RESEARCH ARTICLE

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Evaluation of Heavy Metals Resistance in Biofilm Cells of Native *Rhodococcus spp.* Isolated from Soil

ElhamAdhami[a], Seyed Soheil Aghaei[a*], Mohammad-Reza Zolfaghari[a]

[a]Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran.
***Correspondence** should be addressed to Dr. SoheilAghaei, **Email:** aghaei@qom-iau.ac.ir

A-R-T-I-C-L-EI-N-F-O

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A-B-S-T-R-A-C-T

Background & Aims of the Study: *Rhodococci* according to possess large genome, their active metabolism and survival under extreme conditions, are highly regarded for biodegradation and bioremediation of different pollutants especially heavy metals in the environment. Biofilms are more resistant to heavy metals than free-swimming organisms. The aim of this study was biofilm formation of two native *Rhodococcus* strains (*Rhodococcus rhodochrous* and *Rhodococcus rhodnii*) and comparative evaluation effects of heavy metals such as lead, copper, zinc, chromium and cadmium in various concentrations against biofilm and planktonic cells that carried out at the first time.

Materials & Methods: Bacterial strains used in this investigation were isolated from agricultural soils in Qom, Iran. Biofilm formation was performed at 30 °C and 37 °C in tryptic soy broth (TSB) and brain heart infusion (BHI) broth, at 24,48,72 and 96 hours in 96-well microplate. Minimum inhibitory concentration (MIC) of heavy metals in various concentrations for planktonic cells was determined, using microdilution and macrodilution methods. Minimum biofilm inhibitory concentration (MBIC) and Minimum biofilm eradicating concentration (MBEC) in various concentrations of heavy metals for biofilm cells were evaluated by microdilution method. The result of this study confirmed, using scanning electron microscope (SEM).

Results: The best condition for biofilm formation of native *R.rhodochrous* isolate was determined after incubation at 37°C in BHI broth, at 96 hours and the best condition for biofilm formation of native *R.rhodnii* isolate was determined at 30°C in BHI broth, at 96 hours. MIC of heavy metals for planktonic cells of isolates for cadmium, zinc and lead was 8 mM and assayed for copper and chromium respectively 4 and 1 mM. MBEC of heavy metals for biofilm cells of isolates for cadmium, zinc and lead was 16 mM and assayed for copper and chromium respectively 8 and 4 mM. The results of present study showed biofilm cells of native *Rhodococcus* isolates were 2 times more resistance to lead, copper, zinc and cadmium than planktonic cells while biofilm cells were 4 times more resistance than planktonic cells to chromium.

Conclusions: Biofilm formation of *R.rhodochrous* and *R.rhodnii* and their high resistance to various concentrations of heavy metals especially cadmium show that inoculation of these native *Rhodococcus* isolates to contaminated agricultural soils with heavy metals, have an effective role for bioremediation.

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Background

Heavy metals are omnipresent pollutants that are entering the environment by human activities such as mining and industrial waste (1). In the future the amount of generated waste will increase; 27 billion tons in 2050 (2). Some metals such as copper, zinc, nickel and chromium are necessary micronutrients for plants, animals and microorganisms (3).While others (for example: cadmium, mercury and lead) have unknown biological function

(4).Generally, increasing the concentrations of these metals is higher than the threshold prevent the activity of microorganisms by relocation of essential metal ions and blocking essential functional groups (5).

Resistant microorganisms such as bacteria can be used as biological cleaning factors. Compared with other methods, biological cleaning is less costly and more promising for clean water and contaminated soil (6,7). Naghizaded.et al used from membrane bioreactor (MBR) for biological wastewater treatment (8).Some bacterial species are resistant to high concentrations of heavy metals in toxic metals contaminated environments. In addition, soil microorganisms have different mechanisms to resistant against stress of heavy metals including: efflux the metal ions out of cells, accumulation of metal ions within the cell, reduce the toxicity of heavy metals (9) biofilm formation and exopolysaccharide production (10).

Biofilm formation is regulated by several genetic and environmental factors. Genetic studies suggest that the bacterial cell membrane proteins, polysaccharides and extracellular signaling molecules are important in biofilm formation. Stages of biofilm formation, including reversible binding, irreversible binding, maturation I, maturation II and dispersion (11).

Bacterial biofilm are placed in an extracellular polymer (EPS), the matrix consists of polysaccharides, proteins and nucleic acids. Biofilms are more resistant to antimicrobial factors compared with planktonic cells (1). Mechanisms of biofilm resistance to heavy metals are included: (1) slow growth (Facultative anaerobic cells that are near the biofilm depth in areas without oxygen and these cells are slow-growing, this slow growth is leading to a tolerance to antibiotics), (2) persister cells, (3) Quorum sensing (QS) (12), (4) Reducing the influence of metals, (5) Phenotypic variation (13).

The genus *Rhodococcus* as soon as are considered to be one of the organisms for biodegradation compounds that are not easily degradable by other organisms (14). For heavy metal resistance genes in the genus *Rhodococcus*, arsenic resistance operon (arsADC1RBC2C3C4C5) and eight isolated arsenate reductase genes, mercuric reductase gene merA, alkylmercury lysase merB and eleven transporter gene for lead, cadmium, zinc and mercury were diagnosed in the genome. In addition to the two sets of genes for resistance to cobalt, zinc and cadmium cszD, two sets of cadmium transporter gene cadD, two sets of cobalt and nickel transporter gene TauX, Cobalt and manganese transporter gene corA and copper resistance genes copC and copD also discovered (15).

Aims of the study:

The aim of this research was to assess biofilm formation of *Rhodococcus rhodochrous* and *Rhodococcus rhodnii* and evaluation of heavy metals (such as lead, copper, zinc, chromium and cadmium) resistance in biofilm cells and planktonic cells of native strains *Rhodococcus* spp. isolated from soil that carried out at the first time.

Materials & Methods

Bacterial strains and growth conditions

Native *Rhodococcus* strains (*Rhodococcus rhodochrous* and *Rhodococcus rhodnii*) used in this investigation were isolated from agricultural soils in Qom, Iran (16). Bacterial strains were activated, using cultivation on different culture media (Trypticase soy agar, Brain heart infusion agar, Bennet's agar, ISP Medium No. 5 and Luria Bertani Agar).

Biofilm formation Assay

Strains were cultured on Brain Heart Infusion agar (BHI; Merck, Germany) and were incubated at 30 °C for 72 hours. After incubation isolates separately were inoculated in BHI broth and Triptic soy broth media (Merck, Germany), adjusted to 0.5 McFarland standards. Then, suspension of isolates was

diluted separately1:100 into sterile BHI broth and TSB. Wells of microplate were filled with bacterial suspension and biofilm formation was examined for different temperature including: 37 °C and 30 °C for 24, 48, 72 and 96 hours (17).Negative control is containing BHI broth and also TSB broth without bacteria. 4-8 replicate wells were used for qualitative evaluation. The microplates were evacuated to remove planktonic cells and the wells were rinsed with sterile saline solution. Biofilm cells were stained with 1% w/v Crystal violet for 20 minutes. The excessive stains were removed by washing the wells with sterile saline solution. Stained attached cells (Biofilm cells) were detached by dimethyl sulfoxide (DMSO) and solubilized biofilms measured, using microplate reader (SUNOSTIK) at 450, 492 & 630 nm. Evaluation of biofilm formation was determined, using this formula: high biofilm former: O.D> 0.5; moderate O.D≥0.3-0.5 and poor biofilm former OD<0.3 (18).

Determination of Minimum Inhibitory Concentration (MIC)

Macro dilution method

Concentration of stock solution of heavy metals (Zn, Cr, Cu, Pb and Cd) was 512 mM.1.0 ml of sterile Mueller Hinton Broth (Merck, Germany) was added to all tubes and then 1.0 ml of stock solution of heavy metals was added to the first tube. 1.0 ml from the first tube was transferred to the second tube, mix the contents of this tube and transfer 1.0 ml to the third tube. This method was continued until tenth tube. 1.0 ml from tenth tube was poured out. 100 µl of 0.5 McFarland bacterial suspensions were added to 10 tubes. Eleventh tube was positive control that contained Mueller Hinton Broth and bacterial suspension and twelfth tube was negative control that contained Mueller Hinton Broth and heavy metal. Tubes were incubated at 30°C and growth of isolates was monitored every 24 hours. The highest dilution without growth is considered as MIC (19). All of the above mention tests were performed in triplicate for each heavy metal.

Micro dilution method

We used sterile 96-well microtitre plates. Using a pipette 100 µl of sterile Mueller Hinton Broth was added to all wells of the microplate.100 µl of heavy metals stock solution (512 mM) was added to the microplate wells in the first column (Pb: row A, Cu: row B, Zn: row C, Cr: row D, Cd: row E).100 µl was removed from column 1 and added this to column 2 then 100 µL from second column were transferred to third column. This method was continued until tenth column and then 100 µl from tenth column was poured out. 10 µl of 0.5 McFarland bacterial suspension was added to 10 columns. The columns 11 and 12 had the positive control and the negative control. Microplate was incubated at 30°C and growth of isolates was monitored every 24 hours (20).The test was repeated three times for each heavy metal.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

This method was done, using BHI broth instead of Mueller Hinton broth. The microplates were incubated at 30°C and 37°C respectively for *R.rhodnii* and *R.rhodochrous* isolates for 24 hours. Then, the microplates were washed three times with sterile saline solution and were exposed to air-dry. 200 µl of 1% crystal violet were added to all wells .The microplates were incubated at room temperature for 20 minutes; they were washed in sterile saline solution and air-dried. The biofilm cells in the wells were dissolved with dimethyl sulfoxide (DMSO); then, solubilized biofilms were measured by microplate reader at 630nm (18,21). The test was repeated three times for each heavy metal.

Determination of Minimum Biofilm Eradicating Concentration (MBEC)

The cultured of the isolates in microtitre plates containing BHI broth were incubated at 30 °C and 37 °C to biofilm formation for *R.rhodnii* and *R.rhodochrous* isolates respectively. Microplates were incubated for 96 hours and wells were washed with sterile saline solution

to remove of suspended (planktonic) cells. The remained 96 hours biofilms in the each well were treated with two-fold serial dilution of heavy metals solution as described above. The microplates were incubated at 30°C and 37 °C for 24 hours (22). Biofilm cells rinsed in sterile saline and stained with crystal violet and quantified, using Elisa Reader as described above.

Scanning Electron Microscope (SEM)

Biofilm formation of the isolates confirmed, using Scanning Electron Microscope (SEM) method. The wells of the microplates were rinsed with sterile saline solution to remove planktonic cells. Before performing SEM characterization, the samples must be clean and completely dry. The next step is gold coating; the gold sputter coater is a machine that we used to coat the specimens in gold before they go into the SEM (PHILIPS XL30).

Results

Bacterial strains and growth conditions

The best condition for growing and activating *R.rhodochrous* and *R.rhodnii* was growing in BHI agar and TSA and incubated them for 72 hours at 30 °C.

Biofilm formation

The best condition for biofilm formation of *R.rhodochrous* was determined after incubation at 37 °C in BHI broth, at 96 hours and the best condition for biofilm formation of *R.rhodnii* was determined at 30 °C in BHI broth, at 96 hours.

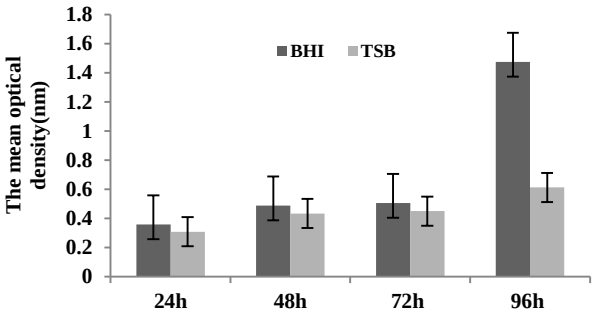


Figure (1-a)

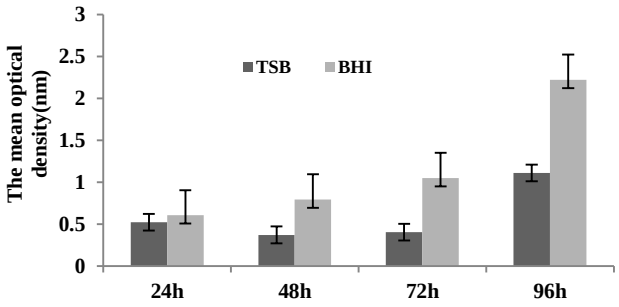
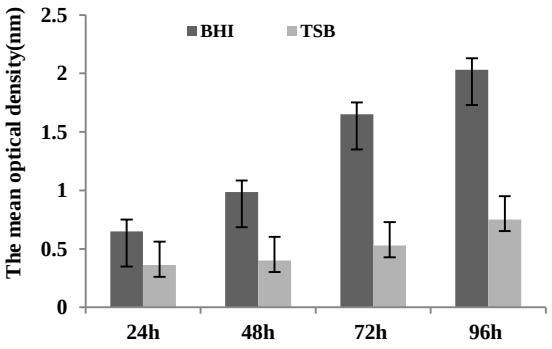


Figure (1-b)

Figure 1) (a) The mean optical density of biofilm formation of native *R.rhodochrous* isolate at 30 °C, 630nm during 24-96hrs. (b)The mean optical density of biofilm formation of native R. rhodochrous isolate at 37 °C 630nm during 24-96hrs.



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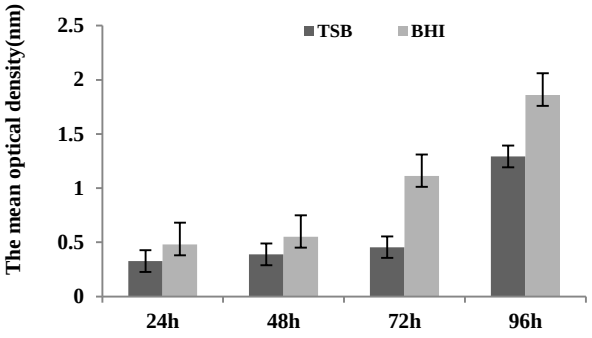

Figure (2-b)

Figure 2) (a) The mean optical density of biofilm formation of native *R.rhodnii* isolate at 30 °C 630nm during 24-96hrs (b) The mean optical density of biofilm formation of *R. rhodnii* isolate at 37 °C 630nm during 24-96hrs.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of heavy metals for planktonic cells of isolates for cadmium, zinc and lead was 8 mM and assayed for copper and chromium 4 and 1 mM, respectively.

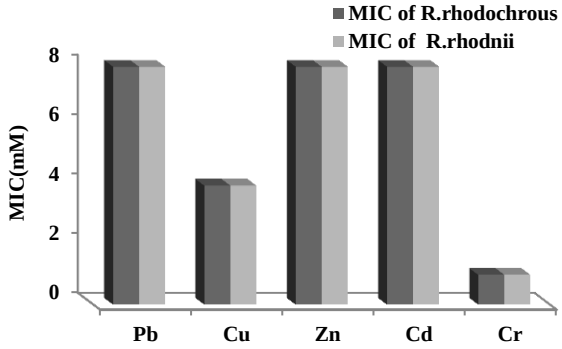

Figure 3) MIC determination of heavy metals for native *Rhodococcus* isolates

Determination of Minimum Biofilm Eradicating Concentration (MBEC)

MBEC of heavy metals for biofilm cells of isolates for cadmium, zinc and lead was 16 mM and assayed for copper and chromium 8 and 4 mM, respectively.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

MBIC of native *R.rhodochrous* isolate for lead and cadmium was 4mM and for zinc, copper and chromium was 2, 1 and 0.5mM while MBIC of native *R.rhodnii* isolate for copper and lead was determined 2mM and for zinc, cadmium and chromium was obtained 1, 4 and 0.5mM, respectively.

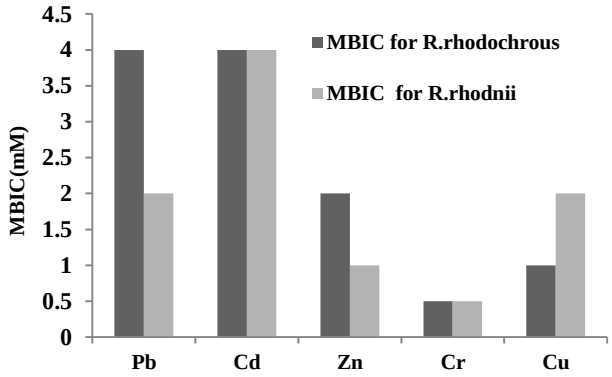

Figure 4) MBIC determination of heavy metals for native *Rhodococcus* isolates

Determination of Minimum Biofilm Eradicating Concentration (MBEC)

MBEC of heavy metals for biofilm cells of isolates for cadmium, zinc and lead was 16 mM and assayed for copper and chromium 8 and 4 mM, respectively.

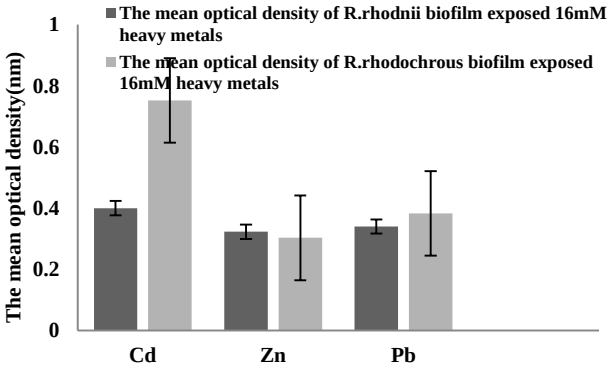

Figure (5-a)

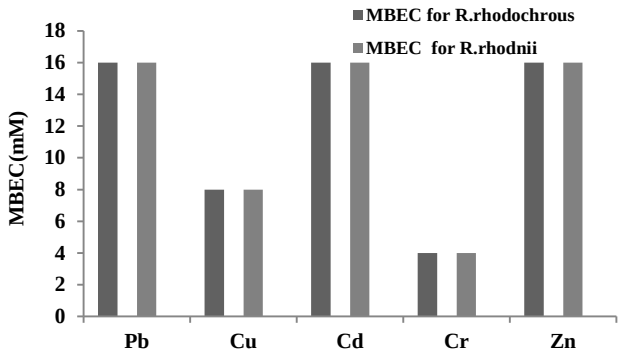

Figure (5-b)

Figure 5) (a) The mean optical density of biofilm cells in native *R.rhodnii* and *R.rhodochrous* isolates exposed 16 mM heavy metals (b) The minimum biofilm eradicating concentration of native *R.rhodnii* and *R.rhodochrous*

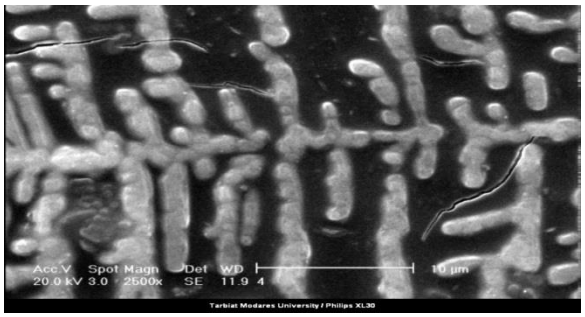

Figure (6-a)

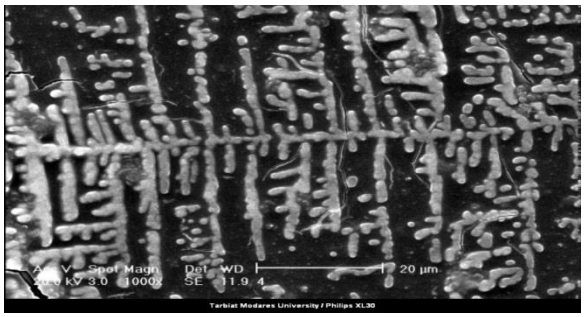

Figure (6-b)

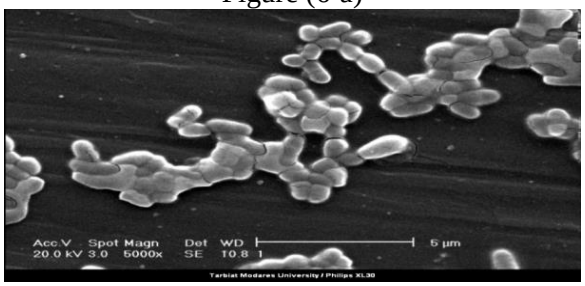

Figure (6-c)

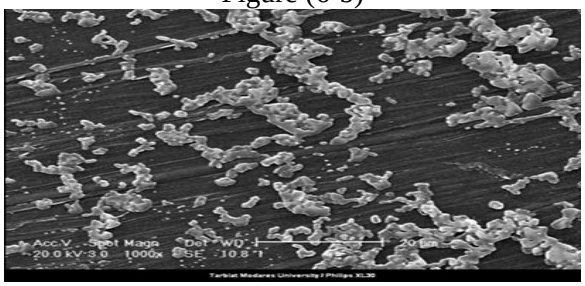

Figure (6-d)

Figure 6) (a,b) Biofilm of native *R.rhodochrous* at 37° C after 96 hrs (c,d) Biofilm of native *R.rhodnii* at 30 ° C after 96 hrs

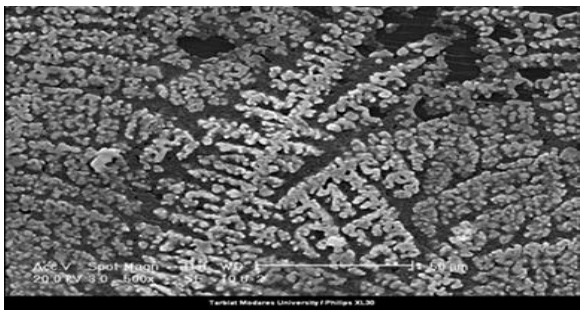

Figure (7-a)

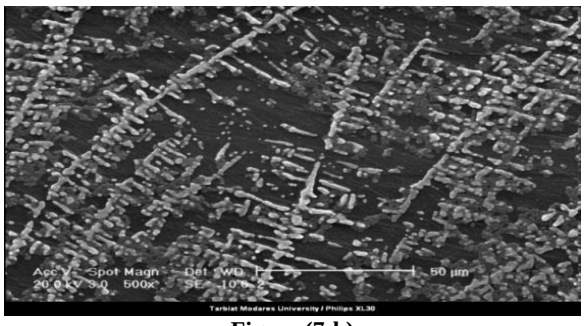

Figure (7-b)

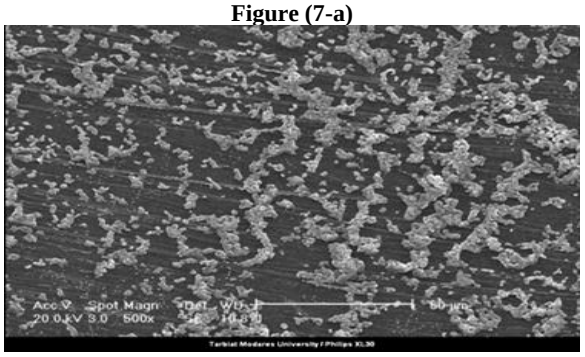

Figure (7-c)

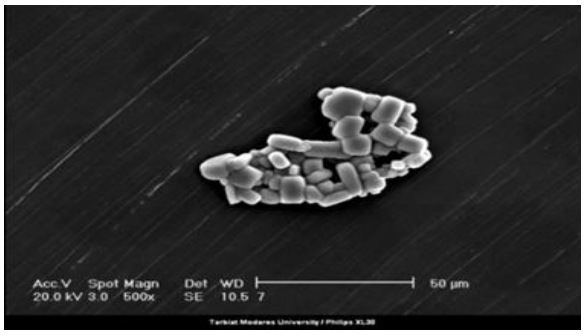

Figure (7-d)

Figure7) (a) *R.rhodochrous* biofilm before exposure to cadmium (b) *R.rhodochrous* biofilm after exposure to cadmium (c) *R.rhodnii* biofilm before exposure to cadmium (d) *R.rhodnii* biofilm after exposure to cadmium

Discussion

Increase of heavy metals in soil and absorption of them by plants is an environmental concern, unlike other pollutants that are degraded it is very difficult to remove heavy metals from polluted environment (23). Cadmium inhibits the growth of stems and roots of plants and

often accumulate in the important agricultural products (24).

Resistant microorganisms including bacteria could be used as bioremediation factors (21). Bioremediation by biofilm cells is better compared to planktonic cells because biofilm cells have the ability to adapt and increased survival in stressful conditions and are protected in the matrix (25).

Metabolic ability of many species in the genus of *Rhodococcus* in the sense that they can destroy the whole range of environmental pollutants and convert them to other materials or produce compounds with useful applications. Therefore, we can consider native *Rhodococcus* isolates for bioremediation and biodegradation of chemical pollutants such as heavy metals (26).

We need to do a lot of researches on bacterial resistance to toxic metals. This study focused on native isolates of *R.rhodochrous* and *R.rhodnii* for biofilm formation and their ability to be resistant to heavy metal stress.

In our research, heavy metals susceptibility(MIC, MBIC and MBEC) was evaluated, using micro dilution methods. The results showed that planktonic cells of native *R.rhodochrous* and *R.rhodnii* isolates were more susceptible to the heavy metals than biofilm cells. MIC of cadmium, zinc and lead for *R.rhodochrous* and *R.rhodnii* was 8 mM and for copper and chromium was 4 and 1 mM, respectively .Due to the results, these isolates were almost 4 times more resistance to cadmium than *Rhodococcus* strains was studied by Belimov.et al (22) and also native *R.rhodochrous* and *R.rhodnii* were 2 times more resistance to cadmium than *Rhodococcus* strains was studied by vela-Cano et al (27).

In this study, the *Rhodococcus* strains showed the greatest resistance to lead, cadmium and zinc and showed the highest sensitivity against chromium while Kalantari studied about assessment of toxicity of iron, chromium and cadmium on Bacillus cereus growth and resulted that chromium had partial inhibitory effects on the growth of bacteria and cadmium was very toxic (28).

Because microplate method is easier and less costly, in this study biofilm formation was performed, using microtiter plates and also Presterl et al used from 96-wells microplate in their research (15).

The best medium and time for biofilm formation were BHI broth and 96hrs while Gilan.et al formed biofilm of *Rhodococcus* ruber in Nutrient broth during 24hrs (29).

The best temperature for biofilm formation was at 30 °C and 37 °C respectively for *R.rhodnii* and *R.rhodochrous* while Mor.et al formed biofilm of *Rhodococcus* ruber at 35 °C (30).

The results of this investigation showed biofilm cells of native *Rhodococcus* isolates were 2 times more resistance to lead, copper, zinc and cadmium than planktonic cells while biofilm cells were 4 times more resistance than planktonic cells to chromium.

Conclusion

In this regard the use of microorganisms including bacteria is better and less costly than other methods. In this research, native *R.rhodochrous* and *R.rhodnii* isolates were used to be measured their resistance against heavy metals that eventually showed high resistance to heavy metals especially to cadmium. Biofilm cells of isolates were more resistance to heavy metals than planktonic cells. Inoculation of these native *Rhodococcus* isolates to agricultural soils, have an effective role for bioremediation of toxic heavy metals and promoting soil fertility. Therefore, these native *Rhodococcus* isolates considered as one of the best candidate for removing toxic metals from contaminated agriculture soils and prevention of disease such as poisons and gastrointestinal cancers in human.

Footnotes

Conflict of Interest:

The authors declared no conflict of interest.

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