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Investigation of Some Biotechnological Aspects of Two Novel *Bacillus* species Isolated from Natural and Man-made Alkaline Ecosystems in Egypt

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Alkaliphiles are unique microorganisms, with great potential for microbiological and biotechnological exploitation for the environmental applications. In the present study, two bacterial strains were isolated from two different sites in Egypt. The first is Bani Salama Lake at Wadi El-Natrun characterized by pH about 11.5 and high salinity level. The second is Abu Qir Bay, one of the most highly polluted areas in Alexandria surrounded by many factories discharging alkaline waste. Seventy-nine alkaliphilic strains were isolated (33 from Bani Salama lake and 46 marine strains from Abu Qir Bay). The strains were screened for the production of six extracellular degradative enzymes namely mannanase, pectinase, amylase, xylanase, protease and cellulase. The majority of isolates possessed significant enzyme activities. Based on genotypic and phenotypic characterization, the two potent strains were classified as *Bacillus cereus* N1 and *Bacillus licheniformis* A26. The study was extended to investigate the efficiency of the two strains to remove metal cations of copper, cobalt and lead from solutions at pH range 8-9. Cells of *B.cereus* N1 were able to absorb cobalt and lead at 0.4 mM, absorbing about 90.5 % and 92%, respectively, while *B. licheniformis* A26 was able to absorb copper and cobalt at metal concentrations of 0.2 and 0.4 mM, sequestering 24.5%, 64% for copper and 23%, 89% for cobalt, respectively. Moreover, *B.cereus* N1 showed antimicrobial activity against *Klebsiella pneumonia and Candida albicans* with inhibitory zones of 4 and 15 mm, respectively. Whereas, *B.licheniformis* A26 showed antagonistic activity against *Klebsiella pneumonia, Staphylococcus aureus* and *Candida albicans* with inhibitory zones of 4, 4 and 10 mm, respectively.

Key words: Alkaliphilic bacteria, *Bacillus cereus*, *Bacillus licheniformis*, soda lakes.

Alkalophilic and alkalitolerant bacteria can be found mostly in alkaline environments including soda soils, soda lakes and deserts, neutral environments and deep-sea sediments[1,2]. Man-made alkaline environments such as effluents from food, textile, tannery, and potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources[3, 4].

The soda lakes are characterized by the presence of large amounts of carbonate minerals which can generate pH values >11.5 [3,5]. Extremely alkaline lakes, for example, Lake Magadi in Kenya and Wadi El-Natrun in Egypt, are probably the most stable highly alkaline environments on Earth, with a consistent pH of 10.5 to12 depending on the site.

Alkaliphiles and alkalitolerant bacteria are reported to be a rich source of alkaline active

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enzymes, such as, amylase, protease, cellulase, xylanase, mannanase and other enzymes that have numerous applications in many industrial processes due to an interest in their physiological adaptation to high pH[6, 7]. The advances in the application of alkaliphilic- or alkalitolerant-based biomolecules during the past 20 years are due to the introduction of proteolytic enzymes into detergent industry[5, 8]. Industrial applications of alkaliphiles have been investigated and some enzymes have been commercialized [7]. Some new antibiotics were produced by certain bacteria grown in alkaline medium (pH 9 to 10.5) [9, 10]. The discovery of these bioactive compounds provides evidence that organisms from such environments are also capable of producing antibiotic-type compounds[9, 10].

The use of alkaliphilic bacteria in bioremediation has been investigated. Uptake of heavy metals, such as Cd, Cr, Fe, Pb, Co and Zn, has been reported by *Bacillus* species [11]. Binding of metal cations on the outer surface of bacterial cells has become one of the most attractive means for bioremediation of industrial wastes and other metal polluted environment [12, 13, 14].

It was thus aimed in the present work to isolate alkaliphilic bacteria from two local sites ; Bani Salama Lake (Wadi El-Natrun) representing a natural alkaline ecosystem and Abu Qir Bay as a man made ecosystem. The potentiality of selected strains to produce extracellular enzymes and antmicrobial agents as well as their efficacy to remove metal ions was investigated.

MATERIALS AND METHODS

Growth media

The composition of the media used throughout the work is described below in g/l unless otherwise stated. All media were prepared with distilled water and sterilized by autoclaving at 121°C for 20 minutes.

Laury broth medium (LB)15: was used for maintenance of bacteria. It contained: peptone, 10; yeast extract, 5; and sodium chloride, 5; bacteriological agar, 20; pH value 10.5 ± 0.2.

Horikoshi-I media[16]: was used for isolation of alkaliphilic bacteria. It contained: glucose, 10; yeast extract, 5; peptone, 5; sodium chloride, 15; potassium dihydrogen phosphate, 1; magnesium sulfate heptahydrate, 0.2; and sodium carbonate, 10. Sterile sodium carbonate was added after the sterilization process aseptically (pH value 10.5 ± 0.2). For solid media 15g/l of agar were added.

Sampling sites

Water samples were collected from two sites; Bani Salama Lake (Wadi El-Natrun) representing natural soda lake and Abu Qir Bay characterized by a high input of industrial wastes. Wadi El-Natrun and its alkaline inland saline lakes is an elongated depression, about 90 km northwest of Cairo (Fig.1). The lakes had a pH value of 8.5-11 and salinity ranging from 283 to 540 g/l [17], while the pH of Abu Qir bay was found to be in the range 8.2-8.6. The samples were stored as fast as possible at temperature 4°C transferred immediately to the lab.

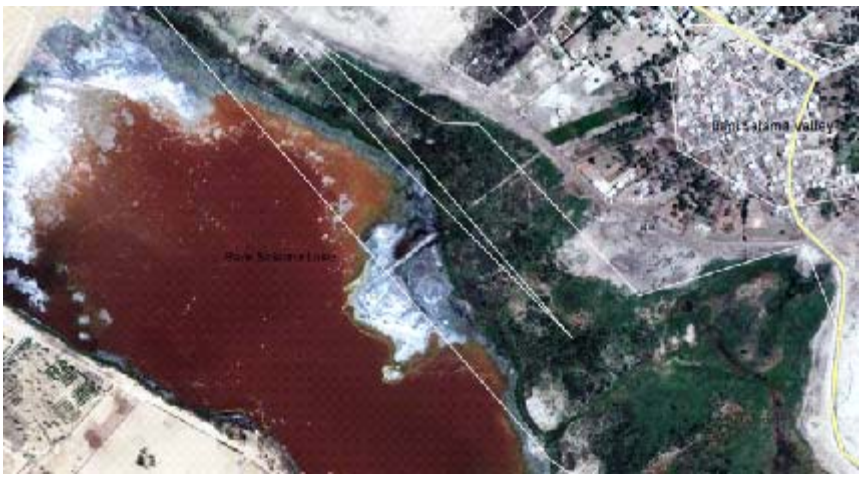

Fig. 1. Location of Bani Salama Lake at Wadi El-Natrun

Isolation and maintainance of alkaliphilic bacteria.

Serial dilutions were made for both sediment and water samples. One ml from each dilution was transferred into sterile Horikoshi-I medium agar plates then incubated at 37°C for 48 h. Morphologically different colonies were purified and maintained on Horikoshi-I medium agar plates applying the code (N) for isolates of Bani Salama Lake (Wadi El-Natrun) and the code (A) for isolates of Abu Qir Bay. All isolates were purified and stored at 4°C with regular monthly transfer.

Enzymatic profile of bacterial isolates.

The ability of bacterial isolates to produce extracellular degradative enzymes was examined on Horikoshi-I medium agar plates. The plates were amended with either starch, skimmed milk, galactomannan, pectin, xylan or carboxymethylcellulose at 4g/l to detect the production of amylase, protease, mannanase, pectinase, xylanase or cellulose, respectively. Formation of halo zone around the colonies, resulting from polymer hydrolysis, was taken as evidence of hydrolytic activity [18]. Enzymatic activity was calculated as the ratio of the diameter of the clearing zone to the diameter of the colony.

Phenotypic identification of bacterial isolates.

Cell morphology was studied by phase-contrast microscopy. Gram staining was performed according to Murray *et al,* (1994)[19]. Physiological and biochemical tests were determined and compared to phenotypic data of known organisms described in the Bergey's Manual of Systematic Bacteriology [20, 21].

Bacterial isolates were tested for growth at different pH values (5-13) and temperature degrees (20, 25, 35, 45, 55 and 60°C). The inoculum size of 0.1 ml (approximate 108 CFU/ml) was aseptically transferred to 25 ml of Horikoshi-I broth for each test. The optical density at the wavelength of 550 nm was used for evaluating bacterial growth.

The susceptibility of the two selected bacterial isolates to 11 clinically relevant antibiotics in Egypt was evaluated using commercial antibiotic discs. The disc diffusion method described by Kirby *et al* (1959)[22], was employed. Inocula were taken from a freshly prepared culture (18-24 h) then incubated at 37°C for 18 h.

Catalase test was applied using 3% hydrogen peroxide. Oxidase test was also applied using filter paper (Whatman no. 40) wetted with 0.5 ml of 1% dimethyl-p-phenylenediamine dihydrochloride.

Molecular characterization

Molecular characterization of the two isolates was done by 16S rDNA sequence analysis. DNA was isolated from cells using standard procedures [23]. The purity of the isolated DNA was confirmed by gel electrophoresis. Amplification of 16S rDNA gene was performed using F 5'AGAGTTTGATCMTGGCTCAG3' and R 5'TACGGYTACCTTGTTACGACTT3' as forward and reverse primers. The PCR amplification products were analyzed by electrophoresis on a 1% agarose gel and purified. Each amplified product of 16S rDNA was sequenced using an ABI PRISM 377 DNA Sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing (Perkin Elmer). The 16S rDNA sequence was uploaded to NCBI database using BLASTN program (http://www.ncbi.nlm.nih.gov/blast/; version 2.0) and compared with sequences available in the GenBank database. Sequences of most close members were aligned using CLUSTALW program (http://www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed using the phylogeny inference package (PHYLIP, version 3.6).

Antagonistic activity against some indicator microorganisms

Production of bioactive substances was tested against *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Candida albicans* using the agar diffusion method according to Kirby-Bauer bioassay technique [24]. Pathogens were kindly provided by the Botany and Microbiology Department, Faculty of Science, Alexandria University. Nutrient agar plates seeded with pathogens were spot inoculated with each of the bacterial strains and incubated at 37°C for 24-48h. The inhibition zones formed were measured in mm.

Bioremediation of heavy metals

Preparation of heavy metal solutions

Metal cations were used as sulphate salts. Heavy metal solutions of 0.05, 0.2 and 0.4 mM concentrations of copper, cobalt and lead were prepared by adding the equivalent weight for each metal in definite volume of dionized water, adjusting pH using NaOH (0.01N) or HCl (0.01N) and sterilization by filtration [25].

Metal uptake experiments

Bacterial cultures (24-48h) grown in medium broth at pH 9 were harvested, washed twice with distilled water, and 0.05g dry weight was re-suspended in 250 ml conical flasks each containing 100 ml of different metal solution (0.05, 0.2 and 0.4 mM for copper, cobalt and lead, respectively). The flasks were incubated on orbital shaker at 37ºC and 180 rpm for 2 h. Thereafter, the metal solutions were centrifuged for 15 min at 5000 rpm. The supernatant was used to determine the concentration of residual metal using atomic absorption spectrophotometry [25],[26].

$$\% \text{ uptake} = \frac{\text{Initial metal concentration} - \text{Final metal concentration}}{\text{Initial metal concentration}} \times 100$$

RESULTS AND DISSCUSION

Isolation of bacterial strains

Alkaliphilic bacteria are found in natural and man-made alkaline environment [3, 27, 28 29]. In this study, seventy nine bacterial isolates were obtained on Horikoshi-I media, pH 10. Samples from Abu Qir Bay yielded 46 isolates representing 58.23% of the total number. This percentage was higher than that obtained from Bani Salama Lake (Wadi El-Natrun) samples which resulted in 33 isolates (41.77%) (Fig.2). The isolates grew well at pH 10 and poorly at pH 7 and at temperature up to 37°C. Based on definition of Horikoshi (1999a)[3], who applied the term alkalophile for microorganisms that grow optimally or very well at pH values above 9.0, but can not grow or only grow slowly at neutral pH values, the isolates were then characterized as alkaliphilic.

All isolates were Gram positive long and short rods, except for one isolate (N12) which was coccus in shape. Endospores were observed in all cells, except for three short rods (A11, N3, N11) and the coccoid (N12).

Production of extracellular hydrolytic enzymes

The majority of isolates (58.7%) expressed significant enzyme activity when applied on substrates like soluble starch, mannnan, pectin, cellulose, xylan, and casein. Based on the screening experiment, 10 isolates (A2, A6, A12, A14, A19, A25, A26, A40, N1 and N23) were selected to define enzyme production. Selection was based on their potentiality to produce more than one enzyme. Data in Fig. 3 depict that the variation of enzymatic profile was dependent on bacterial species and nature of substrate. Based on the data obtained, isolate N1 and A26 representing Bani Salama Lake (Wadi El-Natrun) and Abu Qir Bay respectively, were selected for identification .

Alkaliphilic enzymes are of great industrial importance [30]. They have been isolated from different habitats and from different organisms [30]. Several studies reported the production of hydrolytic enzymes from alkaliphilic bacteria. Ibrahim *et al* (2007)[5] studied the isolation and identification of alkaline protease producing alkaliphilic bacteria from an Egyptian Soda Lake (Wadi El-Natrun). Abou-Elela *et al* (2011)[7] detected alkaline protease production by alkaliphilic marine *Bacillus cereus* isolated from Marsa-Matrouh. Alkaline β-mannanase production by alkaliphilic *Bacillus* sp. N16-5 isolated from sediment of Wudunur Soda Lake in Inner Mongolia, China was published by Lin *et al* (2007)[31]. Mannan, glucomannan, galactomannan, and

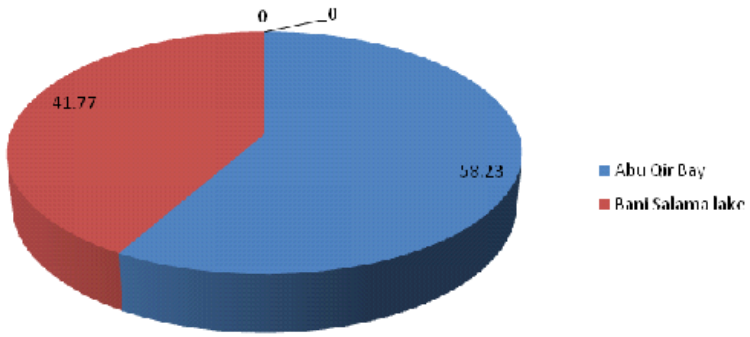

Fig.2. Pie chart representing the percentage of bacterial isolates recovered on Horikoshi-I agar plates (pH 10) inoculated from two alkaline samples (Abu Qir Bay and Bani Salama Lake)

galactoglucomannan are the major polysaccharides that constitute hemicellulose. The hemicelluloses are the second richest renewable energy substances on earth [32].

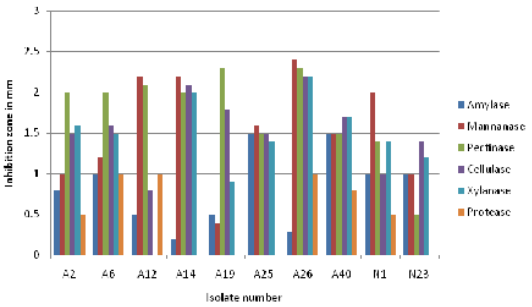

Fig.3. Enzymatic profiles of selected bacterial isolates

Extracellular enzymes like amylase, lipase, protease and cellulases produced by *Bacillus cereus, B.firmus, Enterococcus caseliflavus, B.fusiformis, B.cohnii, B.horikoshii* were isolated by Tambekar and Tambekar (2012)[33] from water and sediment of alkaline Lonar Lake located in the Buldhana district of Maharashtra State, India.

Identification of two bacterial isolates N1 and A26. Phenotypic characterization

Phenotypic characterization which is complementary to genotypic analysis, includes mainly colony and cell morphology, pigmentation, biochemical and physiological tests and others. Table.1 summarizes some of these characters for the two strains N1 and A26. When grown on Horikoshi-I agar medium N1 produced white large sphere colonies with dull surface and undulate margins. Cells were Gram positive rod-shaped forming endospores. Positive for oxidase and catalase tests. Capable of degrading casein, xylan and pectin, cellulose, soluble starch and mannan. On the other hand, A26 produced moist, flat and white colonies with irregular configuration. Cells were Gram positive rod-shaped with endospore. Oxidase and catalase positive, degrades casein, xylan, pectin, cellulose, mannan and to some extent soluble starch.

Regarding their Gram staining reaction and the presence of endospores, one could state that both strains belong to genus *Bacillus*.

Antibiotic sensitivity is shown in Fig. 4.a & 4.b. The degree of resistance and sensitivity

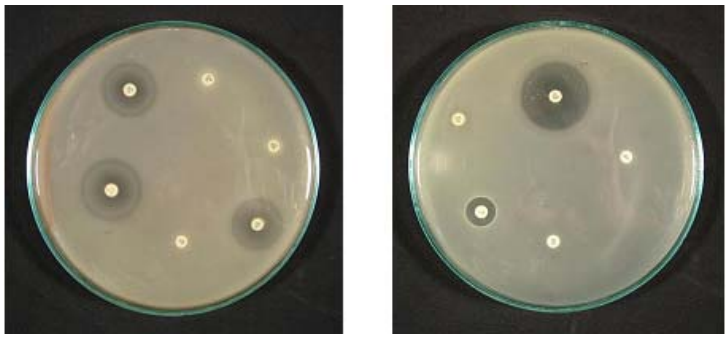

Fig. 4.a. Antibiotic sensitivity test of isolate A26.

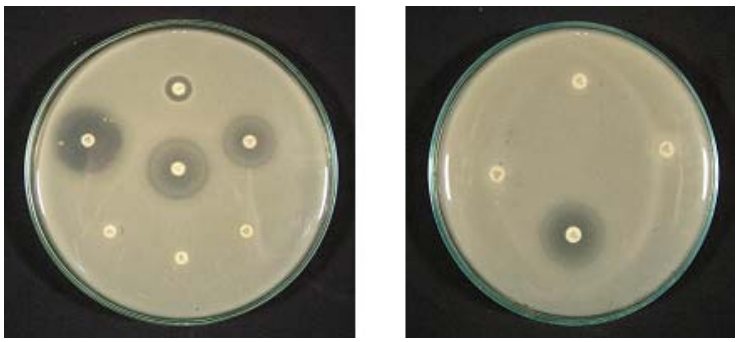

Fig. 4.b. Antibiotic sensitivity test of isolate N1.

Table 1. Morphological and physiological characteristics of the selected strains

Characteristic	N1	A26
Colony color	White	Milky white
Colony margin	Irregular	Irregular
Colony elevation	Convex	Flat
Colony configuration	Lobate	Moist
Gram stain	Positive	Positive
Presence of Endospore	Positive	Positive
Cell shape	Rod-shaped	Rod- shaped
Physiological characteristics		
Optimum pH	9-10	8
Optimum temperature	45°C	37°C
Amylase	-ve	+ve
Catalase	+ve	+ve
Oxidase	+ve	+ve
Pectinase	+ve	+ve
Xylanase	+ve	+ve
Protease	+ve	+ve
Cellulase	-ve	+ve
Mannanase	+ve	+ve
Antibiotics		
Gentamicin CN10	S	S
Ciprofloxacin CIP5	S	S
Imipenem IPM10	S	S
Eryrromycin E15	S	S
Flucloxacillin FL5	R	R
Norfloxacin NOR10	S	S
Ampicillin / Sulbactam SAM20	R	R
Ceftazidime CAZ30	R	R
Ampicillin AM10	R	R
Cephalerxin CL30	R	R
Cefadroxil CFR30	R	R

of these bacteria to antibiotics differs. It was found that both of two strains were sensitive to about 45.5% of the antibiotics mentioned in the result and were resistant to about 54.5 % of the antibiotics used.

The genus *Bacillus* comprises a heterogenous group of chemo-organotrophic, aerobic and rod-shaped microorganisms, including mesophilic and thermophilic species as well as acidophiles and alkalophiles. They are known as commercial sources of a number of enzymes [34, 35].

Ibrahim *et al* (2012a)[2] was able to isolate alkaliphilic *Amphibacillus sp*. NPST-10 from sediment and water samples collected from hyper saline soda lakes in Wadi El-Natrun. Jameel and Mohd (2011)[36] stated that alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, and potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources [37].

Tseng *et al* (2002)[27] reported the isolation of an alkaliphilic *Bacillus ûrmus* from a waste water treatment plant of a pulp and paper manufacturer in Bang-Pre, Thailand. The alkaline conditions were prevailing in the sea water of the study area (Abu-Qir Bay) near Rakta Company so that the detection of alkaliphiles was reasonable.

Vargas *et al* (2005)[38], succeded in isolating a new species of *Bacillus bogoriensis*, a novel alkaliphilic, halotolerant bacterium from Bogoria soda lake in Kenya. Whereas, Borsodi *et al* (2008)[39] characterized three alkaliphilic and moderately halophilic strains designated K1-5T, K1-10 and B1-

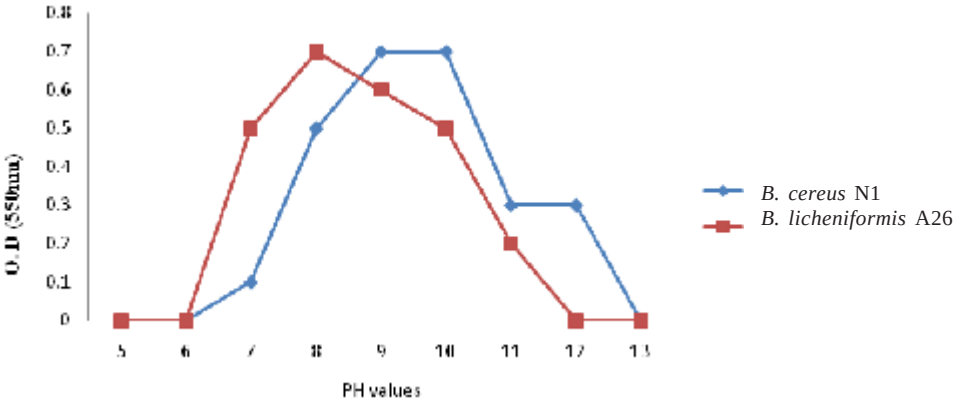

Fig. 5. Effect of different pH values on bacterial growth of N1 and A26

1, characterized by optimal growth at pH 9.0-10.0 and at 3-7 % (w/v) NaCl, isolated from extremely shallow, alkaline soda lakes located in Hungary.

Optimum pH and temperature

Both strains showed no growth in acidic medium i.e. below pH 7. Poor growth was observed at pH 7 for N1 and increased with the increase in pH reaching a maximum value (OD 0.7 at pH 9 and 10). On the other hand, isolate A26 grew well at pH 7 and showed mximal OD (0.7) at pH 8 **(Fig.5).** We can thus safely conclude that N1 is an alkaliphile whereas A26 is an alkalitolerant.

Khalil (2011)[40], who isolated three thermophilic bacterial strains, identified as *Bacillus* sp, *Brevibacillus borstelenesis and Deinococus geothermals* from Al-Khoba and Al-Arida hot springs in Saudi Arabia found that the three isolates were either alkalitolerant (showed very good to excellent growth from pH 7 to 9, but no growth at pH11) or alkalophilic (showed very good and excellent growth between pH 7 and 11), while they showed less cellular yield at pH 6.

Dodia *et al* (2006)[41] isolated and characterized eight moderately halophilic-alkaliphilic bacteria from a saline habitat in western India. They grew in presence of 20% (w/v) NaCl and pH 10, with optimum pH for of 9.

The two strains of the present study grew well at temperature range from 25-50ºC with optimum recorded as 45 ºC and 37 ºC for N1 and A26, respectively.

Asoodeh and Lagzian (2012)[42], isolated thermophilic alkaliphilic *Bacillus subtilis* DR8806 from a hot mineral spring in Iran. They reported that the optimum temperature for growth was 60p C, while an alkaline pH range around pH 9 is preferred for the organism growth.

Phylogenetic analysis

Over the years, a data base of 16S rRNA gene has been constructed and it was successfully used in the differentiation of bacteria [43]. Genotypic identification emerged as a complement to established phenotypic methods. Typically, genotypic identification of bacteria involves the use of conserved sequences within phylogenetically informative genetic targets, such as the small-subunit (16S) rRNA gene [44]. The bacterial ribosomal operon has been used as a genetic marker to study the evolution and phylogeny of microorganisms [45, 46].

Table 2. Shows GenBank accession numbers of 16S rRNA gene partial sequences of the two isolates N1 and A26, the highest sequence similarity as well as the closest neighbour(s).

Table 2. Accession number and similarity percent to the nearest neighbors of the tested isolates

Isolate	GenBank accession number	Similarity	Nearest neighbor(s)
N1	KF164288	99%	*Bacillus cereus* CM100B
A26	KF164289	97%	*Bacillus licheniformis* UY138

Nucleotide sequences of the N1and A26 were affiliated according to their 16S rDNA gene sequences to members of genus *Bacillus*. N1 showed the highest sequence homology (99%) to *Bacillus cereus* strain CM100B (Fig.6.a) and was designated as *Bacillus cereus* N1. Whereas, 16S r DNA sequence of A26 showed a similarity of 97% to *Bacillus licheniformis* strain UY138 (Fig.6.b)

Antimicrobial activity

Screening for new antibiotics from natural sources is becoming increasingly important for the pharmaceutical industry as pathogenic bacteria are quickly becoming resistant to commonly used therapeutic agents [47]. Secondary metabolites from microorganisms have a diverse chemical structure and biological activities and are produced only by some species of the genus *Bacillus*. Marine environment is a source of novel secondary metabolites [48].

The antibiotics produced by *Bacillus* species are more effective against Gram-positive organisms, however, compounds such as polymyxin, colistin, and circulin exhibit activity against Gram negative organisms. Lipopeptides produced by *Bacillus* also demonstrate anti-fungal activity [49].

As shown in Fig. 7, *B. cereus* N1 showed antimicrobial activity against two tested pathogens *Klebsiella pneumonia* and *Candida albicans* with inhibitory zones of 4 and 15 mm in diameter,

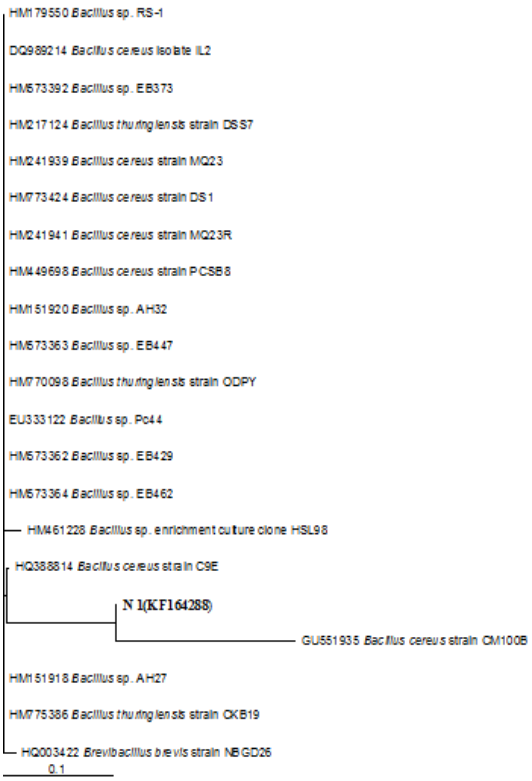

Fig.6.a.16S rDNA-based dendogram showing the phylogenetic position of isolate N1

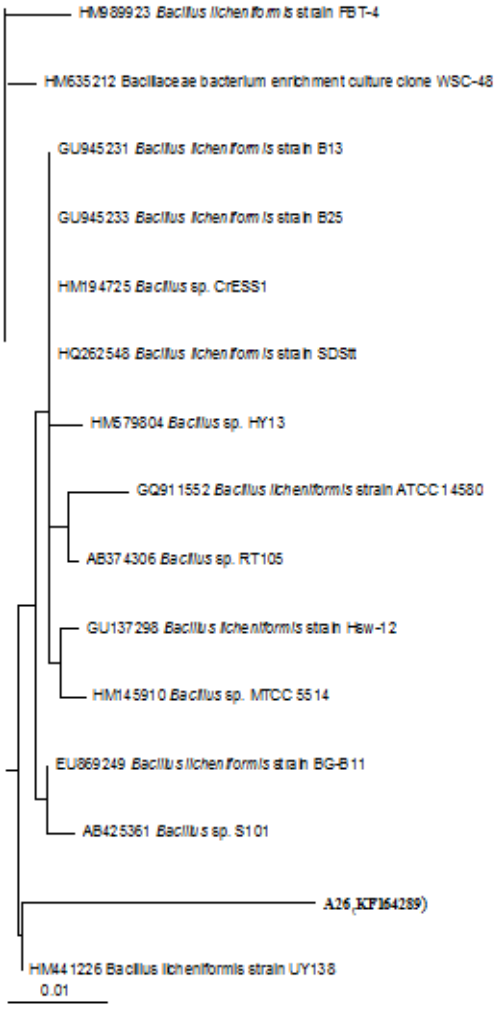

Fig.6.b. 16S rDNA-based dendogram showing phylogenetic position of isolate A26

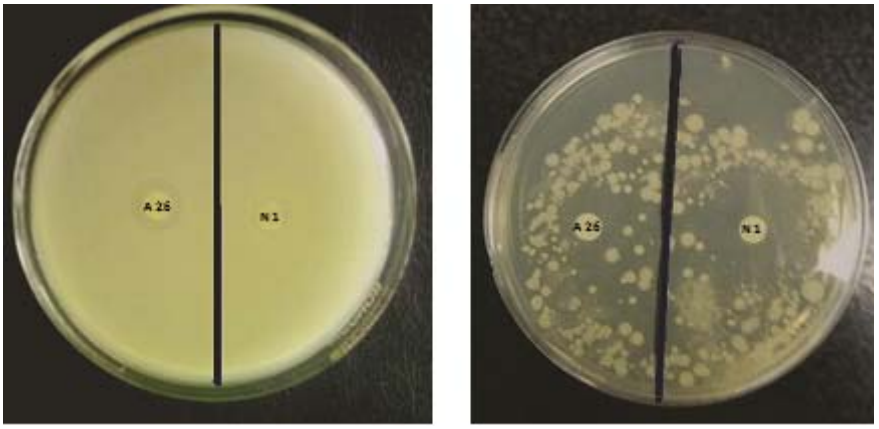

Fig. 7. Antimicrobial activity of *B.licheniformis* A26 and *B.cereus* N1 against *Staphylococcus aureus* and *Candida albicans*, respectively

respectively. However, *B. licheniformis* A26 showed antimicrobial activity against three tested pathogens *Klebsiella pneumonia*, *Staphylococcus aureus* and *Candida albicans* with inhibitory zones of 4, 4 and 10 mm in diameter, respectively

Al-Ajlani and Hasnain (2010) [49] isolated *Bacillu*s species from 6 different soil samples from different parts of Pakistan. The selected strains were identified as *Bacillus subtilis*, *Bacillus amyloliquefaciens, Bacillus cereus,* and *Bacillus licheniformis*. Identified strains showed interesting biological activities e.g. inhibiting the growth of clinical isolates (*Klebsilla* species), strong antifungal and anti-algal activities.

Bioremediation of heavy metals

One of the currently most important environmental problems is the global contamination of the environment by toxic substances. Heavy metals are among the most alien substances found in water and air, representing potential hazard of the contamination of aquifers and food chain, with subsequent impact on human health [13, 50].

Applying remediation procedures using biological systems at redevelopment of soils and waters contaminated with toxic metals appeared as perspective economic and ecologic alternative of physical-chemicl technologies. Advantage of this access is especially low price, minimal amount of secondary wastes and simulateously minimal disturbance of environment [51, 52].

Therefore, it was aimed in this part of the work to evaluate the heavy metals bioaccumulation ability for the two bacterial isolates.The amount of metals taken up by the cells was determined according to Nakajima and Sakaguchi (1986)[53]. It could be noticed that *Bacillus cereus* N1 had high ability to accumulate lead, cobalt and copper cations at 0.4 mM concentration. On the other hand, it was noticed that *Bacillus licheniformis* A26 had high ability to bioaccumulate copper and cobalt cations at 0.2 and 0.4 mM, respectively, while it had no ability to accumulate lead at any of the mentioned concentrations (Table 3.a & 3.b).

Table 3.a. Percentages of metal uptake by *B. cereus* N1 using different concentrations of metal cations at 37°C and pH 9

Concentration of metal cation/mM	Cu	Co	Pb
0.05	4.5	3	0.3
0.2	7.4	15	9
0.4	25.4	90.5	92

Table 3.b. Percentages of metal uptake by *B. licheniformis* A26 using different concentrations of metal cations at 37°C and pH 9

Concentration of metal cation/mM	Cu	Co	Pb
0.05	15	2.8	0
0.2	24.5	23	0.7
0.4	64	89	3.5

The use of *Bacillus* species cells for metal removal was previously reported [54]. Also Issazadeh *et al* (2011)[13] reported that *Bacillus licheniformis* accumulated from 0 to 1.1 mol/g biomass of lead, and, from 0.1 to 5.8 mol/g biomass of copper. On the other hand, the uptake of metal by *B.cereus* ranged from 0 to 0.60 mol/g biomass for lead; from 0.1 to 6.1 mol/g biomass for copper, respectively.

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