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APPLICATION OF METAL RESISTANT BACTERIA BY MUTATIONAL ENHANCMENT TECHNIQUE FOR BIOREMEDIATION OF COPPER AND ZINC FROM INDUSTRIAL WASTES

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ABASTRACT
In this research, using mutation in the metal resistant bacteria, the bioremediation of the copper and zinc from copper factory effluents was investigated. Wastewater effluents from flocculation and rolling mill sections of a factory in the city of Kerman were collected and used for further experiments. 20 strains of *Pseudomonas* spp. were isolated from soil and effluents surrounding factory and identified by microbiological methods. Minimum inhibitory concentrations for copper (Cu) and zinc (Zn) were determined by agar dilution method. Those strains that exhibited highest minimum inhibitory concentrations values to the metals (5mM) were subjected to 400-3200 mg/L concentrations of the three mutagenic agents, acriflavine, acridine orange and ethidium bromide. After determination of subinhibitory concentrations, the minimum inhibitory concentrations values for copper and zinc metal ions were again determined, which showed more than 10 fold increase in minimum inhibitory concentrations value (10 mM for Cu and 20 mM for Zn) with $P \leqslant 0.05$. The atomic absorption spectroscopy of dried biomass obtained from resistant strains after exposure to mutagenic agents revealed that strains 13 accumulate the highest amount of intracellular copper (0.35% Cu/mg dried biomass) and strain 10 showed highest accumulation of zinc (0.3% Zn/mg dried biomass) respectively with $P \leqslant 0.05$. From above results it was concluded that the treatment of industrial waste containing heavy metals by artificially mutated bacteria may be appropriate solution for effluent disposal problems.

Key words: Pollution, bioremediation, copper and zinc waste effluents, *pseudomonas*

INTRODUCTION
Today due to fast development of industry and production of different toxic compounds containing heavy metals, the environment surrounding these industries are heavily polluted and cause destruction of living ecosystem in these area (Diels *et al.*, 1999). One of the main environmental pollutants in Kerman province in Iran is copper produced by copper factories. These factories also excrete zinc as product. There are microscopic organisms in environment like bacteria that have ability to grow and absorb effluents containing heavy metals (Lovely and Coates, 1997). Uptake of zinc and copper by halophilic bacteria isolated from the Dead Sea shore in Jordan was studied by Al-Momani *et al.*, (2007). Maximum bioremediation for Zn was achieved by culture 7 at 11.2%, 1.0%, 38.4%, and 8.4%, respectively infact, the heavy metal resistant bacteria capable of bioaccumulation of high concentrations of metals like Ag, Cu, Pb or Cd can play an important role in clean up or bioremediation of the effluents from heavy metal industries (Shakibaie *et al.*, 1999). For many heavy metals the d orbital of their atoms are not completely filled and react with other compounds in the form of oxide or sulfide (Shakibaie and Harati, 2004) and cause production of free radicals and cellular damage. Human is exposed to heavy metals since long back, however, within recent 50 years has been augmented due to rapid development of industry (Wood and Wang, 1985).

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For example in USA, each year thousands of tons of pollutants like arsenic , Zn, Cd enter in to soil or water and then to human food chain. Therefore, bioremediation of heavy metals are very important for environmental health.

There are several researches carried out on the removal of heavy metals from industrial wastes. Soltan in 2001, in Egypt isolated 240 *Psudomonas areuginosa* strains which were resistant to Pb, Cd, Hg, Zn, Ag and Cu. He found that some strains could accumulate high concentration of these metals.

Hifelie *et al.,* (1984) used one *Pseudomonas stutzeri* isolated from silver mine which could accumulate 2 mg/g biomass of Ag. Similarly, Wood and Wang (1985) used an strain of *Peudomonas putida* capable of biosorption of 6.5% of Cu. Cooksay and Azad (1992) used a strain of *Peudomonas syngeri* which could remove 115 mg/g biomass of Cu. Shakibaie *et al.,* (1999) using a genetic engineered *Acinetobacter baumannii* could remove 2.5 mg/g biomass of Ag from effluents of film industry. Similarly Shakibaie (2002), isolated one *Pseudomonas* strain from burn patient that contained one conjugative plasmid and that could remove considerable quantity of Pb. Bioaccumulation of copper by *Trichoderma viride* was studied by Anand *et al*., 2006. At a concentration of 100 mg/L of $CuCl_2.2H_2O$, 81% of Cu (II) was removed by 3.4 g/L of the biomass in 72 h. Similarly, tolerance and biosorption of copper and zinc by *Pseudomonas. putida* CZ1 isolated from metal-polluted soil showed that it was capable of removing about 8.72% of Cu and 9.8% of Zn during the active growth cycle (XinCai *et al*., 2006).

In- situ accumulation of copper, chromium, nickel, and zinc in soils used for long-term waste water reclamation studied by Lin *et al.,* (2008). They found that copper accumulated only in the 0 to 1 m top soil layer, with concentration increase of 0.28 to 0.76 mg/kg.

The objective of this research was to study the increase in removal of copper and zinc from copper and zinc waste effluents by metal resistant stains of *Pseudomonas* isolated from copper factory using mutational enhancement technique.

MATERIALS AND METHODS

Sampling

20 L of the effluents were collected in 40 L capacity Can from both foacculation (exogen) and rolling mill (before any treatments) sections of the copper factory in Kerman Iran, and transferred to High-Tech research center for further analysis. The pH of the samples was determined from 100mL of surface of effluents using blotting papers. The pH was measured using pH meter (Hana HI 8314 membrane pH meter).

Determination of concentrations of Cu and Zn in the effluents

For measurement of concentrations of Cu and Zn, 300 mL of effluent from rolling mill and foacculation sections were collected and filtered using blotting papers. The concentration of these heavy metals in the effluents then determined by atomic absorption spectrophotometer attached to a graphite analyzer (Beckman, USA).

Isolation of bacteria from effluents and soil near factory

For isolation of bacteria from effluent and soil surrounding copper factory, one gram of soil and effluents were weighted carefully and transferred to 10 mL of sterile D/W. 5 mL of the supernatant were transferred to tube containing 10 mL sterile D/W (10^{-2} dilution). Dilution was repeated till 10^{-8}. 0.1 mL of each dilution was spread on to sterile Muller- Hinton agar and incubated at 35°C for 24 h. The colonies grown on medium were then identified by microbiological and biochemical methods (shakibaie *et al.,* 1999).

Determination of MIC

Minimum inhibitory concentration (MIC) of above heavy metals for isolated strains was determined by agar dilution technique (XinCai *et al*., 2006). For MIC experiment 20 isolated Pseudomonas strains were grown for 8 h in 20 mL sterile Muller-Hinton broth separately and 0.1 mL log phase (10^8 cells/mL) cultures were inoculated into serially diluted Muller-Hinton agar containing 0.5, 1.0, 5.0, 10, and 20 mM concentrations of copper and zinc. The sensitivity of the isolates was determined by number of colonies grown in the above plates after 24-48 h at 35°C.

Induction of mutation

The ability of the organisms to grow in higher concentrations of copper and zinc were augmented by exposing the isolates to different mutagenic agents like acridine orange, acriflavine and ethidium bromide. These compounds have ability to intercalating with DNA and cause frame shift mutation. For induction of mutation, we used two methods 1) Gradient plate technique (GPM) 2) Subihibitory concentration (SIC). For GPM, initially 40 mg of above reagents were dissolved 100 mL D/W and mixed properly. Then 10 mL of solution were added to 10 mL of melted nutrient agar medium and immediately plated into Petri plates and kept as gradient. After solidification of the medium, 5 mL soft agar was poured on to the medium and allowed to solidified, by this method a gradient concentration of mutagenic agent was created. 0.1 mL of the bacterial culture was then spread into the medium and incubated for 24 h at 35°C. The colonies which grown in the highest gradient concentration of the medium were selected for further investigation. In case of SIC, the serial dilution of mutagenic agents was prepared from 400 mg/L to 3200 mg/L in nutrient broth medium. 0.1 mL of each culture was inoculated in medium and incubated for 24 h at 35°C. The SIC was then determined as highest concentration of mutagenic agent that bacterial strains could grow.

Heavy metal measurement

Bioremediation of above heavy metal contents in the dried biomasses grown in the highest concentration of above heavy metals after exposure to mutagenic agents were determined by atomic absorption spectrophotometer as described previously (shakibaie *et al.*, 1999). Briefly, 20 mg bacterial cell biomasses which previously grown in 200 mL of sterile Muller – Hinton broth over night were inoculated in to effluents 10 and 20 mM of the Cu and Zn respectively and after 0.0, 0.2, 0.4, 0.6, 0.8, 1.2, 1.8 and 16.4 h the cells were harvested by centrifugation at 12,000 rpm for 10 min at room temperature and biomass was dried in oven at 60°C. 10 mg dried residues were dissolved in 1 mL of concentrated nitric acid and diluted to 10 mL with DD/W. Blanks were treated in the same way and analyzed by atomic absorption spectrophotometer. Calibration curve of each heavy metal was drawn with working standard solution before testing.

Statistical analysis

All the statistical analyses were carried out using SPSS version 7.5 (Norusis, 1993). Chi-square and fisher tests were used for determination of significance of association. The $p<0.05$ was considered significant.

RESULTS

pH and concentrations of Cu and Zn in floacculation (Exogen) and rolling mill sections of the copper factory in Kerman city, Iran are shown in Table 1 and Table 2. In rolling mill section, the copper concentration was 56.255 ppm with pH= 4.08, while, in foacculation section the copper concentration was 20.130 ppm with pH=5.53. Among bacteria isolated from soil and effluents, *Pseudomonas* strains were predominant. There was one *Xantomonas* which could not grow in presence of high concentrations of Cu and Zn and eliminated from this study.

Table 1: The pH of the effluent samples collected from copper factory

Rolling mill section	Foacculation (exogen)	Sample collected
4.08	5.53	pH

Table 2: The concentrations of copper and zinc in the effluents

Zn concentration ppm	Cu concentration ppm	Sample collected
25.237	20.130	Foacculation (exogen)
145.86	56.255	Rolling mill section

Sensitivity of 20 *Pseudomonas* strains which were isolated in soil and effluent of surrounding factory were determined by agar dilution technique. Strains 2, 6, 8, 10, 11, 13, 14, 16, and18 showed highest MIC value for Cu and Zn (0.5 mM). Among them, strains number 10, 13 and 16 were selected for bioremediation purpose. Table 3 shows the sensitivity and growth of the above strains to mutagenic agents. Among the mutagenic compounds, acridine orange and acriflavine had maximum effect on the bioremediation of these metals and increased the MIC of above strains from 0.5 mM to 10 mM for Cu and 20 mM for Zn with P⩽0.5, while ethidium bromide did not exert such effect as shown in Table 4. Figs. 1 and 2 shows the percentage of bioaccumulation of Cu and Zn by the resistant strains of *Pseudomonas* (dried biomass) after exposing to high concentration of mutagenic agents. Strains 13 could remove o.35% Cu/mg dry biomass, while,strain 10 could remove 0.3% Zn mg/biomass respectively in dry weight with P⩽0.5 and standard deviation ± 0.016 respectively. This was further supported by a considerable decrease in amounts of Cu and Zn in the effluents (Figs. 1 and 2). Simultaneous addition of Cu and Zn in to effluents revealed considerable decrease in accumulation of Cu and Zn.

Table 3: Subinhibitory concentrations (SIC) of mutagenic agents acridine orange and acriflavine for *Pseudomonas* strains isolated from soil and effluents of copper factory

Isolate No.	Concentrations (mg/L)				
	400	600	800	1600	3200
2	+	+	+	+	-
6	+	+	+	+	-
7	+	+	+	+	-
8	+	+	+	+	-
9	+	+	+	+	-
10	+	+	+	+	-
11	+	+	+	+	-
12	+	+	+	+	-
13	+	+	+	+	-
14	+	+	+	+	-
15	+	+	+	+	-
18	+	+	+	+	-

+ = growth, - = no growth

Table 4: The MIC values of each *Pseudomonas* strain to Cu and Zn before and after exposure to mutagenic agents

Bacterial isolates	Cu concentrations (mM)					
	Before mutation			After mutation		
	2.5	5	10	2.5	5	10
2	+	-	-	+	+	+
8	+	-	-	+	+	+
13	+	-	-	+	+	+
14	+	-	-	+	+	+
16	+	-	-	+	+	+
	Zn concentrations (mM)					
Bacterial isolates	Before mutation			After mutation		
	10	15	20	10	15	20
6	-	-	-	+	+	+
7	-	-	-	+	+	+
8	-	-	-	+	+	+
9	-	-	-	+	+	+
10	-	-	-	+	+	+
12	+	-	-	+	+	-
13	-	-	-	+	-	-
14	-	-	-	+	-	-
16	+	-	-	+	+	-

The above results are repeated twice and similar observation was made (P⩽0.5)

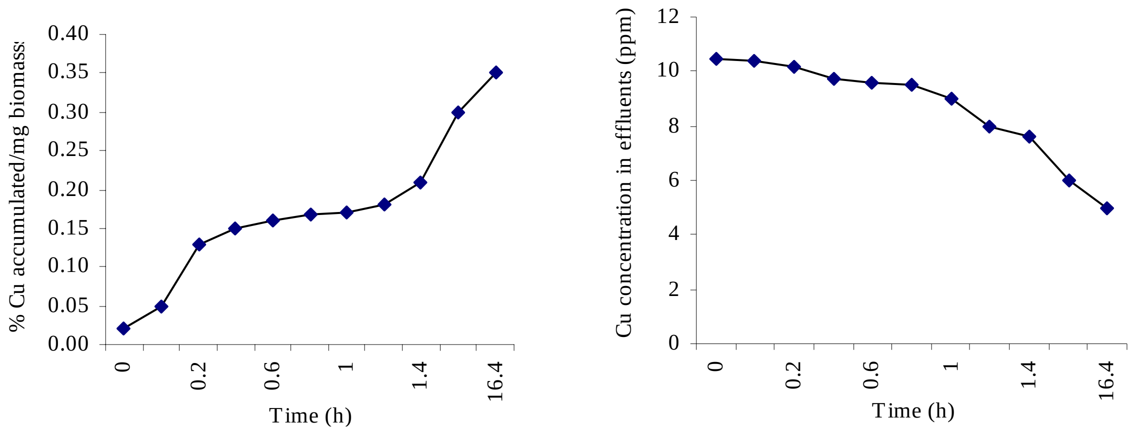

Fig. 1: Accumulation of copper in form of %Cu/mg biomass in dry weight bacteria by highly resistant strains of *Pseudomonas* after exposure to mutagenic agents

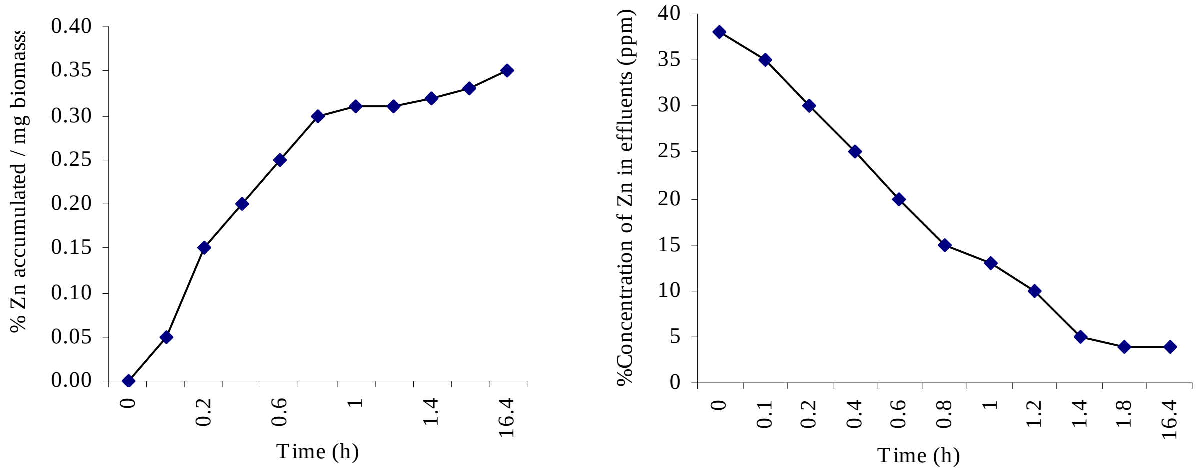

Fig. 2: Accumulation of zinc in form of %Zn/mg biomass in dry weight bacteria by highly resistant strain of *Pseudomonas* after exposure to mutagenic agents

DISCUSSION

In this study, we tried to remove copper and zinc heavy metals from the effluents of the copper factory in Kerman provenience, Iran using mutational enhancement technique. For this reason 20 liter samples were collected from rolling mill and exogen sections of the factory and the concentration of these heavy metals were determined by atomic absorption spectrophotometer. Bacterial samples were collected from soil and effluents near factory and subjected to identification by microbiological and biochemical methods. Pseudomonas strains were predominant bacteria which could tolerate high concentrations of the Cu and Zn.

The isolated strains were subjected to different concentration of Cu and Zn, some could grow up to 2.5 mM Cu and 5 mM of Zn. The MIC decreased considerably when we used two metals simultaneously. The biosorption of Pb (II), Cr (III) and Cu (II) metal species using *Rhodococcus opacus* strain (Bueno *et al.*, 2008). Capacity of the Pb (II) ion was found to be reduced by the presence of the other competing metal ions. Similar observation was made by us when we used both heavy metals simultaneously the bioremediation of the Cu and Zn were drastically decreased. This indicates that both metals may use the same entry channel, and compete for each other.

In order to increase the MIC values for the above metal ions and enhance the accumulation process, we exposed the bacterial cells to different concentrations of mutagenic agents, acridine orange, acriflavine and ethidum bromide. These agents are intercalating dyes and are capable to bind to DNA of bacteria induce frame shift mutation, therefore they are strong mutagenic agents. In our research we found that acridine orange and acriflavine had profound effect on the ability of isolates to grow in very high concentrations of Cu (10 mM) and Zn (20 mM), while ethidium bromide either had negligible or no effect in this process.

Study on bioremediation of copper and zinc metal ions revealed that isolates 13 could accumulate 0.35% of copper per mg dry weight of biomass while, isolate 10 could accumulate 0.3% per mg dry weight of biomass.

Many investigations were carried out regarding accumulation of heavy metals from effluents. Wood *et al.*, (1985) isolated *Pesudomonas putida* strains from copper factory in Canada that could accumulate 6.5% of Cu in dry biomass. Mclean *et al.*, (2001) isolated a *pseudomonas* strain that reduce chromate (VI) to chromites (III). Geesy and Lang, (1989) studied interaction between ions and capsular polymers. They found that capsular polymer was remove considerable quantity of metals from waste water. Slawson and Trevors (1992), were studied bioaccumulation of silver in *Pesudomonas. Stutzeri*. Similarly, Shakibaie *et al.*,(1999) isolated an *Acinetobacter baumannii* BL54 which removed 0.25% silver per gram biomass of bacteria from photographic waste water effluents in film industry.

Finally, the data presented in this paper clearly indicated that with use of mutation in metal resistant bacteria we can considerably enhance the bioremediation of heavy metals from effluents of the factories and improve the disposal problems of the waste with little expense.

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