17% (v/w) concentration of NaCl by *Planococcus maritimus* in Yoon *et al.* (2003) survey and the same result in up to 12% NaCl (v/w) by the strains of *Planococcus* sp. in Dunlap *et al.* (2017) study showed that this genus is halotolerant.

Thus, based on our findings, we conclude that the identified halophilic bacteria isolated from the sediments of the mangrove forests in Qeshm Island, South of Iran, were mostly from genus *Bacillus* and *Planococcus*, which possess variant species and strains with different degrees of tolerance to salinity. Our phylogenetic analysis showed a close relationship between different identified strains with previous studies and recorded strains. Strains PG-1-MS, PG-3-MS and, PG-4-MS in Cl1, Strain PG-2-MS in Cl2 and Strain PG-5-MS in Cl3 lie in monophyletic groups. The noted strains generally formed clades in a phylogenetic tree with more than 98% bootstrap value protection.

The results presented in our spot survey in the mangrove sediments of Qeshm Island, South of Iran, confirm that this ecosystem hosts a peculiar diversity of halophilic bacteria with bio-ecological and functional characteristics either similar or different from those of similar strains isolated from other environments. The results presented here suggest that the prokaryotic diversity of this peculiar ecosystem deserves further attention, also in order to identify microorganisms that could possess a valuable potential in bioremediation and better growth of halophile plants.

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REFERENCES

- Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NAH, 2008. Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. *Appl. Biochem. Biotechnol.* 150:289-303.
- Arjmand MM, Rezaee A, Nasser S, Eshraghi SS, 2016. Removing *Bacillus subtilis* spores from drinking water using a bipolar electrochemical method. *Int. J. Electrochem. Sc.* 11:10080-10086.
- Boone DR, Liu Y, Zhao ZJ, Balkwill DL, Drake GR, Stevens TO, Aldrich HC, 1995. *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *Int. J. Systematic Bacteriol.* 45:441-448
- Dias AC, Andreote FD, Dini-Andreote F, Lacava PT, Sá ALB, Melo IS, Azevedo JL, Araújo WL 2009. Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment. *World. J. Microbiol. Biotechnol.* 25:1305-1311.
- Donio MBS, Ronica SFA, Viji VT, Velmurugan S, Jenifer JA, Michaelbabu M, Citarasu T, 2013. Isolation and characterization of halophilic *Bacillus* sp. BS3 able to produce pharmacologically important biosurfactants. *Asian. Pac. J. Trop. Med.* 6:876-883.
- Dunlap CA, Kwon SW, Rooney AP, Kim SJ, 2015. *Bacillus paralicheniformis* sp. nov., isolated from fermented soybean paste. *Int. J. Syst. Evol. Micr.* 65:3487-3492.
- Dunlap CA, Schisler DA, Perry EB, Connor N, Cohan FM, Rooney AP, 2017. *Bacillus swezeyi* sp. nov. and *Bacillus haynesii* sp. nov., isolated from desert soil. *Int. J. Sys. Evol. Micr.* 67:2720-2725.
- Fan B, Blom J, Klenk HP, Borriss R, 2017. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus*

- siamensis* form an “operational group *B. amyloliquefaciens*” within the *B. subtilis* species complex. *Front. Microbiol.* 8:22.
- Farrar WE Jr, 1963. Serious infection due to “non-pathogenic” organisms of the genus *Bacillus*. Review of their status as pathogens. *Am. J. Med.* 34:134-141.
- Feinberg L, Jorgensen J, Haselton A, Pitt A, Rudner R, Margulis L, 1999. Arthromitus (*Bacillus cereus*) symbionts in the cockroach *Blaberus giganteus*: dietary influences on bacterial development and population density. *Symbiosis* 27:109-123.
- Fry JC, 2004. Culture-dependent microbiology, p. 80-87. In: A.T. Bull (ed.), *Microbial diversity and bioprospecting*. American Society of Microbiology, Washington, DC.
- Ghosh A, Dey N, Bera A, Tiwari A, Sathyaniranjan KB, Chakrabarti K, Chattopadhyay D, 2010. Culture independent molecular analysis of bacterial communities in the mangrove sediment of Sundarban. India. *Saline Systems* 6:1-11.
- Guixa-Boixareu N, Calderón-Paz J, Heldal M, Bratbak G, Pedrós-Alió C, 1996. Viral lysis and bacterivory as prokaryotic loss factors along a salinity gradient. *Aquat. Microb. Ecol.* 11:215-227.
- Holguin G, Gonzalez-Zamorano P, De-Bashan LE, Mendoza R, Amador E, Bashan Y, 2006. Mangrove health in an arid environment encroached by urban development - a case study. *Sci. Total. Environ.* 363:260-274.
- Javor BJ, 2012. *Hypersaline environments: microbiology and biogeochemistry*. Springer -Verlag: 328 pp.
- Jasim B, Sreelakshmi S, Mathew J, Radhakrishnan EK, 2016. Identification of endophytic *Bacillus mojavensis* with highly specialized broad spectrum antibacterial activity. *3 Biotech.* 6:187.
- La Jeon Y, Yang JJ, Kim MJ, Lim G, Cho SY, Park TS, Suh JT, Park YH, Lee MS, Kim SC, Lee HJ, 2012. Combined *Bacillus licheniformis* and *Bacillus subtilis* infection in a patient with oesophageal perforation. *J. Med. Microbiol.* 61:1766-1769.
- Janek T, Łukaszewicz M, Rezanka T, Krasowska A, 2010. Isolation and characterization of two new lipopeptide biosurfactants produced by *Pseudomonas fluorescens* BD5 isolated from water from the Arctic Archipelago of Svalbard. *Bioresour. Technol.* 101:6118-6123.
- Jørgensen JN, Laguna JS, Millán C, Casabuena O, Gracia MI, 2016. Effects of a *Bacillus*-based probiotic and dietary energy content on the performance and nutrient digestibility of wean to finish pigs. *Anim. Feed Sci. Technol.* 221:54-61.
- Kaewtapee C, Burbach K, Tomforde G, Hartinger T, Camarinha-Silva A, Heinritz S, Seifert J, Wiltafsky M, Mosenthin R, Rosenfelder-Kuon P, 2017. Effect of *Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs. *J. Anim. Sci. Biotechnol.* 8:37.
- Kaur I, Das AP, Acharya M, Klenk HP, Sree A, Mayilraj S, 2012. *Planococcus plakortidis* sp. nov., isolated from the marine sponge *Plakortis simplex* (Schulze). *Int. J. Syst. Evol. Micr.* 62: 883-889.
- Kiran GS, Thomas TA, Selvin J, Sabarathnam B, Lipton AP, 2010. Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. *Bioresour. Technol.* 101: 2389-2396.
- Kumar S, Stecher G, Tamura K, 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870-1874.
- Li L, Mendis N, Trigui H, Oliver JD, Faucher SP, 2014. The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.* 5:258.
- Lane DJ, 1991. 16S/23S rRNA sequencing, p. 115-175. In: E. Stackebrandt and M. Goodfellow (eds.), *Nucleic acid techniques in bacterial systematics*. Wiley & Sons, Chichester.
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947-2948.
- Larsen N, Thorsen L, Kpikpi EN, Stuer-Lauridsen B, Cantor MD, Nielsen B, Brockmann E, Derkx PM, Jespersen L, 2014. Characterization of *Bacillus* spp. strains for use as probiotic additives in pig feed. *Appl. Microbiol. Biotechnol.* 98:1105-1118.
- Lee SY, Dunn RJK, Young RA, Connolly RM, Dale PER, Dehayr R, Lemckert CJ, McKinnon S, Powell B, Teasdale PR, Welsh DT, 2006. Impact of urbanization on coastal wetland structure and function. *Austral. Ecol.* 31:149-163.
- Liang JB, Chen YQ, Lan CY, Tam NF, Zan QJ, Huang LN, 2007. Recovery of novel bacterial diversity from mangrove sediment. *Mar. Biol.* 150:739-747.
- Ma C, Zhou S, Lu Q, Yang G, Wang D, Zhuang L, Li F, Lei F, 2013. Decolorization of Orange I under alkaline and anaerobic conditions by a newly isolated humus-reducing bacterium, *Planococcus* sp. MC01. *Int. Biodeter. Biodegr.* 8:17-24.
- Lima TM, Fonseca AF, Leão BA, Mounteer AH, Tótola MR, Borges AC, 2011. Oil recovery from fuel oil storage tank sludge using biosurfactants. *J. Bioremed. Biodegrad.* 2:10-4172.
- Mandell GL, Bennett JE, Dolin R, 2010. *Mandel, Douglas and Bennett's principles and practice of infectious diseases*, 7th ed. Churchill Livingstone, Philadelphia.
- Martínez Cruz P, Ibáñez AL, Monroy Hermosillo OA, Ramírez Saad HC, 2012. Use of probiotics in aquaculture. *ISRN Microbiology* 2012;2012:916845.
- Meidong R, Doolgindachaporn S, Jamjan W, Sakai K, Tashiro Y, Okugawa Y, Tongpim S, 2017. A novel probiotic *Bacillus siamensis* B44v isolated from Thai pickled vegetables (Phak-dong) for potential use as a feed supplement in aquaculture. *J. Gen. Appl. Microbiol.* 63:246-253.
- Meng QW, Yan L, Ao X, Zhou TX, Wang JP, Lee JH, Kim IH, 2010. Influence of probiotics in different energy and nutrient density diets on growth performance, nutrient digestibility, meat quality, and blood characteristics in growing-finishing pigs. *J. Anim. Sci.* 8:3320-3326.
- Mondal KC, Pati BR, 2000. Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. *J. Basic Microbiol.* 4:223-232.
- Nakagawa Y, Sakane T, Yokota A, 1996. Emendation of the genus *Planococcus* and transfer of *Flavobacterium okeanokoites* Zobell and Upham 1944 to the genus *Planococcus* as *Planococcus okeanokoites* comb. nov. *Int. J. Syst. Evol. Microbiol.* 46: 866-870.
- Nicholson WL, 2002. Roles of *Bacillus* endospores in the environment. *Cell. Mol. Life. Sci.* 59:410-416.

- Pacwa-Plociniczak M, Plaza GA, Piotrowska-Seget Z, Cameotra SS, 2011. Environmental applications of biosurfactants: recent advances. *Int. J. Mol. Sci.* 12:633-654.
- Palmisano MM, Nakamura LK, Duncan KE, Istock CA, Cohan FM, 2001. *Bacillus sonorensis* sp. nov., a close relative of *Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. *Int. J. Syst. Evol. Micro.* 51:1671-1679.
- Por FD, 1980. A classification of hypersaline waters, based on trophic criteria. *Mar. Ecol.* 1:121-131.
- Ruiz-Garcia C, Bejar V, Martinez-Checa F, Llamas I, Quesada E, 2005. *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain. *Int. J. Syst. Evol. Microbiol.* 55: 191-195.
- Salehipour Milani A, Jafar Beglu M, 2012. Satellite-based assessment of the area and changes in the mangrove ecosystem of the Qeshm Island, Iran. *J. Environ. Res. Dev.* 7:1052-1060.
- Stenfors Arnesen LP, Fagerlund A, Granum PE, 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* 32:579-606.
- Stoica C, Sorescu I, 2017. ABIS online - Advanced Bacterial Identification Software, an original tool for phenotypic bacterial identification. *Regnum Prokaryotae* 10:1-13. Available from http://www.tgw1916.net/bacteria_logare_desktop.html
- Thu PM, Populus J, 2007. Status and changes of mangrove forest in Mekong Delta: Case study in Tra Vinh, Vietnam. *Estuar. Coast. Shelf Sci.* 71:98-109.
- Thwaite JE, Atkins HS, 2012. *Bacillus*: Anthrax; food poisoning, p. 237-244. In: D. Greenwood, M. Barer, R. Slack and W. Irving (eds.), *Medical microbiology*. Elsevier, Amsterdam.
- Valentine DL, 2007. Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat. Rev. Microbiol.* 5:316-323.
- Vasavada SH, Thumar JT, Singh SP, 2006. Secretion of a potent antibiotic by salt-tolerant and alkaliphilic actinomycete *Streptomyces sannanensis* strain RJT-1. *Curr. Sci.* 91:1393-1397.
- Ventosa A, 2006. Unusual micro-organisms from unusual habitats: hypersaline environments, p. 223-254. In: N.A. Logan, H.M. Lappin-Scott and P.C.F. Oyston (eds.), *Proceedings SGM Symposium 66: Prokaryotic diversity – mechanisms and significance*. Cambridge University Press, Cambridge;.
- Wei X, Ji Z, Chen S. 2010. Isolation of halotolerant *Bacillus licheniformis* WX-02 and regulatory effects of sodium chloride on yield and molecular sizes of poly- γ -glutamic acid. *Appl. Biochem. Biotechnol.* 160:1332-1340.
- Yan L, Guizhu C, 2007. Physiological adaptability of three mangrove species to salt stress. *Acta Ecol. Sinica* 27:2208-2214.
- Yeon SH, Jeong WJ, Park JS, 2005. The diversity of culturable organotrophic bacteria from local solar salterns. *J. Microbiol.* 43:1-10.
- Yoon JH, Weiss N, Kang KH, Oh TK, Park YH, 2003. *Planococcus maritimus* sp. nov., isolated from sea water of a tidal flat in Korea. *Int. J. Syst. Evol. Microbiol.* 53:2013-2017.
- Yoon YJ, Im KH, Koh YH, Kim SK, Kim JW, 2003. Genotyping of six pathogenic *Vibrio* species based on RFLP of 16S rDNAs for rapid identification. *J. Microbiol.* 41 312-319.
- Zahed MA, Rouhani F, Mohajeri S, Bateni F, Mohajeri L, 2010. An overview of Iranian mangrove ecosystems, northern part of the Persian Gulf and Oman Sea. *Acta Ecol. Sinica* 30:240-244.
- Zeigler DR, Perkins JB, 2015. The genus *Bacillus*, p. 309-337. In: E. Goldman and L.H. Green 2nd (eds.), *Practical handbook of microbiology*. CRC Press, Boca Raton.
- Zhang X, Gao J, Zhao F, Zhao Y, Zhanshuang L, 2014. Characterization of a salt-tolerant bacterium *Bacillus* sp. from a membrane bioreactor for saline wastewater treatment. *J. Environ. Sci.* 26:1369-1374.