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Molecular Characterization of Some Novel Marine *Alicyclobacillus* Strains, Capable of Removing Lead from a Heavy Metal Contaminated Sea Spot

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Abstract: Sea water from heavy metal contaminated area in the Mediterranean, was analyzed for its heavy metal contents and their concentrations. It was observed that lead has the highest concentration (0.48 ppm) among the remaining heavy metal concentrations. Four different Gram-positive, rod-shaped and spore forming *Alicyclobacillus* (formally *Bacillus*) isolates were isolated from the same sea spot. Phenotypic characterization of pure cultures were examined for motility, Gram reaction, spore morphology, catalase and oxidase production. Scanning electron micrograph showed that cells of both strains were occurring singly or in short chains. Randomly Amplified Polymorphic DNA (RAPD) analysis showed a great deal of differentiation among the isolates, revealing that each of them has its own DNA fingerprint. A dendrogram showing the genetic similarity among the sea isolates, clustered them into two main groups at 30% of genetic similarity. Partial sequencing of the 16S rDNA of 2, representative isolates revealed that both of them are novel *Alicyclobacillus* strains S2 and S4. The isolates had the ability to remove lead from contaminated solutions. A promising strain, S4, showed a valuable uptake levels, 64 and 65.3% at 0.5 and 0.9 ppm of pb^{2+}, respectively, after only 2 h of exposure to lead. This strain can be later used efficiently for the bioremediation of lead in contaminated water bodies.

Key words: Bioremediation, *Alicyclobacillus*, Lead, RAPD, 16S rDNA

INTRODUCTION

Toxic heavy metals have been released to biosphere through industrial activities and spread into a variety of environments causing a severe threat to the humans, animals and environmental balances. Lead is one of these metals, which causes hazardous effects on humans (Volesky, 1990). It is extremely toxic and can damage the nervous system, kidneys and reproductive system, particularly in children (Sheng *et al*., 2004).

As a result of development in the field of environmental microbiology, recent studies have focused on the use of microbial-based potential biosorbents such as bacteria and fungi. They are capable of removing heavy metals from waste streams (Gadd, 1990; Volesky and Holan, 1995; Kapoor and Viraraghavan, 1995; Pradhan and Rai, 2001; Liu *et al*., 2004). This biological phenomenon defined as biosorption, seems to be a good alternative to the existing methods since it doesn't produce chemical sludge, more efficient, easy to operate and cost effective. Members of genus *Bacillus* have been recognized as biosorbents of lead and their biomasses have been successfully used for lead removal from polluted environment (Pan *et al*., 2007).

Randomly Amplified Polymorphic DNA (RAPD) method (Williams *et al*., 1990), which is an arbitrary primed PCR (AP-PCR) procedure, was used to type a wide range of bacteria (Corney *et al*., 1997). In addition, RAPD technique was used to type species of *Alicyclobacillus* (Yamazaki *et al*., 1997). This technique is relatively simple, easy and has a wide genome coverage and does not require prior sequence information for amplification (Pattanayak *et al*., 2001). Phylogenetic analysis based on a sequence comparison of the 16S rDNA 5`end hyper-variant region (HV region) has been successfully used as an index for the identification or grouping of *Bacillus* species (Goto *et al*., 2000). However, more recent study has subjected 24 strains of *Alicyclobacillus* to a sequence comparison of the HV region, for the phylogenetic analysis (Goto *et al*., 2002). The results emphasized the efficiency of the HV region as a rapid index for identifying or grouping *Alicyclobacillus* species.

In this study, four different spore-forming rods were isolated from a heavy metal polluted sea spot. Genetic diversity among them was elucidated using RAPD analysis. The 5`end of HV region of the 16S rDNA was used as an index for identification of 2 representative

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isolates. Moreover, the ability of these 2 isolates to remove lead from a polluted solution has also been investigated.

MATERIALS AND METHODS

Spore forming bacillus isolation: Sea samples were collected from a heavy metal-polluted spot in the Egyptian Mediterranean Sea, eastern port, March 2007. One milliliter of the sample was suspended in 50 mL of 2*SG sporulation medium (Schaeffer *et al*., 1965), then incubated at 37°C under shaking conditions (170 rpm) for 24 h. Vegetative cells were killed by adding chloroform (1% v/v), vortexing and keeping for 24 h at room temperature. Spores were germinated by platting 0.1 mL of the prepared spore suspensions with and without dilutions, into plats containing solidified 2×SG medium. Isolated colonies were further purified by streaking on the same solid medium.

Phenotypic analysis: Pure cultures were examined for motility, Gram reaction, spore morphology, catalase and oxidase production after plating into solidified 2×SG medium.

Electron microscopy: For Scanning Electron Microscopy (SEM), cells grown in LB, were harvested by mild centrifugation, washed with phosphate buffer and fixed with 2% glutraldehyde followed by 1% osmium tetroxide treatment. After completion of fixation, samples were washed in buffer solution and the washed cells were dehydrated in ascending order of ethanol concentrations. The samples were dried completely in a critical point dryer and finally coated with gold in JEOL-JFG1100 E ion-sputter-coater. The specimens were viewed in JEOL- JSM 5300 scanning electron microscope operated at 20 kV with a beam specimen angle of 45°.

Heavy metal analysis of sea water: The content of heavy metal concentration in sea water was determined by atomic absorption spectrophotometer.

Preparation of Pb^{+2} stock solution: Metal salts used for the batch adsorption experiment were of analytical reagent grade: Pb $(NO_3)_2$ stock metal solutions were made at a level of 1000 mg L^{-1} by dissolving an appropriate amount of individual metal in water with nitric acid. Working standards with a range of metal concentrations were prepared by diluting the stock solution.

Culture conditions and analysis of residual Lead: Inocula from fresh slants of isolating spore forming *Bacillus* were used to initiate preculture at 30°C. At late logarithmic phase of growth (A_{550} = 1), inocula of one mL were transferred and allowed to grow in 50 mL volume of the growth culture medium which has the following composition (g L^{-1}): peptone, 10; yeast extract, 1; KCl, 0.36; $MgSO_4.7H_2O$, 4.75 and NaCl, 10 in 250 mL conical flask at 30°C on a rotary shaker (250 rpm). At A_{550} = 1.0, aliquots (0.2 g mL^{-1}) were harvested by centrifugation for 10 min at 5000 x g and washed with sterile glass distilled water. This biomass were suspended in 10 mL distilled water amended with 0.5, 0.9 and 1.3 ppm pb^{2+} at pH 7.4. These metal solutions were incubated on a rotary shaker at 150 rpm for 2 h, after which the content were centrifuged and the supernatants were analyzed for residual pb^{2+}. Residual pb^{2+} was determined by atomic absorption spectrophotometer, as previously described. Control tube corresponding to each organism using biomass suspended in 10 mL sea water was also tested, in order to compare the efficiency of lead removal from sea water content.

Molecular characterization: DNA preparation and PCR amplification for Randomly Amplified Polymorphic DNA (RAPD):

Bacterial cells were obtained from late exponential cultures, shaken in LB medium at 37°C. Genomic DNA was prepared using the method of Wang *et al*. (2001), then purified using Wizard genomic DNA purification kit (Promiga, Co., USA). For RAPD analysis, six different decameric primers were used. The used primers were synthesized for random amplifications in bacterial species (Pharmacia Biotech., USA). The PCR reaction mixture contained 200 µM of each dNTP, 0.5 µM primers, 20 mM tris-HCl pH 8, 2 mM $MgCl_2$, 50 mM KCl, 5 µL Taq polymerase and about 100 ng of template DNA (Pharmacia Biotech., USA). PCR amplification was performed as following: PCR mixture was incubated for 4 min at 95°C, then subjected to 45 cycles by using the following temperature profile: 94°C for 1 min, 36°C for 1 min and 72°C for 1 min. Samples were then incubated at 72°C for 10 min.

PCR amplification and partial sequencing of the 16S rDNA: Primers used for amplification and sequencing are shown in Table 2. These primers were used to amplify the 16S rDNA gene and to sequence about 300 bp of its 5`end. The PCR reaction mixture contained 200 µM of each dNTP, 0.5 µM primers, 10 mM tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 U Taq polymerase and about 100 ng of template DNA. Amplicons were obtained with a PCR cycling program of 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C for 30 sec and 72°C for 30 sec. At the end, the reaction was incubated at 72°C for 7 min.

Amplicons produced for both RAPD and 16S rDNA amplification were analyzed by electrophoresis and the DNA was detected by UV transilluminator after staining with ethidium bromide. The molecular sizes of the amplified DNA fragments were estimated by comparison with a 100 or 1000 bp ladder DNA.

Data analysis: for partial sequencing of the 16S rDNA, PCR fragments were purified by QIA quick PCR purification reagents (Qiagen), labeled with Big Dye Terminator Cycle Sequencing Kit and DNA sequences were obtained using ABI PRISM 3730 sequencer (Perkin Elmer, Applied Biosystem, USA). Sequencing service provided by Macrogen Company, Seoul, Korea.

Homology search was performed against DDBJ (DNA Data Bank Japan) using Blast program to find the sequences producing significant alignment. Multisequence alignment and molecular phylogeny were performed using ClustalW (distance-based analysis program). The resulted tree topology was evaluated using bootstrap analysis (Felsenstein, 1985) of the neighbor-joining method based on 1000 resamplings.

For RAPD analysis, electrophoretic profiles were compared using total lab program version 1.11 (www.totallab.com). The dendrogram was obtained using paste program version 1.2 (www.paste.com) as recommended by Hoffman and Frodsham (1993).

RESULTS AND DISCUSSION

Sea water which was collected from heavy metal contaminated area was analyzed for its heavy metal contents and their concentrations. Table 1 recorded ten different heavy metal concentrations (ppm) in sea water. It was observed that lead has the highest concentration (0.48 ppm), thus lead was selected for the bioremediation experiment. However, all remaining heavy metal concentrations were in their permissible range as suggested by Archana *et al*. (2005).

Four different *Bacillus* isolates have been isolated from a heavy metal polluted sea spot. The isolates were gram, catalase and oxidase positive, motile and oval spore-forming rods. In addition, they differ among each other regarding colony morphology (data not shown). The 16S rDNA analysis, which has been performed later, showed that the isolates are belonging to genus *Alicyclobacillus* (formally *Bacillus*) (Wisotzkey *et al*., 1992).

Table 1: Concentrations of different metals (ppm) in sea water

Metal	Concentration (ppm)
Pb	0.48
Cr	0.012
Zn	0.020
Ni	0.002
Cd	0.041
Cu	0.043
Fe	0.114
Mn	0.050
Co	0.249
Mg	0.103*1/10000

A representative sort of genetic fingerprint was performed to express the genetic diversity among the isolates. The method of randomly amplified polymorphic DNA (RAPD) has been applied to type a wide range of organisms including bacteria (Busch and Nitschko, 1999; Svensson *et al*., 2004). In this research, RAPD analysis was performed using 6 different decameric primers (Table 2). Each band produced in RAPD reaction was treated as an individual character (present or absent). The profiles obtained were scored and dendrograms were generated based on Unweighted Pair-Group Method using Arithmetic Average (UPGMA). The band patterns and the dendrogram are shown in Fig. 1-3.

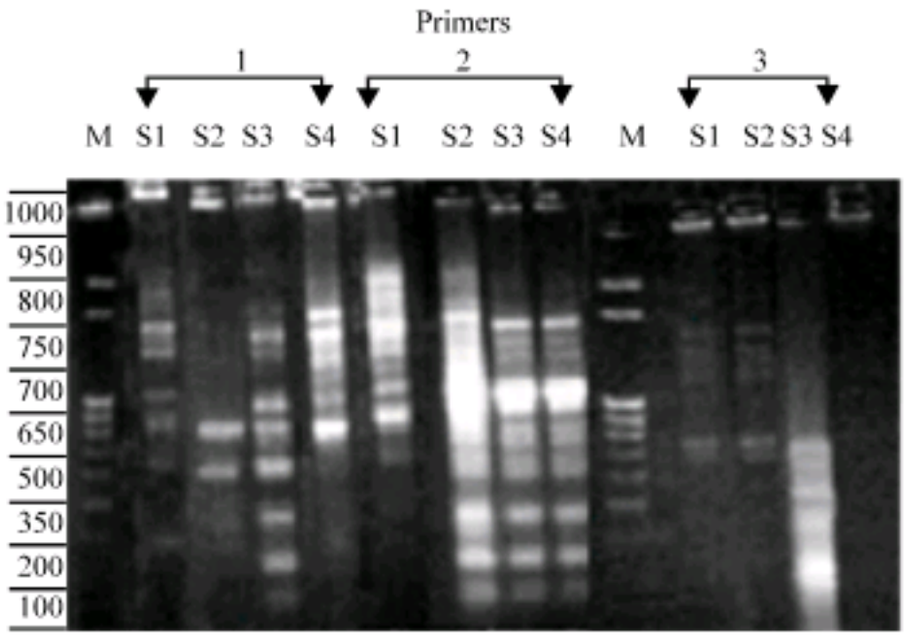

Fig. 1: RAPD analysis of the isolates using primers 1, 2 and 3. M, marker; S1, S2, S3 and S4 are *Alicyclobacillus* sea isolates 1, 2, 3 and 4, respectively.

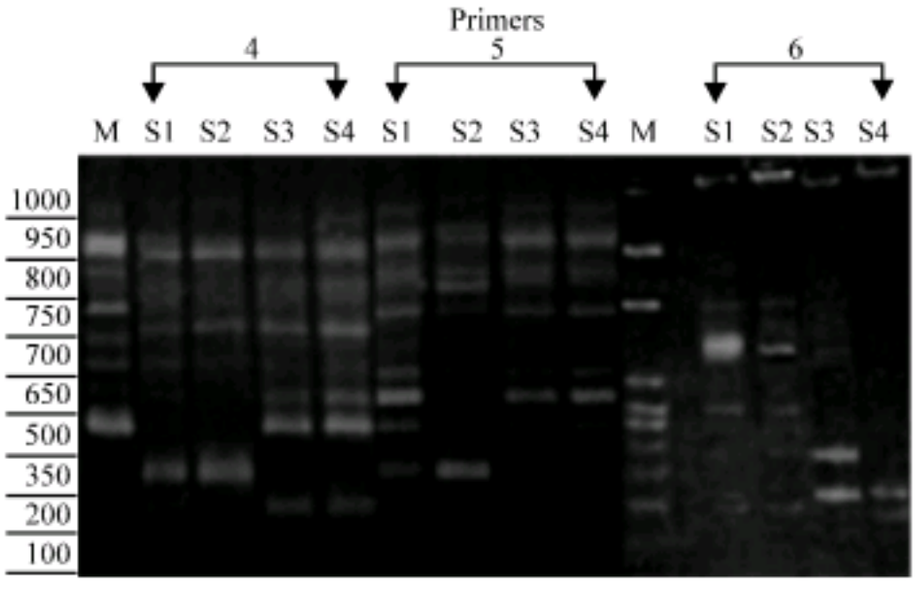

Fig. 2: RAPD analysis of the isolates using primers 4, 5 and 6. M, marker; S1, S2, S3 and S4 are *Alicyclobacillus* sea isolates 1, 2, 3 and 4, respectively

Table 2: Primers used for PCR amplification and sequencing

Primer	Sequence 5` to 3`
1	GTTTCGCTCC
2	AACCTGGCTG
3	GGCTATCGGA
4	TATCCGCGGA
5	AAGAGCCCGT
6	CCCTGGATCA
7	TGTAAAACGACGGCCAGTGCCTAATACATGCAAG TCGAGCG
8	CAGGAAACAGCTATGACCACTGCTGCCTCCCG TAGGAGT

Primers 1, 2, 3 and 4 were used for RAPD analysis. Primers 7 and 8 for the amplification of the 16S rDNA gene

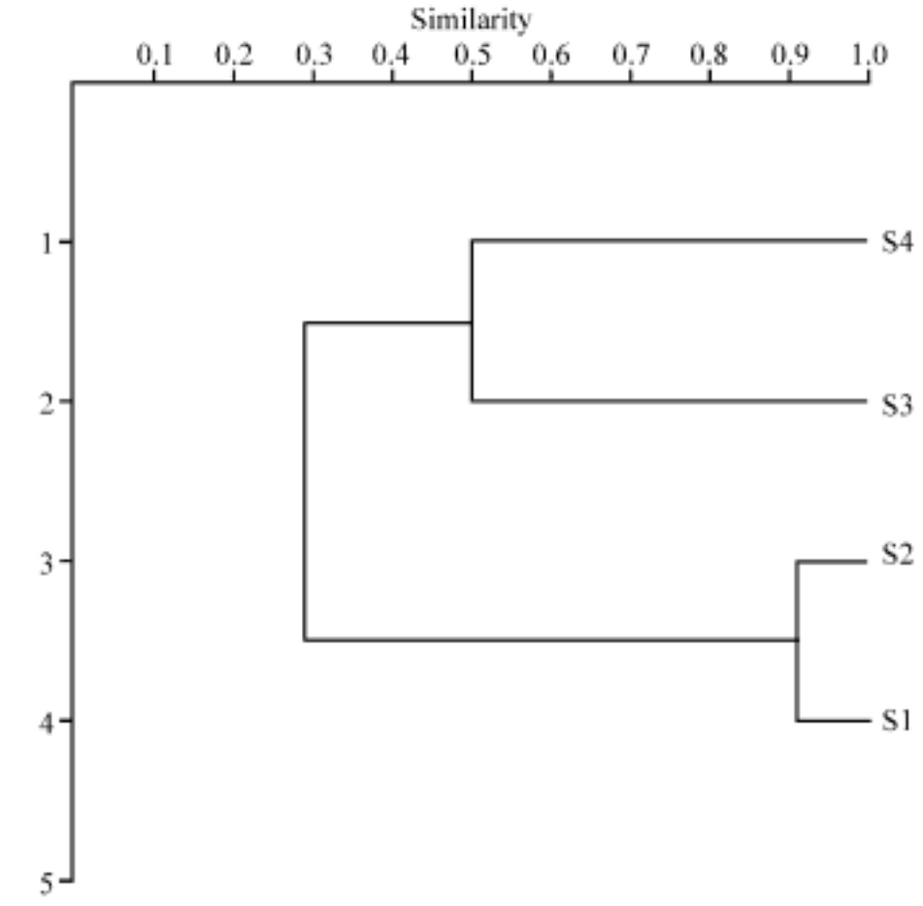

Fig. 3: Dendrogram showing the genetic similarity among the *Alicyclibacillus* isolates based on RAPD data analysis. S1, S2, S3 and S4 are *Alicyclobacillus* sea isolates 1, 2, 3 and 4, respectively

According to these patterns and their analyses, noticeable genetic variations can be observed among the isolates. The total number of bands and polymorphic fragments produced by the primers were 135 and 43, respectively. However, primer 6 showed the highest percentage of polymorphism, although it produced the smallest number of bands (Table 3).

A dendrogram showing the genetic similarity analysis among the *Bacillus* isolates was illustrated in Fig. 3. According to this dendrogram, the isolates were divided into two main clusters at 30% of genetic similarity. Accordingly, the results showed a great deal of differentiation among the *Bacillus* isolates revealing that each one has its own reproducible DNA fingerprint.

RAPD-PCR analysis demonstrated a high genetic heterogeneity in this study and in other previous studies. It has been successfully used to distinguish among

Table 3: Percentages of polymorphism produced by the six used RAPD primers

Primer	Bands total No.	No. of polymorphic bands	Polymorphism (%)
1	22	5	22.7
2	27	10	37.0
3	17	4	23.5
4	24	8	33.3
5	31	10	32.3
6	14	6	42.9
Total	135	43	31.9

Table 4: Similarity matrix of the 16S rDNA partial sequences

Organism	1-18**	L3**	S4*	S2*	NcHUali5**	TK25***
1-18						
L3	97					
S4	97	98				
S2	98	98	98			
NcHUali5	98	99	98	98		
TK25	99	97	98	98	97	

*S2 and S4 are 2 sea *Alicyclobacillus* isolates; **1-18, L3 and NcHUali5 are 3 different species belonging to *Alicyclobacillus*; ***TK25 is *Alicyclobacillus acidoterrestris* strain TK25

species belonging to *Bacillus* (Matarante *et al*., 2004; Konecka *et al*., 2007; Mahadtanapuk *et al*., 2007). Moreover, this technique exhibited a differentiating tool among members of *Alicyclobacillus* (Yamazaki *et al*., 1997).

Partial sequencing (approx. 300 bp) of the 16S rDNA of two representative isolates, S2 and S4, resulted in two different nucleotide sequences, 98% similarity (Table 4).

The sequences were submitted to the GenBank and their accession numbers are EU528673 and EU528674, respectively. The phylogenetic position of these two isolates, based on these sequences, are shown in Fig. 4. S2 and S4 are phylogenetically belonging to *Alicyclobacillus* sp. 18-1 and *Alicyclobacillus* sp. L3, respectively, with a similarity percentage of 98% for each of them.

This and other studies emphasize that the 16S rDNA hyper-variant region is an efficient index for identification and grouping of members belonging to *Bacillus* and *Alicyclobacillus* (Goto *et al*., 2000, 2002).

Scanning electron micrograph showed that Cells of both strains were occurring singly or in short chains Fig. 5. This micrograph shows a normal typical shape of *Bacillus* sp.

De Jaysankar and Vardanyan (2008) have illustrated that, *Alcaligenes faecalis* (seven isolates), *Bacillus pumilus* (three isolates), *Bacillus* sp. (one isolate), *Pseudomonas aeruginosa* (one isolate) and *Brevibacterium iodinium* (one isolate) removed over 70% of Cd and 98% of Pb within 72 and 96 h, respectively from growth medium that had initial metal concentrations of 100 ppm.

In this study, Lead uptake values obtained after 2 h (as recommended by El-Sersy and El-Sharouny, 2007) by only 0.2 g mL^{-1} of *Alicyclobacillus* S2 and S4. were illustrated in Table 5.

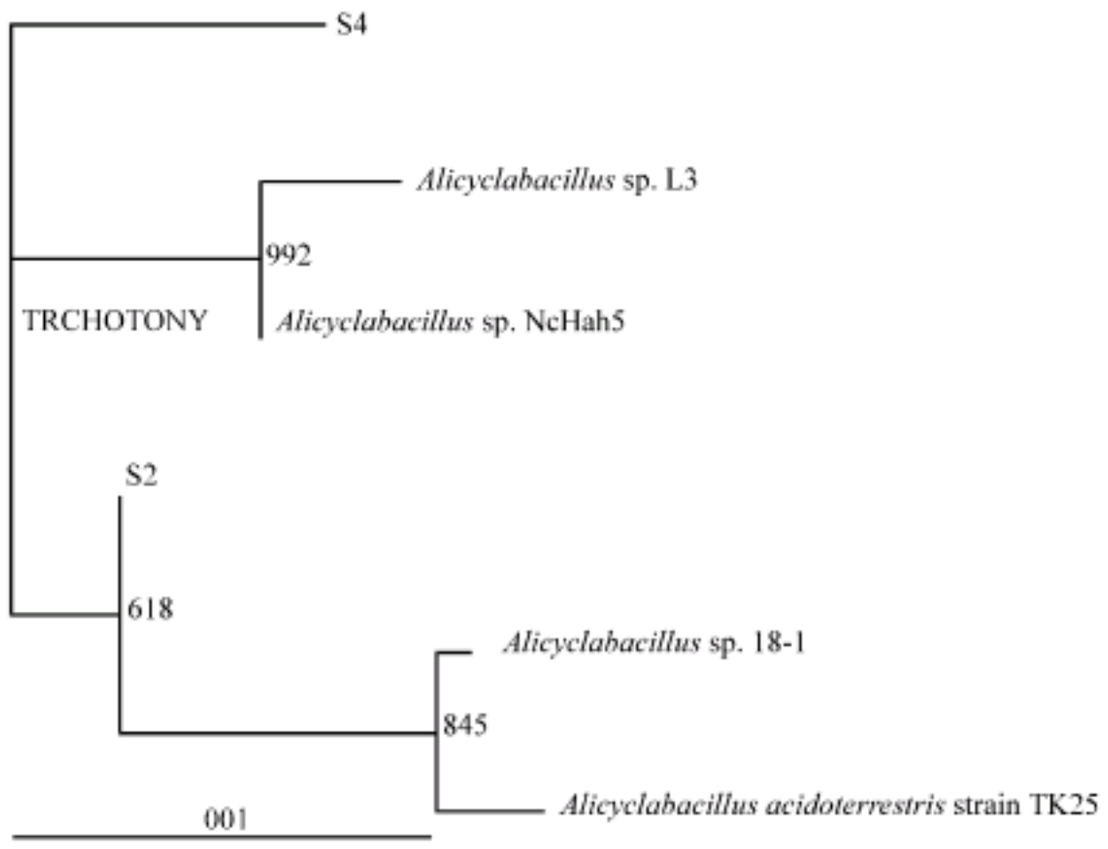

Fig. 4: Phylogenetic position of S2 and S4 *Alicyclobacillus* isolates based on partial sequencing of the 16S rDNA. The scale indicates substitutions per site

Table 5: Lead accumulation by the two novel isolates of *Alicyclobacillus* S2 and S4

Isolated sp.	Pb^{++}initial conc (ppm)	Pb^{++}final conc (ppm)	Uptake (%)
S4	0.5	0.180	64.00
	0.9	0.312	65.30
	1.3	0.540	58.46
S2	0.5	0.250	50.00
	0.9	0.520	42.20
	1.3	0.721	44.50

Results show that isolate S4 is generally more potent in bioaccumulation of lead than S2. The S4 uptake percentages of lead at initial concentrations of 0.5 and 0.9 ppm were higher than that at 1.3 ppm. The biosorption capacity may reach its maximum value at lead concentration of 0.9 ppm then decreases at higher concentrations. These results suggest some form of cooperation with respect to interactions between the metal and the biomass. As such, these results suggest that the initial interaction between the biomass and the metal results in the formation of a layer of metal on the biomass surface. This surface may then become hyper-reactive to other metal ions, resulting in a stacking effect nucleated by the initial layer of metal on the surface of the biomass. This nucleation phenomenon is not unique and has been suggested for uranium biosorption by certain forms of biomass (El-Sharouny and ElSersy, 2005).

Above these equilibrium (optimal) metal concentrations, an observed reduction in metal sequestering efficiency was noticed. One possible reason for this observation, especially for our promising strain, S4, may be that at concentrations of the metal above 0.9

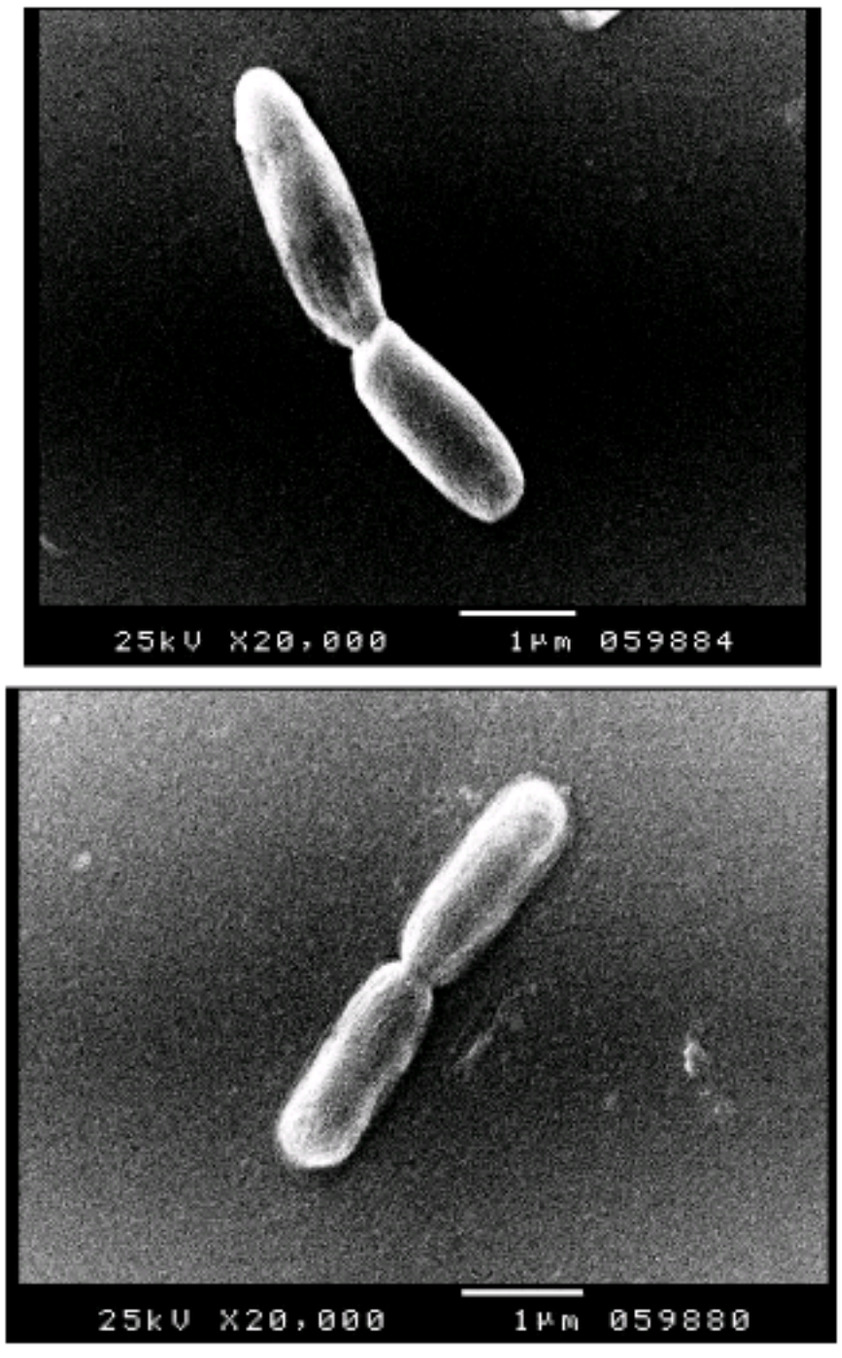

Fig. 5: Electron micrograph showing S2 upper and S4 lower of *Alicyclobacillus* sp.

ppm ion-ion repulsion become prevalent and these interactions may result in decreased affinity of the metal for binding sites on the biomass surface (El-Sharouny and ElSersy, 2005).

Moreover, testing lead removal from sea water using biomass of S4 and S2 isolates resulted in 60 and 54% of lead uptake (from initial concentration of 0.5 ppm) respectively, which reveal a quite near efficiency of lead removal using distilled water amended with metal concentration.

These results indicate that these isolated strains were good potential for application in bioremediation of toxic heavy metals.

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