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HEAVY METAL TOLERANCE AND REMOVAL EFFICIENCY OF THE *Rhodotorula mucilaginosa* AND *Saccharomyces boulardii* PLANKTONIC CELLS AND BIOFILM

Sandra M. Grujić, Ivana D. Radojević*, Sava M. Vasić, Ljiljana R. Čomić, Aleksandar M. Ostojić

University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Republic of Serbia
*Corresponding author; E-mail: ivana@kg.ac.rs

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ABSTRACT. The impact of heavy metals, cadmium (Cd^{2+}), zinc (Zn^{2+}) and nickel (Ni^{2+}) on planktonic cells and biofilm of *Rhodotorula mucilaginosa* and *Saccharomyces boulardii* was examined. The metal tolerance testing was performed by MBEC™-HTP assay. The minimum inhibitory concentration (MICp) and minimum lethal concentration (MLCp) were determined as well as the minimum biofilm eradication concentration (MBEC). Biofilm was more tolerant on the presence of heavy metals than the planktonic cells. The planktonic cells of *R. mucilaginosa* were tolerant to high concentrations of Cd^{2+}, Zn^{2+} and Ni^{2+}, while the planktonic cells of *S. boulardii* tolerated Zn^{2+}, exclusively. The *R. mucilaginosa* biofilm was tolerant to all of the tested metal concentrations and the obtained results were confirmed by fluorescence microscopy. *S. boulardii* did not show ability of biofilm formation. Metal removal efficiency of the *R. mucilaginosa* planktonic cells and biofilm were also tested. The *R. mucilaginosa* biofilm showed higher efficiency in metals removing compared to the planktonic cells. Until now, the heavy metal tolerance and the removal efficiency (Cd^{2+}, Zn^{2+} and Ni^{2+}) analyzes were performed solely on planktonic cells of *Rhodotorula* species. In this study, we investigated the metal removal efficiency of *R. mucilaginosa* planktonic cells and biofilm and compared the obtained results.

Keywords: biofilm, metals, tolerance, removal, *Rhodotorula mucilaginosa*, *Saccharomyces boulardii*.

INTRODUCTION

An understanding of the nature of heavy metals, their relationships and toxicity or deficiency problems associated with them, is important for environmental protection. As more and more analytical data become available in the world literature, it is evident that considerable areas in many parts of the world have been contaminated with heavy metals, which present potential toxicity problems (ALLOWAY, 1995).

A wide range of methods for the heavy metals removal from contaminated environment are being used. Most of them are not efficient in removing low concentrations of metals, have high energy requests, lead to accumulation of toxic sludge and other waste products, therefore requiring a careful disposal of waste (AHALYA *et al.*, 2003). With increasing ecological awareness, search for effective alternative technologies is essential. Microbial biomass is considered as an alternative for the heavy metals removal (ALLURI *et al.*, 2007).

Some authors reported nickel tolerance of planktonic cells *Rhodotorula mucilaginosa* (SAN and DÖNMEZ, 2012) and *Rhodotorula glutinis* (SUAZO-MADRID *et al.*, 2011). Cadmium tolerance was tested by planktonic cells of *Rhodotorula* Y11 (LI and YUAN 2006, 2008) and *Rhodotorula rubra* (SALINAS *et al.*, 2000). The heavy metal tolerance of biofilms are taking a great attention, since they could be applied in bioremediation of polluted environments. HARRISON *et al.*, (2006) reported that *Candida tropicalis* could survive in the most adverse environmental conditions, thanks to the ability to form a biofilm.

Heavy metal tolerance is associated with the ability to remove heavy metals from the environment (FAZLI *et al.*, 2015). Recently, it was reported that *R. rubra* (planktonic cells) have a potential application in degradation and bioleaching of heavy metals (REZZA *et al.*, 2001). The accumulation of lead and cadmium by *R. rubra* biomass was tested (SALINAS *et al.*, 2000), as well as the removal of nickel by planktonic cells of *Rhodotorula* sp. (LI and YUAN, 2008).

In previous studies, the heavy metal tolerance and the removal potential for Cd^{2+}, Zn^{2+} and Ni^{2+} have been focused on planktonic cells of *Rhodotorula* species, exclusively (SALINAS *et al.*, 2000, REZZA *et al.*, 2001, LI and YUAN, 2008, SUAZO-MADRID *et al.*, 2011, SAN and DÖNMEZ, 2012). Only one study has reported heavy metal removal efficiency for Zn^{2+} by *Candida rugosa* and *Cryptococcus laurentii* biofilms (BASAK *et al.* 2014). In our previous investigations, we concluded that *R. mucilaginosa* biofilm was few times more tolerant and had a higher potential for removing Hg^{2+}, Cu^{2+} and Pb^{2+} ions than planktonic cells (GRUJIĆ et al. 2017a). Furthermore, the *R. mucilaginosa/Escherichia coli* mixed biofilm was more efficient in removing heavy metals than their mono-species biofilms (BUZEJIĆ *et al.*, 2016, GRUJIĆ *et al.*, 2017b). These findings led to the further development of studies on the Cd^{2+}, Zn^{2+} and Ni^{2+} ion tolerance and removal efficiency of planktonic cells and biofilm of *R. mucilaginosa* and *Saccharomyces boulardii*, including comparative analysis with the previously obtained results.

MATERIALS AND METHODS

Microorganisms and growth conditions

Two species of yeast were used – *R. mucilaginosa* (isolated from the environment) and *S. boulardii* (commercial probiotic). The *R. mucilaginosa* was identified by the test for rapid identification of yeast API 20 C AUX (Biomerieux, France). Tryptic soy broth (TSB) was chosen as the growth medium for all metal tolerance assays (HARRISON *et al.*, 2006). For the metal removal assays, YPED medium was used (MUNEER *et al.*, 2007). All serial dilutions were carried out using 0.9% saline.

Cultivation of biofilms

Growth of the selected yeasts in the presence of heavy metals was tested by quantitative assay in the MBEC-HTP device (MBEC BioProducts, Innovotech, Canada) as previously described (CERI *et al.*, 1999). Plastic lid with 96 pegs that fits inside a standard 96-

well microplate was used. The peg lid was immersed into a sterile solution of 1% L-lysine in distilled water (dH_2O) and incubated at room temperature for 16 h.

Cryogenic stocks cultures of *R. mucilaginosa* and *S. boulardii* were streaked out twice on TSA and incubated at 26°C for 48 h. The growth was monitored throughout 48 h. This culture was used for inoculum preparation for setting MBEC-HTP device. Inoculum was prepared in TSB to match a 1.0 McFarland standard and diluted 30-fold in TSB. 150 µL of inoculum was transferred into each well of a 96-well microtiter plate. The dried, L-lysine-coated peg lids were then inserted into 96-well microtiter plate containing this inoculum, and placed for 48 h in incubator at 26ºC.

Preparation of metal solution

Tolerance of the planktonic cells and biofilms was tested in the presence of Cd^{2+}, Zn^{2+}, and Ni^{2+} metal ions originating from the $CdSO_4$, $ZnSO_4$, $NiSO_4$ salts (Sigma). All metal compounds were dissolved in the sterile distilled water. Stock solutions were filtered using the 0.2 µm syringe filter. Work solutions of metals were diluted in TSB from stock solutions, to prepare challenge media, no more than 60 minutes before the exposure. Used concentrations were in accordance with the concentrations used in the study of AL-ENZI and AL-CHARRAKH (2013). Range of concentrations for nickel was from 1.30 to 20.67 mM; for cadmium and zinc was 0.1, 1, 10 and 100 mM. Range of concentrations for amphotericin B was: 0.24; 0.47; 0.94; 1.89; 3.78; 7.57; and 15.15 µg/mL. This antimycotic was a control for yeast cells susceptibility, not to compare with metals. The Zn^{2+} and Cd^{2+} ions were neutralized using 10 mM reduced glutathione while the Ni^{2+} ions were chelated with 0.5 mM reduced glutathione (HARRISON *et al.*, 2006). Based on the previous studies on metal removal potential, selected concentration was 100 µg/mL for each metal (BASAK *et al.*, 2014).

Tolerance of planktonic cells to heavy metals

The tolerance of the *R. mucilaginosa* and *S. boulardii* planktonic cells was determined according to the method described by CERI *et al.* (1999). The culture inoculum was prepared in McFarland 1.0 and diluted 30-fold in TSB for setting MBEC-HTP device. Each well of a 96-well microtitre plate was set with 150 µL with plastic lid with 96 pegs. After 48-h incubation at 26 °C, biofilm was formed on peg and planktonic cells left in wells were both used for metal challenge.

Tolerance of biofilms to heavy metals

Tolerance of biofilms was evaluated as previously described by HARRISON *et al.* (2006). The peg lid (with the formed biofilms) was immersed in the 96-well microtiter plates containing TSB with metal salt in the appropriate concentrations. The challenge plates were incubated at 26ºC for 48 h.

After exposure period, pegs with biofilm were removed from the challenge plates and washed twice with sterile 0.9% saline. Plastic lid with pegs, was transferred to a new plate with TSB containing neutralizer (200 µL per well). After neutralization, plastic lid with pegs was transferred to a plate with TSB and the entire plate was exposed to the ultrasonic waves, the frequency of 20 kHz to 400 kHz for 5 min in a water bath for sonification (Aquasonic 250 HT Ultrasonic Cleaner, VWR International, Radnor, PA, USA). This microtiter plate was marked as recovery plate and it was incubated for 48 h at 26ºC. After the incubation period, minimum biofilm eradication concentration (MBEC) was obtained using ELISA microplate reader (OD_{650}) (Rayto, China).

Fluorescence microscopy

Fluorescence microscopy was used to evaluate the effect of metals on the *R. mucilaginosa* biofilm according to the method described by KRONVALL and MYHRE (1977) with some modifications. The content of the recovery microtiter plate was removed. 50 µL of methanol was added in each well of microtiter plate. Microtiter plate was incubated at room temperature until methanole vaporized. 50 µL of acridine orange (5 mg/mL) was added in each well. After 2 min., the microtiter plate was washed with sterile distilled water. The *R. mucilaginosa* biofilm was observed on the Olympus BX51 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) and analyzed using Cytovision 3.1 software package (Applied Imaging Corporation, Santa Clara, California, USA).

Metal removal efficiency using planktonic cell

Metal removal efficiency was analyzed according to the method described by MUNEER *et al.* (2007). The cells were grown in 250 ml Erlenmeyer flasks containing 100 ml of YPED medium. One flask was the control and other three contained YPED medium, suspension, and metals with concentration of 100 µg/ml. The flasks were incubated at 26 °C. Growth of the *R. mucilaginosa* planktonic cells was determined by reading optical density at 520 nm (OD520) after 12, 24, and 48 h. At the same time, from the flasks with tested metal, 5 ml of aliquots was taken out and cells were separated by centrifugation. The supernatant (samples and controls) were subjected to spectrophotometer (357.9 nm) analysis for residual metal concentration. All experiments were performed in triplicates and their mean value was calculated.

The metal removal percentage (%) was calculated from the following equation (1):

$$E(\%) = \frac{(Ci - Cr)}{Ci} \times 100 \quad (1)$$

where C*i* is the initial concentration of metal ion (µg/mL) and C*r* is the final concentration of metal ion (µg/mL).

Metal removal efficiency using biofilm

Metal removal efficiency was analyzed according to the method described by BASAK *et al.* (2014). Biofilm was formed on 22 × 22 mm polyvinyl plastic coverslips placed in each well of a 6-well culture plate. Fifty microliters of suspension (McFarland 1.0) was added to each well with 5 ml YPED medium. Coverslips with formed biofilm were placed in the new 6-well plate that contained tested metals individually, with concentration of 100 µg/ml. After 12, 24, and 48 h incubation period, 1.5 mL aliquots were taken and centrifuged at 10000 rpm for 5 min. The supernatant (samples and controls) were subjected to spectrophotometer (520 nm) analysis for residual metal concentration. All experiments were performed in triplicates and their mean value was calculated.

The metal removal percentage (%) was calculated from the equation 1.

RESULTS AND DISCUSSION

Tolerance of planktonic cells on heavy metals

Heavy metal tolerance of *R. mucilaginosa* and *S. boulardii* planktonic cells, for the exposure period of 48 h, was analyzed. The planktonic cells of *R. mucilaginosa* showed high tolerance in the presence of metals (Cd^{2+}, Zn^{2+} and Ni^{2+}), while *S. boulardii* showed tolerance toward Zn^{2+} only. The results are presented in the Table 1.

Table 1. Heavy metal tolerance of *R. mucilaginosa* and *S. boulardii* planktonic cells at exposure period of 48 h.

Species	Test substance	[1]MICp	[2]MLCp
R. mucilaginosa	Cd^{2+}	10	100
R. mucilaginosa	Zn^{2+}	10	100
R. mucilaginosa	Ni^{2+}	5.17	10.33
R. mucilaginosa	Amphotericin B	15.15	>15.15
S. boulardii	Cd^{2+}	<0.1	<0.1
S. boulardii	Zn^{2+}	0.1	1
S. boulardii	Ni^{2+}	<1.3	<1.3
S. boulardii	Amphotericin B	<0.24	<0.24

[1]MICp - Minimum inhibitory concentration of planktonic cells;
[2]MLCp - Minimum lethal concentrations of planktonic cells;
given as mM for metals and µg/mL for antimycotic.

Cadmium tolerance of *Rhodotorula* sp. Y11 was reported by LI and YUAN (2006, 2008), with the highest tolerated concentration of 0.1 mM. In our study, *R. mucilaginosa* tolerated cadmium concentration up to 10 mM. A possible reason for the disparity may be the species difference, even though they belong to the same genus. The *R. mucilaginosa* species in this study was isolated from environment, which also may influence the obtained results. In another study, *R. rubra* tolerated cadmium to concentration of 10 mM (SALINAS *et al.,* 2000), which is in accordance with our results.

The tolerance of *R. mucilaginosa* to the presence of 50 mg/L nickel was previously reported by SAN and DÖNMEZ (2012). Furthemore, the tolerance of another species, *R. glutinis* to the presence of nickel under concentration range from 10 to 400 mg/L was reported by SUAZO-MADRID *et al. (*2011). In our study, the range of concentrations was significantly higher, ranging from 100 to 3200 mg/L. The MIC was observed at the 400 mg/L, which implies the similar metabolic response to heavy metal impact by two different species.

GRUJIĆ *et al*. (2017a) have tested the influence of heavy metals (Hg^{2+}, Cu^{2+}, and Pb^{2+}) on the *Rhodotorula mucilaginosa* and *Saccharomyces boulardii* biofilm and planktonic cells. The *R. mucilaginosa* planktonic cells showed the tolerance in the presence of all tested metals, while while the *S. boulardii* planktonic cells only tolerated Pb^{2+} (MICp 0.43 mM).

Tolerance of biofilms to heavy metals

The heavy metal tolerance of *R. mucilaginosa* and *S. boulardii* biofilms was analyzed. *R. mucilaginosa* formed the biofilm after 48 h of exposure, while *S. boulardii* did not exhibit the biofilm formation ability. The results of *R. mucilaginosa* heavy metal tolerance is presented in the Table 2.

Table 2. Heavy metal tolerance of the *R. mucilaginosa* biofilm at exposure period of 48 h.

Test substance	[1]MBEC
Cd^{2+}	>100
Zn^{2+}	>100
Ni^{2+}	>20.67
Amphotericin B	>15.15

[1]MBEC - minimum biofilm eradication concentration;
given as mM for metals and µg/mL for antimycotic.

The obtained results showed a significant difference in metal tolerance between the *R. mucilaginosa* biofilm and planktonic cells. The *R. mucilaginosa* biofilm was more tolerant in the presence of all tested metals, compared to planktonic cells. This is due to the extracellular polymeric substances (EPS) that surround the cells in biofilms. Furthermore, it is confirmed, and our results were similar to the results of HARRISON *et al.* (2006), who examined the effect of heavy metal (AsO_4^{3-}, Cd^{2+}, Pb^{2+}, Ni^{2+}, SeO_3^{2-}, CrO_4^{2-}, Mn^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Zn^{2+}, Hg^{2+}, Al^{3+}, AsO_2^{-}, SeO_3^{2-}, Te_3^{2-}) on *Candida tropicalis* biofilm.

Fluorescence microscopy

The fluorescence microscopy was used as visual confirmation of already obtained results through MBEC. The impact of heavy metals and amphotericin B on the *R. mucilaginosa* biofilm were observed and results were shown in Figure 1-4.

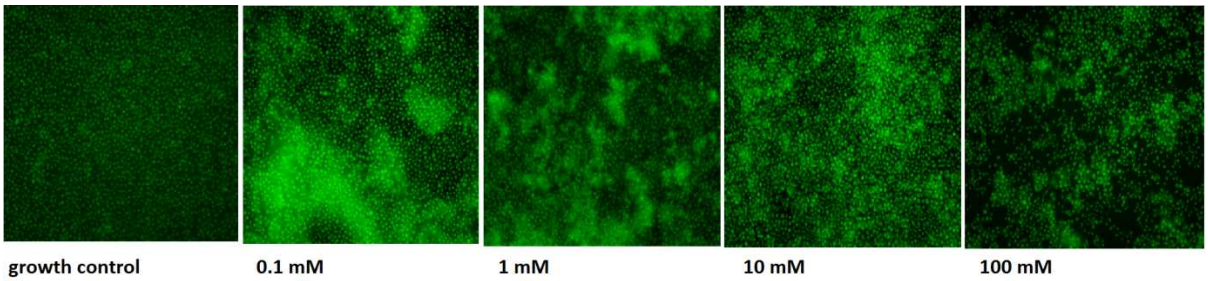

Figure 1. The effect of Cd^{2+} on the *R. mucilaginosa* biofilm.

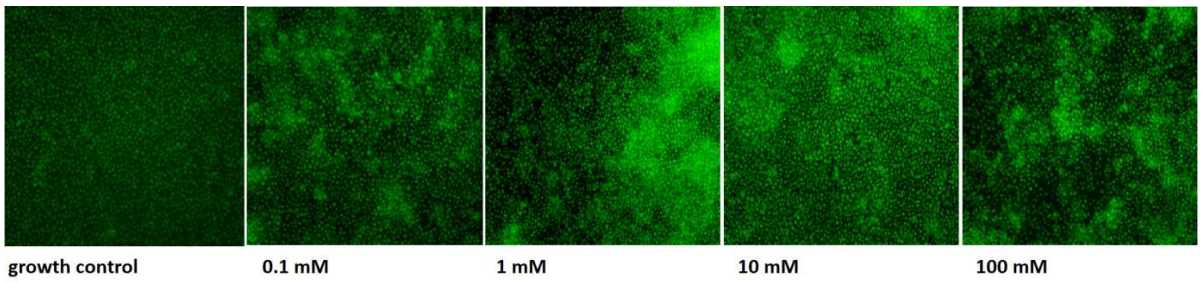

Figure 2. The effect of Zn^{2+} on the *R. mucilaginosa* biofilm

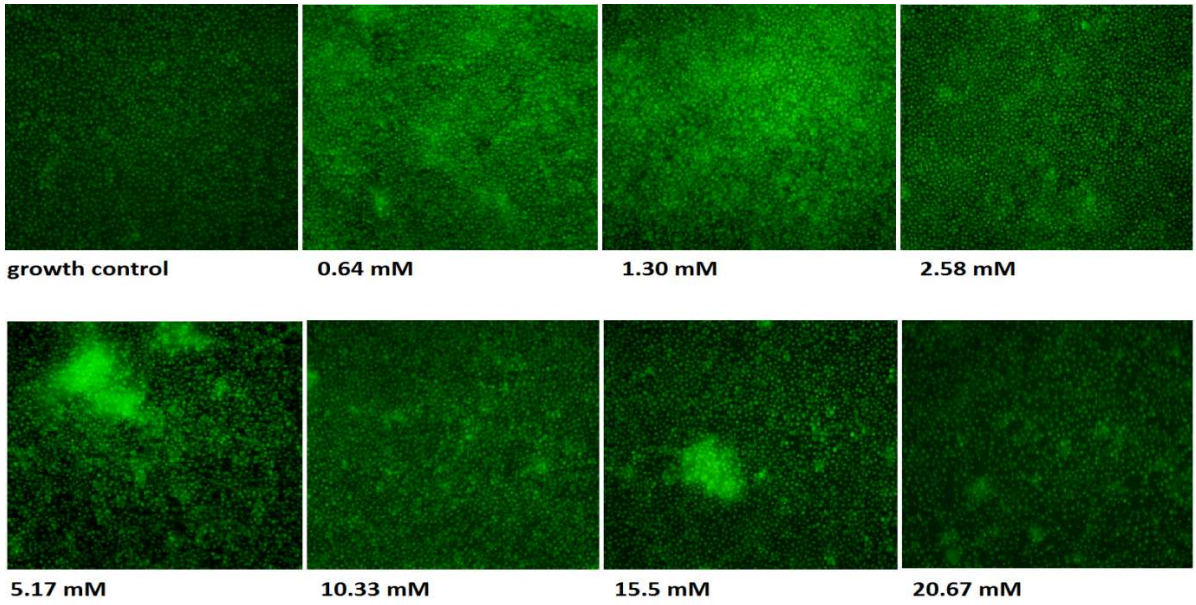

Figure 3. The effect of Ni^{2+} on the *R. mucilaginosa* biofilm

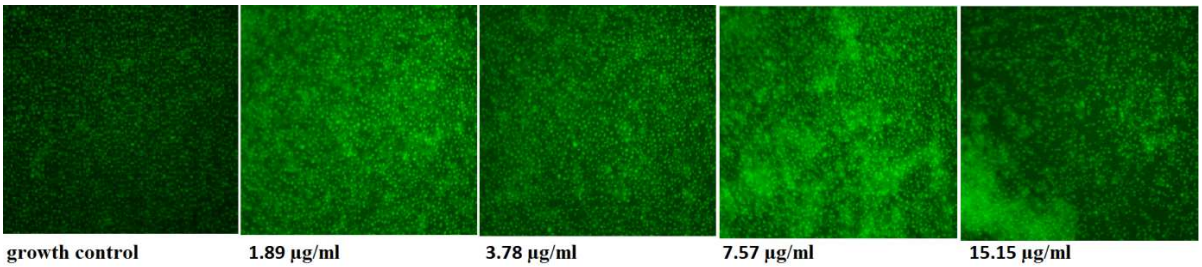

Figure 4. The effect of amphotericin B on the *R. mucilaginosa* biofilm

The results of reading the optical density at microplate rider were in accordance with the results of fluorescence microscopy.

Metal removal efficiency using planktonic cells and biofilm

The percentage of heavy metals removal by *R. mucilaginosa* planktonic cells after 48 hours of incubation is shown in the Table 3. The removal percentage of Cd^{2+}, Zn^{2+} and Ni^{2+} were 2.11; 4.99; 29.25%, respectively.

Table 3. Metal removal efficiency using *R. mucilaginosa* planktonic cells (%).

Time	Cd^{2+}	Zn^{2+}	Ni^{2+}
12 h	1.11	2.57	2.42
24 h	1.49	3.15	3.03
48 h	2.11	4.99	29.25

The efficiency in Ni^{2+} removal by *Candida* spp. (planktonic cells) isolated from sewage was determined by DÖNMEZ and ZÜMRIYE (2001), at initial concentration of 100 µg/mL. Percentage of Ni^{2+} removal, after 5-15 days was 29-57%. In our study, *R. mucilaginosa* removed 29.25% Ni^{2+} after 48 h, at initial concentration of 100 µg/mL. The

results obtained for Ni^{2+} in the mentioned study are similar with the results obtained in this study.

Removal of Cd^{2+}, Zn^{2+} and Ni^{2+} ions using the *R. mucilaginosa* biofilm was tested. The results are presented in the Table 4.

Table 4. Metal removal by *R. mucilaginosa* biofilm (%).

Time	Cd^{2+}	Zn^{2+}	Ni^{2+}
12 h	81.12	82.22	77.85
24 h	83.49	85.04	87.23
48 h	90.71	89.62	91.24

The metal removal efficiency of the *R. mucilaginosa* biofilm was better, compared to the planktonic cells. Obtained results showed that the *R. mucilaginosa* biofilm removed over 90% of every tested metal after 48 h. These results are in accordance with the results of BASAK *et al.* (2014), who reported 88% and 72.2% Zn^{2+} removal by *Candida rugosa* and *Cryptococcus laurentii* biofilm, respectively, for 24 h. The percentage of Zn^{2+} removal in our study after 24 hours was 85.04%, which was in accordance with results of mentioned studies.

CONCLUSION

Our findings suggest that biofilm and planktonic populations show different levels of tolerance to heavy metals. Understanding this difference is significant for understanding the microbial ecology of environments polluted with heavy metals, as well as the basics of biofilm tolerance to antimicrobial agents in general. This study gives an insight about the ability of *R. mucilaginosa* to form biofilm on coverslips and remove metal ions (Cd^{2+}, Zn^{2+}, Ni^{2+}) as an inexpensive and alternative method to traditional techniques for removal of heavy metals from waste waters. Our results indicate that biofilm has a higher ability to remove heavy metals compared to planktonic cells, which suggests that biofilm has a better potential for application in the environment remediation. The ability of the *R. mucilaginosa* biofilm to remove Cd^{2+}, Zn^{2+} and Ni^{2+} ions could be used in some future examinations on real effluent.

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