Desalination and Water Treatment
www.deswater.com

doi:10.5004/dwt.2017.21483

93 (2017) 139–144
October

Isolation and characterization of copper and cadmium resistant bacteria from industrial wastewaters and evaluating the biosorption of selected bacteria

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Received 25 April 2017; Accepted 21 September 2017

ABSTRACT

Heavy metals are considered as the most serious environmental contaminations because of their high stability in the environmental matrices. Biological activities are imperative themes because of their relationship with mitigation of heavy metals toxicity as well as removing them from the environment. The purpose of this study was to isolate and purify bacteria resistant towards copper and cadmium available in industrial wastewater, and also to evaluate the biosorption of these metals by selective bacteria. In this research the resistant bacteria were isolated at different concentrations of copper and cadmium sulfate salts. Thereafter, the minimum inhibitory concentration(MIC) and minimum bactericidal concentration(MBC) of isolated bacteria were assessed via three repetitions. Analysis of variance (ANOVA)was performed for isolated bacteria according to MBC values and significant differences were observed between data. Then, comparison between the means values of samples by using Duncan method was achieved and the resistant bacteria was selected. Out of 31 resistant bacteria were isolated, purified and gram stained, the most percentage of resistance to copper and cadmium belonged to gram-negative bacteria. Four bacterial species were resistant to high concentration of each copper sulfate and cadmium sulfate. Bacteria were obtained from *Pseudomonas* spp., *Alcaligenes* spp., *Acinetobacter* spp. and *Achromobacter* spp. *Pseudomonas* sp. had the highest ability to absorb copper (78.31%) from 2 mM copper sulfate while *Achromobacter* sp. got the maximum potency to absorb cadmium (53.38%) from 5 mM cadmium sulfate. This work demonstrated that biosorption of copper was higher than cadmium by resistant bacteria and bioaccumulation played a vital role in the removal of cadmium. Consequently, using these bacteria could really considers as a systematic and effective approach in order to heavy metals removal from industrial wastewaters by biosorption and bioaccumulation.

Keywords: Minimum inhibitory concentration (MIC); Minimum bactericidal concentration (MBC); Heavy metals; Biosorption; Wastewater

1. Introduction

Since the industrial revolution, the marked increase in industrial wastewater disposal into the environment and water bodies [1–5]. It can causes a severe damage to the environmental components, in particular the aquatic organisms[6]. The effluent of such facility contains a considerable amount of toxic metals such as cadmium, nickel, cobalt, zinc, copper and lead into the environment [7]. These toxic metals lead to environmental imbalance which influencing seriously on the ecosystem as a whole [8]. However,copper for instance in its ionic form is necessary for eukaryotic organisms and human in trace amounts but when exceeds

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the permissible limit transitions into toxins. The toxicity lies in its intrinsic properties of copper, as free copper ions undergo redox cycling reactions alternating between Cu (I) and Cu (II) as well as transfer of electrons to hydrogen peroxide which usually results a concomitant generation of hydroxyl radicals that readily caused irreparable harm to cellular systems of bio-molecules [9,10].

Cadmium, as a hazardous pollutant is another example commonly present in the natural environment. The importance of detecting of cadmium levels before they entered the human food chain because it conceals a real to human health represented in some of undesirable side effects such as (oxidative stress, changes in some enzymes activities, and interactions with biomolecules including DNA and RNA) [11].

Soluble metal ions are considered as one of most obvious inorganic contaminants risks causes to the environment. Variety of methods physical and chemical techniques have been used in order to detect the heavy metals concentrations in the environmental matrices, however, they have not been as effective as biological methods. Biotechnology plays pivotal role in removing unwanted heavy metal ions from water and soil. Furthermore, using biodegradable polymers like chitosan is a sufficient method to absorb heavy metals from industrial wastewater [12–15].

Numerous bacteria, fungi, yeasts, algae, plants and other aquatic organisms have been studied biological absorbents of heavy metals [16–18]. Absorption and toxicity of heavy metals by bacteria at different environmental conditions has been studied.

Researchers concluded that there was a direct relationship between resistance of bacteria to heavy metals, and concentration of metals, environmental pH and duration of contact time as a relevant factors with heavy metals adsorption process [19].

In order to achieve the highest proportion of heavy metals absorption and economically viable at once, threshold of bacterial resistance and concentration of heavy metals has to be considered [20].

In this work, isolation and purification of bacterial resistance to copper and cadmium was carried out and then MIC and MBC of bacteria were determined. The most resistant bacteria species was identified using biochemical tests. Finally, biosorption of metals by selective bacteria was evaluated.

2. Materials and methods

2.1. Sampling and isolation of bacterial resistant to Cadmium and Copper

Industrial wastewater samples were collected from the fifth largest copper and charcoal factories Kerman, Iran.

Industrial wastewater samples were collected from the fifth largest copper and charcoal factories Kerman, Iran. The grab sampling method was used; the samples were kept in standard containers.

Isolation and purification bacterial resistance to cadmium and copper was done in nutrient agar Petri dishes containing different concentrations of metal salts [copper sulfate ($CuSO_4{\cdot}5H_2O$) and cadmium sulfate ($CdSO_4{\cdot}8H_2O$)], pH value was adjusted to 7 [21–23]. Cultures were prepared by adding metal salts with concentration from 0.5 to 12 mM/l (by increasing 0.5 mM/l) for copper and cadmium sulfate, separately. After that, each sample was cultured in concentration in pour plate method during two repetitions, separately and they were incubated for three days at temperature of 30°C. Colonies which grew up in different concentrations were purified and they were kept inside the tubes in refrigerator for further tests.

2.2. Determination of MIC and MBC for isolated bacteria to against of metal salts, Copper and Cadmium sulfate

Nutrient broth and agar cultures were used to determine MIC and MBC of 31 isolated bacteria from industrial wastewaters [22]. In this way, the isolated bacteria from previous cultures were placed inside the nutrient broth with the same metal salts and primary concentration. Thereafter, they were cultured on nutrient agar without metal salts, after 24 h of incubation at 30°C, by spread plate method and the growth rate was investigated. Concentration increased steeply by 0.5 mM/l in nutrient broth when bacteria was growing. This experiment was repeated continuously, until growing stopped in nutrient broth. Thus, bacteria MIC was determined. Tubes were used to determine bacteria MBC on nutrient agar using spread plate method. This trial was continued until there was increase in the concentration of metal salt in nutrient broth. Finally, bacteria did not grow in nutrient agar considered as MBC. Therefore, MIC and MBC were determined for each bacterium via three repetitions for copper and cadmium sulfate [21–24]. ANOVA of isolated bacteria was investigated according to MBC values using SPSS software (SPSS version 20). It showed that there is a significant difference between bacteria in terms of MBC. Afterward, comparison between mean values of samples and the most resistant bacteria was employed using. The genera of these bacteria were identified routinely by biochemical reactions according to Bergey's Manual of Systematic Bacteriology [25].

2.3. Determining the ability of selective bacteria for biosorption of copper and cadmium

Bacterial suspension were provided for absorption studies of selected resistant bacteria (four copper resistant bacteria and four cadmium resistant bacteria) in LB broth [26]. Copper and cadmium sulfate solutions were provided in three different concentrations in LB broth and their pH was adjusted to 7, with regard to bacteria MIC. Three solutions with concentrations 2, 4 and 8 mM/l were adopted to study absorption ability of 4 bacterial resistances to copper sulfate,- Similarly, three solutions with concentrations 1.25, 2.5 and 5 mM/l were provided to study absorption ability of 4 bacterial resistances to cadmium sulfate. Each bacterium with different concentrations were putted in incubator shakers at 30°C and 160 rpm for 24 h. A sample without bacterium was considered for each concentration. After 24 h, samples were centrifuged in 5000 rpm for 10 min and the solution obtained was used to determine the metal present using flame atomic absorption system with Specter AA 220. The ability of bacteria to absorb and remove copper and cadmium was calculated by fraction of primary metal concentration from supernatant concentration and absorption percentage of each metal was determined by selective bacteria.

3. Results

The results in Table 1 show the mean of three repetitions of MIC and MBC for each of isolated bacteria for metal salts, copper and cadmium sulfate in mM/l. Out of 31 resistant bacteria were isolated, purified and gram stained, the most percentage of resistance to copper and cadmium belonged to gram-negative bacteria.

ANOVA of the isolated bacteria was achieved according to MBC for copper and cadmium sulfate and interestingly, significant difference was observed between them. Moreover, comparison between MBC means was done using Duncan method, the isolated bacteria based on MBC for copper sulfate were divided into 8 groups,Since group "h" includes 4 bacteria; C_3, A_4, A_6 and $A_8$was with the highest resistance (Fig. 1). Likewise, the isolated bacteria according to MBC for cadmium sulfate were divided into 7 groups. So meanwhile, group "g" includes 4 bacteria; C_3, C_1, C_2 and A_8 got uppermost resistance (Fig. 2). Subsequently, 4 bacterial species with maximum resistance to both copper sulfate and cadmium sulfate were selected among 31 isolated bacterial species.

Outcomes of calculated MIC concentrations of selected bacteria indicated that three concentrations were chosen to determine biosorption of copper and cadmium. The minimum MIC were 8.167 and 5.333 mM/l for bacterial resistant to copper sulfate in group "h" and cadmium sulfate in group "g", respectively.

Tables 2 and 3 present the biosorption rate of each selected bacterium for each metal in specific concentrations after 24 h at 30°C and pH 7.

Two bacterial species C_3 and A_8 were witnessed in both groups of bacterial resistant to copper and cadmium. The genera of 8 selected bacteria were identified. Bacterial species of A_4, A_6were from *Pseudomonas* spp., A_8, C_1were from *Alcaligenes* spp., and C_2 and C_3were from *Acinetobacter* sp. and *Achromobacter* sp., respectively.

Table 1
Morphology of isolated bacteria and means of three repetitions of their MIC and MBC against copper and cadmium sulfate

Bacteria	Shape	MIC (mM) $CuSO_4·5H_2O$	MBC (mM) $CuSO_4·5H_2O$	MIC (mM) $CdSO_4·8H_2O$	MBC (mM) $CdSO_4·8H_2O$
A1	Bacillus G+	0.333	0.833	0.167	0.333
A2	Coccobacillus G–	6.333	8.500	6.667	7.833
A3	Coccus G+	0.167	1.833	0.167	0.167
A4	Coccobacillus G–	8.333	10.833	6.667	7.667
A5	Coccus G+	0.333	0.833	0.167	0.500
A6	Coccobacillus G–	8.167	10.333	6.500	8.000
A7	Bacillus G–	0.333	1.000	0.167	0.333
A8	Filamentous G–	8.333	10.000	6.833	8.333
A9	Coccobacillus G–	5.833	7.500	4.333	5.667
A10	Coccus G+	0.167	1.667	0.167	0.167
A11	Coccus G+	0.167	1.000	0.167	0.167
B1	Coccus G+	<0.5	1.167	No growth	No growth
B2	Coccus G+	0.167	1.167	No growth	No growth
B3	Coccus G+	<0.5	1.000	No growth	No growth
C1	Coccobacillus G–	7.667	9.000	6.667	9.000
C2	Coccobacillus G–	6.833	8.500	5.333	8.500
C3	Bacillus G–	8.167	10.833	6.667	9.000
C4	Bacillus G–	8.000	9.000	6.500	7.667
C5	Coccobacillus G–	6.833	8.333	6.333	7.833
C6	Coccobacillus G–	6.333	7.667	6.000	7.833
C7	Bacillus G–	0.500	0.833	No growth	No growth
D1	Coccobacillus G–	6.667	8.000	6.000	7.500
D2	Coccobacillus G–	5.333	6.500	4.333	7.000
D3	Bacillus G–	0.167	0.667	No growth	No growth
E1	Bacillus G–	4.333	6.333	4.333	5.667
E2	Bacillus G–	4.167	6.333	5.000	7.667
E3	Coccus G+	0.167	0.667	0.167	0.167
E4	Bacillus G–	4.667	6.000	6.000	6.333
E5	Coccobacillus G–	4.333	5.333	5.667	6.000
E6	Coccus G+	0.167	1.000	0.167	0.167
E7	Coccus G+	0.167	0.833	0.167	0.167

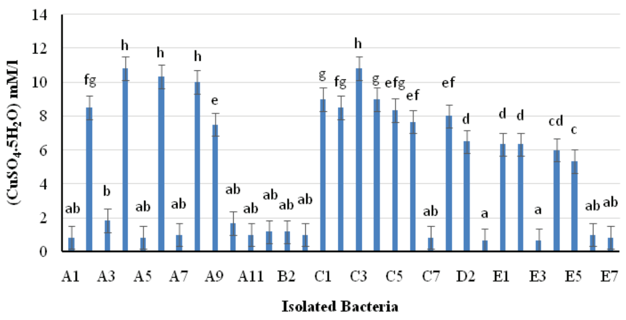

Fig. 1. Comparison between MBC of isolated bacteria toward copper sulfate and their classification using Duncan method.

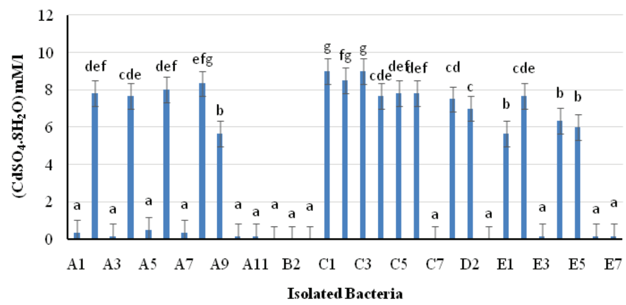

Fig. 2. Comparison between MBC of isolated bacteria toward cadmium sulfate and their classification using Duncan method.

As shown in Table 2,the highest absorption rate of copper was obtained in concentration of 2 mM with mean valueof 77.65% by bacteria,whereas bacterium A_4 (*Pseudomonas* sp.)had the utmost percentage of absorption with 78.31%.

Table 3 captures the highest rate of cadmium absorption was attained at concentration of 5 mM with percentage of 52.47% by bacteria and bacterium C_3 (*Achromobacter* sp.) had the highest rate of absorption with percentage of 53.38% .

Metals affect the growth and metabolism of bacteria directly and indirectly. Bacteria absorb metals through biosorption and bioaccumulation actively and inactively, respectively. Biosorption has shown high potencyand consideredas the most practical method [27]. In this study, absorption after 24 h of both types were studied. Thus, bacteria started absorption through this time atdifferent concentrations actively and inactively. By the findings acquired, inactive absorption was observed for cadmium in higher concentrations, because the activity of bacteria flourishes in low concentrations, accordingly, absorption and removal of this metal be less. Additionally, by increasing the concentration and cell death, bioaccumulation increases. Eventually, active and inactive absorption flourished for copper effectively.

4. Discussion

Today, environmental contamination caused by industries is a serious concernat the increasing rate of wastewater disposable into the environment directly without or with partial treatment, as such effluents contain toxic materials particularly heavy metals [28].

According to previous research, genera of bacteria such as *Pseudomonas* spp., *Alcaligenes* spp., *Klebseill* spp.,

Table 2
The amount of copper in mg/l in supernatant and percentage of its absorption by selected resistance bacteria during 24 h

Bacteria	Concentration (mM)		
	2	4	8
Control sample (mg/l)	399.344	770.569	1553.72
A4 mg/l (absorption %)	86.599 (78.31%)*	180.391 (76.59%)	352.080 (77.3%)
A6 mg/l (absorption %)	96.670 (77.3%)	186.986 (75.73%)	372.680 (76%)
A8 mg/l (absorption %)	92.346 (76.88%)	182.445 (76.32%)	379.832 (75.5%)
C3 mg/l (absorption %)	87.571 (78.1%)	188.402 (75.6%)	387.570 (75%)

*Percentage of copper absorption from solution during 24 h by bacteria.

Table 3
The amount of cadmium in mg/l in supernatant and percentage of its absorption by selected resistance bacteria during 24 h

Bacteria	Concentration (mM)		
	1.25	2.5	5
Control sample (mg/l)	196.936	369.421	1537.176
A8 mg/l (absorption %)	170.007 (13.67%)*	321.008 (13.11%)	737.3 (52.04%)
C1 mg/l (absorption %)	178.029 (9.6%)	317.946 (13.93%)	746.312 (51.45%)
C2 mg/l (absorption %)	172.876 (12.22%)	346.051 (6.33%)	799.232 (48%)
C3 mg/l (absorption %)	188.824 (4.12%)	350.526 (5.11%)	639.844 (58.38%)

*Percentage of cadmium absorption from solution during 24 h by bacteria.

Serratia spp., *Escherichia coli*, *Flavobacterim* spp.,*Staphylococcus* spp., *Achromobacter* spp., *Acinetobacte* spp. etc. were resistant bacteria to heavy metals like copper and cadmium, and they were isolated and identified from different places, they have shown the ability to absorb and decrease concentration of heavy metals [29–31]. In this research four genus of bacteria including; *Achromobacter* spp., *Acinetobacter* spp., *Alcaligenes* spp. and *Pseudomonas* spp. were isolated from industrial wastewaters and they were identified as copper and cadmium resistant bacteria. They were gram-negative bacilli of Non-fermentation bacteria.

Shakoori and Muneer [21] isolated copper resistant bacteria by studying wastewater and they introduced gram-negative bacteria with 400 mg/l MIC as most resistant bacteria to copper. Andeazza et al. [32] studied copper resistant bacteria isolated from Vineyard soils and mineral wastewater and they introduced two genera of bacteria; *Staphylococcus* sp. and *Bacillus* sp. as most resistant and powerful absorbent bacteria to copper. In this research, two genera of bacteria; *Pseudomonas* sp. and *Achromobacter* sp. had the highest resistance to copper with MIC and MBC means (8.333 and 10.833 mM) and (8.167 and 10.833 mM), respectively.

Sinha et al. [33] introduced *Pseudomonas aeruginosa strain KUCd1* resistance to cadmium (8 mM). It exhibited high Cd accumulation under *in vitro* aerobic condition and demonstrated a significant ability to remove more than 75 and 89% of the soluble cadmium during the active growth phase from the growth medium. In this research, *Alcaligenes* sp. and *Achromobacter* sp. with mean of MIC and MBC (6.667, 9 Mm) exhibited highest rate of resistance to cadmium.

Regarding the fact that microorganisms are the best option to reduce metal contaminations in aquatic and soil environments, it is necessary to isolate and identify them from wastewaters and contaminated soils containing heavy metals. Researches carried out by Ansari and Malik [22] showed biosorption of cadmium and nickel by isolated resistant bacteria from contaminated soils. They demonstrated the effect of concentration, pH and contact time of bacteria on biosorption. Andreazza et al. [32] indicated that some bacteria have high ability to remove copper from the environment. Regarding the absorption carried out by selected bacteria in different concentrations of copper, this research demonstrated that biosorption and bioaccumulation of copper was more than that of cadmium. Removal of cadmium was slow and less because of its toxic substance and the active bacteria prevent from entering it into the cells. However, in high concentrations of cadmium, bioaccumulation increases in dead cells and leads to the removal of cadmium from environmental. Therefore, living and non-living biomass of bacteria, act together to absorb and remove heavy metals.

5. Conclusion

As a matter of fact, cadmium is one of the most dangerous metal for human and ecosystem health, since; small amounts can causes devastating effects. On the other hand, as a result of widespread uses of copper, it had been difficult to control entrance of such metals to water and soil matrices. It would therefore be necessary to remove the extra amount these metals from the natural environment.This research demonstrated that biosorption of copper by resistant bacteria was higher than cadmium and also it showed that bioaccumulation had an important role to absorb of cadmium. So, using living and non-living biomass of resistant bacteria can be an effective approach to absorb heavy metals from the environment based on biosorption and bioaccumulation concepts.

Acknowledgments

This research was supported by an educational grant for doctoral thesis from Islamic Azad University of Karaj (grant number: 11530513931005). Authors wish to thank Mrs. Afrooshteh for her assistance in the identification of the bacteria. Special thanks to the laboratory technicians in Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology (Kerman, Iran) for their sincere helps in doing experiments.

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