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Original Article

بررسی جذب زیستی سرب توسط باکتری های مقاوم به سرب جداشده از پساب کارخانجات شهرک صنعتی آق قلا

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چکیده

سابقه و هدف: سرب یک فلز سنگین و یک آلاینده پایدار در طبیعت است که از طریق پساب های صنعتی به محیط وارد می شود. این مطالعه با هدف جداسازی باکتری های مقاوم به سرب از پساب های صنعتی و بررسی جذب زیستی سرب انجام شد.

مواد و روش ها: این مطالعه توصیفی بر روی سه نمونه پساب انجام گرفت. برای جداسازی باکتری های مقاوم به سرب از نوترینت آگار حاوی غلظت های مختلف استات سرب استفاده شد. سپس خصوصیات ۲۸ کلنی بررسی گردید. از این تعداد ٦ کلنی با بیشترین مقاومت به سرب انتخاب شدند و با استفاده از اسپکتروفتومتری جذب اتمی میزان جذب سرب توسط این جدایه ها اندازه گیری گردید. ٤ جدایه برتر که دارای بیشترین جذب سرب بودند به کمک روش مولکولی شناسایی شدند.

یافته ها: ۷۸/۵ درصد باکتری های مقاوم جدا شده گرم مثبت و ۲۱/۵ درصد گرم منفی بودند. با استفاده از آزمون های بیوشیمیایی مشخص شد که ٤٦/۵ درصد از جدایه ها به جنس *باسیلوس* تعلق دارند. از این میان جدایه T5 بیشترین جذب سرب را داشت. همچنین نتایج تعیین توالی جدایه های برتر نشان داد که این سویه ها متعلق به گونه های *اشریشا کلی* سویه ۷۸۹، *انتروباکتر کلواکه* سویه GGT036، *باسیلوس تکوئی لنسیس* سویه KM30 و *کورتیا* اس. پی سویه VITA1 (T5) هستند.

نتیجه گیری: یافته های این مطالعه نشان می دهد که باکتری های جدا شده از پساب آلوده به سرب پتاسیل زیادی برای حذف این فلز از محیط های آلوده دارند و می توانند کاندیداهای خوبی برای پاکسازی و تصفیه زیستی باشند.

واژگان کلیدی: سرب، باکتری های مقاوم، باکتری های جاذب، زیست پالایی.

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Original Article

Study on lead biosorption by lead resistant bacteria isolated from AqQala industrial park wastewater

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Abstract

Background & Objectives: Lead is a heavy metal and persistent pollutant in the environment that enters through industrial wastewaters. The aim of this study was to isolate lead resistant bacteria from the industrial wastewaters, and to study the lead absorption by the isolated bacteria.

Materials & Methods: This descriptive study was performed on three wastewater samples. Nutrient agar medium containing different concentrations of lead acetate were used to isolate the lead resistant bacteria. Overall, 28 colonies were selected and their characteristics were determined. Of these, six colonies with the highest lead resistance were selected. The rate of lead absorption of these strains was measured using atomic absorption spectrophotometry. Four strains with higher lead absorption was identified using molecular method.

Results: 78.5% of the isolated resistant bacteria were Gram positive and 21.5% of the bacteria were Gram negative bacteria. Biochemical tests showed that 46.5% of the isolates were belonged to *Bacillus* sp. Of these, T5 had the highest lead absorption. Also, sequencing results revealed that the selected isolates are belong to *Escherichia coli* strain 789, *Enterobacter cloacae* strain GGT036, *Bacillus tequilensis* strain KM30 and *Kurthia* sp. VITA1 (T5).

Conclusion: The result of this study showed that the bacteria isolated from lead-containing wastewaters are highly potent for removal of this metal from polluted environments, and therefore can be appropriate candidates for bioremediation and biological treatment.

Keywords: Lead, Resistant bacteria, Absorbing bacteria, Bioremediation.
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Introduction

In recent years, heavy metal pollution of industrial wastewaters has become a serious environmental problem. The main sources of the heavy metals are the industrial activities such as metal processing, mining and electroplating, tanning, carpet washing, dyeing, surface finishing industry, energy and fuel production, fertilizer and pesticide industry, metallurgy, iron and steel, electrolysis, electro-osmosis, leather industry, photography, electric appliance manufacturing, metal surface

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treating, aerospace and atomic energy installation (1, 2).

The release of untreated industrial wastewater into the environment has become a major concern as these metals cannot be degraded and persist in the ecosystem (3, 4). Although some of the heavy metals (cobalt, chromium, nickel, iron, manganese, zinc, etc.) are among the trace elements, and are essential for the organisms but some of them have no biological role and are hazardous to human health and animals even at very low concentrations (cadmium, copper, lead, etc) and cause ecosystem imbalance at high concentration (4-6).

Hence the study of methods to remove these contaminants from the wastewaters is essential (7). The use of physicochemical methods, such as ion exchange, chemical precipitation, electrochemical treatment, reverse osmosis and evaporation for this purpose is very expensive. Moreover, these methods have low efficiency at low metal concentrations, so bioremediation has currently received attention of researchers and removal of the heavy metals from the wastewaters by microorganisms has become a promising approach (8). The most common biological techniques of bioremediation are biosorption and bioaccumulation.

Biosorption is an inactive metal absorption by inactive or dead materials originated from a biological source, but bioaccumulation is heavy metal absorption by microorganisms (9). The results of recent studies have shown that isolated microorganisms from the heavy metals -polluted areas have a high potential to remove these metals from the environment (7).

Among the microorganisms, the heavy metal resistant bacteria have been proposed to be efficient and economical in bioremediation of wastewaters with heavy metals contamination (3). These bacteria are able to clean-up the industrial wastewaters with the processes like biosorption, bioaccumulation and bioprecipitation (1).

As lead (Pb) is a major waste pollutant and is highly toxic to the organisms, many studies have focused on the isolation and identification of lead resistant bacteria from polluted environments (10).

The reported isolates were *Pseudomonas* sp., *Klebsellia* sp., *Staphylococcus* sp., *Proteus* sp., *Bacillus* sp (6), *Salmonella choleraesuis* strain 4A, *Proteus penneri* strain GM10, *Bacillus subtillis* strain GM02, *Pseudomonas aeruginosa* strain 4EA, *Proteus penneri* strain GM03, *Providentia rettgeri* strain GM04 (11), *Delftia tsuruhatensis*is (12), *Kurthia* genus, *Enterobacteriacae* (13), as well as *Halomonas eurihalina strain* D (14).

This study was aimed to isolate and identify lead resistant bacteria from wastewaters plants located in AqQala industrial park, Gorgan, Golestan province. Moreover, lead biosorption by resistant bacterial isolates was assessed, and the effects of pH, initial lead concentration, and time of lead exposure was examined on lead absorption.

Materials and Methods

Sample Collection and Isolation of Lead Resistant Bacteria

To perform this descriptive study, three samples were collected from metal processing, paper recycling and dyeing plants located in AqQala industrial park, Gorgan, Golestan province, Iran. First, the lead content of

the samples was measured. The samples were cultured in nutrient agar (pH ±7.0) plates containing lead acetate, with increasing concentrations from 1 mM to 7 mM, and incubation at 37 ˚C for 24 h (8). Then, colonies differing in both the shape and colors were selected and used for further studies.

Morphological and Biochemical characteristics of the lead absorbing isolates

Morphological characteristics of the isolates were determined by examination of Gram's reaction and spore formation. Then, the isolates were analyzed by biochemical tests including MR-VP test, citrate utilization, sulfide-indole-motility test (SIM medium), casein hydrolysis, starch hydrolysis, urea hydrolysis, gelatin hydrolysis, TSI agar, as well as catalase and oxidase activities (3).

Quantification of Lead Absorption

The selected isolates were cultured in nutrient broth enriched with 0.3% yeast extract and incubated at 37 ˚C for 72 h. Then, the bacterial suspension was centrifuged for 20 min at 5000 rpm; the pellet was washed twice with double distilled water and centrifuged the same as the previous step. After that, 0.5 g of the pellet was added to a conical flask containing 2 mM lead acetate solution with pH 5.5 and was incubated at shaker incubator at 180 rpm, at 37 ˚C for 3 h. The solution was centrifuged for 20 min at 5000 rpm and the supernatant was collected. The amount of lead in the initial solution and the supernatant was measured with atomic absorption spectroscopy (AAS) and the percentage of absorption was calculated (7, 15).

Table 1: Primers used in this study (16).

Primer name	Primer sequence	PCR product
16S-F	5'-AGAGTTTGATCCTGGCTCAG-3'	1500 bp
16S-R	5'-ACGGCTACCTTGTTACGACTT-3'	

16S rDNA gene amplification

The bacterial isolate was cultured in the nutrient broth overnight. Then, the genomic DNA of the isolate overnight culture was extracted using Geno Plus TM Genomic DNA Extraction Miniprep System (Viogene, China). The quality and quantity of the extracted DNA was determined by agarose gel electrophoresis (1%). The genomic DNA was used as a template for *16S rDNA* gene amplification using consensus primers (16).

To carry out PCR, 2X Master Mix (Thermo scientific, USA) was applied. The reaction mixture was prepared by adding 1 µl of each primer (20 pmol), 5 µl of DNA template, and 19 µl of double-distilled water (DDW). The PCR amplification was performed with 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 56 ˚C for 30 s, and extension at 72 ˚C for 30 s. The initial denaturation and final extension were 94 ˚C for 3min and 72 ˚C for 10min respectively. The primers used in this study are mentioned in Table 1.

The *16S rDNA* gene was successfully amplified with consensus primers, and anticipated fragments with 1500 bp were observed on 1% agarose gel. Then, the purified PCR products were sent to Macrogen (South Korea) for sequencing. The obtained nucleotide sequences were searched for homology in the NCBI nucleotide database

Table 2: Morphological and biochemical characteristics of the selected isolates for lead absorption.

Isolates	T6	T5	B8	B7	K7	K2
Morphology	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	-	+	-	+	+	+
Spore formation	-	-	-	+	+	+
Motility	+	+	+	-	-	+
Catalase	+	+	+	+	+	+
Oxidase	-	-	+	+	+	+
Casein	-	-	+	+	+	+
Gelatin	-	-	-	+	+	+
Starch	+	+	+	+	+	-
Urea	-	-	+	+	-	-
Indole	+	+	-	-	-	+
MR	+	+	-	-	-	+
VP	-	-	+	+	+	-
Citrate	-	+	-	+	+	+
TSI	A/A	ALK/A	A/A	A/A	ALK/A	A/A
H_2S	-	+	-	-	-	-

using BLAST tool (17). The *16S rDNA* gene sequences were aligned against the reference nucleotide sequences retrieved from Gen Bank.

Effect of pH on the lead absorption

To examine the effect of pH on the lead absorption, two sets of flasks containing 25 ml of 2 mM lead acetate solution were prepared. The pH was adjusted from 2.5 to 5.5 using 1 N HCl and 1 N NaOH, respectively.

One set of flasks was inoculated with the isolate with the highest lead absorption rate, and and incubated at shaker incubator at 150 rpm; at 37˚C for 3 h. Next, the lead absorption was evaluated. The other set was used as the control and no inoculum was added (7).

Effect of lead concentration on its absorption

To evaluate the effect of lead concentration on the absorption, different concentrations of lead acetate solution including 1, 1.5, 2, 2.5 and 3 mM at optimum pH of 5.5 were prepared. After inoculation, the incubation was performed the same as the previous step, and finally the lead absorption was calculated (7).

Effect of exposure time on the lead absorption

The effect of lead exposure time on the lead absorption was assessed in a 2 mM lead acetate solution with pH of 5.5 following 30, 60, 120, 180, 240 and 300 min incubation period at 150 rpm, 37˚C. After that, the lead absorption was measured (7).

Results

Isolation and Identification of lead resistant bacteria

After 24 h of incubation, out of 85 colonies appearing in the plates containing different concentrations of lead acetate, 28 isolates were selected and their morphological and biochemical characteristics were determined. No colony was formed in the plates containing 7 mM lead acetate. Morphological studies showed that the percentage of Gram positive bacteria is higher than Gram negative ones so that 78.5% of the resistant bacterial isolates were Gram positive, and 21.5% of them were Gram negative bacteria.

The results of biochemical tests showed that 46.5% of the isolates were belonged to

Bacillus sp. Out of the 28 colonies, 6 colonies with the highest lead resistance in each of the three samples were selected and used for further studies.

Quantification of Lead Absorption

The quantity of lead absorption by the 6 selected isolates was examined. The results showed that T5 had the highest lead absorption as high as 88% amongst the isolates, so this strain was used for the next experiments and K2 and B7 isolates with the lowest lead absorption were excluded in this step (Table 3).

PCR and sequencing results

The results of the genomic DNAs PCR in some of the bacterial strains is presented in Figure 1. The results of the alignment of nucleotide sequences with already available database were as follows: T5, *Kurthia* sp. (99% similarity to *Kurthia* sp.VITA1, accession number JQ398850.1); T6, *Escherichia coli* (99% similarity to *Escherichia coli* strain 789, accession number CP010315.1); B8, *Enterobacter cloacae* (98% similarity to *Enterobacter cloacae* strain GGT036, accession number CP009756.1); K7, *Bacillus tequilensis* (99% similarity to *Bacillus tequilensis* strain KM30, accession number JF411311.1).

Effect of pH on the lead absorption

The results indicated that *Kurthia* sp.VITA1 has no lead absorption at pH of 2.5 without so much difference between the lead absorption at pH 3.5 (71%) and 4.5 (76%).

The highest lead absorption was measured at pH of 5.5 (88%).

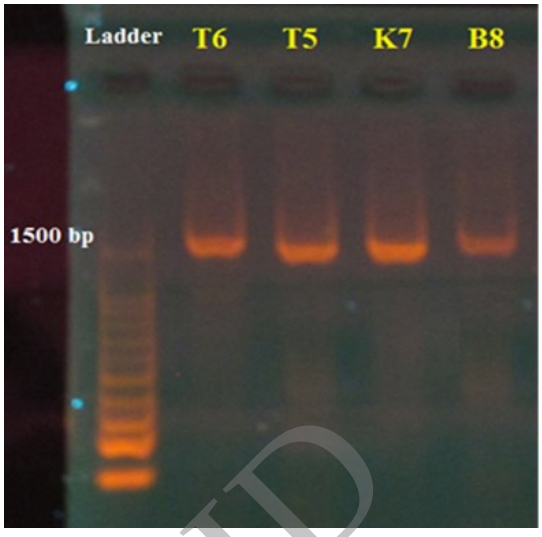

Figure 1: Lane 1: DNA ladder Mix as a size standard, Lanes 2-5: PCR result on the genomic DNAs of T6, T5, K7 and B8 strains.

Effect of lead concentration on the absorption

The results showed that with increasing lead concentration from 1 to 2 mM, the quantity of lead absorption by *Kurthia* sp. VITA1 will be increased. The quantity of lead absorption at 2 mM concentration was higher than 2.5 mM and 3 mM concentrations.

Effect of exposure time on the lead absorption

After 30 min exposure with lead solution, 63% of the existing lead was rapidly absorbed. After that, the lead absorption

Table 3: Quantification of lead absorption by the selected isolates.

Strains	Remaining lead in solution (mg/l)	Initial concentration (mg/l)	Percentage lead of absorption
K2	216/5	342/6	%37
K7	166/8	342/6	%51
B7	233/4	342/6	%32
B8	101/1	342/6	%71
T5	40/9	342/6	%88
T6	85/6	342/6	%75

speed was reduced and the lead absorption reached to 88% after 4 h. Then after, it remained constant, so that the maximum amount of lead absorption for *Kurthia* sp. VITA1 was 88% at 2 mM lead acetate concentration with pH of 5.5 after 4 h (Figure 2).

Discussion

Contamination of the environment with heavy metals has become a serious problem for the human health and other creatures. Industrial wastewaters containing the heavy metals are a major source of pollution to the ecosystems. Absorption of the heavy metals by microorganisms has led to special attention of the researchers to identify the various types of them, with the ability of removal of these metals from the environment especially from the effluent of industrial plants and contaminated sewage. In the present study, lead resistant bacteria were detected in the industrial wastewaters of AqQala industrial park, Gorgan, Golestan province, Iran.

Then, the lead absorption by the isolates was studied. Based on the results, the percentage of Gram positive bacteria was higher than Gram negative ones so that 78.5% of the isolated resistant bacteria were Gram positive and 21.5% of them were Gram negative.

Most of the isolates (46.5%) were belonged to *Bacillus* sp. This result is consistent with the findings of Kafilzadeh *et al*. (2012) that studied on indigenous bacteria isolated from lead contaminated soils near gas stations. They reported 67% of the resistant bacterial isolates as Gram positive and 33% as Gram negative, where the highest percentage was belonged to *Bacillus* sp. (100%). Moreover, this bacteria was recognized as one of the most resistant bacterial strains against lead element (18).

Nath *et al*. (2012) isolated thirty cadmium and lead resistant bacteria from the sewage of industrial effluents, garages and petrol pumps against. The predominant isolates were identified as *Pseudomonas* sp., *Klebsellia* sp., *Staphylococcus* sp., *Proteus* sp. and *Bacillus* sp , with *Bacillus* sp showing the highest resistance pattern against cadmium (1800 µg/ml) and lead (1200 µg/ml) (6).

In a research by Naik *et al*. (2012), lead-resistant bacterial strains including *Salmonella choleraesuis* strain 4A, *Proteus*

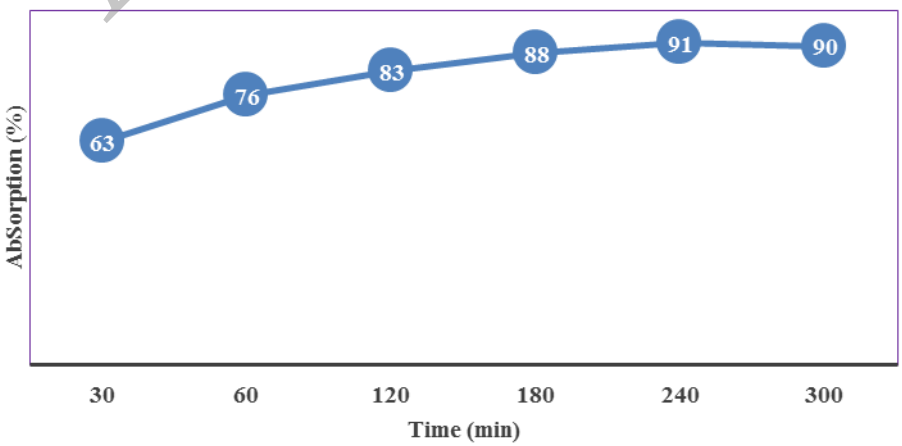

Figure 2: Effect of exposure time on the lead absorption.

penneri strain GM10, *Bacillus subtillis* strain GM02, *Pseudomonas aeruginosa* strain 4EA, *Proteus penneri* strain GM03 and *Providentia rettgeri* strain GM04 were isolated from soil contaminated with car battery waste (10).

According to the report of Lugauskas *et al*. (2005), *Bacillus* sp. bacteria are the most abundant Gram positive bacteria in the soil (19), not surprising that they have been reported as the most abundant isolates in the lead-polluted environments.

It seems that continuous exposure of lead to the environment has adapted these bacteria to this metal. In current research, the results of biochemical and molecular analyses indicated *Kurthia* sp. VITA1, *Escherichia coli* strain 789, *Enterobacter cloacae* strain GGT036 and *Bacillus tequilensis* strain KM30 as lead-resistant isolates.

The highest concentration of the lead acetate to which the isolates showed resistance was 6 mM. Dorian *et al*. (2012) isolated *Delftia tsuruhatensis*is from mine tailings that was resistant to 6 mM lead concentration (11). However, there are some reports on bacterial isolates with higher lead resistance.

Malik *et al*. (2002) reported that the bacterial strains isolated from the industrial soils are able to tolerate 7.23 mM lead and cadmium concentrations (20).

Meanwhile, bacterial isolates from heavy metal contaminated soils reported by Abou Shanab and his colleagues were resistant to 15 mM lead concentration (21).

Karim salmani *et al*. (2011) isolated three bacterial strains that were belonged to *Bacillus* sp. and were able to tolerate lead nitrate concentration of up to 15 mM (7).

Various studies have been carried out on the quantity of lead absorption by bacteria. In the present study, *Kurthia* sp. VITA1 had the most absorption rate among the lead resistant isolates and could absorb up to 88% of the initial concentration of lead after 3 h exposure to lead.

In a research by Sowmya *et al*. (2013), isolated heavy metal resistant halophilic bacteria belonging to *Kurthia* genus and *Enterobacteriacae* could absorb 99% of lead in the presence of 10% and 15% salt, respectively (12).

Karim salmani *et al*. (2011) reported that one of the isolates absorbed 98% of the initial lead nitrate concentration (150 mg/l), while Tunali and his colleagues (2005) reported that the lead absorption by *Bacillus* sp. isolate is 92.27 mg/gdw out of 250 mg/l initial lead concentration (7, 22).

Amouzegar *et al*. (2009) isolated a halotolerant strain, *Halomonas eurihalina strain* D, that following 48 h aerobic culturing in SW-5 medium could reduce the amount of lead nitrate from 60 mg/l to 5.29 mg/l (13).

One of the important factors that affect the biosorption of the metals is pH. In this study, the highest lead absorption by the *Kurthia* sp. VITA1 was at pH 5.5.

Nayeri *et al.* (2011) also reported the same result for their two isolates, including *Pseudomonas* sp. P-II *Bacillus* sp. Q-III, but Gabor *et al*. (2008) reported that the optimum pH for the absorption of Ni and Pb by *Pseudomonas aeruginosa* ASU 6a was 6 and 7 respectively (9, 23).

According to the result of the present study and other ones we can conclude that the

maximum lead absorption occurs at weak acidic or neutral pH. The other important factor in lead absorption is its concentration. In this study, the *Kurthia* sp. VITA1 had the highest absorption in lead solutions with 2 mM, lead acetate concentration. In fact with increasing the lead concentration up to 2 mM the rate of lead absorption was increased but in the upper concentrations it was reduced.

The time of exposure with lead also affects the efficacy of metal removal. The result of this research showed that 63% of lead absorption of *Kurthia* sp. VITA1 occurred after 30 min exposure and it reached to 88% after 4 h; but the rate of absorption remained constant after 4 h. Ray *et al*. (2005) reported that the lead absorption of *Bacillus cereus* M_{16} after 10min exposure was 95% and it came into equilibrium after 30 min (24) but in a study by Tunali *et al*. (2005) the lead absorption of *Bacillus* sp. (ATS-1) came into balance after 15 min exposure period (21).

According to the results of other studies, we found that *Kurthia* sp. VITA1 needs more exposure time for higher lead absorption. As optimal conditions for the lead absorption differ in various types of bacteria, we examined the optimal conditions for *Kurthia* sp. VITA1. The results showed the optimal conditions for *Kurthia* sp. VITA1 as 2 mM lead concentration at pH 5.5 after 4 h in which it can absorb 88% of the initial lead concentration. In the research of Karim salmani *et al*. (2011) the optimal conditions for their isolate was reported as 150 mg/l lead concentration at pH 4 after 4 h (7), while in the study of Nayeri *et al*. (2011) the optimal conditions for their isolates was at pH 5.5 after 2 h (9).

Comparing the results of different studies, we conclude that the optimal conditions for the lead absorption vary in different bacteria, so finding the optimal conditions for the lead- absorbing bacteria would be helpful to improve the bioremediation process.

Conclusion

The result of this study showed that the isolated bacteria from lead-containing wastewaters have a high potential for removing this metal from polluted environments and could be good candidates for bioremediation and biological treatment. In addition, the bioremediation process will be improved when the optimal conditions for growth of these bacteria are provided.

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