

Bioremediation Activity of Pb (II) Resistance *Citrobacter* sp. MKH2 Isolated from Heavy Metal Contaminated Sites in Iran

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Abstract

Heavy metal pollution all around the world has become a major environmental concern and the bioremediation of polluted environments is an increasingly popular strategy due to both its efficiency and safety. In this study, a bacterial strain resistant to heavy metal was isolated from metal contaminated sites. Strain MKH2 was removed Pb (II) with 95.06%. This isolate was also resistant to zinc, copper, nickel and cadmium. The results of morphological and physiological characteristics showed that MKH2 was belonging to *Citrobacter* sp. Moreover, 16S rRNA sequencing and phylogenetic analysis indicated that MKH2 was similar to *Citrobacter freundii* with 99% homology. Investigation to determine if the gene resistance to heavy metal is located exclusively on a plasmid, curing was achieved. The results suggested that the resistance to heavy metal in the MKH2 is possibly conferred chromosomally. The isolated heavy metal resistant bacterium could be useful for the bioremediation of heavy metal contaminated sites.

Keywords: Pb (II); Heavy metal; Biosorption; *Citrobacter*.

Introduction

One of the most important pollutions that threaten human health is heavy metals [1]. Heavy metals such as lead, cadmium, copper, zinc and nickel are very toxic for living organisms even in small quantities [2]. One of the acute toxic metals for human is lead. Impaired biosynthesis of hemoglobin and anemia, high blood pressure, kidney damage, abortion and neonatal deaths, neurological disorders, brain damage, infertility, decreased learning and behavioral disorders in children

are the main adverse effects due to increasing of lead concentrations in human [3-5]. Although the inorganic form of lead is a general metabolic poison and enzyme inhibitor, organic forms are even more poisonous [6]. Therefore, removal of heavy metals, particularly lead metal from contaminated wastes is absolutely necessary. Conventional methods that have been used to remove heavy metals from wastewaters, are include chemical precipitation, ion exchange, membrane separation, reverse osmosis, evaporation and electrolysis [7]. These

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methods have their disadvantages. For example, Chemical precipitation cause to the production of toxic sludge. These techniques are not suitable for effluents containing less than 100 mg L^{-1} of targeted heavy metals [8, 9]. In addition, ion exchange processes are too expensive due to the high cost of synthetic resins [10]. Therefore, methods are expensive and harmful to the environment.

One of the best ways to remove heavy metals from the environment, which recently have attracted a lot of interest, is the use of microorganisms [11, 12]. Bioremediation is the most efficient and least method for treating of heavy metal contaminated soils [13]. Recent studies focused on the biological treatments capable to remove heavy metals from contaminated sites [14, 15]. The use of microorganisms not only low cost and safe for the environment, but also has a high yield and is readily available [16].

This study aimed to isolate and identify bacteria resistant to lead from contaminated wastes. Strain that showed the high removal lead was selected. In addition, to determine if the gene resistance to heavy metal is located exclusively on a plasmid, curing was achieved.

Materials and Methods

All chemicals and culture media were purchased from Merck Chemical Co. (Germany), through a local agency.

Screening of lead resistance bacteria

For screening of lead resistance bacteria, the contaminated wastewater samples were collected then 1% inoculated into LB medium (containing g L^{-1} distilled water: tryptone, 10.0; yeast extract, 5.0; NaCl, 5.0, and glucose, 1.0) containing of 200 mg L^{-1} lead. The flasks were incubated on an orbital shaker at 28°C and 100 rpm. After 24 hours, $100 \mu\text{L}$ of the culture medium transferred to fresh nutrient agar plates supplemented with 500 mg L^{-1} lead then incubated at 28°C for 24 hours. The bacterial colonies differing in morphological characteristics were selected and checked for purity and used for further studies. The isolates were stored in 15% (v/v) glycerol solution at -86°C .

Measuring the elimination rate of lead

In order to obtain the high concentration of metals uptake by the isolated bacteria, the strains were cultured into 100 mL of LB medium containing 80 mg L^{-1} lead. The flasks were incubated on orbital shaker at 30°C , 150 rpm for 24 hours. Then the cultures were centrifuged at 6500 rpm for 15 minutes. The Pb (II) concentrations of the supernatants were determined

using Atomic absorption spectroscopy (AAS) (Thermo Electron, USA). Therefore the superior strains were selected for further lead biosorption studies. All experiments were repeated three times [7, 17].

MIC determination of heavy metals

The cross heavy metal resistance of bacterial isolate was evaluated as minimum inhibitory concentration (MIC) of metals such as lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd) and nickel (Ni). MIC has been defined with the agar dilution method [18, 19]. All metal salts were added to the nutrient agar after autoclaving and cooling to 50°C from filter sterilized stock solutions. The metal salts used for this study included $\text{Pb}(\text{NO}_3)_2$, $\text{Cd}(\text{NO}_3)_2$, NiCl_2 , ZnSO_4 and CuSO_4 . For evaluating the resistance of the bacteria, the sterile metal stock solutions were added into nutrient agar to give a final concentration of 0.5–12 mM as required. Then the bacteria cultured radially in the plates and incubated at 28°C for 48 hours. The absence of bacterial growth indicated its sensitivity, while the presence of bacterial growth indicated that bacteria were resistant.

Growth inhibitory by cyanide

In addition, cyanide growth inhibitory was determined according to the method described by Babu *et al.* 1996 [20]. Isolated strain was grown in LB medium contain 0.5-8.0 mM NaCN. The tubes were incubated at 28°C and 120 rpm for 24 hours.

Plasmid curing

Curing was performed using LB medium as described by Smejkal *et al.* (2001) and Turnbull *et al.* (2001) [21, 22]. A 1 mL aliquot of an overnight culture of strain MKH2 was inoculated into 100 mL of fresh LB medium and incubated at 28°C for 30 days. Every 24 hours for 30 days, successive subcultures were performed from the culture to the nutrient agar plates supplemented with 4 mM lead and incubated at 28°C .

Antibiotic resistance to ampicillin was done for parent and cured bacteria. Fresh LB medium containing various concentrations of antibiotic ampicillin ($1\text{--}200 \mu\text{g mL}^{-1}$) was prepared. Both parent and cured bacteria were inoculated into medium and observed after 24 hours.

Identification of Pb resistant isolate

For identification of the bacterial isolate, morphological and physiological characteristics was undertaken as described in *Bergey's manual of systematic bacteriology* [23]. The shape and colors of the colonies were examined then morphology of isolate

Table 1. Minimum inhibitory concentration of heavy metals (mM) on growth of MKH2 using agar dilution method.

| Isolate | Concentration of heavy metals (mM) | | | | |
|---------|------------------------------------|-----|------|------|------|
| | Pb | Cd | Ni | Zn | Cu |
| MKH2 | 9.5 | 0.8 | 12.0 | 10.0 | 11.5 |

was examined under the microscope after Gram staining.

16S rRNA PCR and phylogenetic analysis

For molecular identification of the isolate, genomic DNA was extracted using a standard bead beating method [24]. The extracted DNA was examined using 0.8% (w/v) agarose gel electrophoresis. The *16S rRNA* gene was PCR amplified using universal bacterial *16S rRNA* primers (PA 5'-AGAGTTTGATCCTGGCTCAG-3' and PH 5'-AAGGAGGTGATCCAGCCGCA-3'). The thermocycler was programmed to denature at 95°C for 5 minutes followed by 35 cycles of 1 minute at 95°C, 1 minute at 50°C and 72°C for 2 minutes with a final extension step for 5 minutes at 72°C. The PCR products were purified using a GeneJet PCR purification kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. Purified products were sequenced by GATC Biotech (Germany). The *16S rRNA* gene sequences was analyzed against those available from the National Centre for Biotechnology Information (NCBI) database using the BLAST search system to identify the most similar sequence alignment [25].

For phylogenetic analyses, all reference sequences were obtained from NCBI. Then the sequences were aligned using the multiple sequence alignment program

ClustalX. Neighbor joining phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis package (MEGA5) [26].

Results

Lead resistance bacteria

In this study, twelve isolates were screened from LB medium supplemented with initial level of lead. Bacterial isolates, including Gram-positive and Gram-negative bacteria, were examined for lead uptake capacity using AAS after 24 hours incubation at 28°C (Figure 1). The results indicated that the isolate MKH2 were removed Pb (II) 95.06% and lead concentration was reduced from 80.0 mgL⁻¹ to 3.952 mgL⁻¹. Therefore, the Gram-negative isolate MKH2 was shown high capacity for removal of heavy metal lead in the environment then selected for further studies.

Evaluation of heavy metal and cyanide resistance

To examine the heavy metal resistance, MIC was undertaken using different metal concentration 0.5-13.0 mM. The results demonstrated that isolate MKH2 was shown high resistance to all tested heavy metals (Table 1). MKH2 was more resistant to Pb²⁺ (9.5 mM) and Ni²⁺ (12.0 mM). The isolate was tolerant to multiple metal ions (data not shown). In addition, to determine cyanide

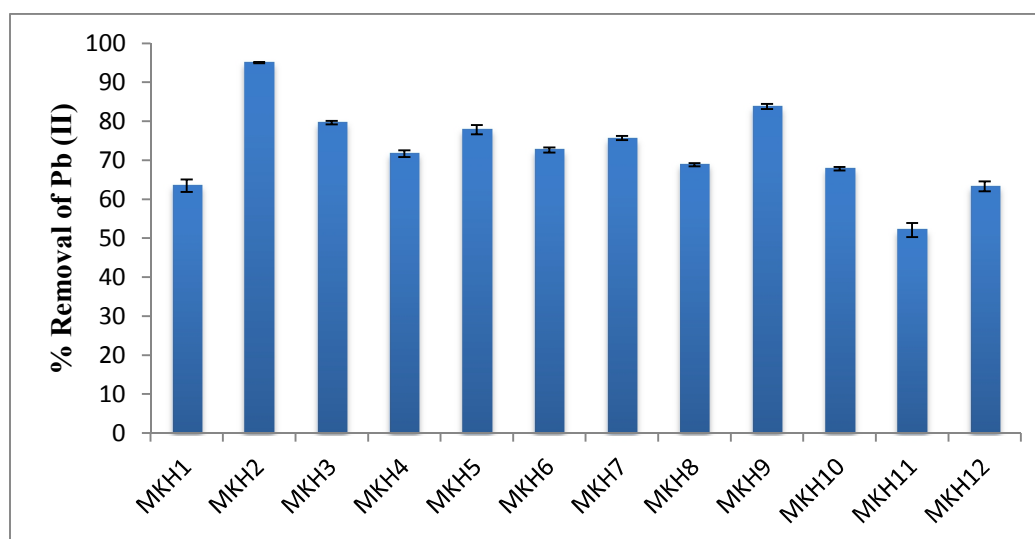


Figure 1. Percentage of lead removal by isolated bacteria MKH1-12 in a growth medium containing 80 mgL⁻¹ lead using AAS. Each data point represents the average and standard deviation of three replicates.

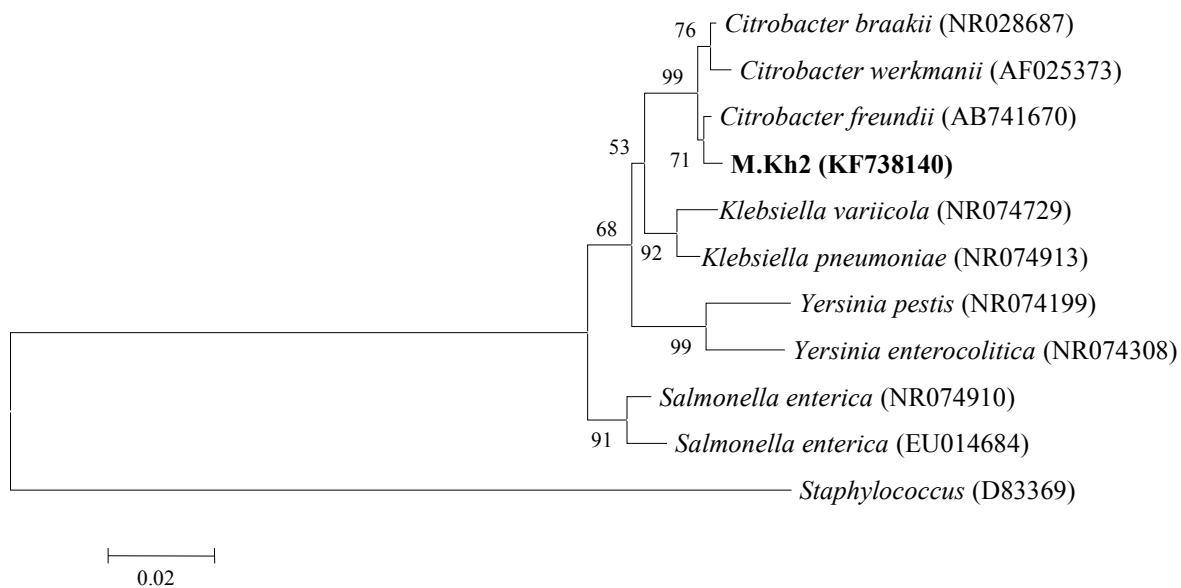


Figure 2. Neighbour-joining tree showing phylogenetic relationships of *16S rRNA* sequences isolate MKH2 to closely related sequences from GenBank. Bootstrap values of >50% are shown. Scale bars indicate Jukes-Cantor distances.

growth inhibitory, the isolate was cultured with NaCN. The results indicated that MKH2 was able to grow at high cyanide concentration (7 mM) after 24 hours.

Plasmid curing

Plasmid curing is carried out to confirm whether the genes for resistance are encoded by genomic DNA or plasmid DNA. There was no change for heavy metal resistance in MKH2 after curing. These results demonstrate that the resistance to heavy metals in the MKH2 is possibly conferred chromosomally.

The isolates were examined for ampicillin resistance to confirm plasmid curing experiments. The parent MKH2 was resistance to $100 \mu\text{g mL}^{-1}$ ampicillin but the cured strain was unable to grow at this condition. The results revealed that the isolate lost the resistance plasmid after curing experiment. Therefore the cured MKH2 lost the antibiotic resistance function whereas the parent MKH2 was resistant to ampicillin. These results suggested that the bacterial plasmid lost during curing.

Identification and phylogenetic analysis

For identification of Gram negative, rod shaped isolate MKH2, partial sequences of *16S rRNA* fragments were performed. The *16S rRNA* fragments were analysed using BLAST subroutine to search for related sequences in NCBI (National Center for Biotechnology Information) database. The BLAST query revealed that the isolates MKH2 was most similar to *Citrobacter*

freundii with 98% homology. Phylogenetic analysis confirmed that the isolate was found to be similar to *Citrobacter*, belong to γ -Proteobacteria (Figure 2). In addition, the *16S rRNA* sequences for the Pb resistance isolate *Citrobacter* sp. MKH2 was deposited in the NCBI GenBank under accession number KF738140.

Discussion

Heavy metals pollution in the environment has become a major problem because its toxicity, non-biodegradability and bio-accumulation. Bioremediation is the best affected choice to cleaning up the contaminated sites with advantages such as low cost and large available quantities and environmental friendly. Lead is one of the dangerous metals that can threaten human health. Many studies have been focused on toxic effect of lead on human health [27-29]. These studies indicated that are necessary to remove lead from the environments. Many microorganisms have been reported to remove lead such as *Bacillus*, *Citrobacter*, *Pseudomonas*, *Plectonema*, *Saccharomyces* and *Aspergillus* [27]. In this research, a bacterium resistance to heavy metals was isolated from contaminated area. This isolate had the ability to remove up to 90% of lead after 24 hours. Due to high resistance and removal of heavy metals, the isolate MKH2 can be used for remediation of lead hazardous metal from environments. Heavy metals resistance bacteria have been reported in both gram-negative and gram-positive

strains that isolated from lead-contaminated sites. Roane (1999) isolated Pb-resistant *Pseudomonas marginalis* and *Bacillus megaterium* from contaminated soils and indicated that *P. marginalis* sequestered Pb extracellularly, while *B. megaterium* accumulated Pb intracellularly [30]. A Pb-resistant *P. aeruginosa* WI-1 was isolated from contaminated site that accumulated high amount of Pb intracellularly by metallothionein [31]. Shin *et al.* (2012) isolated *Bacillus* sp. MN3-4 from metal hyper accumulator roots that resists high concentrations of lead via extracellular sequestration and intracellular accumulation [19]. MKH2 that isolated in this study was gram-negative. BLAST subroutine of *16S rRNA* was shown that MKH2 was most similar to *Citrobacter freundii*. Isolate MKH2 was grown at the presence of 9.5 mM Pb (II). In addition, MKH2 was resistance to other heavy metals including zinc, nickel, copper and cadmium.

Cyanide compounds are widely used in various chemical industries including metals, mining, plating, evaporation and coal. These compounds are highly toxic to living organisms, particularly in inactivating the respiration system by tightly binding to terminal oxidase [32-35]. The isolate was also resistance to 7 mM cyanide. The results demonstrated that this isolate could remove heavy metals from environments while contaminated with cyanide.

Mechanisms for resistance and removal of heavy metals in bacteria may be encoded by chromosomal genes; however most resistance systems appeared to be associated with plasmids [36]. Curing is the process of removing plasmids from a bacterial cell. Raja *et al.* (2009) reported that the isolate *Pseudomonas aeruginosa* exhibited resistance to heavy metals such as cadmium, chromium, nickel and lead [37]. Their results, after plasmid curing, showed that nickel and ampicillin resistance gene was conferred by plasmid DNA. Cadmium resistant gene was present on chromosomal DNA along with the gene for chromium resistance. According to their finding, lead resistance genes was shown to be present on the chromosomal DNA rather than the plasmid DNA as the cured and parent cultures remained similar in lead resistance. The results of study by Samanta *et al.* (2012) indicated that the genes for antibiotic and heavy metal resistance of the *Bacillus* sp. may be reside on plasmid DNA [38]. The results of our study indicated that resistance to ampicillin was lost whereas resistance to lead was remained after plasmid curing in MKH2. Therefore, lead resistance in MKH2 was probably related to chromosomal genes. A similar finding was observed from a plasmid curing determinant in *Citrobacter freundii* [39]. This study suggested that ampicillin resistance were conferred by

plasmid DNA, while lead resistance seems to be encoded by genes of the bacterial chromosome.

Conclusion

In this study, MKH2 was isolated with high resistance activity to heavy metals, lead. In addition, due to the resistance to cyanide, the isolate may useful for treatment of wastewater co-contaminated with heavy metals and cyanide. Our results indicated that resistance to heavy metal was chromosomally in MKH2. Therefore, this isolate may be employed as a suitable model of microorganism for removal of hazardous metal in high-risk contaminated environmental sites.

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