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Pb AND Hg HEAVY METAL TOLERANCE OF SINGLE- AND MIXED-SPECIES BIOFILM (*Rhodotorula mucilaginosa* AND *Escherichia coli*)

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ABSTRACT. The aim of this study was to examine heavy metal tolerance (lead (Pb^{2+}) and mercury (Hg^{2+})) of single- and mixed-species biofilms, formed by yeast *Rhodotorula mucilaginosa* and bacteria *Escherichia coli* LM1. Single- and mixed-species biofilms were quantified by crystal violet test and the absorbance was measured using microplate reader (OD_{570}). The minimal inhibitory concentration (MIC) and the minimal lethal concentration (MLC) were determined and the results were confirmed by fluorescence microscopy.

The significant difference in lead tolerance was observed between the mixed- and the single-species biofilms. The MIC of lead (Pb^{2+}) for the examined biofilms (*E. coli* LM1, *R. mucilaginosa* and *R. mucilaginosa / E. coli*) was recorded at concentrations of 4000 µg/ml, 4000 µg/ml and 16000 µg/ml, respectively. The MIC of mercury (Hg^{2+}) for the biofilms was noticed at concentrations of 31.25 µg/ml, 250 µg/ml and 250 µg/ml, respectively. Standard antibiotics (amphotericin B and tetracycline) were used as positive control. Results obtained for single-species biofilms were compared in between and with the results obtained for mixed-species biofilm.

The tolerance of the mixed- species biofilm was higher in comparison to the single-species biofilms and the results were confirmed by a fluoresecence microscope. The obtained results suggest that the *R. mucilaginosa / E. coli* biofilm may have a potential to be used in bioremediation of wastewaters contaminated with lead and mercury.

Key words: *Rhodotorula mucilaginosa*, *Escherichia coli*, biofilm, mixed-species, heavy metals, tolerance.

INTRODUCTION

The increase of heavy metal concentration in wastewaters is a consequence of industrial development (AHLUWALIA and GOYAL, 2007). Heavy metals present a major problem for the environment and the human health. They are easily accumulated in body cells which leads to an increase of their concentration throughout the food chain (AHLUWALIA and GOYAL, 2007). Typical physical-chemical methods used for the removal of heavy metals from waste waters are inadequate, expensive and produce large quantities of harmful chemical sludge. Bioremediation is considered more suitable and cost-effective method because it involves the use of microorganisms for the purpose of removing heavy metals from wastewater (AHLUWALIA and GOYAL, 2007).

Microorganisms in natural environments usually form sophisticated communites surrounded by an extracellular matrix called a biofilm (YANG *et al*., 2011). Biofilm formation can be established by one or several different microbial species, with the latter being the more frequent case. In recent decades a number of studies examined the impact of antimicrobial agents on individual biofilms and it was confirmed that the microorganisms within the biofilm were more tolerant to the effects of antimicrobial agents (HARRISON *et al*., 2005, 2006; TEITZEL *et al*., 2003; ELIAS and BANIN, 2011). Numerous studies have examined tolerance of microbial biofilms and planktonic bacterial cultures on the presence of heavy metals (TEITZEL *et al*., 2003; HARRISON *et al*., 2005). The results obtained in these studies proved microbial biofilm to be two to 600 times more tolerant to the heavy metal influence.

Since the previous studies have reported a high heavy metal tolerance of yeast (HARRISON *et al*., 2006) and bacterial biofilms (TEITZEL *et al*., 2003; HARRISON *et al*., 2005), the aim of our study was to investigate lead and mercury (Pb^{2+} and Hg^{2+}) tolerance of a mixed biofilm consisting of the *Escherichia coli* LM1 and the *Rhodotorula mucilaginosa* strains isolates from the environment.The heavy metal tolerance of different *E. coli* strains (*E. coli* HM22, *E. coli* HM21 and *E. coli* JM109) is already known (HARRISON *et al*., 2005). However, the previous studies on the heavy metal impact on *Rhodotorula* species were conducted only on the solitary planktonic cells (SALIES *et al*., 2000; LI *et al*., 2008). Because of that, we decided to examine the impact of heavy metals on *R. mucilaginosa* biofilm.

Studies on the mixed-species biofilms are scarce and based on testing the impact of antibiotics (clinical isolates, infective agents) (ADAM *et al*., 2002; AL-FATTAN and DOUGLAS, 2006). To the authors' knowledge, only one study on the effect of heavy metals on the mixed bacterial biofilm was published (GOLBY *et al*., 2014). For this reason, the aim of our study was to examine heavy metal tolerance of the mixed-species biofilm (yeast and bacteria) and compare the effect with the single-species biofilms.

MATERIALS AND METHODS

Microorganisms and growth conditions

Two species of microorganisms isolated from environment were used in this study - the yeast *R. mucilaginosa* and the bacteria *E. coli* LM1.The *E. coli* LM1 strain is a gift from the Institute for Public Health, Kragujevac, Serbia. The *R. mucilaginosa* strain was identified by the test for rapid identification of yeast API 20 C AUX (Biomerieux, France). Based on the available literature Tryptic Soy Broth (TSB, Difco) was chosen as the growth medium for the both strains (ADAM *et al*., 2002).

Biofilms formation

Tested biofilms (*R. mucilaginosa, E. coli* LM1 and *R. mucilaginosa / E.coli*) were formed in polystyrene microtiter 96 well plates (Sarstedt, Germany) according to the method described by ALMEIDA *et al*. (2011) with certain modifications. The 100 µl of suspension (OD_{520}=0.8) was added in every well of the plate. To form the mixed *R. mucilaginosa / E. coli* biofilm, an equal amount of suspension was mixed immediately before use.

Preparation of metal solutions

Metal tolerance of single- and mixed-species biofilms was tested in presence of two metal ions Pb^{+} i Hg^{2+}originating from the $Pb(NO_3)_2$ and $HgCl_2$ salts (Sigma-Aldrich, St.

Louis, MO, USA). Stock solutions were filtered using the 0.22 mm syringe filter in glass vials and stored in the fridge. Work solutions were prepared in TSB medium from stock solutions, no more than 60 min before use. Since the biofilm of any *Rhodotorula* species was not tested, range in which heavy metals effect the *R. mucilaginosa* biofilm was unknown. For this reason, a number of different concentrations were tested, and the range was selected, in which the lowest concentration does not lead to a significant response (compared to control) and the highest concentration causes a 100% test response of the organism. The effect range of lead (Pb) and mercury (Hg) concentrations is shown in Table 1. Standard antibiotics amphotericin B and tetracycline were used as a control to verify the susceptibility of the *R. mucilaginosa*and *E. coli* LM1strains isolated from the environment.

Table 1. Range of concentrations (µg/ml) of tested substances

Column numbers	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Pb	2 000	4 000	8 000	16 000	32 000	64 000	128 000
Hg	7.81	15.62	31.25	62.5	125	250	500
Amphotericin B	7.81	15.62	31.25	62.5	125	250	500
Tetracycline	7.81	15.62	31.25	62.5	125	250	500

Heavy metal tolerance of tested biofilms

After the incubation period of 48h, the tested biofilms (*R. mucilaginosa*, *E.coli* LM1 and *R. Mucilaginosa / E.coli*) were treated with heavy metals and antibiotics. First, the contents of the plate (where the biofilms were formed) were removed. In the each well of the plate 100 µl of fresh TSB medium was added and the front wells were treated with 100 µl of metal ions and antibiotics in separate wells. Using eight-channel pipette a series of double dilution was made (Table 1). The microtiter plates were placed in an incubator at 26°C. After 24h, 48h and 72h quantification was performed using CV (crystal violet) assay according to the method described by ALMEIDA *et al*. (2011) with certain modifications. Content from the plates was removed after 24h, 48h and 72h, and 50 µl of methanol 98% (vol/vol) was added. After 15 minutes the methanol was removed and the plates were allowed to dry at room temperature. Furthemore, 50 µl of crystal violet (CV) was added to each well. After 5 min the plates were washed three times with sterile distilled water and stored at room temperature to dry. 100 µl of 33% (vol/vol) glacial acetic acid was added to each well of the plate. Following, the optical density (OD) was measured at 570 nm using a microplate reader (Rayito, China). All the tests were performed in triplicates and their mean value was calculated. Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) were determined. The minimal inhibitory concentration (MIC) is the lowest concentration of an antibiotics or metal ions that will inhibit the visible growth of biofilm populations. The minimal lethal concentration (MLC) is defined as the concentration of an antimicrobial agent that kills 95-100% of biofilm populations (HARRISON *et al*., 2005).

Fluorescence Microscopy

Fluorescence microscopy was used to examine the influence of heavy metals on tested biofilms according to the method described by KRONWALL and MYHRE (1977) with certain

modifications. Tested biofilms were 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).

The content of the microtiter plate was removed. In each well of a microtiter plate 50 µl of methanol was added in order to perform fixation of the biofilm to the walls of the plate. Thus prepared microtiter plate was incubated at room temperature to vapors of the methanol. After incubation, 50 µl of acridine orange stain (5 mg/ml) was added in microtiter plate. After 2 min the microtiter plate was washed with sterile distilled water. Tested biofilms were then observed using a fluorescent microscope.

RESULTS

Biofilm formation after 24h, 48h and 72h

In this study we tested ability *R. mucilaginosa* and *E. coli* LM1 to form single- and mixed-species biofilm in 96-well microtiter plates. The formation of single- and mixed-species biofilm was tested using crystal violet. The results are shown in Figure 1.

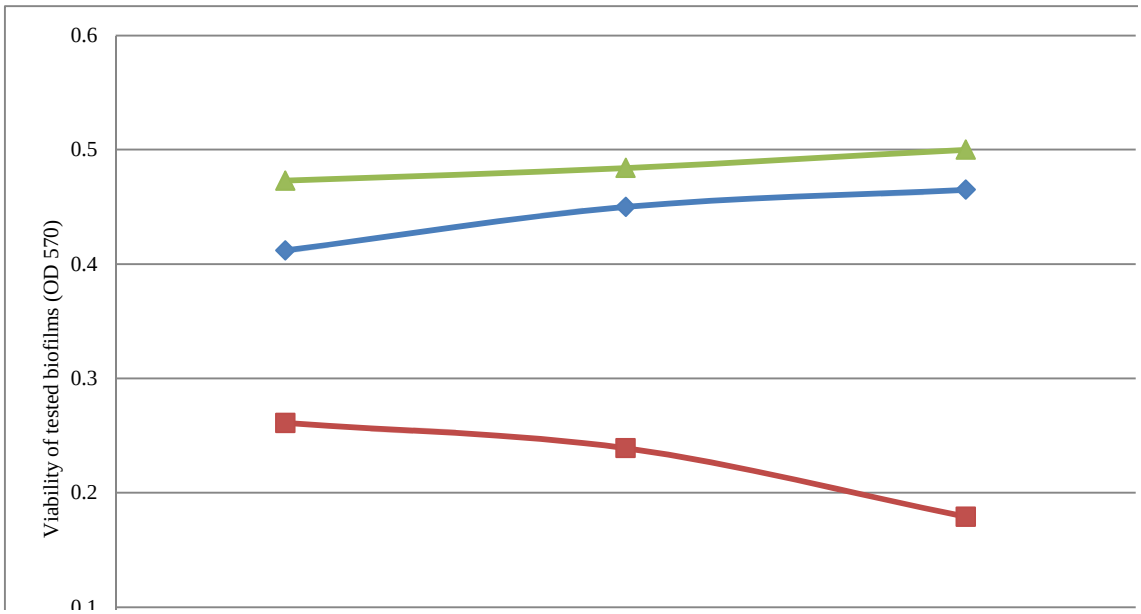

Figure 1. Growth control of *R. mucilaginosa*, *E. coli* LM1
and *R. mucilaginosa / E. coli* biofilms during 24h, 48h and 72h.

Figure 1. shows growth control of the *R. mucilaginosa*, *E.coli* LM1 and *R. mucilaginosa / E. coli* biofilms formed in 96 well microtiter plate during 24h, 48h and 72h period. The growth control of the mixed-species biofilm was better compared to the single-species biofilms.

Heavy metal tolerance of tested biofilms

The MIC and the MLC of single- and mixed-species biofilm were determined. MIC for single-species biofilm *E. coli* LM1 was determined after 24h and MLC after 48h. MIC for single-species biofilm *R. mucilaginosa* and mixed-species biofilm were determined after 48h and MLC after 72h. The results were shown in Table 2.

The obtained results were shown a significant difference in lead tolerance between the mixed- and the single-species biofilms. There was no difference in mercury tolerance between the mixed-species biofilm and the *R. mucilaginosa* biofilm**.**

Table 2. Heavy metal tolerance of single- and mixed-species biofilm

Tested substance	Single biofilm				Mixed biofilm	
	Rhodotorula sp.		*E. coli* LM1		*Rhodotorula*/*E. coli*	
	MIC*	MLC**	MIC	MLC	MIC	MLC
Pb	4000	64 000	4000	16 000	16 000	32 000
Hg	250	500	31.25	62.5	250	500
Amphotericin B	7.81	62.5	7.81	31.25	250	500
Tetracycline	62.5	125	15.25	62.5	500	500

*MIC-minimal inhibitory concentration; **MLC- minimal lethal concentration. Values in the table are in µg/ml.

Fluorescence Microscopy

The impact of heavy metals and antibiotics amphotericin B (pictures on the figures marked with A) and tetracycline (marked with a T) on the test biofilms was monitored for 24h, 48h and 72h. The results are shown in Figures 2-10. Numbers from 1 to 7 are marking a range of concentrations (Table 1).

Considering that the impact of heavy metals was monitored during the various incubation periods, it was noticed that the heavy metal tolerance of the biofilms decreased with time.

DISCUSSION

Heavy metal tolerance of tested biofilms

Heavy metal tolerance of single- and mixed-species biofilm were tested in our study. The MIC and MLC for tested biofilms were determined. The MLC of Hg and Pb for the *R. mucilaginosa* biofilm was observed at concentrations of 500 µg/ml and 64 000 µg/ml, respectively. HARRISON *et al*. (2006) reported that a *Candida tropicalis* biofilm was more tolerant on the presence of heavy metals compared to the planktonic cells of this species. In their study the MLC_{100} for the *C. tropicalis*biofilm in the presence of Hg and Pb was observed at concentrations of 515 µg/ml (1.9 mM) and >20 728 biofilm µg/ml (>77 mM), respectively. The results of our study were in accordance with the MLC results of previous study, especially for the Hg test. The obtained MIC results for Pb were partially in accordance with the mentioned study, since the authors did not determine the MLC of Pb.

HARRISON *et al*. (2005) examined the effect of chromium (CrO^{2-}_4), arsenate (AsO^{3-}_4), arsenite (AsO^{-}_2), selenite (SeO^{2-}_3), telluride (TeO^{2-}_4) and tellurite (TeO^{2-}_3) on the biofilm and planktonic cells of *E. coli* JM109. The *E. coli* JM109 biofilm exhibited high tolerance to the presence of tested metal anions which was in accordance with our results obtained for the *E. coli* LM1 biofilm (Table 2).

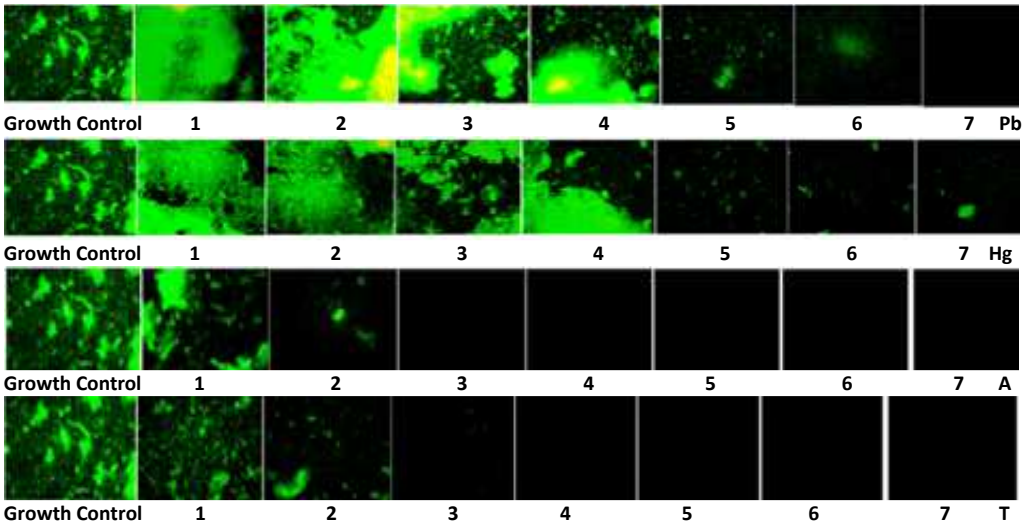

Figure 2. Effects of tested substances on the *E. coli* LM1 biofilm after 24h.

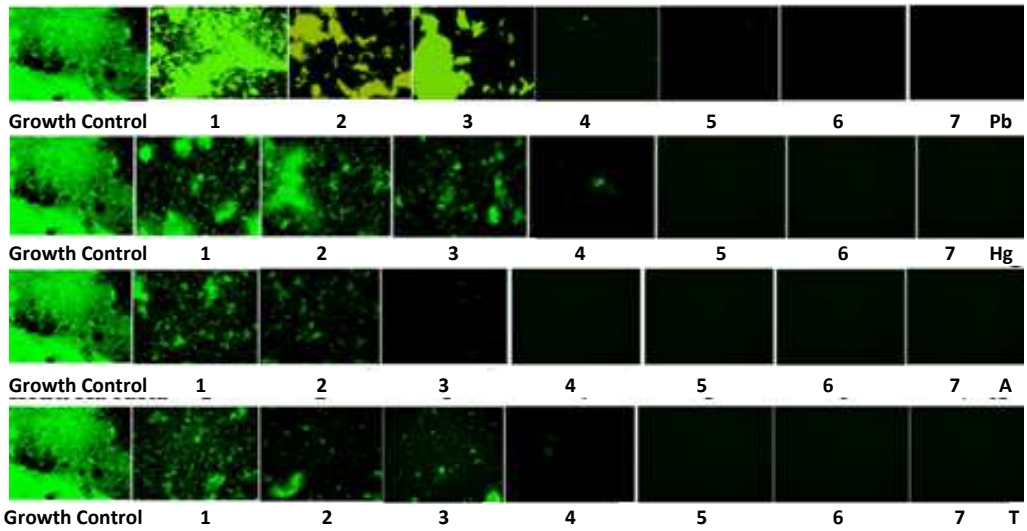

Figure 3. Effects of tested substances on the *E. coli* LM1 biofilm after 48h.

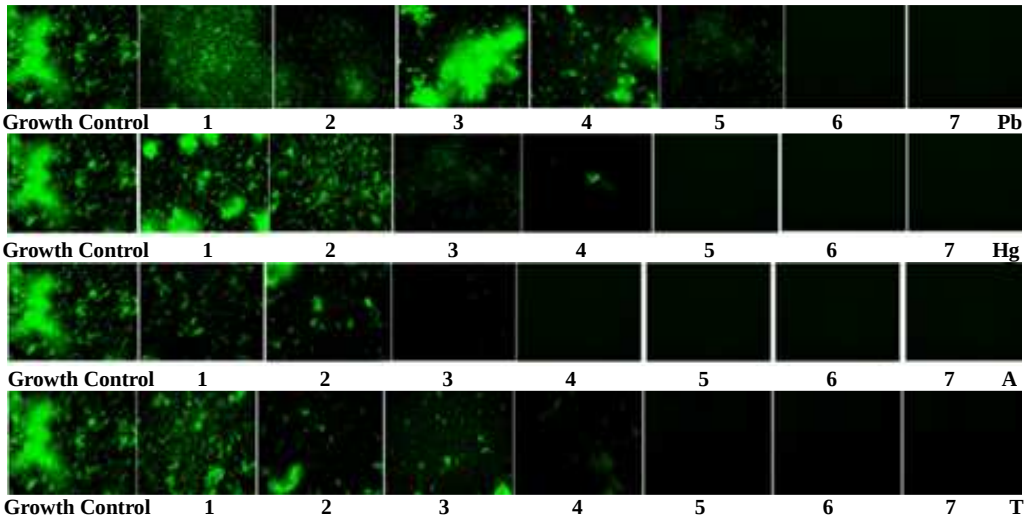

Figure 4. Effects of tested substances on the *E. coli* LM1 biofilm after 72h.

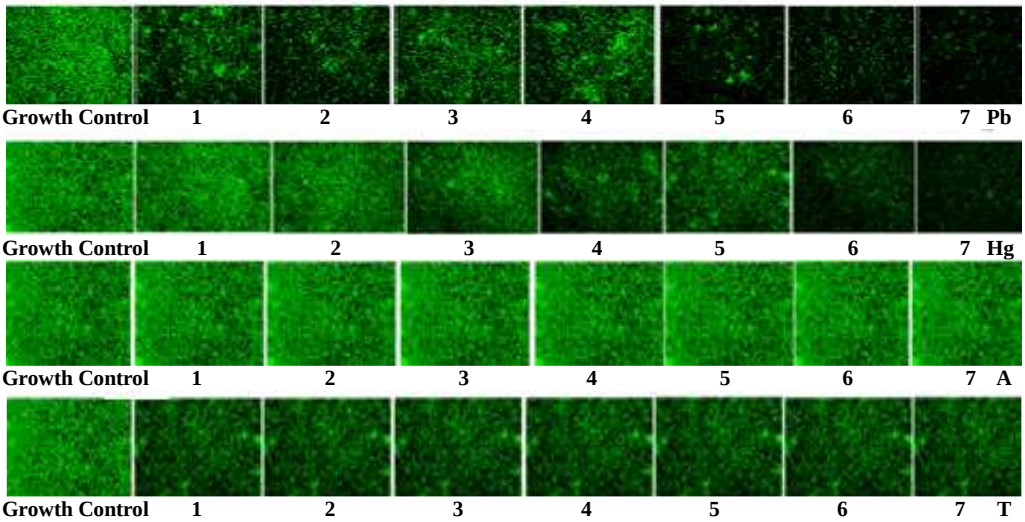

Figure 5. Effects of tested substances on the *R. mucilaginosa* biofilm after 24h.

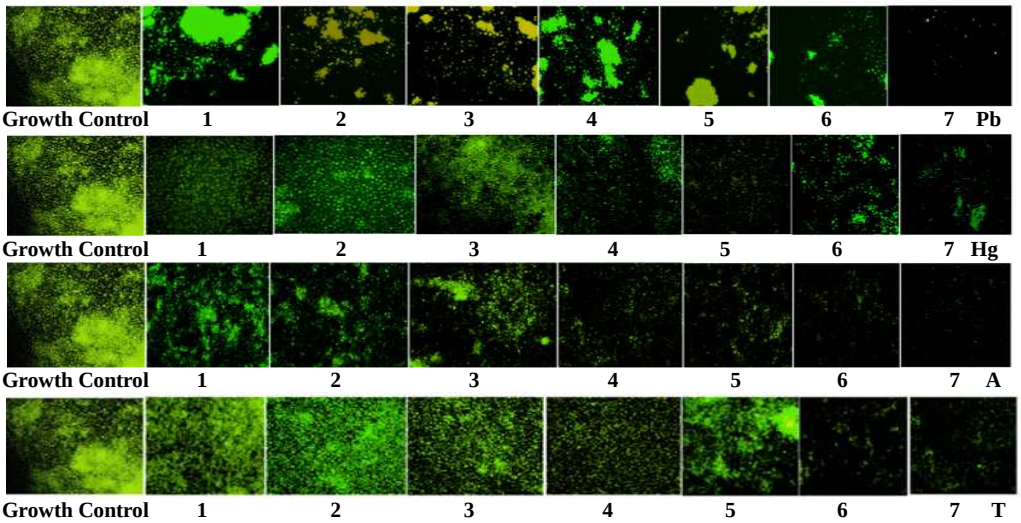

Figure 6. Effects of tested substances on the *R. mucilaginosa* biofilm after 48h.

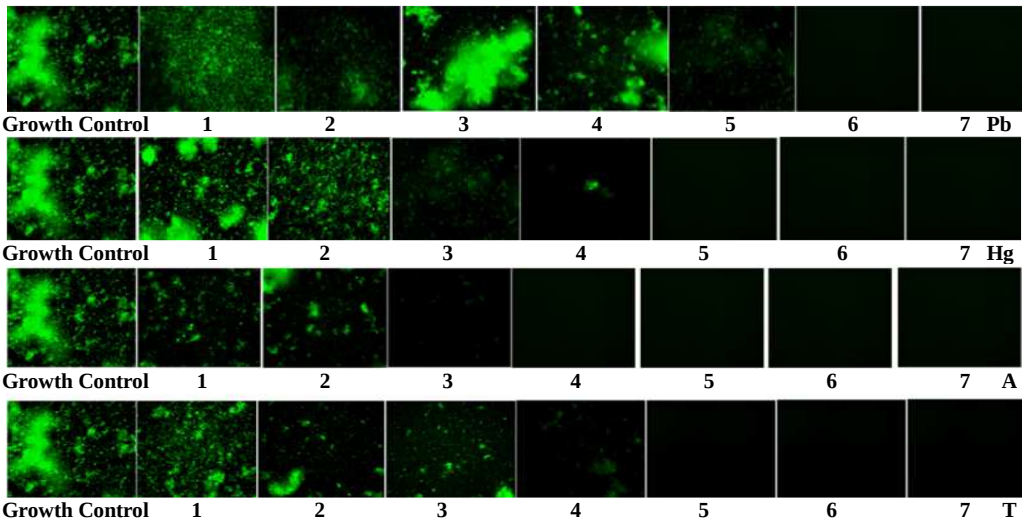

Figure 7. Effects of tested substances on the *R.mucilaginosa* biofilm after 72.

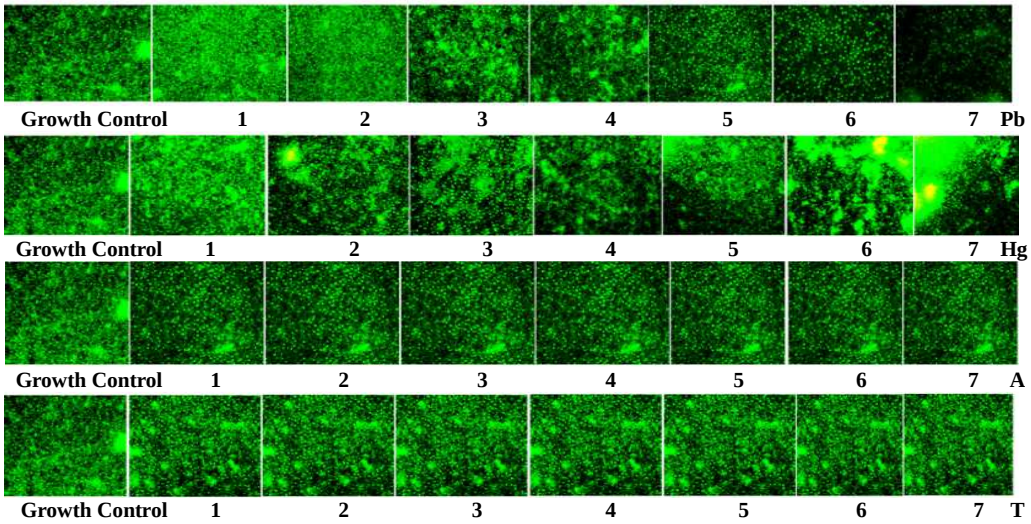

Figure 8. Effects of tested substances on the mixed-species biofilm after 24 h.

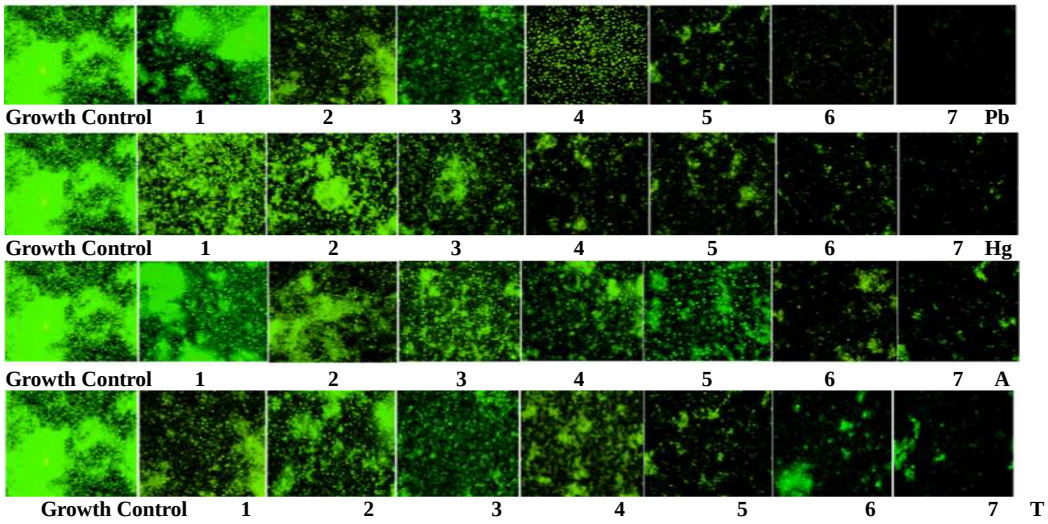

Figure 9. Effects of tested substances on the mixed-species biofilm after 48h.

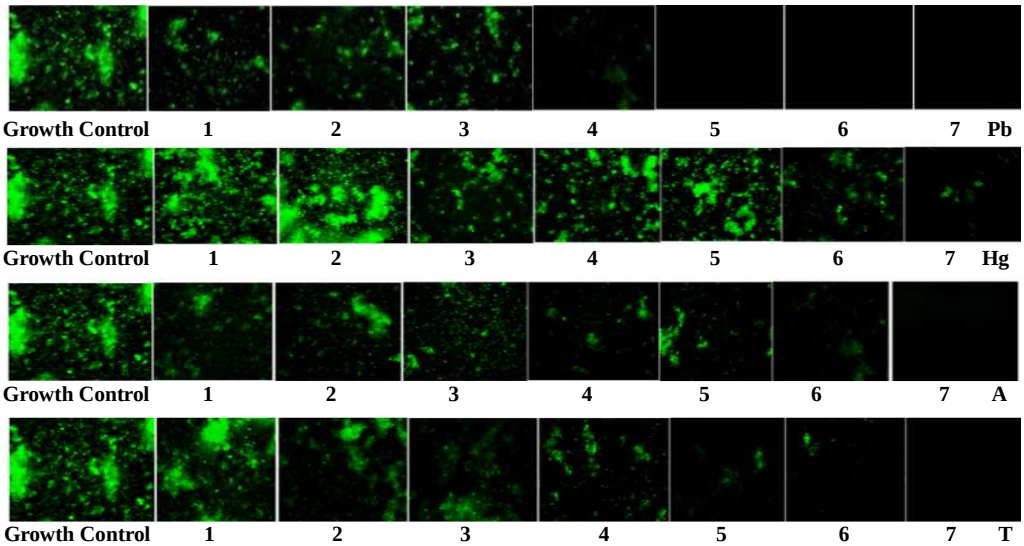

Figure 10. Effects of tested substances on the mixed-species biofilm after 72h.

The mixed-species biofilm showed to be more tolerant to antimicrobial treatment in comparison with single-species biofilms (LERICHE *et al*., 2003). The results of our study showed that heavy metal tolerance of mixed-species biofilm was higher compared to the single-species biofilms, which is in accordance with the results reported in mentioned study. The largest difference in lead tolerance was observed between the mixed and the single species biofilms. The MIC of Pb^{2+} for the examined biofilms (*E. coli* LM1, *R. mucilaginosa* and *R. mucilaginosa / E. coli*) was recorded at concentrations of 4000 µg/ml, 4000 µg/ml and 16000 µg/ml, respectively. The MIC of Hg^{2+} for the biofilms was noticed at concentrations of 31.25 µg/ml, 250 µg/ml, 250 µg/ml, respectively.

Heavy metal tolerance of mixed-species biofilms was also examined by GOLBY *et al*., (2014). The biofilm used in this study was isolated from the sludge tailings in North Alberta (Canada) and its tolerance on the presence of metal ions including Cu, Ag, Pb, Ni, Zn, V, Cr, and Sr was tested. The obtained results showed that the mixed bacterial biofilm was extremely resistant to the applied metal ions. The reported tolerance values were as follows; over 20 mg/l for Pb, 16 mg/l for Zn, 1000 mg/l for Sr, and 3.2 mg/l for Ni. In our study, the MIC of Pb for the mixed-species biofilm was observed at 16 000 µg/ml. In the study of GOLBY *et al*. (2014) mixed bacterial biofilm showed resistance to the effect of Pb in concentration over 20 mg/l, which is partially in accordance with the results of our study.

Furthermore, ADAM *et al*. (2002) examined the effect of antibiotics on mixed-species biofilm consisting of yeast *Candida albicans*and bacteria *Staphylococcus epidermidis*. Both species are pathogenic and infection causers. In this study, results showed that bacteria and yeast ensure the survival of each other when forming a mixed biofilm. The results of ADAM *et al*. (2002) study were in accordance with the results of our study where the mixed biofilm was about 65 times more tolerant to the effect of antibiotics compared to single biofilms (Table 2).

CONCLUSION

Heavy metals influence on single- and mixed-species biofilms composed by yeast *R. mucilaginosa* and bacteria *E.coli* LM1 strains, isolated from the environment was examined in this study. The tolerance of the mixed-species biofilm was higher in comparison to the single-species biofilms.The results suggest that mixed-species biofilms could be more effective in the process of bioremediation than single-species biofilms, which opens the possibility for future tests of *R. mucilaginosa / E. coli* biofilm in the remediation of contaminated water.

Acknowledgment

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