

Comparison of the *Rhodotorula mucilaginosa* Biofilm and Planktonic Culture on Heavy Metal Susceptibility and Removal Potential

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Abstract This study compares the effect of heavy metals (Hg^{2+} , Cu^{2+} , and Pb^{2+}) on the *Rhodotorula mucilaginosa* and *Saccharomyces boulardii* biofilm and planktonic cells. A MBECTM-HTP assay was used to test the levels of tolerance to heavy metals. The minimum inhibitory concentration (MICp) and minimum lethal concentration (MLCp) of the *R. mucilaginosa* and *S. boulardii* planktonic cells were determined, as well as minimum biofilm eradication concentration (MBEC). Metal removal efficiency was determined by batch biosorption assay. Previous studies had focused on heavy metal tolerance and removal efficiency of planktonic cells from *Rhodotorula* species only. Hence, our study presents and compares results for metal tolerance and removal efficiency of the *R. mucilaginosa* planktonic cells and biofilm. Biofilm tolerance was higher than the planktonic cells. The *R. mucilaginosa* planktonic cells showed the tolerance in the presence of Hg^{2+} (MICp 0.08 mM), Cu^{2+} (MICp 6.40 mM), and Pb^{2+} (MICp 3.51 mM), while the *S. boulardii* planktonic cells only tolerated Pb^{2+} (MICp 0.43 mM). The *R. mucilaginosa* biofilm showed the highest tolerance in the presence of Hg^{2+} (MBEC >0.31 mM), Cu^{2+} (MBEC >12.81 mM), Pb^{2+} (MBEC >7.12 mM), and obtained results were confirmed by fluorescence microscopy. *S. boulardii* did not show

potential in biofilm formation. The *R. mucilaginosa* biofilm exhibited better efficiency in removal of all tested metals than the planktonic cells. Metal removal efficiency was in the range from 4.79–10.25% for planktonic cells and 91.71–95.39% for biofilm.

Keywords *Rhodotorula mucilaginosa* · *Saccharomyces boulardii* · Biofilm · Planktonic cells · Heavy metal

1 Introduction

The environmental pollution by metals is a serious problem which negatively affects natural ecosystems (Chipasa 2003). On the other hand, it represents great potential for studying the interactions between microorganisms and metals (Gadd 2004). Heavy metal polluted environments are a source of undiscovered microbial diversity and specific microbial strains and understanding the processes which are involved in natural biogeochemical cycles, already fundamental for environmentally friendly biotechnologies (Pieper and Reineke 2000; Gadd 2010).

Microbes and metals interact at all spatial scales for centuries (Ehrlich 1997; Gadd 2004). Metals support microbial growth by providing essential nutrients, microbial activity alters metal solubility and their oxidation state (Ehrlich 1997). Nowadays, when our reliance on metals increases, also does their amount in the environment and living systems. The dichotomy between metabolic requirements for metals by microorganisms and the potential associated toxicity has created an

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interesting and broad set of metal homeostasis and resistance systems required to maintain a delicate balance (Ehrlich 1997). Metal toxicity is significant to metal mobilizing microbes, which must resist toxic heavy metals released into the environment as a result of their metabolism (Gadd 2004). However, exposure to high toxic concentrations can trigger also various specific responses, involving metal resistance (Gadd and Griffiths 1977). Knowledge of metal-microbe interactions and the consequences of these interactions are necessary to understand metal resistance mechanisms of microorganisms (Hafner and Kothe 2007).

Microbes, as predominant population in nature and environments, are present in two forms of life—as planktonic cells and biofilm. The most of microbes exist in surface-attached communities called biofilms, whose metabolism differ from their planktonic cells (Booth et al. 2011). Researches confirmed that biofilms were more resilient on antibiotic than their planktonic cells (Ceri et al. 1999). Teitzel and Parsek (2003) studied heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa* and reported that biofilms were from 2 to 600 times more resistant to heavy metal stress than free-swimming cells. Harrison et al. (2004) examine the metalloid oxyanions toxicity against biofilm and planktonic cells of *P. aeruginosa* and *Staphylococcus aureus* and got the same results. The tolerance to antimicrobials depends on metabolic heterogeneity or the existence of a “persister” cell phenotype (Harrison et al. 2004). Also, differential metabolic shifts play a role in biofilm-specific multimetal resistance and tolerance (Booth et al. 2011). Persister cells of *Escherichia coli* biofilm, known for high tolerance on antibiotics, were also resistant to metal oxyanions (Harrison, et al. 2005). The comparison of tolerance between bacterial planktonic cells and biofilms to metal ions has been documented in a number of studies. No study to date has examined the susceptibility of yeast biofilms and planktonic cells to heavy metals, except for *Candida tropicalis* (Harrison et al. 2006).

Considering that yeasts are often predominant microbial species in environments contaminated with pollutants (Hagler and Mendonça-Hagler 1981) and that the planktonic cells of *Rhodotorula mucilaginosa* are known for high levels of resistance to the metal ions (Garza et al. 2016), we decided to test heavy metal susceptibility and removal efficiency of biofilm and planktonic cells of yeasts *R. mucilaginosa* isolated from environment and *Saccharomyces boulardii* (commercial probiotic) and make comparison of the obtained results.

2 Materials and Methods

2.1 Strains and Growth Media

Two species of microorganisms were used in this study. *R. mucilaginosa* (isolate from the environment, food spoilage, Serbia) and *S. boulardii* (commercial probiotic). The *R. mucilaginosa* strain was identified by the test for rapid identification of yeast API 20 C AUX (Biomerieux, France). Tryptic Soy Broth (TSB, Difco) was chosen as the growth medium for the both strains and for all metal susceptibility assays (Harrison et al. 2006). For metal removal studies, YPED medium was used (Muneer et al. 2007). All serial dilutions were performed using 0.9% saline.

2.2 Biofilm Cultivation

The *R. mucilaginosa* and *S. boulardii* biofilms were formed in the MBEC-HTP device (MBEC BioProducts) according to the manufacturer’s instructions and as described previously Ceri et al. (1999). Cryogenic stock cultures of *R. mucilaginosa* and *S. boulardii* were streaked out twice on TSA and incubated at 26 °C for 48 h. After incubation, period culture was used to prepare inoculum (McFarland 1.0 and diluted 30-fold in TSB) for setting MBEC-HTP device. One hundred fifty microliters of inoculum was transferred into each well of a 96-well microtitre plate, and plastic lid with 96 pegs was immersed into it. After 48-h incubation at 26 °C, biofilm was formed on peg, which was used for metal challenge.

Biofilm formation on peg lid was confirmed by sonication (Aquasonic 250 HT Ultrasonic Cleaner, VWR International, Radnor, PA, USA) of peg with lid in the new 96-well plate with fresh TSB media (biofilm formation confirmation). Evaluation of biofilm growth was obtained by reading the optical density of released biofilm at 650 nm (OD₆₅₀) using ELISA microplate reader (Rayto, China).

2.3 Stock Metal Solutions

The susceptibility testing of the planktonic cells and biofilms was tested in the presence of ions; Hg²⁺, Cu²⁺, and Pb²⁺ originating from Pb(NO₃)₂, CuSO₄, and HgCl₂ (Sigma). All metal compounds were dissolved in sterile distilled water. Stock solutions were filtered using a 0.22-mm syringe filter into sterile glass

vials and stored at fridge until use. Working solutions of metals were diluted in TSB from stock solutions, to prepare challenge media, no more than 60 min before exposure. Used concentrations for metal susceptibility assays were in accordance with the concentrations used in other study (Al-Enzi and Al-Charrakh 2015). Range of concentrations for lead (Pb^{2+}) was from 0.11 to 7.03 mM; for copper (Cu^{2+}) was from 0.40 to 12.81 mM and for mercury (Hg^{2+}) was from 0.01 to 0.32 mM. Twofold dilutions were made in the wells of a sterile 96-well microtiter plate (the challenge plate), leaving the first wells of each row for sterility control (media only), and the second as a growth control (without metal ion).

Concentration for metal removal studies was 100 $\mu\text{g/ml}$, which was in accordance with the concentrations used in other studies (Li and Yuan 2006; Muneer et al. 2007; Basak et al. 2014).

2.4 Neutralization

The susceptibility testing of the planktonic cells and biofilms used neutralizing step as previously described Harrison et al. (2006). Pb^{2+} was neutralized using 10 mM reduced glutathione (TwinLab), Cu^{2+} was chelated with 0.5 mM sodium diethyldithiocarbamate (Sigma) and Hg^{2+} with 10 mM l-cysteine (Scitec Nutrition).

2.5 Metal Susceptibility Testing of Biofilm

Metal susceptibility testing of biofilm was performed as described previously (Harrison et al. 2006). The peg lid (with the formed biofilms) was immersed in the 96-well microtitre plates containing TSB with metal salt in the appropriate concentrations. The challenge plates were incubated at 26 °C for 48 h.

After 48 h exposure, plastic peg lid was removed from the challenge plate 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 biofilm was disrupted by the ultrasonic waves (frequency of 20 to 400 kHz for 5 min in a water bath for sonication) into recovery medium. After incubation, minimum biofilm eradication concentration (MBEC) was obtained by reading the optical density at 650 nm (OD_{650}) of the recovery plate using ELISA microplate reader.

2.6 Metal Susceptibility Testing of Planktonic Cells

Experiments with *R. mucilaginosa* and *S. boulardii* planktonic cells were prepared according to the manufacturer's instructions and as described previously (Ceri et al. 1999).

Challenge plates were prepared as described above. Fourteen-microliter aliquots of the planktonic cultures from challenge plate were transferred into the new 96-well microtitre plate (which contained neutralizing agents). Twenty-microliter aliquots of the neutralized planktonic cultures were placed into the 96-well microplate with TSB. MICp and MLCp values were obtained after 48- and 72-h incubation at 26 °C by reading the optical density at 650 nm (OD_{650}) using a ELISA microplate reader.

2.7 Fluorescence Microscopy

Fluorescence microscopy was used to evaluate the effect of metals on the *R. mucilaginosa* biofilm as described previously by Kronvall and Myhre (1977) with certain modifications. The contents of the recovery microtiter plate were removed. Fifty microliters of methanol was added in each well. Microtiter plate was incubated at room temperature until methanol vaporized. Fifty microliters of acridine orange (0.1 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).

2.8 Metal Removal Study Using Biofilm

Metal removal study using biofilm was performed by batch biosorption assay according to the method described by Basak et al. (2014) with certain modifications. Biofilm was formed on 22 × 22 mm polyvinyl plastic coverslips placed in each well of a 6-well culture plate. Fifty microliter of suspension (McFarland 1.0) was added to each well with 5 ml YPED medium (Stenberg et al. 2014). Coverslips with formed biofilm were placed in the new 6-well plate that contained tested metals individually, with concentration of 100 $\mu\text{g/ml}$. After 12, 24, and 48 h incubation period, 1.5 ml of aliquots was taken and centrifuged at 10,000 rpm for 5 min. The supernatant (samples and controls) was

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 following Eq. (1):

$$E(\%) = \frac{(C_i - C_r)}{C_i} \times 100 \quad (1)$$

where C_i is the initial concentration of metal ion ($\mu\text{g/ml}$) and C_r is the residual concentration of metal ion ($\mu\text{g/ml}$).

2.9 Metal Removal Study Using Planktonic Cells

The *R. mucilaginosa* planktonic cells were used in metal removal study. Biosorption potential was tested in accordance to the method described by Muneer et al. (2007) with certain modifications. 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 $\mu\text{g/ml}$. The flasks were incubated at 26 °C. Growth of the *R. mucilaginosa* planktonic cells was determined by reading optical density at 520 nm (OD_{520}) 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 (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 Eq. 1.

3 Results and Discussion

3.1 Metal Susceptibility Testing of Biofilm

Susceptibility of the *R. mucilaginosa* and *S. bouldardii* survival preexisting biofilm and planktonic cells in the presence of heavy metal was analyzed. Hg^{2+} , Pb^{2+} , and Cu^{2+} metal ions were used for testing, and obtained results are presented in Table 1.

The obtained results indicated that *R. mucilaginosa* showed potential in biofilm formation. *S. bouldardii* did not show potential in biofilm formation. The *R. mucilaginosa* biofilm showed noticeable resilience in the presence of all tested metals, which was in accordance with the results of Harrison et al. (2006), who reported the high tolerance of *C. tropicalis* biofilm to

Table 1 Susceptibility of the *R. mucilaginosa* biofilm on heavy metals at exposure period of 48 h

| Test substance | MICb (mM) | MBEC (mM) |
|-----------------------------|-----------|-----------|
| Hg^{2+} | 0.31 | >0.31 |
| Pb^{2+} | >7.03 | >7.03 |
| Cu^{2+} | 12.81 | >12.81 |
| ^a Amphotericin B | 15.15 | >15.15 |

^a Amphotericin B values are expressed in microgram per milliliter
MICb minimum inhibitory concentration of biofilm, MBEC minimum biofilm eradication concentration

heavy metal toxicity. In mentioned study, MLCb for Hg^{2+} was obtained at 1.9 ± 0.7 mM. MLCb for Pb^{2+} and Cu^{2+} was obtained at >77 mM and $>1.3 \times 10^2$ mM. Our results were in accordance with the results of Harrison et al. (2006). Within a biofilm, regulatory processes occur (chemical, physical, physiological, and genetic), which allow microbes to develop better mechanism of tolerance and improve their resilience in the presence of metals (Harrison et al. 2007). Teitzel and Parsek (2003) reported Pb^{2+} and Cu^{2+} resistance of biofilm and planktonic *P. aeruginosa*, whereby the MBC for Pb^{2+} was obtained at 0.8 mM and MBC for Cu^{2+} was at 6 mM. In comparison with our results, we could suppose that *R. mucilaginosa* biofilm was more tolerant in the presence of Pb^{2+} and Cu^{2+} metal ions than *P. aeruginosa* biofilm.

Table 2 Susceptibility of the *R. mucilaginosa* and *S. bouldardii* planktonic cells in the presence of heavy metals at exposure period of 48 h

| Species | Test substance | MICp (mM) | MLCp (mM) |
|------------------------|-----------------------------|-----------|-----------|
| <i>R. mucilaginosa</i> | Hg^{2+} | 0.08 | 0.16 |
| <i>R. mucilaginosa</i> | Pb^{2+} | 3.51 | 7.03 |
| <i>R. mucilaginosa</i> | Cu^{2+} | 6.40 | 12.81 |
| <i>R. mucilaginosa</i> | ^a Amphotericin B | 15.15 | >15.15 |
| <i>S. bouldardii</i> | Hg^{2+} | / | / |
| <i>S. bouldardii</i> | Pb^{2+} | 0.43 | 1.75 |
| <i>S. bouldardii</i> | Cu^{2+} | / | / |
| <i>S. bouldardii</i> | ^a Amphotericin B | / | / |

^a Amphotericin B values are expressed in $\mu\text{g/ml}$

MICp minimum inhibitory concentration of planktonic cells, MLCp minimum lethal concentration of planktonic cells, “/” undetermined

3.2 Metal Susceptibility Testing of Planktonic Cells

Susceptibility of the *R. mucilaginosa* and *S. boulardii* planktonic cells was analyzed in the presence of Hg^{2+} , Pb^{2+} , and Cu^{2+} metal ions. The results of heavy-metal susceptibility testing are presented in Table 2.

The *R. mucilaginosa* planktonic cells showed the highest tolerance in the presence of Cu^{2+} (MICp 6.40 mM) than other two metal ions, while the *S. boulardii* planktonic cells only tolerated Pb^{2+} (MICp 0.43 mM). Obtained results indicated that *R. mucilaginosa* was more tolerant to Pb^{2+} than *S. boulardii*. The highest sensitivity *R. mucilaginosa* showed in the presence of Hg^{2+} (MICp 0.08 mM).

Other authors reported the copper tolerance of the *R. mucilaginosa* LM9 (planktonic cells) (Rajpert et al. 2013). In correlation with available literature, Garza-Gonzalez et al. (2016) reported that *R. mucilaginosa* strain, isolated from the water streams, registered as UANL-001 L, was capable to survive heavy metal stress, whereby MIC for Cu^{2+} and Pb^{2+} were obtained at 0.8 g/l and 1 g/l. These results positively correlated with results obtained in our study in which MIC for Cu^{2+} and Pb^{2+} was noted at 6.40 mM (1.02 g/l) and 5.27 mM (1.74 g/l) (Table 2).

Mercury (Hg^{2+}) tolerance of *Rhodotorula rubra* was literature supported and that strain showed tolerance at concentration of 16.8 $\mu\text{g/ml}$ (Ghosh et al. 2006). Results in this study were approximate to our result where *R. mucilaginosa* tolerated mercury at 0.08 mM (21.7 $\mu\text{g/ml}$).

3.3 Differences Between Biofilm and Planktonic Response to Metal Susceptibility

Heavy metal susceptibility of biofilm and planktonic culture revealed that *R. mucilaginosa* biofilm was more

tolerant to all tested metals than its planktonic cells (Fig. 1; Tables 1 and 2).

Fold tolerance¹ for Hg^{2+} was >1.93, for Pb^{2+} was >1, and for Cu^{2+} was >2.01. Biofilms, by their physiology, differ from their planktonic cells and they were up to 600 times more resistant to the effects of heavy metals (Teitzel and Parsek 2003; Harrison et al. 2006). *C. tropicalis* biofilm was up to 65 times more tolerant to metals than corresponding planktonic cultures. Fold tolerance for *C. tropicalis* biofilm for Cu^{2+} was >26; for Hg^{2+} 1.5; and for Pb^{2+} , fold tolerance was not applicable (Harrison et al. 2006). Results of our work were in accordance with mentioned study. Biofilms were less susceptible to antimicrobials than planktonic form, because of structure dependent metabolic heterogeneity or because of the existence of a persister cell phenotype (Harrison et al. 2004). Booth et al. (2011) reported that differential metabolic shifts play a role in biofilm-specific multimetal resistance and tolerance. Biochemical and genetic regulatory processes that occur within a biofilm allow microbes to develop better mechanism of tolerance and improve their resilience in the presence of metals (Harrison et al. 2007).

3.4 Fluorescence Microscopy

The impact of heavy metals on the *R. mucilaginosa* biofilm was observed under fluorescence microscopy, and results are presented in Figs. 2, 3, 4, and 5.

Considering that impact of heavy metals was observed by fluorescence microscopy after 72 h, it was noticed that the biofilm was present at all tested metal concentrations in same quantity, as well as in growth control. Results of MBEC which were obtained by reading the optical density at microplate rider after 72 h, were in accordance with the results of fluorescence microscopy (which was used as visual confirmation).

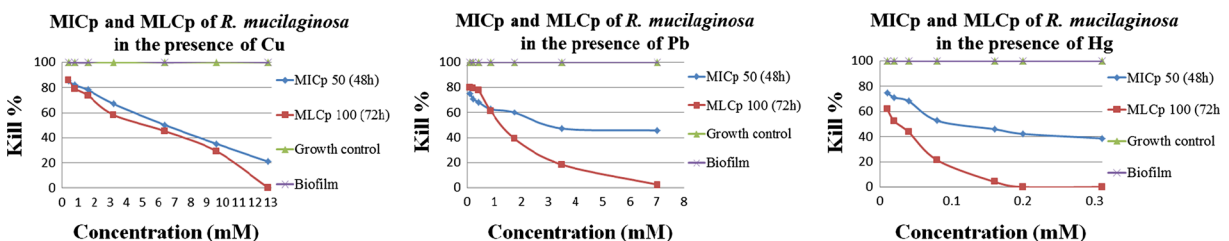


Fig. 1 Comparison of the *R. mucilaginosa* biofilm and planktonic culture survival in percentage

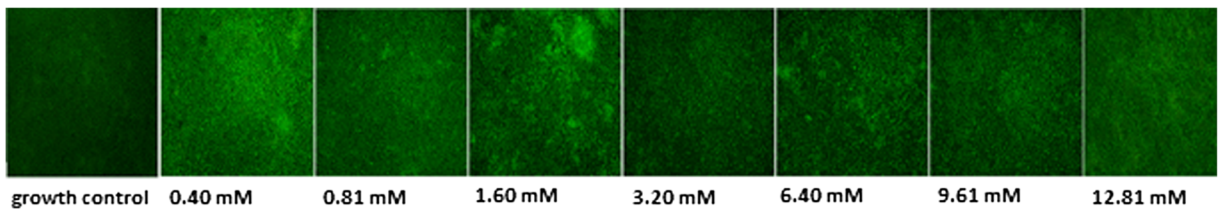


Fig. 2 The effect of copper (Cu^{2+}) on the *R. mucilaginosa* biofilm

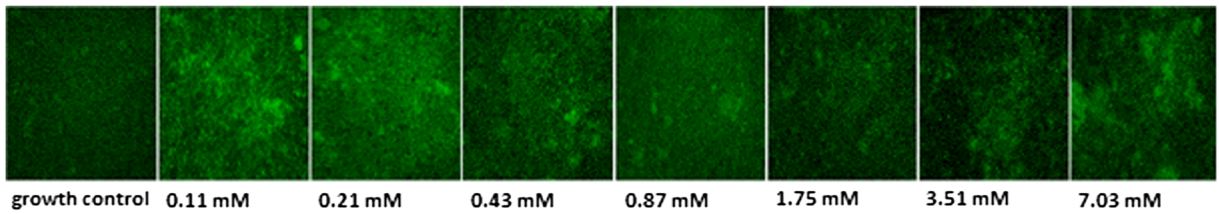


Fig. 3 The effect of lead (Pb^{2+}) on the *R. mucilaginosa* biofilm

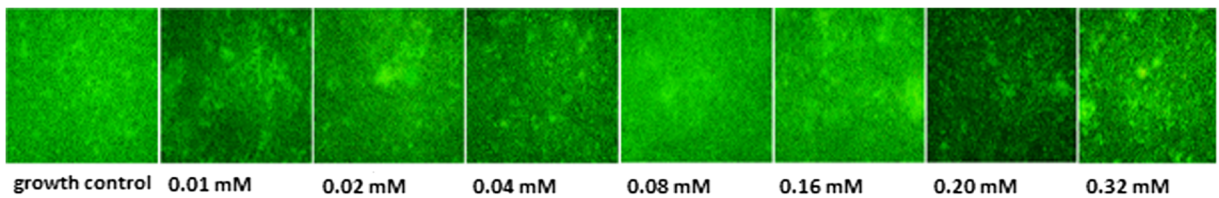


Fig. 4 The effect of mercury (Hg^{2+}) on the *R. mucilaginosa* biofilm

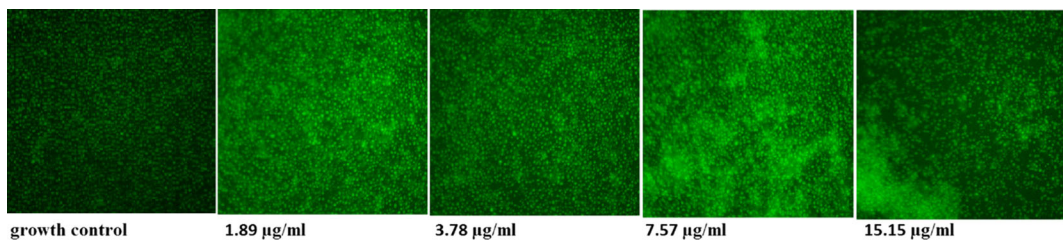


Fig. 5 The effect of amphotericin B on the *R. mucilaginosa* biofilm

Table 3 Metal removal using *R. mucilaginosa* biofilm (%)

| Time | Hg ²⁺ | Pb ²⁺ | Cu ²⁺ |
|------|------------------|------------------|------------------|
| 12 h | 81.58 | 75.37 | 80.89 |
| 24 h | 83.59 | 85.93 | 90.81 |
| 48 h | 95.39 | 94.81 | 91.71 |

3.5 Metal Removal Efficiency Using Biofilm and Planktonic Culture

Removal of Hg²⁺, Pb²⁺, and Cu²⁺ ions using *R. mucilaginosa* preexisting biofilm and planktonic cells was tested, and results are presented in the Tables 3 and 4.

Concentrations of the heavy metal ions in the medium were reducing during testing time, whereby the microbial biofilms showed high efficiency, which was in accordance with the results of Basak et al. (2014) study. Variations in the speed of removing metal ions during time periods are presented in Table 3. It is obvious that the process of the metal removing occurs in two phases. The first phase was extremely fast due to the high initial activity of biofilms as biosorbent. It was observed that the highest efficiency, during the treatment, was during the first day. After that, the slow phase of metal removal occurs, whereby the change in residual metal concentrations was insignificant. Our observation was in accordance with the study of Volesky (2003). Removal of Zn²⁺ using *Candida rugosa* and *Cryptococcus laurentii* biofilms was examined by Basak et al. (2014). The removal of Zn²⁺ ions was found to be 88 and 74.2%. In our study, the percentage of metal removal efficiency for the *R. mucilaginosa* biofilm after 12 h was in the range from 75–80% which was in accordance with the results in mentioned study.

Percentage of heavy metals removal using *R. mucilaginosa* planktonic cells during 48 h are presented in Table 4.

The available literature contains information about copper (Cu²⁺) removal efficiency by planktonic cells of

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

| Time | Hg ²⁺ | Pb ²⁺ | Cu ²⁺ |
|------|------------------|------------------|------------------|
| 12 h | 0.28 | 6.25 | 0.05 |
| 24 h | 4.08 | 9.92 | 1.09 |
| 48 h | 4.79 | 10.25 | 5.49 |

Candida spp., isolated from sewage, in which *Candida* spp. removed 22–52% Cu²⁺ ions after 8 days (Dönmez and Aksu 2001). The removal percentage of copper (Cu²⁺) for the *Saccharomyces cerevisiae* planktonic cells after 4 days exposure was 13–74% (Malik 2004). Our study reported that removal percentage of Cu²⁺ was 5.49 after 48 h/2 days exposure. The affinity of the metals for binding to the yeast cells could be explained by the fact that the cells of microorganisms have active nuclear sites for sorption of metal ions (Volesky 2003).

The obtained results showed a significant difference in metal removal efficiency between the biofilm and the planktonic cells. The best results in removal of all tested metals exhibited the *R. mucilaginosa* biofilm. Metal removal efficiency was in the range from 4.79–10.25% for planktonic cells and 91.71–95.39% for biofilm (Tables 3 and 4).

4 Conclusions

Based on the knowledge about microorganisms for reducing the heavy metal toxicity, it is possible to develop efficient and environmentally friendly (bio) technologies for metal remediation. The main aim of our work was to study metal-microbe interaction in the context of two microbial forms of life (biofilm and planktonic cells). This study also compared metal removal efficiency of the *R. mucilaginosa* planktonic cells and biofilm. The obtained results showed a significant difference in metal tolerance as well as in removal efficiency between the biofilm and their corresponding planktonic cells. The biofilm shows much better results than their planktonic cells, which suggests that biofilm could be used in the development of biotechnologies suitable for metal removal from the environment.

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References

- Al-Enzi, R. M., & Al-Charrakh, A. H. (2015). Heavy metals resistance of *Pseudomonas aeruginosa* isolated from clinical and environmental sources in Hilla city. *Medical Journal of Babylon*, 10(1), 110–119.

- Basak, G., Lakshmi, V., Chandran, P., & Das, N. (2014). Removal of Zn (II) from electroplating effluent using yeast biofilm formed on gravels: batch and column studies. *Journal of Environmental Health Science and Engineering*, 12(8), 1–11. doi:10.1186/2052-336X-12-8.
- Booth, S. C., Workentine, M. L., Wen, J., Shaykhtudinov, R., Vogel, H. J., Ceri, H., Turner, R. J., & Weljie, A. M. (2011). Differences in metabolism between the biofilm and planktonic response to metal stress. *Journal of Proteome Research*, 10(7), 3190–3199.
- Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., & Buret, A. (1999). The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*, 37(6), 1771–1776.
- Chipasa, K. B. (2003). Accumulation and fate of selected heavy metals in a biological wastewater treatment system. *Waste Management*, 23(2), 135–143.
- Dönmez, G., & Aksu, Z. (2001). Bioaccumulation of copper (II) and nickel (II) by the non-adapted and adapted growing *Candida* sp. *Water Research*, 35(6), 1425–1434.
- Ehrlich, H. L. (1997). Microbes and metals. *Applied Microbiology and Biotechnology*, 48(6), 687–692.
- Gadd, G. M. (2004). Microbial influence on metal mobility and application for bioremediation. *Geoderma*, 122(2), 109–119.
- Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology*, 156(3), 609–643.
- Gadd, G. M., & Griffiths, A. J. (1977). Microorganisms and heavy metal toxicity. *Microbial Ecology*, 4(4), 303–317.
- Garza-Gonzalez, M. T., Perez, D. B., Rodriguez, A. V., Garcia-Gutierrez, D. I., Zarate, X., Cardenas, M. E. C., ... & Medina-Ruiz, P. (2016). Correction: Metal-induced production of a novel bioadsorbent exopolysaccharide in a native *Rhodotorula mucilaginosa* from the Mexican northeastern region. *PLoS one*, 11(2). doi: 10.1371/journal.pone.0150522
- Ghosh, S. K., Ghosh, S., Gachhui, R., & Mandal, A. (2006). Mercury and organomercurial resistance in *Rhodotorula rubra*: activation of glutathione reductase. *Bulletin of Environmental Contamination and Toxicology*, 77(3), 351–358.
- Haferburg, G., & Kothe, E. (2007). Microbes and metals: interactions in the environment. *Journal of Basic Microbiology*, 47(6), 453–467.
- Hagler, A. N., & Mendonça-Hagler, L. C. (1981). Yeasts from marine and estuarine waters with different levels of pollution in the state of Rio de Janeiro, Brazil. *Applied and Environmental Microbiology*, 41(1), 173–178.
- Harrison, J. J., Ceri, H., Stremick, C., & Turner, R. J. (2004). Differences in biofilm and planktonic cell mediated reduction of metalloids oxyanions. *FEMS Microbiology Letters*, 235(2), 357–362.
- Harrison, J. J., Ceri, H., Roper, N. J., Badry, E. A., Sproule, K. M., & Turner, R. J. (2005). Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiology*, 151(10), 3181–3195.
- Harrison, J. J., Rabiei, M., Turner, R. J., Badry, E. A., Sproule, K. M., & Ceri, H. (2006). Metal resistance in *Candida* biofilms. *FEMS Microbiology Ecology*, 55(3), 479–491.
- Harrison, J. J., Ceri, H., & Turner, R. J. (2007). Multimetal resistance and tolerance in microbial biofilms. *Nature Reviews Microbiology*, 5(12), 928–938.
- Kronvall, G., & Myhre, E. (1977). Differential staining of bacteria in clinical specimens using acridine orange buffered at low pH. *Acta Pathologica Scandinavica Section B Microbiology*, 85(4), 249–254.
- Li, Z., & Yuan, H. (2006). Characterization of cadmium removal by *Rhodotorula* sp. Y11. *Applied Microbiology and Biotechnology*, 73(2), 458–463.
- Malik, A. (2004). Metal bioremediation through growing cells. *Environment International*, 30(2), 261–278.
- Muneer, B., Shakoori, F. R., Rehman, A., & Shakoori, A. R. (2007). Chromium resistant yeast with multimetal resistance isolated from industrial effluents and their possible use in microbial consortium for bioremediation of wastewater. *Pakistan Journal of Zoology*, 39(5), 289.
- Pieper, D. H., & Reineke, W. (2000). Engineering bacteria for bioremediation. *Current Opinion in Biotechnology*, 11(3), 262–270.
- Rajpert, L., Skłodowska, A., & Matlakowska, R. (2013). Biotransformation of copper from Kupferschiefer black shale (Fore-Sudetic Monocline, Poland) by yeast *Rhodotorula mucilaginosa* LM9. *Chemosphere*, 91(9), 1257–1265.
- Sternberg, C., Bjarnsholt, T., & Shirliff, M. (2014). Methods for dynamic investigation of surface-attached in vitro bacterial and fungal biofilms. *Microbial Biofilms: Methods and Protocols*, 1147, 3–22.
- Teitzel, G. M., & Parsek, M. R. (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, 69(4), 2313–2320.
- Volesky, B. Sorption and biosorption. (ISBN 0-9732983-0-8) BV-Sorbex, Inc. St. Lambert (Montreal), Quebec, Canada. 2003.