African Journal of Microbiology Research Vol. 6(2) pp. 7013-7020, 6 November, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.2055
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Enzymatic potential and characterization of poly-extremophilic bacteria isolated from saline and salt-affected soils in Egypt

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Accepted 8 November, 2012

Fourteen poly-extremophilic bacterial strains were isolated from saline and salt-affected soils in Egypt. Isolation of the strains was done after incubation at 55°C on two different agar media supplemented with 10% NaCl and adjusted at pH 10 (thermohaloalkali philic/tolerant). The isolated extreme bacterial strains were screened for their enzymatic potential. They showed high amylolytic and proteolytic activities but less cellulosic or pectinolytic activities. The fourteen isolates were characterized by studying their morphological, physiological and biochemical characters. Based on their phenotypic characteristics, twelve strains were tentatively identified to four different Gram-positive rod-shaped genera namely, *Alkalibacillus*, *Gracilibacillus*, *Halobacillus* and *Piscibacillus*, and the other two strains could not be identified. Then, the two most active strains with broad spectrum enzymatic activities, designated M201 and M610, were subjected to phylogenetic analyses of the 16S rRNA gene sequences. The phylogenetic results revealed that strain M201 belongs to genus *Gracilibacillus* and strain M610 belongs to genus *Piscibacillus*. The results indicated that the two strains could be classified as new species.

Key words: Extreme bacteria, salt-affected soils, enzymatic potential, characterization, phylogenetic analysis.

INTRODUCTION

The discovery of extremophiles has drastically changed our understanding towards the diversity of life itself and the conditions under which it can be sustained (Atomi, 2005). Extremophiles are organisms that are able to live and reproduce in extreme environments. They are adapted to live at high temperatures in volcanic springs, at low temperatures in the polar regions, under high pressure in the deep sea, at very low and high pH values (pH 0-3 or pH 10-12), or at very high salt concentrations (5-30%) (Fujiwara, 2002; Van den Burg, 2003).

The realization that extreme environments harbor different kinds of prokaryotic lineages has resulted in a complete reassessment of our concept of microbial evolution and has given considerable impetus to extremophiles research (Huber and Stetter, 1998; Rothschild and Mancinelli, 2001).

Saline and salt-affected environments are found worldwide and include a variety of habitats such as salt flats, evaporation ponds, natural inland salt lakes, soda lakes, subsurface salt formations, deep-sea hypersaline basins and others (Sass et al., 2001; Oren, 2002). These environments usually harbor taxonomically diverse bacterial groups, which exhibit some modified physiological and structural characteristics under the prevailing saline conditions.

The majority of saline and salt-affected soils in Egypt are located as salt marshes along the Mediterranean Sea coast such as in Alexandria and Borg El-Arab cities, and in the northern central part of the Nile Delta and its eastern and western sides. Other areas are the inland regions around salt lakes as in Fayoum Governorate, nearby depression lands connected with the evaporation

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of deep salty groundwater with accumulation of salts on the soil surface as in Wadi El-Natrun and the salt-affected soils of secondary origin in the old cultivated areas due to deterioration of these soils as in Beni-Suef and Menia Governorates. However, few microorganisms were described from these soils (Hozzein and Goodfellow, 2008; Sorokin et al., 2008) and few studies were reported (Grant et al., 2004).

The extremophilic microorganisms and their enzymes have been the subject of many recent studies (Demirjian et al., 2001; Van den Burg, 2003; Reyad, 2008). Enzymes from extremophiles have had a great impact on the field of biocatalysis and expected to fill the gap between biological and chemical processes due to their unusual properties (Schiraldi and De Rosa, 2002). In this study, we tend to isolate poly-extremophilic bacteria that can withstand three harsh conditions of temperature, salt and alkalinity and study their enzymatic activities.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected during March 2011 at 0 to 10 cm depth from different salt-affected and saline soils in Alexandria, Beni-Suef, Fayium and Menia Governorates, as well as the marine salt marshes of Borg Al-Arab city in Egypt.

Climate description

Egypt is hot and dry throughout the summer season with temperatures rising to as high as 50°C in some parts of the country (Kassas, 1953), while the weather is moderate in other seasons. El Hadidi (2000) mentioned that extreme temperatures of continental values are recorded in Kharga-Dakhla area of the Western Desert and reaches 52°C during July, where temperature falls to -5°C during January. In general, Alexandria and other Mediterranean coastal cities experience milder weather, but inland cities like Beni-Suef, Fayium and Menia are more hot and dry.

Physicochemical analyses of the soil samples

Moisture content, total soluble salts and electric conductivity were determined according to the method of Jackson (1967). Organic matter content was determined using the modified method of Walkley and Black (1947) as described by Tan (1996). Soil pH value was determined in 1:5 soil water suspensions using a Beckman pH meter. Total carbonates were determined according to the method described by Allen et al. (1974). Total calcium, magnesium and manganese ions were determined as recommended by Piper (1947) using an atomic absorption spectrophotometer (Unicam sp. 1900).

Isolation of the thermohaloalkali philic/tolerant bacteria

Two agar media were used for isolation of the extreme bacterial strains; Sato medium (Sato et al., 1983) and starch nitrate medium (Tadashi, 1975). Both media were supplemented with 10% NaCl and the pH was adjusted at 10.0 by the addition of sodium carbonate, which was sterilized separately. The two media were sterilized by autoclaving for 15 min at 1.5 atm. and 120°C. Inocula consisting of 0.1 ml of the soil serial dilutions were spread over the surface of the isolation plates (Johnson et al., 1959). The plates were then incubated at 55°C for 14 days. Colonies were picked and purified by streak plate technique on the same isolation medium. After pure bacterial cultures were obtained, isolates were routinely grown on the same medium and maintained as suspensions in 20% (v/v) glycerol at -20°C.

Screening for extracellular enzymes

Based on dissimilarities in the colonial appearance, fourteen isolates were selected for further studies. The abilities of the selected extreme bacterial isolates to produce amylases, cellulase, pectinase and protease enzymes were investigated. At the end of the incubation period for each isolate (2- 4 days), the broth cultures of 50 ml grown in 250 ml flasks were centrifuged at 6000 rpm for 15 min. The supernatant was used as a crude enzyme for activity assay. The amylolytic activity was detected by flooding the agar plates containing starch (1 %) with Gram's iodine solution (Slack, 1968). Appearance of clear zones compared with the blue color in the background indicated production of amylases.

Cellulase production was performed in agar plates supplemented with carboxy methyl cellulose (CMC) (0.5 %) as the substrate. The plates were then flooded with congo red and the yellow zones in respect to the red background was considered positive (Yoon et al., 2007). Assay for pectinase production was done in agar plates supplemented with pectin (0.5 %) as the substrate. Hydrolysis zones were detected by flooding the plates with an aqueous solution of hexadecyltrimethylammonium bromide (1% w/v) according to Hankin et al. (1971). The proteolytic activity was visualized by flooding the agar plates containing gelatin (0.4%) with 10% trichloroacetic acid (TCA) solution (Ammar et al., 1991). Appearance of clear zones compared with the white precipitate in the plate was scored as positive.

Characterization of the selected extreme bacterial isolates

Morphological characteristics

Cell morphology was examined using phase contrast and light microscopy. The Gram reaction and presence or absence of endospores was examined using the staining procedures described by Smibert and Krieg (1981) after desalting using the method of Dussault (1955).

Physiological and biochemical characteristics

The effect of different salt concentrations, temperature degrees and pH values on the growth of the isolates was tested on Sato medium by measuring the optical density (OD_{600}). The isolates were grown on nitrate broth for nitrate reduction reaction (Gordon, 1966). Hydrogen sulfide production was detected as described by Küster and Williams (1964). The ability to produce catalase and oxidase enzymes was determined according to Smibert and Krieg (1981). Degradation of casein (1% w/v, skimmed milk) and aesculin (0.1%) was detected in Czapek Dox agar medium. Tween degradation was carried out using Sierra's medium (1957). Urea decomposition was done according to Gordon et al. (1974). Utilization and acid production from different carbohydrates; D-xylose, D-galactose, D-glucose, D-fructose, mannitol, D-trehalose and sucrose, were examined on Czapek Dox agar medium free from sucrose and supplemented with phenol red. In all the experiments, the media were supplemented with 10% NaCl, the pH was adjusted to 8.0 and the results were recorded after 2 to 3 days of incubation at 55°C.

Table 1. Physicochemical characteristics of the collected soil samples and the average bacterial counts (CFU/gm of soil) on Sato medium.

Soil sample	pH	TSS %	E.C (ms)	Cl^- (%)	CO_3^{--} (%)	HCO_3^- (%)	Moisture content (%)	Organic carbon (%)	Bacterial count
A	7.8	1.41	1.06	2.2	12.5	0.047	15.0	0.86	10×10^2
E	8.2	1.40	1.06	1.8	19.0	0.045	19.0	0.44	2×10^2
B	8.0	6.70	18.7	7.4	15.5	0.030	14.2	0.52	30×10^2
F	8.1	9.18	30.0	8.1	18.0	0.039	13.5	0.80	46×10^2
M	7.9	2.87	7.30	2.9	15.0	0.033	12.1	0.18	7×10^2

A = Alexandria, E = Borg El-Arab, B = Beni-Suef, F = Fayoum; and M = Menia.

Table 2. Extracellular enzymatic activities of the isolated poly-extremophilic bacteria.

Isolate code	Enzymatic activities				Isolate code	Enzymatic activities			
	Amylase	Cellulase	Protease	Pectinase		Amylase	Cellulase	Protease	Pectinase
A201	+	-	+	-	F302	+	-	+	-
A202	+	-	+	-	M201	+	+	+	+
A203	+	-	+	-	M406	+	-	+	-
B100	+	-	+	-	M407	-	-	+	-
B200	+	-	+	+	M504	+	-	+	-
B300	+	-	+	-	M604	-	-	+	-
E101	+	-	+	-	M610	+	+	+	+

+ = Positive result, - = Negative result

16S rRNA sequence analysis

Genomic DNA was isolated and purified according to Lyimo et al. (2000). Amplification of the 16S rRNA gene was carried out by polymerase chain reaction (PCR) using the following universal primers:forward primer 5′ CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG 3′ and reverse primer 5′CCCG GGATCCAAGCTTACGGCTACCTTGTTACGACTT 3′. The PCR product was purified and checked for purity. Sequencing of the 16S rRNA gene was performed on a Perkin Elmer automated laser fluorescent DNA sequencer (ABI 310). The gene sequence was then analyzed to assess the phylogenetic position of the strains using nucleotide Blast search tool available at the GenBank sequence database (www.ncbi.nlm.nih.gov) (Altschul et al., 1990). The most similar sequences were selected, aligned and the evolutionary distance matrices were calculated by the method of Jukes and Cantor (1969). Phylogenetic trees were inferred by using the neighbor-joining method (Saitou and Nei, 1987).

RESULTS

The results of the physicochemical properties of the soil samples and the bacterial count are shown in Table 1. The results revealed that the number of bacterial colonies grown under the isolation conditions (10% NaCl, pH 10 and 55^{o}C) was approximately 10^2 CFU per one gram of soil. The results showed that the bacterial count was higher in the salt-affected inland soils (Beni-Suef, Fayoum and Menia) than in the saline costal habitats (Alexandria and Borg El-Arab). The data in Table 1 showed also that the viable bacterial count was positively correlated with the total soluble salts and the organic matter content. In total, 33 bacterial colonies were purified from the isolation plates, and 14 dissimilar isolates were selected to be screened for enzymatic activities.

All the bacterial isolates exhibited positive proteolytic activities; 12 isolates exhibited amylolytic activities, 3 isolates showed pectinolytic activities and 2 isolates had cellulosic activities (Table 2). Colonies of the bacterial isolates under investigation were mainly circular, white, creamy white to creamy in color and non-transparent on solid media. They had entire margins, convex elevation and shiny surfaces.

Morphologically, all the isolated strains were Gram-positive and the cells were found to be rod-shaped with variable lengths and arrangements. In all of them, spherical to ellipsoid endospores were produced. The endospores were either terminal (polar) or central to sub-terminal.

The characteristic and differential phenotypic properties of the selected isolates are given in Table 3. Two isolates could grow at temperatures up to 85°C, 5 isolates up to 65°C, 4 isolates up to 60°C and three isolates up to only 55°C. However, optimal growth temperatures were

Table 3. Differential phenotypic properties of the selected poly-extremophilic bacterial isolates.

Characteristic	Strains													
	A201	**A202**	**A203**	**E101**	**B100**	**B200**	**B300**	**F302**	**M201**	**M406**	**M407**	**M504**	**M604**	**M610**
Spore shape	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Ellipsoidal	Ellipsoidal	Spherical	Spherical	Oval
Spore position	Central	Polar	Polar	Polar	Polar	Polar	Polar	Polar	Polar	Central	Subterminal	Polar	Polar	Polar
Temperature range (°C)	40- 65	40- 85	30- 65	20- 60	30- 55	20- 60	25- 55	20- 55	30- 65	40- 65	30- 60	30- 65	40- 85	30- 60
pH Range	5- 12	3- 12	5- 11	7- 12	5- 11	7- 11	8- 11	8- 11	6- 10	5- 11	5- 11	6- 11	3- 12	6- 10
NaCl Range (%)	0- 25	5- 25	0- 25	0- 25	0- 25	0- 20	0- 25	0- 25	0- 10	5- 30	5- 30	0- 15	0- 20	0- 25
Oxidase reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	-	+	+	-	-	-	+	-	-	+	+	-
H_2S production	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Hydrolysis of:														
Aesculin	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Casein	-	+	+	+	-	+	+	+	+	-	-	-	-	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	-	+	-	+
Tween 80	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Urea	+	+	+	+	+	+	+	+	-	+	+	-	-	+
Acid production from:														
D-xylose	+	+	-	-	-	-	+	-	+	-	+	+	-	+
D-fructose	+	-	-	-	-	-	-	-	+	+	-	+	-	-
D-galactose	-	-	-	-	-	-	-	-	+	-	-	+	-	+
D-glucose	+	-	-	-	-	-	-	-	+	+	+	+	+	-
D-mannitol	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Trehalose	-	-	+	-	-	+	+	-	+	-	+	+	-	-
Maltose	-	-	+	-	-	-	-	-	+	-	-	+	-	-
Sucrose	-	+	-	-	-	-	-	-	+	-	-	+	-	-
Tentative classification	*Halo-bacillus*	NI	*Halo-bacillus*	*Alkali-bacillus*	*Gracili-bacillus*	*Alkali-bacillus*	*Alkali-bacillus*	*Alkali-bacillus*	*Gracili-bacillus*	*Halo-bacillus*	*Halo-bacillus*	*Gracili-bacillus*	NI	*Pisci-bacillus*

All strains have rod-shaped cells. + = Positive result; - = negative result; NI, not identified.

between 45 and 70°C. Two isolates were able to grow over a broad pH range (3 to 12), 2 isolates showed growth in the alkaline medium only at pH from 8 to 11, 2 isolates showed growth in both the alkaline and neutral medium; and the other isolates could grow starting from pH 5 or 6 up to pH 10 or 11. Three strains showed requirement for NaCl for growth; A202 which could grow at 5 up to 25% NaCl and M406 and M407 that showed growth at 5 up to 30% NaCl. Seven strains were tolerating up to 25%, two strains up to 20%, one strain (M504) up to 15% and one strain (M201) up to only 10% NaCl. All isolates were aerobic and exhibited oxidase and catalase production. All isolates also hydrolyzed gelatin. Strains M201 and M504 showed H_2S production and degradation of Tween 80. The same two s trains were also able to produce acids from all tested sugars.

The most two active strains in enzyme production with broad spectrum enzymatic

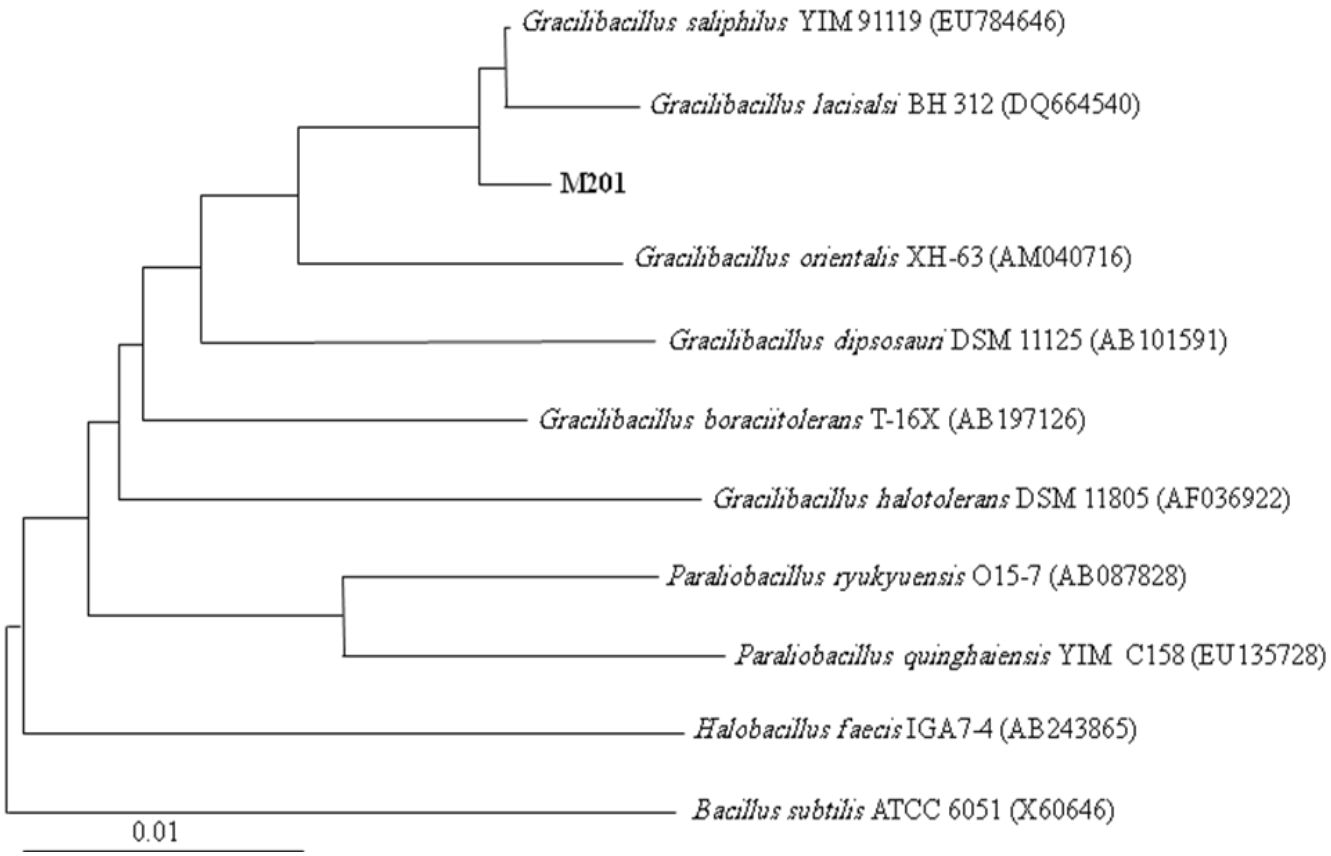

Figure 1. A neighbor-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequence of strain M201 and related taxa. *Bacillus subtilis* was used as an out-group. Accession numbers of the sequences are shown in parentheses. Bar indicated 1 substitution per 100 nucleotide positions.

activities, M201 and M610, were subjected to phylogenetic analysis. The phylogenetic results (Figures 1 and 2) revealed that M201 belongs to genus *Gracilibacillus* and M610 belongs to genus *Piscibacillus*. It was obvious that M201 forms a separate phyletic line within the genus *Gracilibacillus*, where the nearest phylogenetic neighbors were *Gracilibacillus saliphilus* and *Gracilibacillus lacisalsi* with similarity values of 99 and 98%, respectively. On the other hand, *Piscibacillus salipiscarius*, *Filobacillus milosensis* and *Aquisalibacillus elongatus* were the closely related neighbors to strain M610 with the same similarity value of 98%.

DISCUSSION

Extremophiles are microorganisms able to grow optimally in extreme environments of temperature, pH, pressure and salinity, often in places deemed inhospitable to life (Cardoso et al., 2011). Although the molecular strategies employed for survival in such environments are still not fully clarified, it is known that these microorganisms have adapted molecules, as well as different and peculiar biochemical pathways, which are of great interest for biochemical and biotechnological purposes (Alqueres et al., 2007). Previous studies have estimated that only a small percentage less than 1% of organisms present in the natural environment have been cultured (Fujiwara, 2002). Therefore, the aim of the present study was to isolate bacterial strains capable of growing under extreme conditions of temperature, pH and salinity, and to screen them for the production of some industrially important enzymes.

For this purpose, the soil samples were collected from saline and salt-affected habitats in different places of Egypt. To be able to correlate between the bacterial count and the prevailing environmental conditions and soil characteristics, description of the climate was given and some physicochemical properties of the soil samples were determined. The results of the bacterial count (Table 1) revealed that the number of bacterial cells grown under the isolation poly-extreme conditions (10% NaCl, pH 10 and 55°C) was approximately 10^2 CFU per one gram of soil. Similar results were reported by other researchers (Del Moral et al., 1987; Oren, 1991). In our opinion, this count is lower than the real count by two or

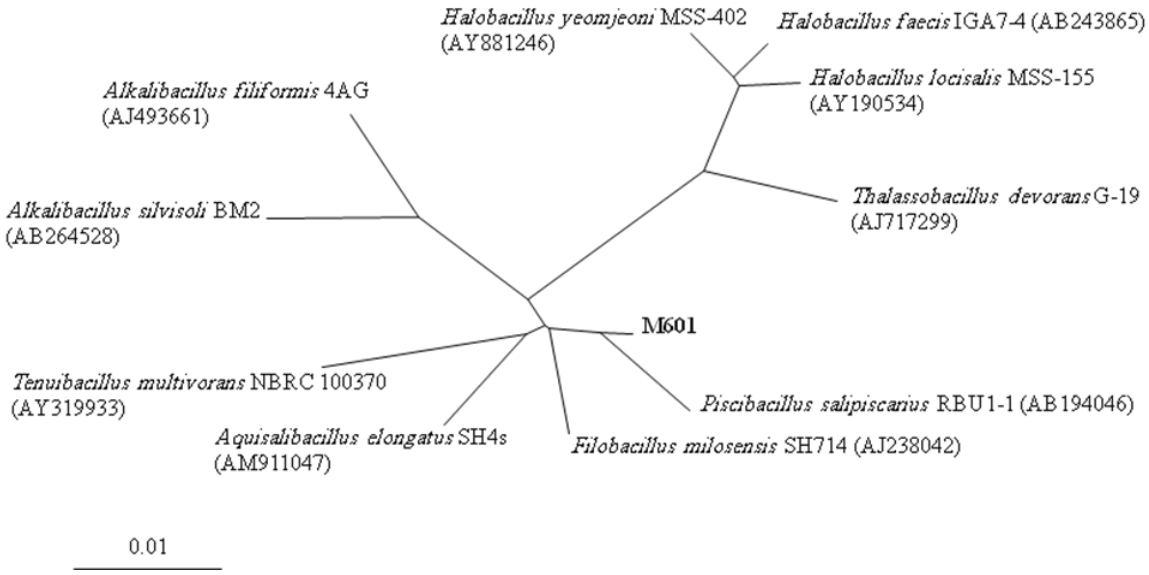

Figure 2. Unrooted phylogenetic tree showing the relationship of strain M601 to some related organisms. The tree was derived from a distance matrix based on a selection of 16S rRNA sequences. Accession numbers of the sequences are shown in parentheses. Scale bar = 0.01 changes per nucleotide position.

more orders of magnitude mainly due to the influence of the medium composition. The results in Table 1 indicated that the bacterial count was higher in the salt-affected inland soils (Beni-Suef, Fayoum and Menia) than in the saline habitats (Alexandria and Borg El-Arab). Similar results were obtained from saline and salt-affected soils in Egypt (Zahran, 1997; Reyad, 2008).

It is well known that the bacterial count and distribution in soil are mainly dependent on the physicochemical characteristics of the soil (Alexander, 1983). The data shown in Table 1 revealed that the viable bacterial count was positively affected by the total soluble salts and the organic matter content. These results are in agreement with those published by other authors (Fujihara and Yoneyama, 1993; Hozzein et al., 2008). In this study, 33 thermohaloalkali-philic/tolerant bacterial strains were isolated and purified. Based on dissimilarities in the colonial appearance and some preliminary morphological observations, fourteen isolates, designated as A201, A202, A203, B101, B201, B301, F302, M201, M406, M407, M504, M604, M610 and E101, were selected to be screened for their enzymatic potential.

All the bacterial isolates exhibited positive proteolytic activities; twelve showed amylolytic activities, three showed pectinolytic activities and two isolates had cellulosic activities. The results in Table 2 indicated that only two strains, namely M201 and M610, showed broad spectrum enzymatic activities. The combined hydrolytic enzymatic activities detected in the two strains could be useful for biotechnological applications. The cultural and morphological characteristics of the fourteen isolates were observed and recorded. The results obtained revealed that they were Gram-positive, rod-shaped and endospore-formers. Previous researchers (Priffner et al., 1986; Bock et al., 1994) reported that most of the microorganisms from hyper saline environments are rod-shaped.

The differential phenotypic properties of the selected isolates are shown in Table 3. The results showed that most strains had wide range of tolerance towards the tested poly-extreme conditions. Characteristically, all strains were aerobic and exhibited oxidase and catalase production. Based on the morphological and biochemical data (Table 3) and according to the Bergey's manual (Holt, 1984), all strains were tentatively identified to belong to the Gram-positive and aerobic rod-shaped extreme genera related to genus *Bacillus*. These findings are in good agreement with previous studies which reported that aerobic organisms present in the saline environments are predominated by Gram-positive and spore forming rod bacteria (Spring et al., 1996; Chen et al., 2008; Amoozegar et al., 2009).

Based on their phenotypic characteristics, 12 strains were tentatively identified to four different Gram-positive rod-shaped genera; namely, *Alkalibacillus*, *Gracilibacillus*, *Halobacillus* and *Piscibacillus*, and the other two strains could not be identified. However, the organisms differed in some physiological and biochemical characters among themselves, but this was in the less distinguishing characters. Further taxonomic studies have to be done to identify the isolates up to the species level, including the molecular analysis of the 16S rRNA gene sequence. Therefore, the most two active strains, M201 and M610, in enzyme production with broad spectrum enzymatic activities were selected for phylogenetic analysis. For this purpose, almost complete 16S rRNA sequences of the

two bacterial isolates were obtained after PCR amplification. These sequences were subjected to comparison with available sequences in the GenBank database and the phylogenetic trees were generated to infer their taxonomic positions.

The phylogenetic results (Figures 1 and 2) confirmed that M201 belongs to genus *Gracilibacillus* and M610 belongs to genus *Piscibacillus*. The phylogenetic results revealed that M201 is associated with *Bacillus* group 1 as defined by Ash et al. (1991), which harbors members of the genera *Gracilibacillus*, *Halobacillus* and *Paraliobacillus*. It was obvious that M201 forms a separate phyletic line within the 16S rRNA gene tree of the genus *Gracilibacillus* and the nearest phylogenetic neighbors were *Gracilibacillus saliphilus* and *Gracilibacillus lacisalsi* with similarity values of 99 and 98%, respectively. On the other hand, the closely related phylogenetic neighbors to strain M610 were *Piscibacillus salipiscarius*, *Filobacillus milosensis* and *Aquisalibacillus elongatus* with the same similarity value of 98%. The results indicated that the two strains could be classified as new species, but further taxonomic studies need to be done to confirm this, especially DNA-DNA pairing.

Indeed, most of the extreme aerobic, spore-forming and rod-shaped bacteria are commonly found in saline soils such as *Halobacillus litoralis* and *Halobacillus trueperi* (Spring et al., 1996), *Gracilibacillus halophilus* (Chen et al., 2008) and *Gracilibacillus ureilyticus* (Huo et al., 2010); or hypersaline lakes such as *Halobacillus alkaliphilus* (Romano et al., 2008) and *Piscibacillus halophilus* (Amoozegar et al., 2009). The strains under study were found to be mainly highly tolerant to the extreme conditions used and more related to halophilic bacterial genera rather than to the alkaliphilic or thermophilic genera.

Conclusion

This study is a preliminary screening report on poly-extremophilic bacteria and their enzymatic potential from saline and salt-affected soils in Egypt. The results are encouraging as the isolated extreme bacterial strains showed a good enzyme potential and a good taxonomic diversity. This study can provide ample scope to assess the biotechnological applications of those newly isolated and poorly studied microorganisms. It is recommended that more detailed and deep studies should be done for proper evaluation and exploration of the biotechnological applications of extreme bacteria from the Egyptian habitats.

ACKNOWLEDGEMENT

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-205.

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