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Isolation and identification of cadmium-resistant bacteria in Soltan Abad river sediments and determination of tolerance of bacteria through MIC and MBC

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ABSTRACT

Biological uptake is one of the most effective ways to remove heavy metals from contaminated areas. Bacteria in contaminated areas get resistant to cadmiumby many ways and become able to remove cadmium. Sultan Abad River, due to its proximity to agricultural land, urban sewage and industrial town of Shiraz suffers from various contaminationswith heavy metals such as cadmium. The purpose of this study is isolation and identification of cadmium-resistant bacteriain Soltan Abad River sediments and determination of MIC and MBC ranges of resistance by those indigenous bacteria. In this study, sediment samples from three stations were taken during two consecutive seasons from Sultanabad River (Autumn and Winter,2012 and 2013). Sediment samples were preparedand studied according to standard methods in the two groups, The first group was used to measure the amount of cadmium by atomic absorption spectrophotometry and the values of pH and temperatureand the second group was used for enrichment and isolation of cadmium-resistant bacteria.LB medium was used for the enrichment and solid nutrient agar medium was used for separation. Bacteria were identified by conventional biochemical tests and tolerance of isolated bacteria by MIC and MBC were evaluated at different concentrations of cadmium chloride. By morphology and biochemical tests, eight Gram-negative bacteria and four Gram-positive cadmium-resistant bacteria were isolated and indentified. The average bacteria counts at all stations show that as compared with other stations, station 3 has more bacteria and the averagebacteria counts was reduced in the presence of 1 mM cadmium chloride ($p<0/001$). The most abundant isolated in this study that were Escherichia cloi and Bacillus, were observed at all stations and the rarest one wasKlebsiella pneumoniaethat was only observed at station 1 and in autumn. The Maximum resistance to cadmium was observed in bacteria Klebsiella pneumoniae with MIC 9 and MBC 10 mM andnext to it, the resistant bacteria Pseudomonas aeruginosa and Bacillus cereus were reported as the most resistant, respectively, with MIC 8 and 6 and MBC 9 and 7 mM. Isolated bacteria, especially bacteria Klebseilla pneumoniae, Pseudomonas aeruginosa and Bacillus cereus are good candidates for treatment and elimination of the cadmium present in polluted rivers.

Keywords: Cadmium-Resistant Bacteria, Soltan Abad River, Biological Removal, *Klebseilla pneumoniae*.

INTRODUCTION

At the present, rivers, and next to them, bays, lakes and oceans are the most polluted waters in that order and about 80% of diseases are water related. River is one of the most important natural resources due to the fact that the ever increasing population are infected especially by heavy metal pollution through which water can enter the body and cause various diseases [1].

The cadmium cycle between water, river, sediments, the vegetation planted in the area and the metal levels in the blood and urine of residents in several studies have proven the direct and indirect impact of environmental pollution

on human health. Cadmium enters the environment from natural resources as well, soil, rock, fossil fuels and volcanic activity have some cadmium levels [2,3]. Natural phosphate layers have between 3-100 $\frac{\mu g}{g}$ cadmium [4].

Cadmium is a heavy metal and among the top ten in the black list of agreements on environmental protection and the World Health Organization (WHO) [5,6]. Cadmium is a toxic pollutant that enters water and sediments in different ways and has a high potential to precipitate in plant and animal tissues.Exessive cadmium inthe presenceof environmental and biological cycles leads to human diseases such as the spongy bone disease, kidney disorders, lung disorders, autoimmune diseases, destruction of red blood cells and some cancers [4,7,8,9].

In a research conducted on water, sediments and periwinkle snails at Elechi River Delta in Nigeria, it was found that cadmium levels in the outer tissues of them is even more than that in the sediments,showing that about two-thirds of the total body cadmium enters the body through food and herbal products [10].

Heavy metals are not biodegradable by chemical reactions and heat and all of them, especially cadmium, have bioacumulation and biomagnification properties as well [7].

Biological removal of heavy metals such as cadmium by microorganisms have been studied in recent decades, which is known as bioremediation [11,12,13].

Sinha *et al* (2009) in India, studied Biological removal of cadmium by *Pseudomonas aeruginosa* KUCD1 and the results showed that this species is a good candidate for elimination of cadmium and has the ability to remove 75 to 89 percent of that metal [14].

Rehman and Shamim (2012) studied biological removal of heavy metals by *Klebsiella pneumoniae* CBL-1 and the results showed that this species is a good candidate for the removal of metals, especially cadmium, at a concentration of 1500 mg / ml [15].

Abyar *et al* (2012), isolated and identified *Achrobacter denitrificans* PQ-1 in the sediments of Persian Gulf and studied the ability of the bacteria tested in removing different concentrations of cadmium [16].

Isolated bacteria from polluted areas have high ability in removing cadmium. Therefore, their use is recommended for wastewater bioremediation and this can lead to more successful cleanup of heavy metals in the environment and this is crucial in order to achieve higher efficiency and more economical feasibility [17].

Soltan Abad River is a seasonal river that is located in the south of thecity of Shiraz, After passing through the industrial town of Shiraz, Soltan Abad River flows into Lake Maharlu. The industrial town of Shiraz has a total area of 1337 ha and new factories and industries in it are full of heavy metals, such as industrial materials and chemical products, rubber and plastics, metal artifacts and ... . Shiraz urban sewage capacity is for 400,000 people and the remaining sewage is flowing into Soltan Abad River, which is rich in cadmium. Of other sources of cadmium in Soltan Abad River is the agricultural land. Cadmium is an element of economic value, including its use in making nickel – cadmium batteries, industrial pigments, stabilizers, glazing and coating of metals such as iron and various alloys. In addition, phosphate fertilizers, fungicides, and pesticides have also significant amounts of the metal [4,7]. Soltan Abad River, due to interference with urban sewage, agricultural land and its vicinity to the industrial town of Shiraz, suffers from various environmental pollutants including heavy metals like cadmium.

The aim of this study was isolation and identification of cadmium-resistant bacteria in Soltan Abad River sediments anddetermination of the resistance spectrum of bacteria by measuring the minimum inhibitory concentration of (MIC) and minimal bacteriocidal concentration (MBC) and the find high-resistant bacteria.

MATERIALS AND METHODS

The Study area (Sampling): River sediment samples were taken from three areas in Soltan Abad. The first area is the interface of sewage with Soltan Abad entrance, The second area is the site of interaction of agricultural land with Soltan Abad River and the third area is the site of interaction of the river with industrial town of Shiraz. Location of the sampling stations was determined by the global positioning system (GPS) (Table 1).

(Table 1) Location of sampling stations

Station	region	Coordinate
1	River sediments under the Soltan Abad entrance bridge	N29º 32′ 11.1″ , E52º 33′ 12.2″
2	River sediments under the Soltan Abad town center bridge	N29º 31′ 12.7″ , E52º 32′ 26.2″
3	River sediments under the bridge of Fasa Road Traffic Police	N29º 29′ 07.6″ , E52º 38′ 17.4″

Surface sediment samples from three stations were taken in two consecutive seasons (autumn and winter, 2012 and 2013), Each season, three samples were taken from each station. The samples were collected in sterile glass containers for culture and for measuring the heavy metal cadmium in polyethylene containers. Sediment sampling was carried out in three to four centimeters from the sediment surface because there is oxygen in this sectionand it would become aerobic beyond it. Sampling was carried out according to standard methodsand all experiments were conducted according to standard reference methods and were repeated three times [18].

Temperature and pH values were measured at each station and the samples were sent immediately to the laboratory within less than 24 hours withstorage at 4 ° C conditions in flasksin contactwith ice.

Measurement of cadmium in the samples: For the measurement of cadmium in sediment samples, sediment samples were first dried in an oven and then milled. Then, 1 g of the sample was taken and 10 ml of hydrochloric acid and nitric acid mixture (with the ratio of three to one) were added and brought to volume with distilled water and the metal content was measured by atomic absorption spectrophotometry [17].

Counting bacteria: Bacteria counts were conducted by viable plate count method. This way: 1 g of each sample was added to 9 ml of normal saline and 1 ml of the suspension was removed and dilution was performed, until a dilution of 10^{-10}. From each of the above mentioned tubes, 0.1 ml was collected and cultured in nutrient agar media containing 1 mM cadmium chloride and nutrient agar without cadmium chloride and incubated for 72-24 h at 30 ° C. After this, the plates were examined and the plates with definite, countable colonies were selected [17].

Enrichment and isolation of cadmium-resistant bacteria: For enriching the cadmium-resistant bacteria, first 5 g of each sample was added to 95 ml of Luria Bertani broth medium containing 0.18 g/l cadmium chloride. Then, all the flasks were incubated in shaker incubator for 2 weeks at 30 ° C. After this, for isolation of cadmium-resistant bacteria, 200µl of the medium containing grown bacteria were removed from the culture and were cultured on the nutrient media containing 0.18 g/lit cadmium chloride surface using glass spread and were incubated for 24-72 h at 30 ° C. After this, the plates were examined and colonies with different morphologies were picked and cultured on blood agar medium and incubated for 24 h at 30 ° C [19].

Identification of the isolated bacteria: Identification of the isolated bacteria using conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology was carried out. Cases were identified by gram staining, morphological characterization, catalase test, oxidase, KOH and other tests for differential diagnosis.

Calculation of minimum inhibitory concentration (MIC)and minimum bactericidal concentration (MBC): Bacterial suspension equivalent to a McFarland standard was prepared for each bacterium and the amount of 1 ml of it was inoculated into tubes containing 1 ml of different concentrations of cadmium chloride metal. For each series of metal dilution, an uninoculated medium as a negative control and an inoculated medium without cadmium as a positive control, was added.Tubes were examined after 3-5 days incubation at 30 ° C, the first tube was clear and no microbial growth was regarded as the minimum inhibitory concentration (MIC). After determination of MIC, the same dilution and the next dilution to 0.1 ml were plated on solid culture medium and the plates were examined after incubation. The concentration of metal in which no colony grew or the number of colonies was reduced by 99.9% as compared with the control plates, was considered as minimum bactericidal concentration (MBC) [15,17,20].

Statistical Analysis
Analysis of the results was performed using SPSS software, and analysis of variance (ANOVA).

RESULTS

Values of temperature, pH and concentration of cadmium in the stations are listed inTable 2.

The Average numbers of bacteria shows that station 3 as compared with other stations has more bacteria significantly ($P< 0.001$). On the other hand, the mean number of bacteria in the presence of 1 mM cadmium salts with metal-free control decreased significantly (Table 3).

(Table 2) Values of temperature, pH and concentration of cadmium in stations in autumn and winter

Season	Station	Temperature	pH	Cd amounts (ppm)
Autumn	1	26	5.9	0.311±0.75
	2	27	7.1	0.298±0.76
	3	27	6.9	0.307±0.77
Winter	1	24	7.1	
	2	25	7.3	
	3	24	7.1	

(Table 3) Average number of bacteria in the presence of cadmium chloride and without cadmium chloride in autumn and winter

Season	Station	cadmium chloride	without cadmium chloride (Control)
Autumn	1	80×10^4	180×10^6
	2	60×10^4	140×10^6
	3	100×10^5	100×10^7
Winter	1	50×10^3	100×10^6
	2	30×10^3	80×10^6
	3	80×10^4	220×10^6

Twelve colonies were examined with different morphologies and by gram staining and biochemical tests, twelve cadmium-resistant bacteria were isolated and identified, Table 4 shows the name of the bacteria and the resistance to cadmium via the MIC and MBC.

(Table 4) Isolation and identification of cadmium-resistant bacteria and maximum resistance to cadmium via MIC and MBC

Bacteria	MIC (mM)	MBC (mM)
Klebseilla pneumoniae	9	10
Pseudomonas aeruginosa	8	9
Bacillus cereus	6	7
Bacillus thuringiensis	4	5
Serratia	3	4
Escherichia cloi	3	4
Corynebacterium	3	4
Flavobacterium	2	3
Staphylococcus	2	3
Achromobacter	2	3
Citrobacter	2	3
Acinetobacter	2	3

Klebseilla pneumoniae bacteria with MIC=9 and MBC=10 mM showed the highest resistance to cadmium.

The most abundant isolated bacteria in this study were *Escherichia cloi* and *Bacillus*t hat were observed at all stations and the rarest one, *Klebseilla pneumoniae* bacteria, was observed only at station 1 and in autumn.

DISCUSSION

The causes of river pollution in Soltan Abad by heavy metals such as cadmium are discharge of sewage into the river, the use of fertilizers and agricultural pesticides on farmland also interactionof the Soltan Abad River with the industrial town of Shiraz. Cadmium can cause contamination of crops and live stock, metal concentrations exceeded the growth and reproduction of plants and reduced the metal uptake by plants and were transmitted to humans and animals [3,21]. 50-75% of the metal accumulates in the liver and kidneys of humans and in various tissues and organs such as liver, kidney, bone, lung and ... . This will cause disturbance and cancer [22].

In the present study, cadmium chloride was added to the culture medium because cadmium chlorideis used in most studies [23,24]. Also, because most studies use nutrient ager used for isolation of cadmium-resistant bacteria, in the present study, too, the same medium was used for isolation of bacteria [6,14].

In the present study, we used an enrichment method for isolation of cadmium-resistant bacteria because in comparison with dilution of bacteria,it makes them more resistant to cadmium [19].

Cadmium is one of the most dangerous heavy metals to humans and aquatic ecosystems [15]. The metal is 0.006 ppb standard for aquatic life, but, unfortunately, in all the stations cadmium content exceeds the limit significantly [17].

In the sections under study, urban, industrial and agricultural activities are regarded as the main causes of pollution in Soltan Abad River. The average number of cadmium-resistant bacteria in the presence of 1 mM cadmium

chloride below the bridge of road traffic police of Fasa (Station 3) was higher than other stations due to direct discharge of wastewater of industrial town of Shiraz.

In the present study, the mean number of bacteria in the presence of cadmium and without cadmium in the autumn was more than that in winter. Also, *Klebseilla pneumoniae* that had the highest resistance to cadmium was observed in autumn so it can be concluded that the optimal conditions for bacterial growth was gone in winter due to increased precipitation.

In the current research, the number of bacteria in a culture medium containing cadmium was lower than the control due to the toxicity of cadmium. The subject was shown in the research of Sharma *et al* (2000) who found that the presence of cadmium reduced the bacterium *Klebsiella planticola* count to fifty times lower [6].

In the present study *E.coli* and *Bacillus* were the most abundant bacteria and *Klebsiella pneumonia* one of the most resistant bacteria in sediments of Soltan Abad River.

In the current research, there were eight gram-negative bacteria and four gram-positive ones indicating that gram-negative bacteria have an outer membrane and a negatively surface charge of LPS as compared with gram-positive bacteria and are less affected by cadmium as compared with Kafilzadeh *et al* (2010) that is consistent with it [17].

Klebseilla pneumoniae bacteria isolated from river sediments in Soltan Abad had the highest resistance to cadmium and it is likely that the structure and the form of bacterial outer membrane makes the uptake of cadmium better. In a study conducted by Rehman and Shamim (2012) they have estimated the amount of resistance to cadmium in *Klebseilla pneumoniae* CBL-1 1500 $\frac{\mu g}{ml}$ as compared with the study of bacterium *Klebseilla pneumoniae* isolates in river sediments of Soltan Abad that is more resistant [15]. Next to *Klebseilla pneumoniae, Pseudomonas aeruginosa* was the most resistant to cadmium; this indicates that resistance to cadmium in gram-negative bacteria is due to the structure of the outer membrane of gram negative bacteria.

Titus and Pfister (1984), Loukidou *et al* (2004), Vullo *et al* (2005) and Singh *et al* (2010) were able to isolate the bacterium *Pseudomonas* from the sediment and various sewage and examined their amount of resistance to cadmium and as compared with the present study, the bacteria were more resistant to cadmium [8,25,26].

Sinha *et al* (2009) and Kafilzadeh *et al* (2010) estimated *Pseudomonas aeruginosa* bacterial resistance to cadmium as 8 and 6 mM, respectively, which is less resistant than *Pseudomonas aeruginosa* isolates with MIC 9 mM isolated in sediments of Soltan Abad River [14,17].

Kafilzadeh *et al* (2010) estimated the amount of MBC in *Pseudomonas aeruginosa* and *Bacillus* Abs as 7 and 5 mM, respectively, while for the bacteria *Pseudomonas aeruginosa, Bacillus cereus* and *Bacillus thuringiensis* they were determined to be 9, 7 and 5 mM respectively. In the present study and thus our isolated bacteria are more resistant [17].

Also Enshaei *et al* (2010) reported the MIC and MBC of the bacterium *Pseudomonas aeruginosa*, 1.5 and 2 mM , respectively, a less resistance as compared with this study [27].

Shakibaei *et al* (2010) after sampling the soil and activated sludge from the factories of Kerman were able to isolate the bacterium *Pseudomonas aeruginosa* that had the highest resistance to cadmium and the bacterial resistance to cadmium was determined after creation of a mutation of 7 mM and before creation of a of 6 mM and despite of the mutation, its resistance was less than that in the present study. Also the bacterium is endemic and does not require genetic manipulation because entrance of manipulated bacteria into the environment the is unauthorized and inappropriate as stated by Global Environmental Protection Agency (EPA) [17,19,28].

Rajbanshi (2008) studied the resistance of isolated bacteria to various heavy metals and found that the amount of cadmium resistance in bacteria *Citrobacter, Flavobacterium* and *Acinetobacter* reported after 10 days of incubation, were, MIC 300, 220 and 150 $\frac{\mu g}{ml}$, which, in comparsion with the current research, that is, the bacteria with MIC 2 mM, have more resistance [29].

CONCLUSION

Due to the growing use of heavy metals, especially cadmium in various industries and replacing bacteria for other methods due to their low cost and low pollution, the results of this study indicate that bacteria isolated, especially

Klebseilla pneumoniae, *Pseudomonas aeruginosa* and *Bacillus cereus* due to the high tolerance to cadmium are a good candidate for the treatment and elimination of cadmium in polluted rivers.

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