

CADMIUM BIOREMEDIATION BY METAL-RESISTANT MUTATED BACTERIA ISOLATED FROM ACTIVE SLUDGE OF INDUSTRIAL EFFLUENT

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ABSTRACT

Bioremediation of metal pollutants from industrial wastewater using metal resistant bacteria is a very important aspect of environmental biotechnology. In this study, three species of *Pseudomonas aeruginosa* were isolated from active sludge of a food factory in the city of Kerman. The bacterial identity was determined by various biochemical tests. Among them, isolate number one could grow on Muller-Hinton agar medium containing 6mM cadmium ion (Cd^{2+}) and was therefore selected for further study. The isolates were subjected to mutation by two mutagenic agents (Acridine Orange and Acriflavine) using gradient plate and SIC techniques. The Minimum Inhibitory Concentration of Cd^{2+} for the isolate one after mutation was increased to 7mM. Removal of Cd^{2+} using mutated and wild type strains of this bacterium was carried out at different time intervals (10-300 minutes). It was observed that within 60 minutes, 94.7% of cadmium was removed in 30mg/L of Cd^{2+} solution. However, with 60mg/L Cd^{2+} solution, only 53.58% and 38.68% Cd^{2+} removed were achieved by mutated and wild type bacteria, respectively. The equilibrium data was fitted by Langmuir isotherm equation and the related parameters for Cd^{2+} were derived. Based on the data obtained in this study, it can be concluded that biomass of this bacterium can be used for bioremediation of cadmium from industrial waste processing plants with high efficiency.

Key words: Bioremediation; Cadmium; Mutation; *Pseudomonas aeruginosa*; Metal resistant bacteria

INTRODUCTION

Along with industrial progress, environmental pollutants like toxic heavy metals are widely spreading throughout the world. This is especially true for developing countries like China and India (Raja *et al.*, 2008). The uncontrolled discharges of large quantity of heavy metal-containing wastes create huge economical and health care burden particularly for the people living near that area (since the effluents of the industries excreted into the environment and through food chain, affect humans and animals from various anthropogenic sources such as industrial wastes, automobile emissions, mining activity and agricultural practices

as well). The important toxic metal pollutants like cadmium, nickel and lead enter to the water bodies through industrial wastewater treatment plants (Denise *et al.*, 1989; Ajmal *et al.*, 1998). Cadmium is the most dangerous metal ion characterized by high stability and toxicity. It is not degradable in nature and will thus, once released to the environment, stay in circulation. Cadmium is known to bind with essential respiratory enzymes (Nies, 2003) causing oxidative stress and cancer (Banjerdki *et al.*, 2005). High concentrations of cadmium is highly corrosion resistant and is widely used to plate metal parts in general industrial hardware as well as in automobiles, electronics, marine and aerospace industries (Herrero *et al.*, 2005).

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Cadmium contamination has been also reported particularly in soils containing waste materials from zinc mines and in sludge amended soils fertilized with cadmium rich phosphate fertilizers (Raskin and Ensley, 2000). The current low world market price of cadmium motivates the development of new applications that by time may develop into new sources of emissions to the environment not covered by existing regulation. Therefore, decontamination of these pollutants through bioremediation process and other biotechnological means are prerequisite for any future decision by the governments.

The potential use of metal-resistant microorganisms in the treatment of heavy metal contaminated wastewater plants has become more important (Shakibaie *et al.*, 2008). Different biomass types, such as bacteria, fungi and algae, have been screened and studied extensively by many authors over the past decades with the aim of identifying highly efficient metal removal biological systems (Viraraghavan, 1995; Vieira and Volesky, 2000; Kapoor and Herrero *et al.*, 2005).

Many efforts have been devoted to the isolation of heavy metal-resistant bacterial strains during the past years. *Staphylococcus aureus* (Novick and Roth, 1968) *Escherichia coli* (Mitra *et al.*, 1975) were found to exclude cadmium ion (Cd^{2+}) from cell surface. Katarina *et al.*, (2004) studied cadmium resistant bacterial community isolated from sewage sludge contaminated by cadmium ions. Among bacteria from bacterial community short cadmium resistant gram-negative rods were predominated. Biochemical tests assigned the eight isolates to six bacterial species, *Alcaligenes xylosoxidans*, *Comamonas testosteroni*, *Klebsiella planticola*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Serratia liquefaciens*. Cadmium-resistant bacterial isolates were able to remove cadmium from solution and the efficiency of cadmium removal correlated with the amount of additionally synthesized proteins in the cell fractions.

Although many researchers have studied the bioremediation of cadmium from industrial waste, none has used mutated cadmium resistant bacteria for this purpose. In this investigation, a cadmium resistant bacteria was isolated from

active sludge processing plant of a food factory near the city of Kerman in Iran. By mutational enhancement technique the bacterial strain was employed for removal of Cd^{2+} using batch bed reactor.

MATERIALS AND METHODS

Effluent sampling and source

500g soil from depth of 0.1 meter and 5L of active sludge effluent in depth of 21cm from waste processing plant of a food factory at Kerman city, Iran, were collected in sterile 15L can and transferred to International Center for Science, High-Technology, Kerman, Iran, for further analysis. The pH of active sludge and soil were measured by pH meter (Metrohm- 691).

Concentrations of cadmium in active sludge and biomass were measured by atomic absorption spectrometry (Philips, PU 9100X). Before analysis of cadmium concentration, all samples were filtered through 0.45 μm pore size hydrophilic membranes filters (Sartorius, Millipore, Germany). The cadmium used was in the form of $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ with 95% purity, purchased from Merck Co. Ltd, (Germany). Muller-Hinton agar and broth were obtained from Hi-Media (Mumbai, India). Mutagenic agents of Acridine Orange and Acriflavine were purchased from Merck Co. Ltd.

Preliminary screening of cadmium resistant bacteria

The preliminary screening of cadmium resistant bacteria was carried out from both active sludge and soil samples by two methods. One method was based on serial dilution technique (Shakibaie *et al.*, 2008) in which 1mL of active sludge was added to a tube containing 9mL of sterile 0.75% normal saline (10^{-1}) and mixed well. 1mL supernatant of this dilution was transferred to another 9mL sterile normal saline (NaOH 0.07%) tube to obtain final volume 10^{-2} . Dilution was repeated till 10^{-8} . 200 μL of each dilution was inoculated on to sterile Muller-Hinton agar (MHA) plates containing 0.5mM $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ solution, spread thoroughly with sterile glass spreader and incubated aerobically at 35°C for 24- 96 hours. For control sample, inoculation was done on Muller-Hinton agar medium without

cadmium sulphate and incubated along other plates. Similarly, all above processes were done for one gram soil sample as well.

In the second method, 1mL of active sludge effluent was inoculated in 10mL tube with 9mL Muller-Hinton Broth medium containing 0.5mM $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ solution and mixed well by agitation and incubated at 35°C for 24 hours. All above processes were repeated for one gram soil sample as well. 200 μL of soil and sludge suspensions were inoculated onto series of 20mL sterile Muller-Hinton agar plates and incubated at 35°C for 24-72 hours. After growth, one loopful of each colony was suspended in 1mL sterile Muller-Hinton broth containing 40% glycerol in 1.5mL Eppendroff tubes, mixed well and stored at -70°C (Shakibaie, *et al.*, 1999).

Determination of cadmium sensitivity

After the preliminary isolation of the cadmium resistant bacteria, the minimum inhibitory concentration (MIC) of Cd^{2+} was determined by the agar plate dilution method as described by Malik and Jaiswal, (2000). 250mL of 0.05M stock solutions of the metal salt ($3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$) were prepared in sterile DD/W to obtain final and concentrations of 1, 2, 3, 4, 5, 6 and 7 mM Cd^{2+} , respectively. The Petri plates were inoculated with 200 μL log phase liquid culture of isolated bacteria and incubated at 35°C for 24-96 hours. The MIC was defined as the lowest concentration of the Cd^{2+} that inhibits the visible growth (number of colonies) of the organisms. The Cd^{+2} sensitivity and resistance of the isolates were calculated according to published papers (Devicente *et al.*, 1990; Sarby *et al.*, 1997; El-Helow *et al.*, 2000).

Induction of mutation

The mutagenic compounds of Acridine Orange and Acriflavine have the ability to bind and intercalate with the DNA and cause frame shift mutation (Glass, 1982). Two methods for induction of mutation were employed in this study. In the first method, Gradient Plate Technique (GPT), stock solutions of mutagenic agents were prepared by addition of 0.1mg of Acridine Orange and Acriflavine into 100mL sterile double distilled water. Various concentrations of mutagenic agents from stock solution were added to 10mL sterile

melted nutrient agar medium, mixed well and kept as sloping condition. After the medium was solidified, 10mL melted soft agar was poured into the sterile Petri plates and kept horizontally till it solidified again.

The cadmium-resistant bacterial strains that were isolated in pervious stages were inoculated throughout gradient plates and incubated at 35°C for 24-48 hours. The colonies that were grown on the highest concentration of the slop, were selected and used for further study. In the second method, Sub-Inhibitory Concentration (SIC) of each mutagenic agent was determined as previously described (Shakibaie *et al.*, 2008). 100 μL of log phase bacteria (8 hours grown cell suspension) that was grown on gradient plates of Acridine Orange and Acriflavine were inoculated onto sterile Muller-Hinton Broth (MHB) medium containing different amounts of the above mutagenic agents (400, 800, 1600, 3200 and 6400 $\mu\text{g}/\text{mL}$). All the tubes were incubated at 35°C for 24-48 hours. 100 μL from each tube which showed visible growth, was streaked on Muller-Hinton Agar (MHA) medium and incubated at 35°C for 24 hours. Individual colony grown on the highest concentrations of the mutagenic compounds were inoculated on MHA medium containing various concentrations of $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ (1-8mM) and incubated for 24-48 hours at 35°C. The bacterial strains that were grown on the highest concentration of $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ containing plates, were then isolated and stored for Cd^{+2} removal study.

Identification of bacterial isolates

Both, Cd^{+2} resistant gram-negative non-fermentative strains (GNNFR) and gram-positive cocci were isolated. The isolates were tested and characterized by several microbiological key conventional tests for basic differentiation of gram-negative and gram-positive bacteria as previously described in Bergeys Manual of Determinative Bacteriology. Further, the isolates were identified on the basis of biochemical tests of commercial identification systems as shown in Table 1.

Mass balance experiment

To determine how much of the Cd^{2+} was

precipitated by the cell in an insoluble form, 1.5mL of the samples were treated with and without cadmium and centrifuged at 10.000rpm for 15 minutes at room temperature. The supernatant and pellet were analysed for Cd²⁺ content by atomic absorption spectrometry.

Table 1: Biochemical tests for identification of *P.aeruginosa* isolated from active sludge and soil near food factory disposal plant

Biochemical tests	Properties
Morphology	Short rods
Gram staining	Gram negative
Motility	+
Growth at 42°C	+
Growth at 4°C	-
Fluorescent pigment	+
Odor	Fruity odor
H2S production in TSI	-
Gas production	-
Growth on Simmon citrate agar	+
Urea test	+/-
Oxidase	+
Arginine dehydrolase	+ (weak)
OF	oxidative
Indole	-
Gelatinase	+

In case of *X.oryzae*, the yellow pigment was observed.

Removal of cadmium by Cd resistant biomass

100mL Erlenmeyer flasks containing 60mL of 30, 45 and 60 mg/L cadmium solutions were prepared. In one set, 0.5g biomass (wet weight) of mutated bacteria and in the other set, 0.5g biomass from wild type were carefully weighted and separately added to each flask. The pH (8.0) and temperature (24°C) of the solution were initially measured before adding the biomass. The flasks were placed on stirrer (100 rpm) and the samples were taken and centrifuged at 10.000 rpm every 5 minutes for the first 20 min, and interval of 30, till 300 minutes. The supernatants and biomasses (biomasses were washed with normal saline (0.75%) and again centrifuged) fractions from each specific time were analyzed for the remaining Cd²⁺ by atomic absorption spectrometry.

For determination of Cd²⁺ concentrations in the biomasses, bacterial cell residues were dissolved in 1mL 95% nitric acid (Merck Co.), mixed well by vortexing and diluted to 10 mL with sterile DD/W. Blanks were treated in the same way and analyzed as described above. Simultaneously, total viable count of the organism was determined each time to see any decrease in the colony count.

RESULTS

pH of active sludge effluent was 8.0 and atmospheric temperature was 25°C, while ambient temperature was 20°C. Several mesophilic gram-negative and cadmium resistant bacteria were isolated. The enrichment was better as compared to direct culture method for the isolation of Cd resistant bacteria in shorter time. Also, the isolates in primary enrichment method could grow on 6 mM concentration of Cd²⁺ containing medium. Majority of the bacterial isolates were belonging to gram-negative non-fermentive *Pseudomonas* (4 isolates). One gram-negative coccus was also capable to grow on 2mM concentrations of Cd²⁺; but on subsequent inoculation, the strain lost its ability to grow on more than 2 mM Cd²⁺ and was

Table 2: Minimum Inhibitory Concentration (MIC) of Cd²⁺ for three *Pseudomonas* strains isolated from active sludge and soil before and after mutation

<i>P.aeruginosa</i> isolates	Source	MIC (mM)	
		Before mutation	After mutation
1	sludge	6	7
2	soil	3.5	4
3	sludge	2.5	3

The above tests were duplicated and similar observation was made.

eliminated from the study. Those strains isolated from soil, exhibited MIC=3.5mM and from active sludge as 6mM for 3CdSO₄.8H₂O, respectively (Table 2).

One *Xanthomonas oryzae* isolated from soil near factory could grow only on 2mM cadmium containing medium; therefore, it was not used for bioremediation study because of low MIC value. Those *P.aeruginosa* isolates grown on the 3.5-6 mM concentration of Cd²⁺ containing medium were exposed to 6400mg/L Acriflavine and 9600

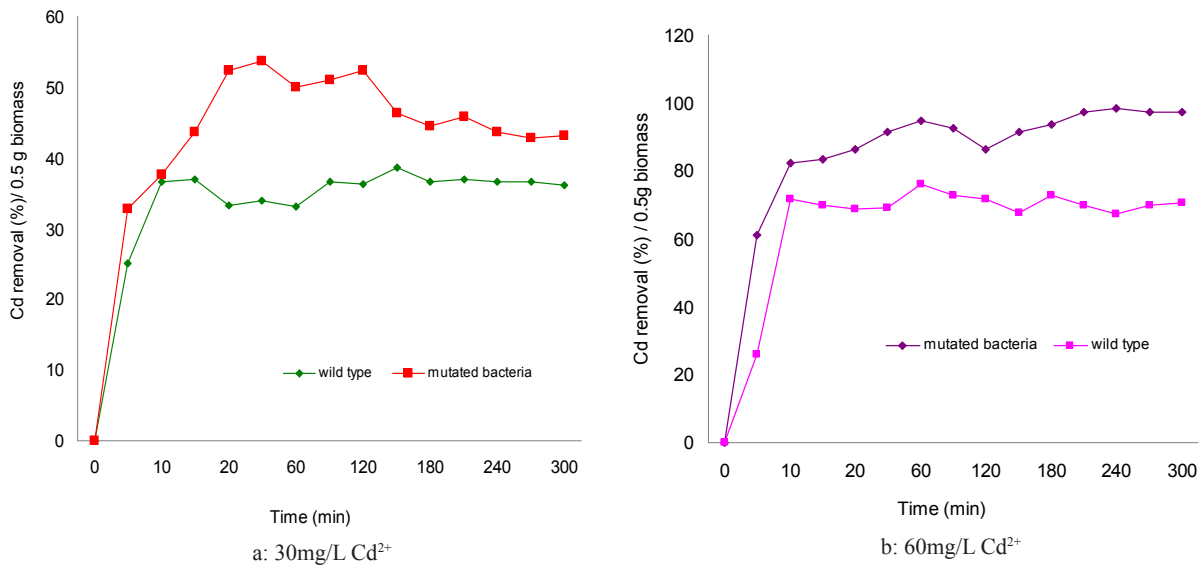


Fig. 1: Removal of Cd²⁺ by *P.aeruginosa* one, isolated from active sludge effluent in solution containing (a) 30mg/L and (b) 60mg/L Cadmium.

mg/L Acridine Orange as shown in Table 3. The isolates could grow well on these concentrations and not any changes in the colony characteristics or bacterial morphology was observed after exposure to these mutagenic agents. Table 2 compares MIC values of Cd²⁺ before and after exposure to these two mutagenic agents. The MIC increased to 7 mM for *P.aeruginosa* isolate 1 after exposure to the above agents.

Fig. 1 (a and b) shows removal of Cd²⁺ by mutated *P.aeruginosa* isolate 1 and wild type in 30 and 60 mg/L of Cd²⁺ solution, respectively. 94.7% removal was achieved till 60 minutes in 30 mg/L of Cd²⁺ by mutated bacteria. However, in 60 mg/L concentration of cadmium, only 53.58% Cd²⁺ was removed by mutated bacteria and 38.68% by wild type. In all cases the Cd²⁺ concentration rapidly decreased during the first 15 minutes in

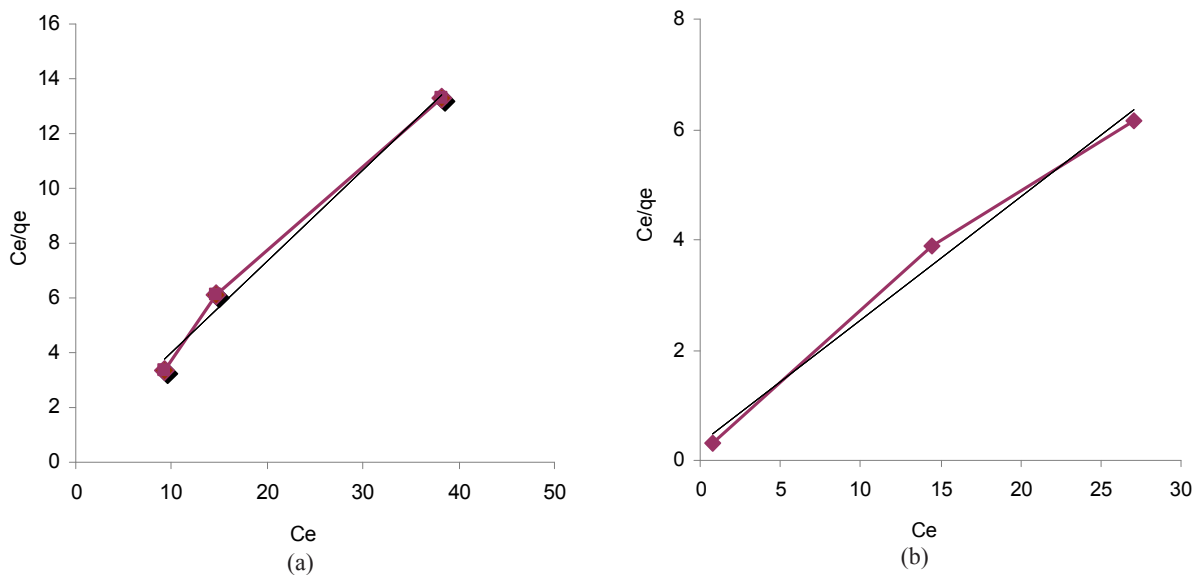


Fig. 2: Langmuir isotherm plot for removal of Cd²⁺ by (a) wild type and (b) mutated bacteria

the supernatants and remained constant during 3 hours. This indicates that as concentration of Cd²⁺ was increased, the total viable count of the organism decreased and was directly related to the amount of Cd²⁺ removed by the biomass. The

Langmuir model was derived based on several assumptions (the surface was homogeneous, adsorption on the surface was localized and each site could only accommodate one molecule or atom).

Table 3: Gradient Plate Method (GPM) and Subinhibitory Concentration (SIC) of mutagenic agents Acridine Orange (AO) and Acriflavine (AF) for *P.aeruginosa* strains 1, 2, and 3 isolated from soil and active sludge processing plant effluent

<i>P.aeruginosa</i> isolates	Concentration (µg/mL)				
	GMP		SIC*		
	AO	AF	AO	AF	
1	9600	6400	6400	3200	
2	9600	6400	6400	3200	
3	9600	6400	6400	3200	

*SIC was defined as maximum concentration of mutagenic agent that bacteria could grow.

Table 4: Langmuir isotherm coefficients for cadmium

Langmuir model for mutated bacteria			Langmuir model for wild type		
q _{max}	k _L	R ²	q _{max}	K _L	R ²
4.47	1.58	0.9945	3.01	0.47	0.9908

q_{max} = the maximum removal capacity of the biomass (mg/g).
The above results are average of two independent experiments.

Fig. 2 (a & b) shows the Cd²⁺ removal isotherm for mutated bacteria as well as wild type which follow Langmuir model. Table 4 shows the parameters of Langmuir isotherm for Cd²⁺ derived in this investigation.

DISCUSSION

The data obtained in this study clearly shows that with employment of cadmium resistant mutated biomass, bioaccumulation of Cd²⁺ from Cd-containing solution considerably increased. *Paeruginosa* isolate 1 could efficiently remove 94.7% in 30 mg/L of Cd²⁺ solution within 60 min. The results were consistent with previously report for strain E1 (Zeng et al., 2009). Strain E₁ with resistance to 18 mM/L cadmium isolated

from Cd-contaminated soil was identified by morphological observation, biochemical and physiological characterization (Zeng et al., 2009). Both living and non-living cells of strain E₁ could remove Cd²⁺ from solution, and living cell had better Cd²⁺ removal than non-living cell (Chelliah et al., 2008).

In the other study, Cd-resistant bacteria were isolated from Cd-contaminated soils by Prapagdee and Watcharamusik (2009). One isolate, TAK1, was highly resistant to cadmium toxicity. TAK1 was isolated from soil contaminated with a high Cd concentration (204.1 mg/kg). The removal of the heavy metal ions by some gram-negative bacterial species such as *E. coli* and *P. syringae* were studied by Cohen et al., (1991) and Cabral (1992), respectively. *P. aeruginosa* was found to detoxify Cd²⁺ through production of intracellular cadmium-binding proteins (Hassen et al., 1998). A Cd²⁺-hyperresistant bacterial strain HQ-1 was isolated from a lead-zinc mine by Qing Hu et al., (2007).

Shakibaie et al., (2008) isolated *Paeruginosa* strains that accumulated high concentrations of copper and zinc after exposure to mutagenic compounds. Similarly, Shakibaie et al., (2003) reported that intracellular accumulation of silver

by *Acinetobacter baumannii* BL54 was an energy dependent process and occurs through binding to a cysteine rich metalloprotein. Paul and Jayakumar (2010) applied an analytical study of the cadmium and humic acids contents of two lentic water bodies in Tamil Nadu, India.

From present study it can be concluded that the strain 1 can be efficiently used for bioremediation and removal of cadmium containing waste pollutants with minimum cost and high efficiency.

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