EPS production and bioremoval of heavy metals by mixed and pure bacterial cultures isolated from Ankara Stream

Nur Koçberber Kiliç, Güliz Kürkçü, Durna Kumruoğlu and Gönül Dönmez

ABSTRACT

This study is focused on isolation of Ni(II), Cu(II) and Cr(VI) resistant bacteria to assess their exopolysaccharide (EPS) production and related bioremoval capacities. Mixed cultures had higher heavy metal removal capacity in media with molasses (MAS) than the control cultures lacking this carbon (AS) containing 50 mg/l of heavy metal. The yields were 32%, 75.7%, and 51.1% in MAS, while the corresponding values were 29%, 55.1%, and 34.5% in AS, respectively. Purification of the strains 1, 5 and 6 present in the mixed cultures decreased the bioremoval capacities of the mixed culture samples, although these strains produced higher EPS amounts in MAS agar. Strain 5 had the highest Cu(II) (69.1%) and Cr(VI) (43.1%) removal rates at 25 mg/l initial concentration of each pollutant with EPS amounts of 0.74 g/l and 1.05 g/l, respectively. This strain was identified as *Stenotrophomonas maltophilia*. The presented data show that especially mixed and also pure cultures of bacterial strains isolated from Ankara Stream could be assessed as potential bioremoval agents in the treatment of Cu(II) or Cr(VI) containing wastewaters.

Key words | bacteria, bioremediation, exopolysaccharide, heavy metal

Nur Koçberber Kiliç (corresponding author)
Güliz Kürkçü
Durna Kumruoğlu
Gönül Dönmez
Faculty of Science, Department of Biology,
Ankara University,
Beşevler, Ankara 06100,
Turkey
E-mail: *nrkilic@ankara.edu.tr*

INTRODUCTION

Heavy metals like Cr(VI), Ni(II) and Cu(II) have many fields of use, however they have toxic effects on organisms like causing allergy, inhibition of metabolism, cancer, etc. (Nies 1999). Wastewaters contaminated with toxic heavy metals can diffuse into the ground waters, spread rapidly into the environment and can enter into the food chain, as well (Al-Jaboobi *et al.* 2014). Fortunately there are numerous chemical and biological treatment methods available offering potential in removing these pollutants (Fu & Wang 2011). Biological treatment methods are preferred for their efficiency, safety, economy, and easiness (Juwarkar *et al.* 2010).

In previous studies on the use of different microorganisms in such treatments, exopolysaccharide (EPS)-producing heavy metal-resistant microorganisms have been often used. (Kılıç & Dönmez 2008; Perez *et al.* 2008). Presence of ionizable functional groups like carboxyl, phosphoric, amine, and hydroxyl groups in EPSs offers an important function to them, acting as a protective barrier against toxic heavy metals (Mikutta *et al.* 2012; Bi *et al.* 2013; Chien *et al.* 2013). As a matter of fact, it was previously shown that *Pseudomonas aeruginosa, Micrococcus* sp. and *Ochrobactrum* sp. developed a Cr(VI) toxicity response by producing EPS (Kılıç & Dönmez 2008). Perez *et al.* (2008) performed a study on the role of EPS of *Paenibacillus jamilae* in the bioremediation efficiency and showed its Pb(II), Cd(II), Co(II), Ni(II), Zn(II) and Cu(II) chelation capacity. In another study carried out by Mikutta *et al.* (2012), they showed that the EPS produced by *Bacillus subtilis* had potential to remove Pb(II), Cu(II), Zn(II), and it had a higher capacity for Pb(II) than the others. Interrelationship in EPS production and heavy metal (Cd (II) and Zn(II)) removal efficiency performed by *Pseudomonas* sp. was also investigated and shown (Chien *et al.* 2013).

In this study, the aim was to obtain a heavy metal resistant mixed bacterial culture from Ankara Stream, to isolate Ni(II), Cu(II) and Cr(VI) resistant pure bacteria from these mixed cultures, to investigate heavy metal removal capacity of the mixed and pure cultures and to investigate the capacity of EPS production and heavy metal removal capacity of the cultures. To the best our knowledge, this is the first report about usage of these bacterial cultures to remove heavy metals and also EPS production of them.

doi: 10.2166/wst.2015.365

MATERIAL AND METHODS

Isolation of mixed bacterial cultures

Mixed bacterial cultures were obtained by culturing the samples taken from Ankara Stream (Ankara, Turkey). They were cultivated in activated sludge medium (AS) containing approximately 25 mg/l Ni(II), Cu(II) and Cr(VI). Composition of AS medium was 1.0 g glucose, 0.12 g Na_2SO_4, 0.4 g KH_2PO_4, 0.15 g $MgSO_4.7H_2O$, 0.005 g $FeSO_4.7H_2O$, 0.042 g NH_4Cl, 0.00021 g $ZnCl_2$, 0.05 g $CaCl_2.2H_2O$, 0.00008 g $NiCl.6H_2O$, 0.017 g beef extract, 0.0001 g H_3BO_3, 0.4 g CH_3COONa in 1 l (Dönmez & Koçberber 2005). Molasses (4% (v/v)) were included in groups of samples labeled as MAS in the series of experiments. MAS medium was prepared by replacing glucose with the 4% (v/v) molasses, which was used as a carbon source for its high reducing power, low cost, availability and ease of storage. Molasses solution is approximately equivalent to 10 g/L sucrose (Dönmez & Koçberber 2005). Microbial inoculation (1 ml) was the same in both media, however carbon source concentrations were 0.1% (w/v) in media including glucose; 0.4% (w/v) sucrose in media with molasses. Heavy metal stock solutions were prepared by dilution of each heavy metal salt solution to a final concentration of 10 g/l metal ion. A total of 1 ml of AS or MAS samples was inoculated onto 20 ml of the media containing one of the heavy metal solutions and were incubated in 50 ml Erlenmeyer flasks at 30 °C on a rotary shaker at 100 rpm for five days.

Heavy metal bioremoval in AS and MAS medium

This series of experiments was performed by using six different media. In the first series, AS media contained 50 mg/l Ni(II), 49.7 mg/l Cu(II) and 56.3 mg/l Cr(VI). In the second MAS media with 41.8 mg/l Ni(II), 47.3 mg/l Cu(II), 55 mg/l Cr(VI) was used. A total of 1 ml of mixed bacterial culture was inoculated into 100 ml AS and MAS media in 250 ml Erlenmeyer flasks and were incubated at 30 °C on a rotary shaker at 100 rpm for five days. At the end of these trials, the optimum medium for heavy metal bioremoval by mixed bacterial culture was determined. Control Erlenmeyer flasks were prepared by adding heavy metals without inoculation, in order to observe the occurrence of any reactions between media and each of heavy metals. All the trials were done in triplicate.

Heavy metal-resistant pure bacterial cultures

A 0.1 ml aliquot of the broth of each growing mixed bacterial culture samples were spread on three different MAS agar [1.5% (w/v)] plates containing 25 mg/l Ni(II), Cu(II), and Cr(VI) at pH 7 and incubated at the same conditions described above for 48 hours. After 48 hours purified colonies were taken from these three different media and transferred onto agar slants; they were kept at 4 °C and were transferred every three months.

The pure cultures tolerating high concentration of each of the heavy metal selected and experiments were done with three different MAS medium including 29.4 mg/l Ni(II), 23.6 mg/l Cu(II), and 25.2 mg/l Cr(VI). Pure cultures were inoculated onto 100 ml of each medium (pH 7) in 250 ml Erlenmeyer flasks at 30 °C on a rotary shaker (100 rpm) for an incubation period of five days.

Identification of the heavy metal-resistant bacteria

Whole cells from the exponentially growing culture of the isolate having the highest heavy metal removal capacity (strain 5) were used for 16S rRNA gene amplification (Lane 1991). Sequencing and phylogenetic analysis was carried out as described elsewhere (Kong *et al.* 2005).

pH effect on bioremoval of heavy metals

These trials were carried out with pure bacterial cultures which succeeded in removing three different heavy metals. To determine the optimum pH level for the highest heavy metal bioremoval, the pH of eight different MAS media containing approximately 25 mg/l of each of the heavy metals [Cu(II) and Cr(VI)] were prepared. Their pH values were adjusted to 6, 7, 8, and 9. The summary of experimental design is shown in Table 1.

EPS production by pure bacterial cultures

In EPS production experiments, pure cultures removing the highest concentration of each heavy metal were inoculated onto MAS agar media. Experiments were done with two different MAS agars with 25 mg/l of each heavy metal at predetermined pH value. The Petri dishes were incubated for 48 hours at 30 °C in a Memmert model incubator (Germany).

Table 1 | Summary of the experimental design

Heavy metal removal with mixed cultures of bacteria		
Medium C_o (mg/l)	**AS**	**MAS**
Ni(II)	50.0	41.8
Cu(II)	49.7	47.3
Cr(VI)	56.3	55.0
Heavy metal removal with pure cultures of bacteria in MAS		
C_o (mg/l)		
Ni(II)	29.4	
Cu(II)	23.6	
Cr(VI)	25.2	
pH effect on removal by strain1, strain 5, and strain 6		
pH	C_o-Cu(II) (mg/l)	C_o-Cr(VI) (mg/l)
6	24.9	24.9
7	23.6	25.2
8	20.5	20.8
9	23.5	25.7

Analytical methods

Heavy metal bioremoval

During the incubation period, 3 ml samples were taken from each flask and were centrifuged to precipitate suspended biomass at 3,421 × *g* for five minutes. The concentration of heavy metals in the supernatant was determined spectrophotometrically at 340 nm for Ni(II), 540 nm for Cr(VI), and 460 nm for Cu(II). Absorbance measurements and centrifugation were performed using a Shimadzu UV 2001 model spectrophotometer (Japan) and Hettich EBA12 model centrifuge (Germany).

Cr(VI) bioremoval experiments were done by using diphenyl carbazide reagent in acid solution as the complexing agent for Cr(VI). Cu(II) and Ni(II) bioremoval experiments were performed by using sodium diethyldithiocarbamate reagent as the complexing agent (Snell & Snell 1959).

Exopolysaccharide (EPS) extraction

EPS isolation was carried out as described by Cérantola *et al.* (2000) with minor modifications. To isolate EPS from the cultivated cultures, colonies were picked up from the MAS agar surface with a glass rod and suspended in an osmolar solution. The suspensions were boiled at 100 °C for 15 minutes. After cooling, trichloroacetic acid (TCA) was added to a final concentration of 4% (w/v) to precipitate proteins. After centrifugation at 10,000 × g for 30 minutes at 4 °C, the supernatant was removed and the precipitate was mixed with cold ethanol. The suspension was centrifuged at 10,000 × g for 30 minutes at 4 °C. The extraction was repeated once. The pellet was removed and dissolved in distilled water. The sugar content of EPS was determined using phenol–H_2SO_4 reagent and glucose standard solutions (Dubois *et al.* 1956).

RESULTS AND DISCUSSION

Heavy metal bioremoval by mixed bacterial culture in different media

The effect of media content on bioremoval efficiency of the mixed culture under nearly 50 mg/l heavy metal is shown in Figure 1. It is clearly seen that molasses had a positive effect on bioremoval. The comparison of Ni(II), Cu(II) and Cr(VI) bioremoval rates in AS and MAS showed that all of the rates found were higher in MAS. Mixed culture removed the applied Ni(II) with a yield of 29% in AS, which was slightly lower than 32% yield in MAS. In AS media with Cu(II), 55.1% of the metal was removed, which was significantly lower than 75.7% removal in MAS. In AS media including Cr(VI), the efficiency of the mixed culture was 34.5%, which was much lower than 51.1% yield found in MAS. Considering the results obtained from these experiments, further tests were carried out in MAS media.

Taking the well-known fact of stimulation of microbial growth by addition of carbon sources into the media into consideration, molasses were added as a supplementary carbon

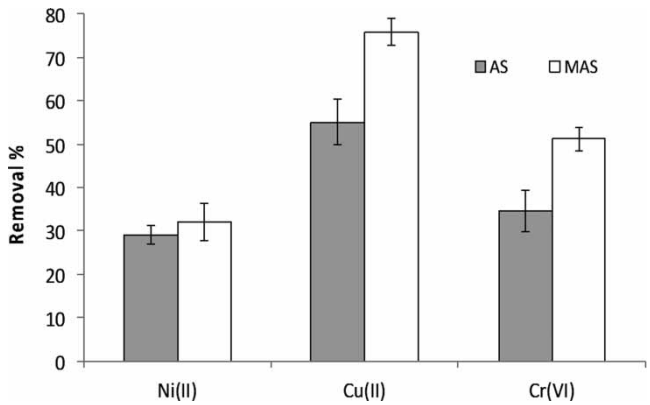

Figure 1 | The effect of different media on bioremoval of Ni(II), Cu(II) and Cr(VI) (C_o for AS media: Ni(II): 50.0 mg/l, Cu(II): 49.7 mg/l, Cr(VI): 56.3 mg/l; C_o for MAS media: Ni(II): 41.8 mg/l, Cu(II): 47.3 mg/l, Cr(VI): 55.0 mg/l; pH: 7; incubation period: 5 days; *T*: 30 °C).

source to increase growth and heavy metal removal. Molasses had approximately equivalent to 10 g/l sucrose (Dönmez & Koçberber 2005). In AS, carbon source concentration was 0.1% (w/v) glucose, while it was 0.4% (w/v) sucrose in MAS. Although inoculation of microbial biomass was the same (1 ml) in both media, we found that by adding molasses into AS media, the microorganisms increased removal of the subject heavy metals. It was pointed out in a previous study out that in media with higher available carbon sources, EPS production was increased (Lee *et al.* 2007). This is in accordance with our results, which leads to the conclusion claiming that metal chelation was improved by the higher EPS production.

Heavy metal-resistant pure bacterial cultures

Six different colony morphotypes were seen on molasses agar plates with 25 mg/l of each metal incubated with the samples taken from the three enriched activated sludge cultures, namely strain 1, strain 2 and strain 3 which were obtained from MAS agar media with Ni(II). Strain 4 and strain 5 were taken from Cu(II) containing MAS agar media. Finally strain 6 was purified from Cr(VI) added MAS agar media.

To select the strain having the highest heavy metal removal capacity, all of the strains were inoculated into three different MAS media with approximately 25 mg/l Ni(II), Cu(II) or Cr(VI). All of the strains could not tolerate Ni(II) and remove it. Strains 1, 5 and 6 had the highest capacity in removing Cu(II) or Cr(VI). Although strain 3 removed Cr(VI) with a yield of 21.4%, it could not tolerate the other heavy metals. The most efficient microorganism in removing Cu(II) or Cr(VI) was strain 5. Cu(II) removal efficiency of strain 5 was 69.1%, while it was 43.1% for Cr(VI). At the end of these trials, strains 1, 5 and 6 were tested for their EPS production under the stress of the heavy metals.

The most efficient bacterium (strain 5) in Cu(II) and Cr(VI) bioremoval was purified and examined under the microscope after Gram staining using standard methods. It was shown that the strain was Gram-negative and rod-shaped. The strain was identified by amplification and sequencing of its 16S rRNA gene. Phylogenetic analysis of the nearly complete sequence data were done by BLAST search. Alignment and further analysis in ARB database revealed that the bacterium had a >99% similarity to *S. maltophilia* (NCBI GenBank Accession number: KT221539).

Effect of pH on bioremoval of the heavy metals

The effect of pH on bioremoval of Cu(II) and Cr(VI) by strains 1, 5 and 6 in media with nearly 25 mg/L of each metal is shown in Figure 2.

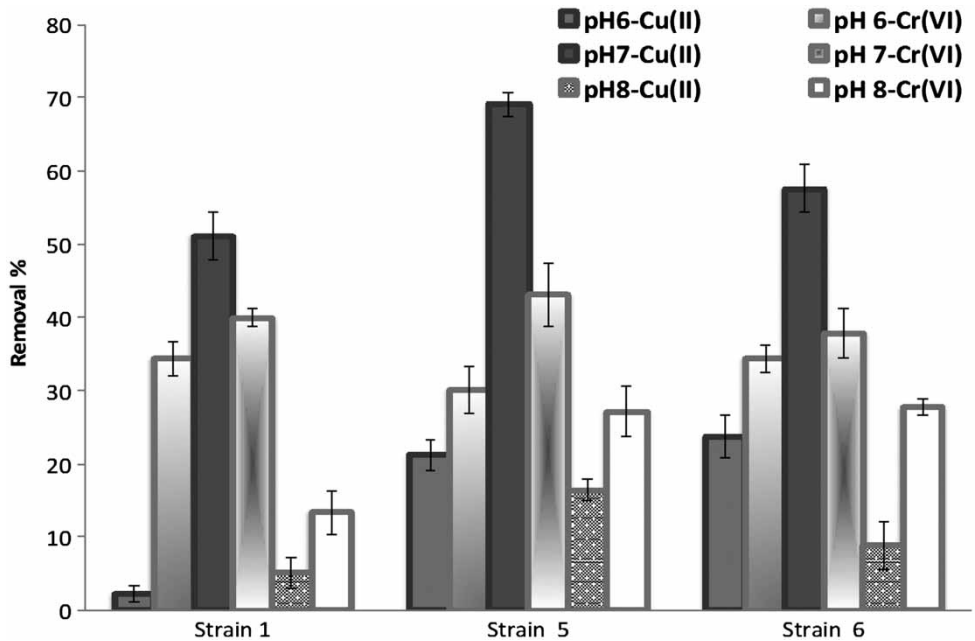

Figure 2 | Effect of different pH levels on bioremoval of Cu(II) and Cr(VI) by strains 1, 5 and 6 in molasses media with approximately 25 mg/l of each metal (incubation period: 5 days; *T*: 30 °C).

Strain 1 was capable of removing Cu(II) with the highest yield of 51.1% at pH 7, but it could remove Cu(II) with yields of 2.2% and 5.1% at pH 6 and pH 8, respectively. At pH 9, strain 1 could not grow. At pH 6, strain 1 removed the applied Cr(VI) with 34.4% efficiency. The highest yield of Cr(VI) removal was found at pH 7 as 39.9%; at pH 8, the capacity was 13.4%.

At pH 6, strain 5 removed Cu(II) with a yield of 21.2%. At pH 7, strain 5 removed Cu(II) with the highest yield of 69.1%. At pH 8, removal yield was 16.4% and at pH 9 strain 5 could not grow at this alkaline media. Chromium (VI) bioremoval efficiency of strain 5 at pH 6 was 30.1%, at pH 7 it was 43.1%. Strain 5 had efficiency of Cr(VI) removal of 27.2% at pH 8.

Strain 6 did also not tolerate pH 9. However, it showed its potential to remove Cu(II) at pH 7 with its highest yield of 57.6%. Strain 6 removed Cu(II) with yields of 23.7% and 8.9% at pH 6 and pH 8, respectively. Removal capacity of strain 6 was 34.4% at pH 6; 37.8% at pH 7 and 27.7% at pH 8 for Cr(VI) samples.

According to the presented results strain 5 was the most resistant against Cu(II) and Cr(VI) with the highest efficiency at pH 7. As a matter of fact, it was also previously reported that optimum pH for Cu(II), Ni(II) or Cr(VI) bioremoval by four *Bacillus* species was 7 (Al-Daghistani 2012). In another study on Ni(II) removal it was reported recently that it was removed by microorganisms at a pH range of 7–9 (Das *et al.* 2014b). The optimum pH of removing Cu(II) ions from MAS media was also found to be pH 7 in the current study, however, it was found to be 6 in a previous study on environmental isolates (Fan *et al.* 2014). Efficient bioremoval of Cr(VI) at pH 7 was also reported by other researchers (Das *et al.* 2014a; Fan *et al.* 2014).

EPS production

EPS production capacities found under 25 mg/l heavy metal stress is summarized in Figures 3 and 4. In Figure 3 it is clearly seen that strain 5 produced the highest amount of EPS as 0.74 g/l. Strain 1 produced 0.15 g/l EPS in media with Cu(II), while strain 6 produced 0.23 g/l at the same conditions, and as expected it removed Cu(II) ions with a higher yield than strain 1. EPS production by the three strains under Cr(VI) stress is presented in Figure 4, which showed that strain 5 had the highest EPS amount (1.05 g/l) and also removed the highest concentration. Strains 1 and 6 produced similar amounts of EPS as 0.123 g/l and 0.145 g/l, respectively. However, strain 1 was slightly more efficient in removing the pollutant than strain 6. These experiments showed that strain 5 had a potential in treatment of Cu(II) or Cr(VI) by removing them through producing EPS. Heavy metal bioremoval by EPSs has been reported for Cr(VI), Cu(II) or Ni(II) ions, and it was proved that microorganisms removed the applied pollutants by producing EPSs (Kılıç & Dönmez 2008; Mikutta *et al.* 2012; Chien *et al.* 2013). In our study, it was also clearly observed that strains 1, 5 and 6 used their EPSs to remove Cu(II) or Cr(VI). In the current study, mixed and pure bacterial cultures were used in Ni(II), Cu(II), and Cr(VI) bioremediation, and it was observed that the mixed ones had much higher capacity in bioremediation of these heavy metals. In a previous study performed with mixed

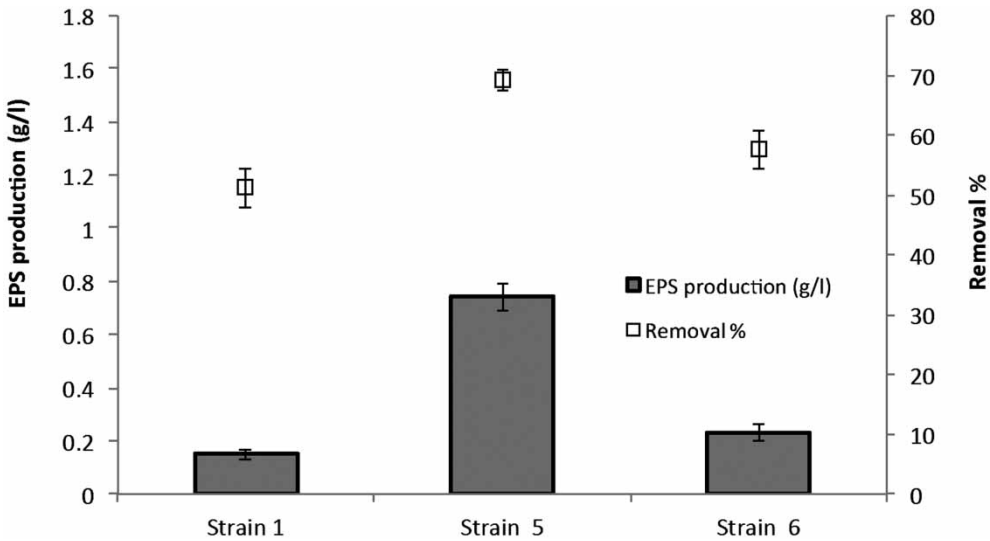

Figure 3 | The relationship between Cu(II) removal and EPS production by three strains in 25 mg/l Cu(II) including MAS media (pH: 7; *T*: 30 °C).

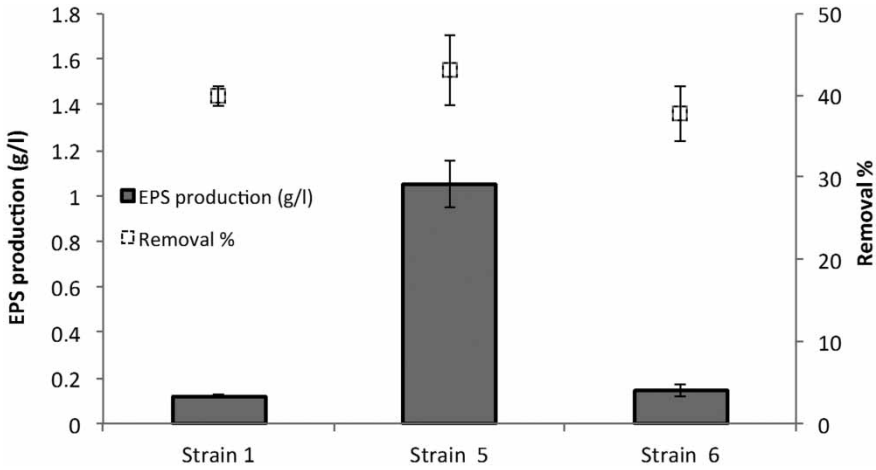

Figure 4 | The relationship between Cr(VI) removal and EPS production by three strains in 25 mg/l Cr(VI) including MAS media (pH: 7; *T*: 30 °C).

and pure cultures of bacteria, authors also showed that mixed cultures were more efficient than pure cultures in removing Cu and As (Nguyen *et al.* 2015).

CONCLUSION

Six heavy metal-resistant strains were purified from Ankara Stream (Turkey). Although none of them could tolerate Ni(II) ions, three strains (strains 1, 5 and 6) produced different amounts of EPS under heavy metal stress and could remove Cu(II) or Cr(VI) efficiently. All of the tested strains had the highest bioremoval capacity in MAS media at pH 7. Among them, strain 5 was the most efficient bacterium in bioremoval process and EPS production under Cu(II) or Cr(VI) exposure. The strain was identified as *S. maltophilia* and had a maximum EPS production as 1.05 g/l under Cr(VI) stress. In parallel to the high EPS production, the bacterium removed the pollutant with the highest yield. Our results demonstrated that as an economical and safe biosorbent, EPSs of strain 5 could be used as an efficient biological agent in bioremoval processes of Cu(II) or Cr(VI).

ACKNOWLEDGEMENTS

Financial support is gratefully acknowledged from the Scientific and Technological Research Council of Turkey (TÜBİTAK and TÜBİTAK-BİDEB 2209-A) and Ankara University research fund.

REFERENCES

Al-Daghistani, H. 2012 Bio-Remediation of Cu, Ni and Cr from rotogravure wastewater using immobilized, dead, and live biomass of indigenous thermophilic *Bacillus* species. *Internet J. Microb.* **10** (1), http://ispub.com/IJMB/10/1/13756.

Al-Jaboobi, M., Zouahri, A., Tijane, M., El Housni, A., Mennane, Z., Yachou, H. & Bouksaim, M. 2014 Evaluation of heavy metals pollution in groundwater, soil and some vegetables irrigated with wastewater in the Skhirat region 'Morocco'. *J. Mater. Environ. Sci.* **5** (3), 961–966.

Bi, X.-D., Zhang, S.-L., Dai, W., Xing, K.-Z. & Yang, F. 2013 Effects of lead(II) on the extracellular polysaccharide (EPS) production and colony formation of cultured *Microcystis aeruginosa*. *Water Sci. Technol.* **67** (4), 803–809.

Cérantola, S., Bounéry, J., Segonds, C., Marty, N. & Montrozie, H. 2000 Exopolysaccharide production by mucoid and non-mucoid strains of *Burkholderia cepacia*. *FEMS Microb. Let.* **185**, 243–246.

Chien, C.-C., Lin, B.-C. & Wu, C.-H. 2013 Biofilm formation and heavy metal resistance by an environmental *Pseudomonas* sp. *Biochem. Eng. J.* **78**, 132–137.

Das, S., Mishra, J., Das, S. K., Pandey, S., Rao, D. S., Chakraborty, A., Sudarshan, M., Das, N. & Thatoi, H. 2014a Investigation on mechanism of Cr(VI) reduction and removal by *Bacillus amyloliquefaciens*, a novel chromate tolerant bacterium isolated from chromite mine soil. *Chemosphere* **96**, 112–121.

Das, P., Sinha, S. & Mukherjee, S. K. 2014b Nickel bioremediation potential of *Bacillus thuringiensis* KUNi1 and some environmental factors in nickel removal. *Biorem. J.* **18**, 169–177.

Dönmez, G. & Koçberber, N. 2005 Isolation of hexavalent chromium resistant bacteria from industrial saline effluents and their ability of bioaccumulation. *Enzyme Microb. Technol.* **36**, 700–705.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. 1956 Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350–356.

Fan, J., Okyay, T. O. & Rodrigues, D. F. 2014 The synergism of temperature, pH and growth phases on heavy metal biosorption by two environmental isolates. *J. Hazard. Mat.* **279**, 236–243.

Fu, F. & Wang, Q. 2011 Removal of heavy metal ions from wastewaters: a review. *J Environ. Manage.* **92** (3), 407–418.

Juwarkar, A. A., Singh, S. K. & Mudhoo, A. 2010 A comprehensive overview of elements in bioremediation. *Rev. Environ. Sci. Bio/Technol.* **9** (3), 215–288.

Kılıç, N. K. & Dönmez, G. 2008 Environmental conditions affecting exopolysaccharide production by *Pseudomonas aeruginosa*, *Micrococcus* sp., and *Ochrobactrum* sp. *J. Hazard. Mat.* **154** (1–3), 1019–1024.

Kong, Y., Nielsen, J. L. & Nielsen, P. H. 2005 Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Appl. Environ. Microbiol.* **71**, 4076–4085.

Lane, D. J. 1991 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics* (E. Stackebrandt & M. Goodfellow, eds). Wiley, New York, pp. 115–175.

Lee, W. Y., Park, Y., Ahn, J. K., Ka, K. H. & Park, S. Y. 2007 Factors influencing the production of endopolysaccharide and exopolysaccharide from *Ganoderma applanatum*. *Enz. Microb. Technol.* **40**, 249–254.

Mikutta, R., Baumgar˙tner, A., Schippers, A., Haumaier, L. & Guggenberger, G. 2012 Extracellular polymeric substances from *Bacillus subtilis* associated with minerals modify the extent and rate of heavy metal sorption. *Environ. Sci. Technol.* **46**, 3866–3873.

Nguyen, V. K., Lee, M. H., Park, J. H. & Lee, J.-U. 2015 Bioleaching of arsenic and heavy metals from mine tailings by pure and mixed cultures of *Acidithiobacillus* spp. *J. Indus. Eng. Chem.* **21**, 451–458.

Nies, D. H. 1999 Microbial heavy-metal resistance. *Appl Microbiol Biotechnol.* **51**, 730–750.

Perez, J. A. M., Garcia-Ribera, R., Quesada, T., Aguilera, M., Ramos-Cormenzana, A. & Monteoliva-Sanchez, M. 2008 Biosorption of heavy metals by the exopolysaccharide produced by *Paenibacillus jamilae*. *W. J. Microbiol. Biotech.* **24**, 2699–2704.

Snell, F. D. & Snell, C. T. 1959 *Colorimetric Methods of Analysis*, vol. 2, 3rd edn. D Van Nostrand Company, New York.

First received 13 February 2015; accepted in revised form 2 July 2015. Available online 15 July 2015