original article

Molecular genetic identification and metal biosorption by a *Geobacillus* genospecies IRKM1 isolated from Deeymand hot spring, Kerman, Iran

Asieh Dokhani, Mohammad Reza Shakibaie[1], Azita Khosravan[2], Zinat Asgarani[3], Majid Moghbeli

Department of Microbiology, Islamic Azad University, Damghan Branch, Damghan, Iran [1]Department of Microbiology and Environmental Health Research Center, Kerman University of Medical Sciences, Kerman, Iran, [2]Department of Material Sciences and Engineering, International Center for Science, High-Technology, Kerman, Iran, [3]Department of Biology, Faculty Sciences, Alzahra University, Tehran, Iran

Address for correspondence:
Dr. Mohammad Reza Shakibaie,
Kerman University of Medical Sciences,
22-Bahman BLVD, 76167-14111, Kerman, Iran.
E-mail: mohammadreza.shakibaie@gmail.com

Access this article online

Quick Response Code:

Website: www.ijehe.org

DOI: 10.4103/2277-9183.102089

ABSTRACT

Aims: This study deals with molecular identification, susceptibility, and metal biosorption of a *Geobacillus* genospecies isolated from Deeymand hot spring at the south east region of Kerman, Iran.

Materials and Methods: Two samples were collected from water and sediment of the hot spring. Genomic DNA was extracted by sucrose lysis technique and polymerase chain reaction (PCR) was performed using the universal primers corresponding to *Escherichia coli* 16S rRNA gene. Both strands of the PCR product were sequenced by dideoxy chain termination method. The susceptibility of the isolate to Cu^{2+} and Ni^{2+} was determined by broth dilution method. Biosorption of dried biomass for the metal solutions was measured at different time intervals (5–300 min). Effect of temperature on biosorption of Cu^{2+} and Ni^{2+} was also determined.

Results: Temperature of hot spring was 73°C and pH was 8. As result of sequencing of 16S rRNA gene, it was found that the organism had 99.8% similarity with member of genus *Geobacillus*. Phylogenetic tree and neighbor-joining phylogeny revealed that the isolate had 99.22% sequence similarity with *Geobacillus uzenensis* and 99.15% with *Geobacillus jurassicus*. The isolate exhibited minimum inhibitory concentration (MIC) of 4 mM to Cu^{2+} and 5 mM to Ni^{2+}. Minimum biosorption of Ni^{2+} occurred at 5 min (0.07%/0.1 g biomass) and maximum biosorption occurred at 120 min (29.6%/0.1 g biomass), while minimum biosorption of Cu^{2+} ion occurred within 5 min (16.6%/0.1 g biomass) and maximum occurred at 210 min (77.8%/0.1 g biomass) ($P < 0.05$). Biosorption of Ni^{2+} was the highest at 27°C (89.8%) and Cu^{2+} biosorption occurred at 65°C (77.8%). *Geobacillus* genospecies IRKM1 did not carry any plasmid.

Conclusions: The above results showed that the isolate was a member of *Geobacillus* spp. and the thermophilic bacteria was moderately resistant to Cu and Ni metals even though there was not any previous contamination of that biological niche. The organism exhibited highest biosorption of Cu^{2+} at 65°C and Ni^{2+} at 27°C. No plasmid was detected in the *Geobacillus* isolate.

Key words: 16S rDNA sequencing, *Geobacillus*, metal biosorption, minimum inhibitory concentration, polymerase chain reaction, thermophilic bacteria

This article may be cited as:
Dokhani A, Shakibaie MR, Khosravan A, Asgarani Z, Moghbeli M. Molecular genetic identification and metal biosorption by a *Geobacillus* genospecies IRKM1 isolated from Deeymand hot spring, Kerman, Iran. Int J Env Health Eng 2012;1:36.

INTRODUCTION

For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological potential in addition to scientific curiosity.[1]

Since many industrial processes using microbial enzymes run at a high temperature to increase the reaction rates, solubility of most chemicals, fluidity, and diffusion rates, thermopiles offer major advantages for industrial and biotechnological processes.[2,3]

Adsorption of metals by cell wall components is one of the more important interaction mechanisms. Metal biosorption reactions by thermophilic microorganisms may therefore differ quantitatively and qualitatively from those of the mesophilic species that have been studied to date.[4] A wide range of geological and anthropogenic thermal environments exhibit high concentrations of dissolved metals. In response to these conditions, microorganisms isolated from these habitats may have unique cell wall structures. Thus, studies of thermophilic microorganisms can increase our present knowledge of metal biosorption, which is completely based on mesophilic organisms.[5,6]

Over a number of years, the large and diverse grouping of bacteria in the genus *Bacillus* have been progressively subdivided into novel genera and, most recently, *Geobacillus*, which represents a phenotypically and phylogenetically coherent group of thermophilic bacilli with high levels of 16S rRNA sequence similarity (96.5–99.2%).[7,8] The members of this genus are widespread in various thermophilic and mesophilic geographic areas on the earth such as oilfields, hay compost, hydrothermal vent, or soils.[9] At present, the members of this genus *Geobacillus* have growth temperatures ranging from 35 to 78°C.[10,11]

Thermophiles harboring intrinsically stable enzymes are suitable for industrial applications. In addition to higher thermostability, proteins from thermophiles often showed higher stability toward organic solvents and higher activity at elevated temperature.[12] The highest tolerance to cadmium ($CdCl_2$), 400–3200μM, was observed for species belonging to the genus *Geobacillus*.[6] The thermophilic gram-positive bacteria *Geobacillus stearothermophilus* and *Geobacillus thermocatenulatus* were selected for further electrophoretic mobility, potentiometric titration, and Cd adsorption experiments to characterize Cd complexation by functional groups within and on the cell wall.[6]

The optimum isolation rate of thermophiles is achieved by culturing on nutrient agar or broth at high temperature (65°C). The phenotypic characterization of those isolates was confirmed by genotypic method using 16S rDNA sequence analysis. Maximal homology of all eight isolates to the genus *Geobacillus* was observed. Five of these isolates showed greater than 98% homology with *G. stearothermophilus* and one isolate showed 100% homology with *G. thermoglucosidasius*. Therefore, 16S rRNA gene sequence analysis can be considered as a valuable genotypic tool for the identification and characterization of thermophilic bacteria at a genus level.[13] To date, very few studies have examined the sensitivity and resistance of *Geobacillus* genospecies to heavy metals. Essential metals are required nutrients, yet at high levels they can be toxic to microorganisms. Examples of essential nutrient metals are cobalt [Co(II)], copper [Cu(II)], nickel [Ni(II)], and zinc [Zn(II)]. This indicates that organisms possess oxido-reduction systems for metal use and regulation. In some cases, these systems and others appear to mediate resistance to high concentrations of essential and nonessential metals.[14]

Previous studies[15,16] investigated the removal and biosorption of silver and copper by *Acinetobacter* and *Pseudomonas spp*. It was found that the biosorption of Ag^{2+} ion in *Acinetobacter* was mainly an energy-dependent process, while in case of Cu^{2+} it involved precipitation of 40-nm particles on the cell surface.

The aims of this study were: 1) biogeography location and isolation of thermophilic bacteria from Deeymand hot spring, Kerman, Iran; 2) molecular identification of the isolate; 3) determining sensitivity of the isolate to metal ions; and 4) to study biosorption of the Cu and Ni metals by the organism.

MATERIALS AND METHODS

Bacterial source and field work

Two samples were collected from water and sediment of the Deeymand hot spring, located at 160 km southeast of Kerman city, Iran. It is a mountainous region and is not polluted with human effluents such as heavy metals wastes. One liter of water was collected from 15 cm depth of the hot spring in a 1.5-L sterile container. Sediment was transferred into 100 mL Erlenmeyer flask and transported to laboratory within 2.5 h after collection. The sampling site and hot spring location map are demonstrated in Figure 1a and b.

Water sample was centrifuged at 8000 rpm (Napco 2028R) for 15 min and the precipitate was dissolved in 5 mL sterile 0.5 N saline. Similarly, 1 g of sediment was weighted precisely with an electronic balance (Sartorius, GE811); 5 mL sterile normal saline was added to this preparation and shaken well. 0.5 mL of supernatant of both samples was added to 10 mL sterile trypticase soy broth (TSB) medium (Merck, Germany) in screw cap tube and incubated at 70°C for 48 h. The bacterial growth was then inoculated in TSB to which 1.5% agar agar was added, covered with plastic or aluminum foil, and incubated at 70°C and 37°C to check the genuinity of the isolate. Bacterial isolate was then preserved at −70°C in sterile TSB medium containing 40% glycerol for further investigation.

Molecular genetic identification

PCR amplification and 16S rDNA gene sequencing

Genomic DNA of thermophilic bacteria of *Geobacillus* sp. was extracted by sucrose- EDTA-Tris [SET] buffer using sucrose lysis technique containing 20% sucrose, 50 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris-HCl, (pH 7.6).Approximately 1465 nucleotides of 16S rDNA gene fragment were amplified using 5 pmol of the universal primers corresponding to *E. coli* 16S rRNA gene, 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-TACGGTTACCTTGTTACGACT-3´). Polymerase chain reaction (PCR) was performed in a Thermocycler (PeQLaB) using 1 U *Taq* polymerase (Cinnagen, Tehran, Iran). The PCR program comprised initial denaturation temperature 95°C for 4 min, followed by 35 cycles each of 94°C for 1 min, 62°C for 45 s, 72°C for 1 min, 72°C for 10 min, and incubation at 4°C for 5 min. PCR products were purified with DNA extraction kit (Bioneer, Seoul, South Korea). Both strands of the PCR product were sequenced by dideoxy chain termination method.

Determination of minimum inhibitory concentration (MIC) of the metal ions

For MIC determination, initially 100 mL stock solutions containing 2.62 g $NiSO_4.6H_2O$ and 2.49 g $CuSO_4.5H_2O$ (Merck, Germany) in DD/W were prepared in two flasks containing 250 mL TSB medium separately.

The volume of 0.1 mL of O/N growth of isolated bacteria from Deeymand hot spring was then inoculated into nine tubes containing 8.9 mL sterile TSB medium and 1 mL of different concentrations of the metal ions. Solution concentrations were varied from 2 to 10 mM and incubated with agitation (100 rpm) at 65°C for 48 h. MIC was defined as the minimum concentration of the metal ion that inhibited the visible growth (turbidity) of the organism within 48 h.

Metal biosorption

For metal biosorption, initially bacterial isolate was grown in 1 L of sterile TSB medium for 48 h at 70°C. Fifty milliliters of 11 mg $NiSO_4.6H_2O$ and 9.8 mg $CuSO_4.5H_2O$ salt solutions were prepared in sterile 250 mL DD/W Erlenmeyer flask separately to obtain 50 mg/L (50 ppm) of the metal salts. To this preparation, 0.1 g of the dried biomass was added and kept in shaker incubator (Multitron, HNFORCE-AG-CH-4103) at 150 rpm. Immediately, 1.5 mL of the microbial solution was transferred to a sterile Eppendorf microtube and centrifuged at 10,000 rpm for 5 min. One milliliter of the supernatant was transferred to another tube containing 9 mL of DD/W to reach a dilution within standard curve. Similarly, the samples were taken and centrifuged every 5 min for the first 30 min and then at an interval of 30 min till 300 min. Fractions from each specific time were analyzed for the presence of remaining Cu^{2+} and Ni^{2+} by flame atomic absorption spectrophotometer (Philips PU9100X). Experiments were done in triplicate for each metal ion side by side and the average values were used in this study.

Effect of temperature on biosorption of CU and Ni by *Geobacillus* spp. IRKM1

In order to find out the effect of temperature on biosorption of the above heavy metals by the isolated thermophile, 0.1 g dried biomass of the isolated bacteria was carefully weighted and inoculated into 50 mL of the metal solutions containing 50 mg/L of each metal ion. After 5 h incubation at three temperatures (27°C, 45°C, 65°C), biosorption of Cu^{2+} and Ni^{2+} was determined by atomic absorption spectrophotometry as described previously by the methods of Shakibaie *et al.*[16]

Plasmid isolation

Rapid isolation of plasmid was carried out acording to method described by Kado and Lui.[17]

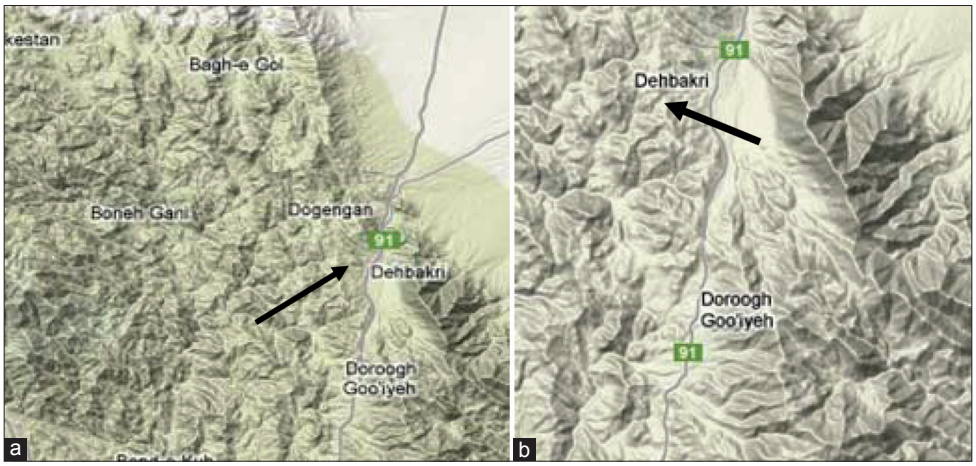

Figure 1: Sampling map and geographic location of the Deeymand hot spring, located at 160 km southeast of Kerman, Iran. a) reperesent the upper most area and b) represent down near the desert plain. The arrow indicates the geographic location of region is at +29°2´8.74″, +57°54´22.28″. It is near Dehbakri mountainous region situated to the southeast of Kerman.The map area was obtained from Google map

Statistical analysis

All analyses were performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). All *P* values were two-tailed; $P \leq 0.05$ was considered statistically significant. Means and standard deviations (SD) were calculated as required for numerical variables.

RESULTS

The geographic map of the region of the Deeymand hot spring is shown in Figure 1a and b. The region is mountainous and situated 160 km southeast of Kerman at +29°2′8.74″, +57°54′22.28″. Since this area is mountainous, it is very difficult to climb; therefore, it is less exposed to contamination by human wastes as compared to the other hot springs.

Temperature of field water was approximately 73°C, while the air temperature was 24°C, and the pH of water was 8.0. The 16S rDNA gene sequence of the isolated *Geobacillus* spp. was compared with those in the NCBI/EZtaxon/Ribosomal Database Project (RPD)/EMBL nucleotide sequence databases using the BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLATS/); a phylogenetic tree and neighbor-joining phylogeny were constructed by using the MEGA soft ware package version 5.0 and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1000 replicates).

It was found by the almost complete 1414 bp 16S rDNA sequencing of the isolated thermophile that it belonged to the genus *Geobacillus* and exhibited highest similarity with the 16S rDNA sequences of *G. uzenensis* U(T) (99.22% sequence similarity) and *G.jurassicus* DS1(T) (99.15% sequence similarity). The phylogenetic tree was constructed by neighbor-joining method [Figure 2]. The isolate had more distance relationship with *G.thermoleovorans* and *G.vulcani* as compared to the above species. It was named as *Geobacillus* genospecies IRKM1.

Since our major concern was to see the overall pattern of sensitivity to metal ions and its significance at a particular site on the biogeographic niche, we decided to perform MIC of Cu and Ni metal ions as shown in Figure 3. The organism exhibited MIC of 5 mM to Ni^{2+} and 4 mM to Cu^{2+} metal ions.

The atomic absorption spectroscopy of dried biomass of the *Geobacillus* genospecies IRKM1 at different time intervals [Figure 4] revealed that for Ni^{2+}, the minimum biosorption occurred at 5 min (0.07%/0.1 g biomass) and reached a maximum at 120 min (29.6%/0.1 g biomass). Beyond that, there was not any change in the biosorption phenomenon, while the minimum biosorption of Cu^{2+} ion occurred within 5 min (16.6%/0.1 g biomass) and reached a maximum at 210 min (77.8%/0.1 g biomass) ($P < 0.05$).

The biosorption of the above metal ions decreased significantly when both Cu^{2+} and Ni^{2+} were added to the medium simultaneously as shown in Figure 5. Similarly, the rate of biosorption of these two metals by dried biomass of *Geobacillus* genospecies IRKM1 was inversely proportional to the temperature of incubation. Maximum biosorption of Ni was achieved at 27°C and Cu at 65°C [Figure 6]. No plasmid was detected from the organism. This indicated that the resistance to Cu^{2+} and Ni^{2+} exerted by *Geobacillus* is not plasmid mediated.

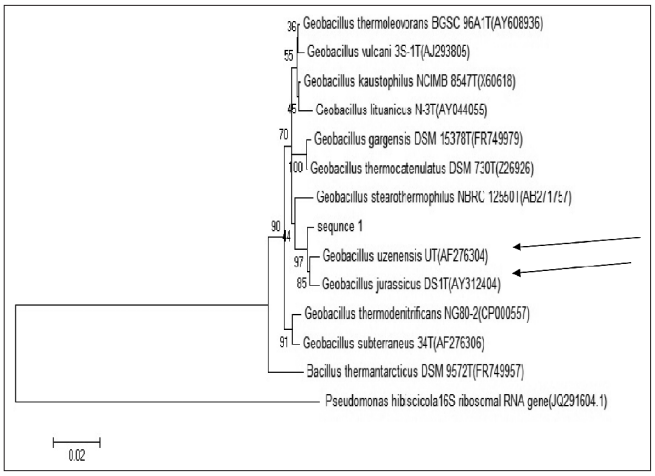

Figure 2: The phylogenetic tree of the genus *Geobacillus* and percentage similarity of each species with our isolate base on 16S rDNA sequencing; values greater than 90% were considered significant. The phylogenetic tree of the genus *Geobacillus* was constructed by neighbor-joining method. Arrows indicates the percent of 16Sr DNA homology between our isolate with two *Geobacillus* genospecies

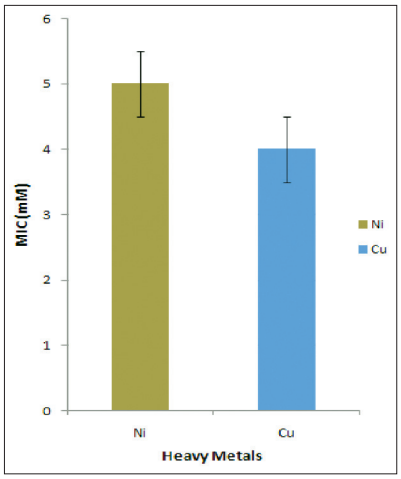

Figure 3: Minimum inhibitory concentrations (MICs) of Cu and Ni heavy metals by *Geobacillus* genospecies IRKM1 isolated from Deeymand hot Spring in Kerman, Iran. For MIC determination, 0.1 mL of O/N growth of isolated bacteria was inoculated into nine tubes containing 8.9 mL sterile TSB medium and 1 mL of different concentrations of both metals (stock solution of 6.28 g $NiSO_4.6H_2O$ and 2.496 g $CuSO_4.5H_2O$/100 mL DD/W) and incubated at 65°C for 48 h

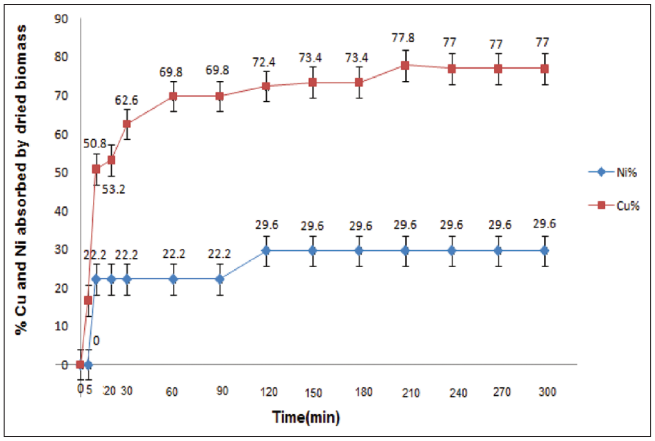

Figure 4: Biosorption of Cu^{2+} and Ni^{2+} by dried biomass of the *Geobacillus* genospecies IRKM1 isolated from Deeymand hot spring, Kerman, Iran. 0.1 g dried biomass was used in this study. The pH of the solution was measured prior to atomic absorption spectroscopy. $P \leq 0.05$ was considered statistically significant. The bar represents average of three simultaneous observations

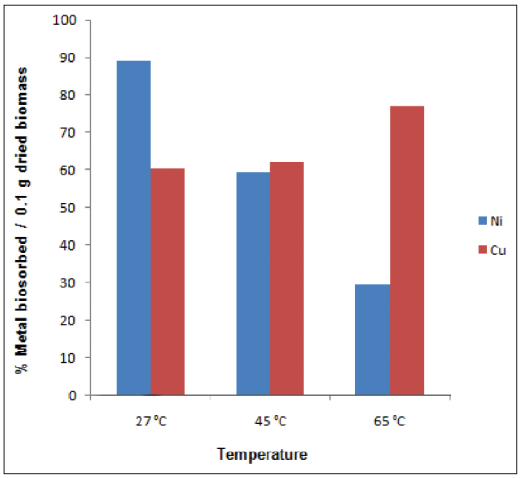

Figure 6: Effect of temperature on biosorption of CU and Ni by *Geobacillus* genospecies IRKM1

DISCUSSION

Several investigations were conducted on the molecular biology and physiology of *Geobacillus*, but a few of them address the metal resistance and biosorption process. In one study by Özdemir *et al*.,[18] the Cd, Cu, Ni, Mn, and Zn resistance and bioaccumulation by thermophilic bacteria, *Geobacillus toebii* subsp. *decanicus* and *G. thermoleovorans* subsp. *stromboliensis* was studied. It was found that the highest metal bioaccumulation was performed by *G. toebii* subsp. *decanicus* for Zn (36.496 mg/g dry weight cell) and the lowest metal bioaccumulation was performed by *G. toebii* subsp. *decanicus* for Ni (660.3 mg/g dry weight cell). In the presence of 7.32 mg/L Cd concentration, the levels of Cd absorbed in live and dead cell membranes were found to be 17.44 and 46.2 mg/g, respectively.

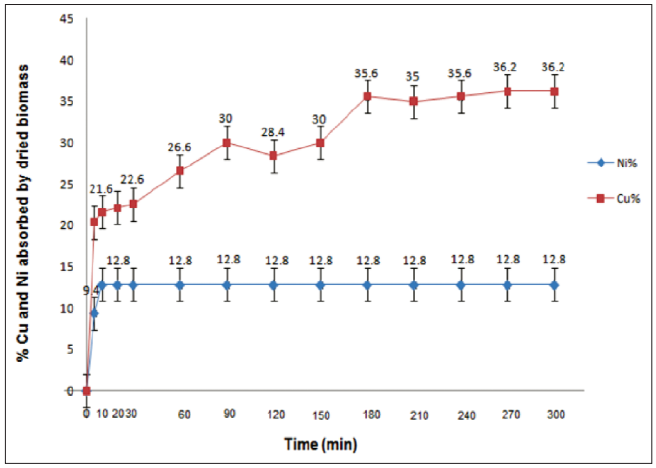

Figure 5: Biosorption of Cu^{+2} and Ni^{+2} by 0.1 g dried biomass of the *Geobacillus* genospecies IRKM1 when both the cations were added simultaneously. The bar represents average of three simultaneous observations

An Iranian native strain of thermophilic *Geobacillus* sp. MKK isolated from a hot spring (pH: 6 and 55°C temperature) at Ramsar – already shown to produce a thermostable DNA polymerase capable of being used in PCR – has been studied and the conditions for its optimized growth with the purpose of obtaining highest bacterial biomass and therefore higher amounts of the desired DNA polymerase production have been determined.[19]

In another study, two proteolytic thermophilic aerobic bacterial strains, PA-9 and PA-5, were isolated from Buranga hot springs in western Uganda. Isolate PA-9 grew at a temperature between 38°C and 68°C (optimum 62°C) and PA-5 grew between 37°C and 72°C (optimum 60°C). Both isolates grew optimally at pH 7.5–8.5. Their 16S rRNA gene sequences indicated that they belong to the newly described genus *Geobacillus*.[20]

The Deeymand hot spring is situated in mountainous area of Kerman city, southeast Iran. At winter it is cold with temperature ranging from 7°C to 10°C and in summer the temperature ranges from 20°C to 25°C. The area is less available to human; therefore, the thermophilic bacteria that are residents of this hot spring must be less susceptible to heavy metals like copper and nickel. To assess the kind of the genus and species of the thermophile and its susceptibility to the metal ions, we performed molecular identification and metal biosorption by a thermophilic bacteria isolated from Deeymand hot spring, Kerman, Iran, using 16S rDNA sequencing.

At the species level, *Geobacillus* isolate had much lower nucleotide diversities with *G. uzenensis* and *G. jurassicus*. With analysis of molecular variance (AMOVA) it was found that there was relatively little sequence resemblance with other *Geobacillus* species. We found much greater genetic

variation in the regions 135 and 254 of 16S rDNA with other species of *Geobacillus* like *G. thermoleovorans* and *G.lituanicus*.

Like other living organisms, microorganisms showed sensitivity to metal pollution.[21,22] Although very low levels of several metals are essential, microorganisms show cation uptake often at concentrations high enough to be detrimental to them.[23] However, some bacteria seem to have adapted themselves to deal with the toxicity of increased concentrations of heavy metals.[24]

In one study, the metal binding capacity of the thermophilic bacteria *G. thermodenitrificans* isolated from Damodar river, India, was assessed using synthetic metal solutions and industrial waste water.[25] Biosorption preference of dead biomass of *G. thermodenitrificans* for the synthetic metal solutions was in the following order: $Co^{+2} > Cu^{+2} > Zn^{+2} > Cd^{+2} > Ag^{+} > Pb^{+2}$. It reduced the concentration of Fe^{2+} (91.31%), Cr^{2+} (80.80%), Co^{+2} (79.71%), Cu^{+2} (57.14%), Zn^{+2} (55.14%), Cd^{+2} (49.02%), Ag^{+} (43.25%), and Pb^{+2} (36.86%) at different optimum pH values within 720 min.

The thermodynamic stability constant *K* for cadmium ion binding was not as high for the *Geobacillus* strains as for the other bacteria described in the literature.[26] A recent study conducted at the University of Waikato evaluated surface complexation models (SCMs) in quantifying metal ion adsorption by activated sludge.[27] It was found that the maximum adsorption capacity drops by 18.3% as the temperature increases from 25°C to 45°C.

In our study, we observed that maximum biosorption of both the metal ions occurred at 120 and 210 min. This indicates that biomass biosorbed the heavy metals actively during the specific period of time, and beyond that the biosorption was stopped after all the surfaces of the biomass were saturated with the above metal ions. Furthermore, the organism exhibited different biosorption pattern of Cu and Ni at different temperatures; in case of Ni, maximum biosorption occurred at 27°C, while in case of Cu it was at 65°C. This suggests that the equilibrium data might not be fitted by Langmuir isotherm equation. In one study by Jabari Nezhad Kermani *et al*.,[28] removal of Cd^{2+} using mutated and wild-type strains of this bacterium was carried out at different time intervals (10–300 min). It was observed that within 60 min, 94.7% of cadmium was removed in 30 mg/L of Cd^{2+} solution. However, with 60 mg/L Cd^{2+}solution, only 53.58% and 38.68% of Cd^{2+} removal was achieved by mutated and wild-type bacteria, respectively. Similarly, biosorption of lead (II) and copper (II) from aqueous solutions by pre-treated biomass of Australian marine algae was studied.[29] Heavy metal resistance of some thermophiles and potential use of a-amylase from *Anoxybacillus amylolyticus* in microbial enzymatic bioassay was also studied by Poli *et al*.[30]

CONCLUSIONS

In conclusion, we isolated thermophilic bacteria belonging to genus Geobacillus from Deeymand hot spring in Kerman, Iran, which was able to grow at 70°C and were identified as genospecies IRKM1. It was moderately resistant to Ni and Cu metals although there was no pervious contact with these metal ions. The organism biosorbed these metals within a specific period of time possibly by surface bonds, and after biosorption it reached a steady state. The important physiological observation in this study was inability of the *Geobacillus* isolate to grow at 37°C. This indicates that protein machinery of the cell was fully adapted to high temperature. Further research must be conducted to study the mechanism of binding of the above heavy metals by this organism and molecular analysis of the genes involved in this process.

ACKNOWLEDGMENTS

Our sincere thanks to authorities of International Center for Science, High-Technology and Environmental Sciences Center, Kerman, Iran, for providing facilities to us to carry out the experiments there and Azad University Damghan branch for help during this research. The authors would like to thank Dr. Shakibaie MR for putting so much effort in this investigation.

REFERENCES

1. Madigan MT, Martinko JM. Brock biology of microorganisms. USA: Pearson Rentice Hall; 2006. p. 151-7.
2. Kristjansson JK, Stetter KO. Thermophilic bacteria. *Thermophilic bacteria*. United States: CRC press; 2000. p. 2-5.
3. Al-Batayneh KM, Jacob H, Emad IH. Isolation and molecular identification of new thermophilic bacterial strain of *Geobacillus pallidus* and *Anoxybacillus flavithermus*. Int J Interg Biolog 2011;11:39-43.
4. Özdemir S, Ersin E, Poli A, Nicolaus B, Güven K. Cd, Cu, Ni, Mn and Zn resistance and bioaccumulation by thermophilic bacteria, Geobacillus toebii subsp. Decanicus and Geobacillus thermoleovorans subsp. Stromboliensis. World J Microbiol Biotechnol 2011;28:155-63.
5. Hussin Akmar N, Asma I, Venugopal B, Yoga Latha L, Sasidharan S. Identification of appropriate sample and culture method for isolation of new thermophilic bacteria from hot spring . Afr J Microbiol Res 2011;5:217-21.
6. Hetzer A, Christopher J, Hugh D, Morgan W. Cadmium Ion Biosorption by the Thermophilic Bacteria Geobacillus stearothermophilus and *G. thermocatenulatus.* Appl Environ Microbiol 2006;72:4020-7.
7. Claus D, Berkeley RC. Genus Bacillus, Cohn 1872, 174AL. In: Sneath PH, editor. Bergey's Manual of Systematic Bacteriology, vol. 2. Baltimore: Williams and Wilkins; 1986. P. 1105-39.
8. Brosius J, Palmer ML, Kennedy PJ, Noller HF. Complete nucleotide sequence of a 16S rRNA gene from *E.coli.* Proc Natl Acad Sci USA 1978;75:4801-5.
9. Nazina TN, Tourova TP, Poltaraus AB, *et al*. Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus* subterraneus gen. nov., sp. nov. and*Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to Geobacillus

as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius and G. thermodenitrificans.* Int J Syst Evol Microbiol 2011;51:433-46.
10. Banat IM, Marchant R, Thahira JR. *Geobacillus debilis* sp. a novel obligately thermophilic bacterium isolated from a cool soil environment, and reassignment of *Bacillus pallidus* to *Geobacillus pallidus*. Int J Syst Evol Microbiol 2004;54:2197-201.
11. Zaliha RN, AbdRahman R, Leow TC, Salleh AB, Basri M. *Geobacillus zalihae* sp. nov. strain T1, a thermophiliclipolytic bacterium isolated from palm oil mill effluent in Malaysia. BMC Microbiol 2007;7:77-81.
12. Schmidt-Dannert C, Rúa M, Atomi H, Schmid RD. Thermoalkalophilic lipase of *Bacillus thermocatenulatus.*I. Molecular cloning, nucleotide sequence, purification and some properties. Biochim Biophys Acta 1996;1301:105-14.
13. Obeidat M, Khyami-Horani H, Al-Zoubi A, Otri I. Isolation, characterization, and hydrolytic activities of *Geobacillus* species from Jordanian hot springs. African Journal of Biotechnology 2012;25:6763-8.
14. Bruins MR, Kapil S, Oehme FM. Microbial resistance to metals in the environment. Ecotoxicol Environ Safety 2000;45:198-207.
15. Shakibaie MR, Dhakephalker PA, Kapadnis BP, Chopade BA. Removal of silver from waste water effluents using Acenitobacterbaumannii BL54. Can J Microbiol 1999; 45:995-1000.
16. Shakibaie MR, Harati A. Metal accumulation in *Pseudomonas aeruginosa* occur in the form of nanoparticles on the cell surface. Iran J Biotechnol 2004;1:55-60.
17. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981;145:1365-8.
18. Ozdemir S, Kiline E, Poli A, Nicolaus B, Güven K. Biosorption of Cd, Cu,Ni, Mn and Zn from aqueous solutions by Thermophilic bacteria, *Gobacillustoebii* subspdecauicus and *Geobacillusthermoleovoraus* subsp. *stroinboliensis* equilibrium kinetics and thermodynamic studies. Chem Eng J 2009;152:195-206.
19. Haji abdolvahabM ,Fooladi J , Gharavi S , Sadeghzadeh M . Growth condition optimization of Iranian thermophilic Geobacillus sp. MKK with the aim of characterizing the DNA polymerase I enzyme and its applications in PCR . World Appl Sci J 2010;11:354-61.
20. Hawumba HF, Theron J, BrÖzel VS . Thermophilic protease-producing Geobacillus from Buranga hot springs in western Uganda . Curr Microbiol 2002;45:144-150.
21. Scott JA, Palmer SJ. Sites of cadmium uptake in bacteria used for biosorption. Appl Microbiol Biotechnol 1990;33:221-5.
22. Verma SK, Singh HN. Evidence for energy-dependent copper efflux as a mechanism of Cu^{+2} resistances in cyanobacterium *Nosioccalciocola.* FEMS Microbiol Lett 1991; 84:291-4.
23. Sar P, Kazy SK, Singh SP. Intracellular nickel accumulation by *Pseudomonas aeruginosa* and its chemical nature. Lett Appl Microbiol 2001;32:257-61.
24. Gadd GM. Metals and microorganisms: a problem of definition. FEMS Microbiol Lett 1992; 100:197-204.
25. Chatterjee SK, Bhattacharjee I, Chandra G. Biosorption of heavy metals from industrial waste water by Geobacillus thermodenitrificans. J Hazard Mater 2010;175:117-125.
26. Yee N, Fein J. Cd adsorption onto bacterial surfaces: a universal adsorption edge. Geochim Cosmochim Acta 2001;65:2037-42.
27. Al-Qodah Z. Biosorption of heavy metal ions from aqueous solutions by activated sludge. Desalination 2006; 5:164-76.
28. JabariNezhadkermani A, FaeziGhasemi M, Khosravan A, Farahmand A, and Shakibaie, MR. Cadmium bioremediation by metal-resistant mutated bacteria isolated from active sludge of industrial effluent. Iran J Environ Health Sci Eng 2012;7:279-86.
29. Matheickal JT, Yu Q. Biosorption of lead(II) and copper(II) from aqueous solutions by pre-treated biomass of Australian marine algae. Bioresour Technol 1999; 69:223-9.
30. Poli A, Salerno A, Laezza G, Di Donato P, Dumontet S, Nicolaus B. Heavy metal resistance of some thermophiles: potential use of alpha-amylase from Anoxybacillus amylolyticus as microbial enzymatic bioassay. Res Microbiol 2009;160:99-106.

Source of Support: Kerman University of Medical Sciences and Islamic Azad University, Damghan Branch, Iran, **Conflict of Interest:** None declared.