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Isolation and phylogenetic analysis of zinc resistant *Acinetobacter* sp. and its potential for bioremediation

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In this study, a gram negative, non- motile short rod-shaped and heavy metal resistant bacterial strain was isolated from effluents. The optimum temperature and pH for the growth of the strain were 33°C and 7, respectively. The minimum inhibitory concentrations (MICs) of zinc (Zn), copper (Cu), chromium (Cr) and mercury (Hg) against the isolate were determined. The isolate showed MICs of 5, 4, 3 and 2 mM when grown on Zn, Cu, Cr and Hg, respectively. The isolate was assessed for its ability to remove zinc from medium amended with different concentrations of zinc. Up to 65% of zinc was removed at the zinc concentration of 1 mM. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belongs to the genus *Acinetobacter* with 98% similarity to *Acinetobacter junni*. It was then designated as *Acinetobacter* sp. HM_AF14. Based on the data obtained in this study, it can be concluded that *Acinetobacter* sp. HM_AF14 can be used for bioremediation of zinc from effluents.

Key words: Heavy metals, *Acinetobacter* sp., bioremediation, 16S rRNA, zinc removal.

INTRODUCTION

Many metal ions are essential as trace elements, but at higher concentrations, they become toxic. Heavy metals are difficult to remove from the environment and are ultimately indestructible, unlike many other pollutants that can be chemically or biologically degraded (Ozaki et al., 2003). Today, heavy metals constitute global environmental hazard. Many industries such as electroplating, tanning, paint and batteries, discharge aqueous effluents containing relatively high levels of heavy metals. The presence of such metals in aquatic environments cause severe damage to aquatic life and kill microorganisms during biological water purification process (Vinodhini and Narayanan, 2008).

Bioremediation is a potential cost effective solution for the remediation of heavy metal-contaminated environment in opposition to the conventional chemical and physical remediation technologies that are generally too costly and often harmful to the environment (Okafor and Opuene, 2007). Microorganisms with unique abilities such as metal absorption, accumulation or resistance can be used for remediation of polluted water and waste streams. The advantages of using microbes for bioremediation include natural occurrence, cheep production, easy availability to treat large volumes of wastewater due to rapid kinetics and high selectivity in terms of removal and recovery of specific metals

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(Genter, 1996). Various microorganisms such as bacteria (Lenung et al., 2001) yeast (Salinas and Melorza, 2000), fungi (Yan and Viraraghovan, 2003) and algae (Ozaki et al., 2003) have been reported to tolerate and remove metals from aqueous solutions. Eze et al. (2009) studied heavy metal resistant bacterial community isolated from sewage. It was shown that bacterial population isolated from effluent were members of the genera *Enterobacter*, *Pseudomonas*, *Proteus*, *Acinetobacter* and *Achromobacter*. Overall tolerance to all metals was shown by *Acinetobacter* sp. and *Pseudomonas* sp. which showed growth on all media containing the different metallic salts. Similarly, Sharma et al. (2000) isolated highly cadmium resistant *Klebsiella* that was found to precipitate significant amounts of cadmium sulfide.

Zinc, the most common metal found as byproducts of processes such as mining and ore processing, as well as industries such as electroplating, paint and battery production is less studied than other metals. Zinc is a highly toxic metal to living organisms. Acute toxicity of zinc may result in sweet taste, throat dryness, cough, weakness, generalized aching, fever, nausea and vomiting. Eating large amounts of zinc, even for a short time, can cause stomach cramps, nausea, and vomiting (Kanawade and Gaikwad, 2011). Long-term zinc intakes higher than the requirements could, however, interact with the metabolism of other trace elements. Copper seems to be especially sensitive to high zinc doses (Yadrick et al., 1989). Anemia has also been observed after higher zinc intakes (Patterson et al., 1985). Despite many reports about microbial resistance to heavy metals, only a few attempts have been made to isolate and molecularly characterize zinc resistant and zinc biosorbed bacteria from effluent in Iran. In the present study, we attempted to isolate and characterize zinc resistant bacteria from effluents that are capable of removing considerable amount of zinc and can thus be employed in bioremediation.

MATERIALS AND METHODS

Isolation of bacteria

The effluent samples were collected from different industrial units in and around Islamshar (Lat: 35.460670, Lon: 51.256714) located in south-west of Tehran, Iran. The samples were collected under aseptic condition in sterilized bottles and transported to laboratory for bacteriological analysis. The samples were directly streaked on nutrient agar plates amended with Zn as $ZnSO_4$ to final concentration of 1 mM using sterile filtered Zn^{2+} stock solutions. Plates were incubated at 30°C for 3 days. Ten zinc resistant isolates representing different colony morphologies were purified on nutrient agar containing 1 mM of Zn^{2+}.

Metal resistance and minimum inhibitory concentrations

The MICs of the metals (Cr, Hg, Zn and Cu) for ten isolates were determined by dilution method (Nieto et al., 1989) in nutrient broth medium with concentrations that ranged from 1 to 6 mM. The minimum concentration of metal in the medium inhibiting complete growth was taken as MIC. The bacterial strain that showed the highest MIC to zinc was selected for further analysis.

Biochemical and physiological properties

Physiological and biochemical characteristics including temperature and pH range for growth and tolerance to different NaCl concentrations were tested. Utilization of different carbon sources and the tests for the presence of enzymes namely oxidase, catalase, gelatinase and amylase were performed. The selected bacterial isolate was identified by morphological and biochemical methods in accordance with Bergey's Manual of Systematic Bacteriology (Holt et al., 1993).

Growth curve of HM_AF14 in the presence of different concentrations of zinc

Exponentially grown culture of the selected bacterial isolate was inoculated into nutrient broth treated with 0 (control), 1, 2 and 3 mM of Zn^{2+} and incubated at 30°C in a rotary shaker (150 rpm) for 24 h. Growth was determined turbidimetrically after interval of each 4 h by measuring optical density (OD) at 600 nm.

Molecular identification and phylogenetic analysis

The isolated bacterium was identified based on the sequencing of 16S rRNA gene. Genomic DNA was extracted from the isolated bacterium using a DNA extraction kit (Roche, Germany). Bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using bacterial universal primers, F (5´ AGAGTTTGATCATGGCTC3´) and R (5´AAGGAGGTGATCCAGCC3´). The PCR mixture contained 10 pmol of each primer, 10 ng template DNA, 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, and 1.25 U Taq DNA polymerase (Fermentas, Lithuania). PCR amplification was performed under the following conditions: 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 60 s at 45°C and 60 s at 72°C using Primus thermal cycler. The resulting PCR product was extended for further 5 min at 72°C. The final PCR product was extracted from a 1% agarose gel (Fermantas, Lithuania) using Gel Extraction kit (Fermantas, Lithuania) according to manufacturer's instruction. The purified PCR product was sequenced using an automated sequencer by Microgene Company (South Korea). BLAST analysis was performed through the National Center for Biotechnological information online services (htt://www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA sequence determined in this study was deposited in the GenBank database under the following accession number JN573359. Multiple sequence alignments were performed using MEGA version 5 (Tamura et al., 2011) after obtaining multiple alignments of data available from public databases by Clustal W (Hall, 1999). Phylogenetic tree was inferred by using neighbor joining tree making algorithm (Saitou and Nei, 1987).

Zinc removal

The isolated microorganism was cultured in NB at 33°C for 24 h and then harvested by centrifugation at 5000 rpm for 15 min. 0.5 g of harvested cells (dry weight) was inoculated into NB medium containing 0.5, 1, 2, 2.5 and 3 mM Zn^{2+}. The inoculated medium was shaken at 150 rpm at 33°C for 24 h. Residual heavy metals in the upper phase following centrifugation at 5000 rpm for 15 min were quantified by atomic absorption spectrometry (Philips, PU 9100X).

Table 1. The major physiological and biochemical properties of *Acinetobacter* sp. HM_AF14.

Characteristic	Result
Gram staining	-
Motility	-
Spore	-
Growth at:	
44°C	-
41°C	+
37°C	+
Gelatin hydrolysis	-
Starch hydrolysis	-
Acid from glucose	-
Utilization of citrate	+
Pigment production	-
Catalase	+
Oxidase	-

Statistical analysis

Each individual experiment was replicated three times. The standard deviation among the replicates was calculated using SPSS 16.0.

RESULTS

Isolation of bacteria and determination of MIC

In the present study, a total of 10 bacterial isolates which differed in color and morphology were selected randomly from Zn^{2+} supplemented nutrient agar. The bacterial isolates were tested for their resistance against zinc in varying concentration from 1 to 6 mM. Finally, strain HM_AF14 which showed higher resistance to zinc was selected for further analysis. The selected isolate also displayed substantial co-resistance against Cu^{2+}, Cr^{6+} and Hg^{2+}. The highest MIC was seen for Zn^{2+} (5 mM). Strain HM-AF14 was able to grow in NB broth containing Cu^{2+}at concentration of 3 mM. This isolate had lower resistance to Cr^{6+} and Hg^{2+} (MICs of 3 and 2 respectively). In the present study, toxicity of metal ions was ranked as follows: Hg^{2+}>Cr^{6+}>Cu^{2+}>Zn^{2+}.

Biochemical and physiological properties

The major physiological and biochemical properties of the isolate are summarized in Table 1. The isolate grew at pH range 6.0 to 10.0, and no substantial growth was observed at pH more than 10 or less than 6. The optimum temperature and pH for growth were obtained at 33°C and 7, respectively. NaCl with concentration more than 2.5% (w/v) inhibited the growth totally.

Phylogenetic analysis

A nearly complete 16S rRNA gene sequence (1276 nucleotides) was obtained for strain HM_AF14. Comparison with the 16S rRNA sequences available in databases revealed that strain HM_AF14 was related to sequences belonging to the genus *Acinetobacter*. A tree depicting the relationships of this isolate within the genus *Acinetobacter* is shown in Figure 1. According to the phylogenetic analysis, strain HM_AF14 and *A. junii* strain NB5 3B were in the same branch supported by a high bootstrap value of 100% (Figure 1). Phylogenetically, the closest relative of strain was *A. junii* strain NB5 3B with similarity value of 98%.

Growth curve of strain HM_AF14

The growth curves for strain HM_AF14 at different time intervals in the presence of 0, 1, 2 and 3 mM of Zn^{2+} are shown in Figure 2. The growth curve for NB medium containing 1 mM Zn^{2+} was similar to that of NB medium without Zn^{2+}, with ordinary lag and exponential phases. Growth curves for the strain in NB medium containing 2.0 or 3.0 mM Zn^{2+} displayed the same trends, but the OD600 value in NB medium containing 3.0 mM Zn^{2+} was lower than in NB medium containing 2.0 mM Zn^{2+}.

Zinc removal

The removal of Zn^{2+} by *Acinetobacter* sp. HM_AF14 in different concentrations of $Zn^{2+}$is shown in Figure 3. Removal of Zn^{2+} by *Acinetobacter* sp. HM_AF14 increased progressively when the concentration of Zn^{2+} in the medium increased from 0.5 to 1 mM. Maximum zinc removal was up to 65% at 1 mM of Zn^{2+}. In contrast, removal of Zn^{2+} by the *Acinetobacter* sp. HM_AF14 decreased as the concentration of Zn^{2+} was raised beyond 1 mM.

DISCUSSION

The effluents provide enriched heavy metal contaminated environment where heavy metal resistant bacteria could be isolated. From the 10 strains, *Acinetobacter* HM_AF14 exhibited high resistance to Zn^{2+}, Cu^{2+}, Cr^{6+} and Hg^{2+}. These results are consistent with previous studies where zinc resistant bacterial isolates were also resistant to other heavy metals (Vullo et al., 2005, 2008).

The results of this study indicate that *Acinetobacter* sp. HM_AF14 was closely related to members of the genus *Acinetobacter* on the basis of 16S rRNA sequence analysis. The major physiological properties of this strain are in agreement with those described for the genus *Acinetobacter* (Holt et al., 1993). As previously described,

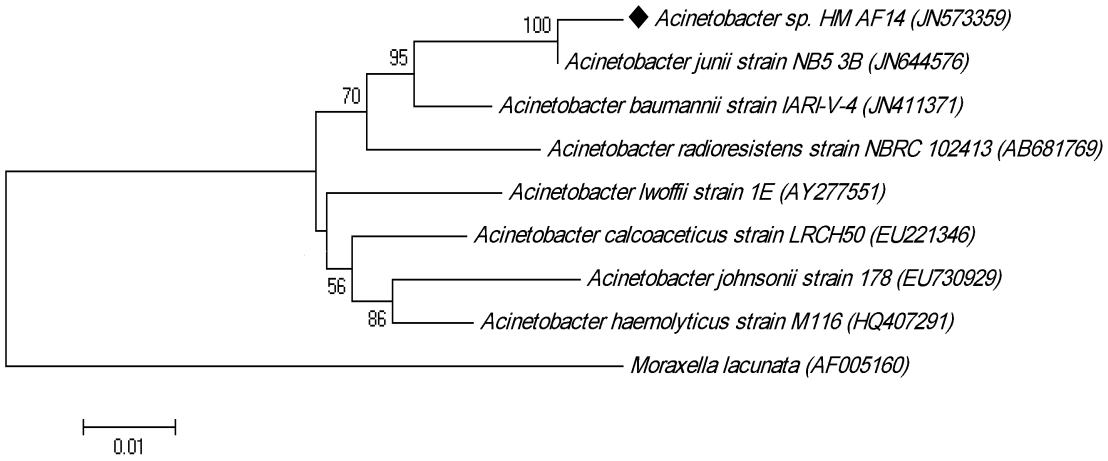

Figure 1. Phylogenetic dendrogram derived from 16S rRNA sequence analysis showing the position of strain HM_AF14 within the genus *Acinetobacter*. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. Only values above 50% are given. *Muraxella lacunata* served as out group (Carr et al., 2003). Accession numbers are given in parentheses. Bar, 0.01 sequence dissimilarity.

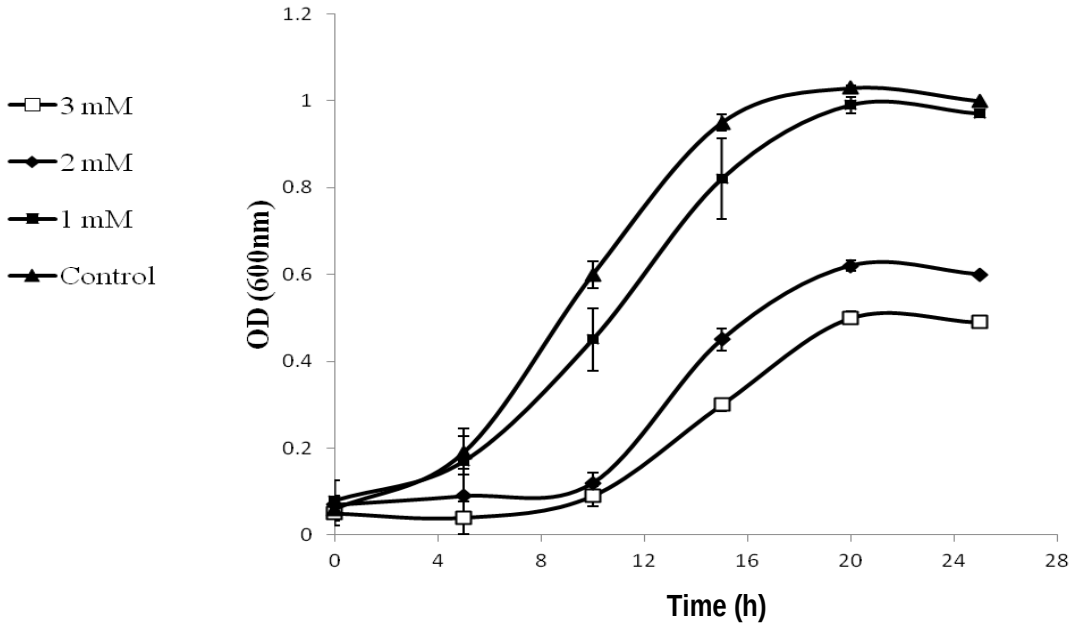

Figure 2. Growth curve of the *Acinetobacter* sp. HM_AF14 at different concentration of Zn^{2+}. Values indicate the means ± SD of three independent experiments.

the members of the genus *Acinetobacter* are strictly aerobic gram-negative coccobacilli that are widely distributed in soil, water and also commonly found in the hospital environment (Dijkshoorn et al., 2007). *Acinetobacter radioresistence* isolated from cotton (Nishimura et al., 1988) and oil degrading *Acinetobacter* (Di Cello et al., 1997) represent two of the genomic species of *Acinetobacter* isolated from the environmental sources. Notable among the habitats occupied by the *Acinetobacter* species is the activated sludge (Carr et al., 2003). The previous reports showed that some strains related to genus *Acinetobacter* have displayed resistance to heavy metals. It was reported that *Acinetobacter baumanii* BL54 had a high level of resistance to silver and was able to remove 0.25% silver per gram biomass of bacteria from photographic wastewater effluents (Shakibaie et al., 1999). Dhakephalkar and Chopade, (1994) isolated 40 strains of *Acinetobacter* from different

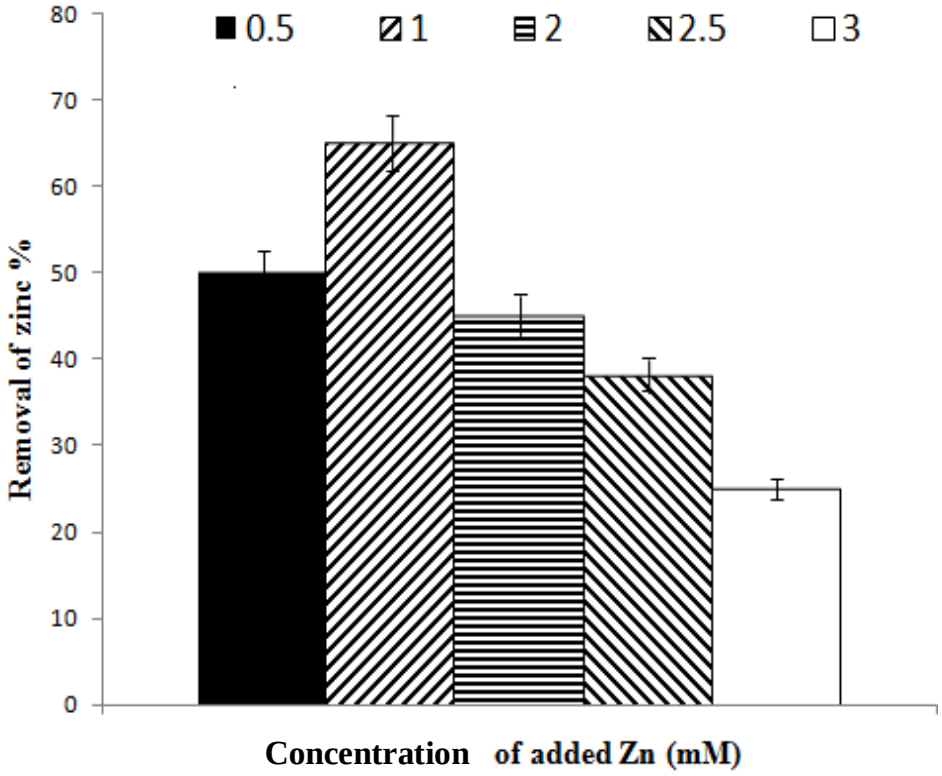

Figure 3. Removal of zinc by *Acinetobacter* sp. HM_AF14 at different concentration of Zn^{2+}. Values indicate the means ± SD of three independent experiments.

environmental sources. It was shown that all environmental isolates of *Acinetobacter* were resistant to multiple metal ions but most of the strains were found to be sensitive to mercury. The results of this study indicated that *Acinetobacter* sp. HM_AF14 was resistant to multiple metals. It also exhibited resistance to mercury (MIC of 2 mM).

The results indicate that *Acinetobacter* sp. HM_AF14 was able to grow well in the presence of 1 mM of Zn^{2+}. The growth curve of HM_AF14 in the presence of different concentrations of Zn^{2+} showed that low concentration of Zn^{2+} (1 mM) had little influence on *Acinetobacter* sp. HM_AF14 but higher concentrations (>1 mM) prolonged lag phase and reduced biomass. These observations are consistent with the previous studies (Wei et al., 2009; Ahemad and Malik, 2012). In the present study, the growth of *Acinetobacter* sp. HM_AF14 significantly decreased when exposed to more than 1 mM of zinc concentrations in the treated media. In the lower concentration, inhibition of bacterial growth was insignificant, which suggests that low concentrations of heavy metals are not toxic to the tested bacterium.

The selected isolate in our study were not only resistant against heavy metals, but also had the extensive capability of taking up zinc. It was found that the amount of zinc taken up by *Acinetobacter* sp. HM_AF14 increased with increase in concentration from 0.5 to 1 mM. This result is in agreement with the findings of Da Costa and Duta (2001) which reported an increase in uptake with higher concentration. Maximum zinc removal was found to be as high as 65% when this strain was exposed to 1 mM Zn^{2+}. Our studies also show that removal efficiency diminished beyond the specific concentrations of Zn^{2+}. As previously reported, this might be because of the saturation of the isolate with metal or due to increased toxicity of metals at high concentrations (Kaewehai and Prasertson, 2002).

In this work, a zinc-resistant bacterium was isolated from effluents and identified as *Acinetobacter* sp. HM_AF14 based on 16s rRNA sequence analysis. It showed significant heavy metal resistance to zinc, copper, chromium and mercury. The results of this study have shown that the removal percentage of zinc by the isolated strain is high. However, the biosorption experiment was performed for only 60 min; it might be possible to obtain higher percentage of zinc removal by increasing the exposure time and the use of optimal conditions for pH and temperature. Findings from this study indicate that the resistant *Acinetobacter* have the potential to remove Zn^{2+} from contaminated water. Therefore, it will be possible to use the strain as inoculants for bioremediation of effluent contaminated with Zinc.

REFERENCES

Ahemad M, Malik A (2012). Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. Bacteriol. J. 2(1):12-21.

Carr El, Kampfer P, Patel BK, Gutler V Seviour RJ (2003). Seven novel species of *Acinetobacter* isolated from activated sludge. Int. J. Sys. Evolut. Microbiol. 53:953-963.

Da Costa ACA, Duta FP (2001). Bioaccumulation of ionic copper, zinc cadmium and lead by *Bacillus* sp. *Bacillus cereus*, *Bacillus sphericus* and *Bacillus subtilis*. Braz. J. Microbiol. 32:1-5.

Dhakephalkar PK, Chopade BA (1994). High levels of multiple metal resistances and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. Biometals. 7(1):67-74.

Di Cello F, Pepi M, Baldi F, Fani R (1997). Molecular characterization of an n-alkane-degrading bacterial community and identification of a new species, *Acinetobacter venetianus*. Res. Microbiol. 148(3):237-249.

Dijkshoorn L, Nemec A, Seifert H (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat. Rev. Microbiol. 5:939-951.

Eze E, Eze U, Eze C, Ugwu K (2009). Association of metal tolerance with multi drug resistance among bacteria isolated from sewage. J. Rural. Trop. Public. Health 8:25-29.

Genter RB (1996). Ecology of inorganic chemical stress to algae. In Algae Ecology. San Diego, Academic Press, USA.

Hall TA, (1999). Bio Edit: A user friendly biological sequence alignment editor and analysis program for Windows 95/ 98/ NT. Nucl. Acids. Symp. Ser. 41:95-98.

Holt GJ, Krieg RN, Sneath AH, Staley T, Williams TS (1993). Bergey's Manual of Systematic Bacteriology. 9rd. Ed. London: Williams and Willkins.

Kaewehai S, Prasertson P (2002). Biosorption of heavy matals. J. Sci. Thecnol. 24:422-430.

Kanawade SM, Gaikwad RW (2011). Removal of zinc ions from industrial effluent by using cork powder as adsorbent. Int. J. Chem. Eng. Appl. 2(3):199-201.

Lenung WC, Chua H, Lo W (2001). Biosorption of heavy metals by bacteria isolated from activated sludge. Appl. Biochem. Biotechnol. 91-93:171-84.

Patterson WP, Winkelmann M, Perry MC (1985). Zinc-induced copper deficiency: megamineral sideroblastic anemia. Ann. Internal. Med. 103:385-386.

Nieto JJ, Fernandes-Castillo R, Marquez MC, Ventoza A, Quesada E Ruiz- Berraquero F (1989). Survey of metal tolerance in moderately halophilic eubacteria. Appl. Environ. Microbiol. 55:2385-2390.

Nishimura Y, Ino T, IIzuka H (1988). *Acinetobacter radioresistens* sp. Nov. isolated from cotton and soil. Int. J. System. Bacteriol. 38(2):209-211.

Okafor EC, Opuene H (2007). Preliminary assessment of trace metals and polycyclic aromatic hydrocarbons in the sediments. Int. J. Environ. Sci. Tech. 4(2):233-240.

Ozaki T, Kimura T, Ohnuki T, Yoshida Z, Francis A (2003). Association mechanisms of Europium (III) and Curium (III) with *Chlorella vulgaris*. J. Environ. Toxicol. Chem. 22:2800-2805.

Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Salinas E, Melorza DO (2000). Removal of cadmium and lead from dilute aqueous solution by *Rhodotorula ruba*. Bioresour. Technol. 72:107-112.

Shakibaie MR, Kapadnis BP, Dhakephalker P, Chopad BA (1999). Removal of silver from photographic wastewater effluent using Acinetobacter baumannii BL54. Can. J. Microbiol. 45(12):955-1000.

Sharma KP, Frenkel A, Balkwill LD (2000). A new *Klebsiella planticola* strain (Cd-1) grows an aerobically at high cadmium concentrations and precipitates cadmium sulfide. Appl. Environ. Microbiol. 66:3083-3087.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). "MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods". Mol. Biol. Evol. 28 (10): 2731-2739.

Vinodhini R, Narayanan M (2008). Bioacumulation of heavy metals in organs of fresh water fish *Cyprinus Carpio*. Int. J. Environ. Sci. Tech. 5(2):179-182.

Vullo DL, Ceretti HN, Hughes EA, Ramirez S, Zalts A (2005). Indigenous heavy metal multiresistant microbiotica of Las catonas stream. Environ. Monitor. Assess. 105:81-97.

Vullo DL, Ceretti HN, Daniel MA, Ramirez S, Zalts A (2008). Cadmium, Zinc and copper biosorption mediated by *Pseudomonas veronii* 2E. Bioresour. Technol. 99:5574-5581.

Wei G, Fan L, Zhu W, Fu Y, Yu J, Tang M (2009). Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China. J. Hazard. Mater. 162:50-56.

Yadrick MK, Kenney MA, Winterfeldt EA (1989). Iron, copper, and zinc status: response to supplementation with zinc or zinc and iron in adult females. Am. J. Clin. Nutr. 49:145-50.

Yan G, Viraraghavan T (2003). Heavy-metal removal from aqueous solution by fungus Mucor rouxii. Water Res. 37(18):4486-4496.