Carbohydrate Polymers 101 (2014) 265–271

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Metal removal of cyanobacterial exopolysaccharides by uronic acid content and monosaccharide composition

Sahlan Ozturk[a,*], Belma Aslim[b], Zekiye Suludere[b], Sema Tan[c]

[a] Nevsehir University, Faculty of Science and Arts, Department of Molecular Biology and Genetics, Nevsehir, Turkey
[b] Gazi University, Faculty of Science, Department of Biology, Ankara, Turkey
[c] Kirikkale University, Faculty of Science and Arts, Department of Biology, Kirikkale, Turkey

ARTICLE INFO

Article history:
Received 28 June 2013
Received in revised form 6 September 2013
Accepted 14 September 2013
Available online 21 September 2013

Keywords:
Exopolysaccharides
FT-IR
Metal removal
Synechocystis sp.
Scanning electron microscopy

ABSTRACT

In the present study, chromium, cadmium and metal mixed (chromium + cadmium) removal and its association with exopolysaccharides and uronic acids production in *Synechocystis* sp. BASO671 were investigated. It was investigated that BASO671 showed different removal ability when exposed to each metal solely and mixed metal. EPS production by BASO671 was increased following exposure to 15 and 35 ppm Cr(VI), Cd(II) and Cr(VI) + Cd(II). Monomer composition of EPS was changed after metal treatment. Uronic acid contents of metal treated cells were higher than control cells of each isolate. Also, glucuronic acid content and galactronic acid content of EPS correlated with uronic acid contents of cells. Scanning electron microscopy and energy dispersive X-ray spectroscopy analysis confirmed that a considerable amount of metals had precipitated on the cell surface. Fourier transform infrared spectrum analysis of EPSs indicated the presence of C—H and C—O group, which may serve as binding sites for divalent cations.

1. Introduction

Toxic metallic trace elements that contaminate the environment are of increasing economic, public health and environmental significance and concern.

Methods of treating metallic trace element contaminated effluents currently consist of chemical precipitation, solvent extraction, dialysis, electrolytic extraction, reverse osmosis, evaporative methods, treatment with ion-exchange resins, carbon adsorption and dilution. In recent years, there has been a significant effort to search for new methods of metallic trace element removal from contaminated sites. Biological methods to remove metals from liquid effluents present many potential advantages. Metallic trace element accumulation processes by biological cells are grouped together under the general term "biosorption" (Salehizadeh & Shojaosadati, 2003).

Many microorganisms are capable of secreting high molecular mass polymers, which can either be released into the surrounding environment (extracellular polysaccharides, exopolysaccharides or extracellular polymeric substances (EPSs)) or remain attached to the cell surface (capsular polysaccharides). ESPs are mainly composed of polysaccharides, proteins, humic substances, nucleic acids, and lipids, containing ionizable functional groups such as carboxylic, phosphoric, amino and hydroxylic groups (Liu & Fang, 2002). These polysaccharides are believed to protect bacterial cells from desiccation, metallic trace elements or other environmental stresses, including host immune responses, and to produce biofilms, thus enhancing the cell's chances of colonizing special ecological niches. In metallic trace element pollution, bacterial exopolymers have become an alternative of interest as metal binding agents in the detoxification of contaminated waters (McEldowney, 2000).

Cyanobacteria, or blue-green algae, are ubiquitous microorganisms that occur naturally and serve as one of the biomaterials with a high capacity for removing metallic trace elements from contaminated waters. They have been known since long as a potential EPS producer. The presence of proteins, uronic acid, pyruvic acid, and O-methyl, O-acetyl and sulfate groups emphasizes the complex nature of cyanobacterial EPS (Bender & Phillips, 2004). The cell surface of cyanobacteria consists of polysaccharides, proteins and lipids, which act as a basic binding site for metallic trace elements. Therefore, it is the most important organism for environment in terms of removing wastes from the water.

In our study, the cyanobacterial strain, *Synechocystis* sp. BASO671, produced exopolysaccharides during the normal growth process. The aim of this study is to determine the metal removal behaviour of *Synechocystis* sp. BASO671 in terms of the relation between metal removals, EPS production. It also aims to determine the effect of Cr(VI), Cd(II) and Cr(VI) + Cd(II) on EPS production, EPS

* Corresponding author. Tel.: +90 0384 228 10 00; fax: +90 312 228 10 43.
E-mail address: sahlan.ozturk@nevsehir.edu.tr (S. Ozturk).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.09.040

monomer composition, uronic acid content of EPS and functional groups of EPS using this cyanobacterial isolate.

2. Materials and methods

2.1. Culture conditions and microorganisms

BASO671 was isolated from Uncali Stream (Antalya), Turkey. Isolation and purification of the isolate were performed by dilution and plating of water samples. Isolate were grown in BG-11 medium: [$NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4{\cdot}7H_2O$, 0.075; $CaCl_2{\cdot}2H_2O$, 0.036; citric acid, 0.006; iron(III) ammonium citrate, 0.006; Na_2-EDTA, 0.001; Na_2CO_3, 0.02 g L^{-1}, 1 mL]; trace elements solution (H_3BO_3, 61; $MnSO_4{\cdot}H_2O$, 169; $ZnSO_4{\cdot}7H_2O$, 287; $CuSO_4{\cdot}5H_2O$, 2.5; $(NH_4)6Mo7O_{24}{\cdot}4H_2O$, 12.5 mg L^{-1}) pH 6.8 at 25 °C with light/dark cycle of 12/12 h by using an incubator shaker (MINITRON), for 20 days. The agitation of the incubator shaker was 100 rpm during incubation. The intensity of light during the light period was 3000 lux.

2.2. 16S rRNA-based identification of cyanobacterial isolate

Genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Cat. No.: 69504, QIAGEN, UK). Cyanobacterial 16S rRNA gene sequences were amplified using cyanobacteria-specific primers as previously described (Nubel, Garcia-Pichel, & Muyzer, 1997): CYA106F (5′-CGGACGGGTGAGTAACGCGTGA-3′) and CYA781R (5′-GACTACAGGGGTATCTAATCCCTTT-3′). Also BACF (5′-GCCAGGGGAGCGAAAGGGATTAGA-3′) and BACR (5′-CATGGTGTGACGGGCGGTGTG-3′) primers which were designed by one of the authors (B. Aslim) were used for amplification. PCR amplifications were performed with a Hybaid thermocycler (ThermoHybaid, UK) and conditions were evaluated as described (Nubel et al., 1997). Sizes of the amplified fragment for CYA106F–CYA781R and BACF–BACR primers were 640 and 629 bp, respectively. The sequencing process was performed via the REFGEN process (Ankara, Turkey), and the sequences obtained were searched against the GenBank DNA database using the blast function.

2.3. Cr(VI) and Cd(II) toxicity

Solutions of different metal concentrations were prepared by dissolving $CdCl_2$ and $K_2Cr_2O_7$ (Merck) in distilled water to reach metal concentrations of 15 and 35 ppm. Cr(VI) and Cd(II) solutions were sterilized by filtering them with a 0.2 μm pore size filter. Experiments were carried out using 100 mL of BG11 in 150 mL glass Erlenmeyer flask Cr(VI) and Cd(II) resistance of cyanobacterial cultures were investigated by determining chlorophyll-a (Hirschberg & Chamovitz, 1994) every 48 h, for a period of 12 days. Also, flasks containing medium lacking Cr(VI) and Cd(II) were inoculated in the same manner to serve as controls. Cr(VI) and Cd(II) resistance were evaluated by comparison with the controls. The main values and the standard deviation were calculated from the data obtained with triplicate trials.

The EC_{50} determined by probit analysis (Finney, 1971) was defined as the Cr(VI) and Cd(II) concentration required to cause 50% mortality within 6 days.

2.4. Cr(VI) and Cd(II) removal

The removal of Cr(VI) and Cd(II) by *Synechocystis* sp. BASO671 was evaluated using a modified method described by Matsunaga, Takeyama, Nakao, and Yamazawa (1999). Isolates (OD_{664}, 2.5) were exposed to 10 ppm Cr(VI), Cd(II) and Cr(VI) + Cd(II) for 7 days in BG11 medium at 25 °C with a light/dark cycle of 12/12 h using an incubator shaker. The intensity of light employed during the light period was 3000 lux. Isolates of 1 mL which were exposed to metals were assayed for 0–7 daytime intervals. Metal removal was determined as metal in the medium, metal adsorbed on the surfaces of the cells, and metal accumulated in the cells. The concentration of Cr(VI) and Cd(II) was measured by an atomic absorption spectrophotometer (AA-6600, Shimadzu). The cromium or cadmium removal rate (%) was calculated as follows: (amount of removed Cr or Cd)/(amount of initial Cr or Cd) × 100. Samples were centrifuged (10,000 rpm) and residual Cr(VI) and Cd(II) in the medium was determined in the supernatant. The pellet was further washed with 1 mL of 10 mM EDTA solution for desorption of Cr(VI) and Cd(II) from the cell surfaces and centrifuged (10,000 rpm) once again. Cr(VI) and Cd(II) adsorbed onto the cell surfaces were separated from this supernatant. The amount of intracellular accumulation of Cr(VI) and Cd(II) were determined by measuring the Cr(VI) and Cd(II) content in the pellet, and resuspended and sonicated (Vibra Cell) at 50 MHz on ice in 1 mL of 1 N HNO_3 using an atomic absorption spectrophotometer.

2.5. Isolation, purification and characterization of exopolysaccharides (EPSs)

EPS was extracted by the modified procedure of Cérantola, Bounéry, Segonds, Marty, and Montrozier (2000). After 20 days of cultivation, cells were harvested at room temperature by centrifugation at 10,000 rpm for 10 min. The supernatant was removed. After the pellet was dissolved in 1 mL deionized distilled water, the solution was boiled for 15 min at 100 °C. It was then kept at room temperature for 10 min and added to 3 μl of 85% TCA. The resultant mixture was centrifuged at 10,000 rpm for 30 min. The supernatant which contained EPS was pooled and equal volume of ethanol was added. The mixture was kept at 4 °C overnight and was centrifuged at 10,000 rpm for 30 min again. Precipitate was then washed two times with 96% ethanol and centrifuged at 10,000 rpm for 30 min. Final precipitate was dissolved in 1 mL deionized distilled water and stored at −20 °C. Total carbohydrate contents of the EPS samples were determined by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) using glucose as a reference standard (Torino, Taranto, Sesma, & de Valdez, 2001). The main values were calculated from the data obtained with triplicate trials.

The monosaccharide composition of freeze-dried extracellular polysaccharide samples was determined with HPLC (VARIAN ProStar) by using Metacarb 87H column (300 mm × 7.8 mm, Cat. No.: 5210). The organic acids were determined with PDA detector (VARIAN 330) (210 nm), while the extracellular polysaccharides were determined with RI detector (VARIAN 350), connected after the PDA detector. The analyze conditions are mobile phase 0.008 N H_2SO_4, flow rate 0.4 mL min^{-1} at 35 °C. Monomer analyze of EPS was carried out at the Central Laboratory, Molecular Biology and Biotechnology R&D Centre, Middle East Technical University.

2.6. Effect of Cr(VI) and Cd(II) on EPS production

Equal biomasses of the isolates were inoculated into 500 mL flasks containing 300 mL of BG-11 with 15 and 35 ppm Cr(VI), Cd(II) and Cr(VI) + Cd(II) concentrations. Isolates were incubated at 25 °C with light/dark cycle of 12/12 h by using an incubator shaker (MINITRON), for 7 days. The intensity of light during the light period was 3000 lux. Initial and final biomass concentrations of the isolates were investigated and equalled by determining chlorophyll-a. Cells were collected by centrifugation at 10,000 rpm for 10 min at room temperature. EPS was isolated as described by Cérantola et al. (2000) and total EPS (mg L^{-1}) was estimated by the phenol-sulfuric method (Cérantola et al., 2000). The main values and the standard deviation were calculated from the data obtained with triplicate trials.

2.7. Determination of uronic acid

To 40 μl of (1 μg μl^{-1}) EPS solution, 40 μl of 4 mol l^{-1} sulfamic acid was added. After mixing well, 2.4 mL of concentrated sulfuric acid was added, and the resulting solution was vortexed and heated in a boiling water bath for 20 min. After the solution was cooled on an ice water bath, 80 μl of m-hydroxy diphenyl was added, mixed well, and incubated for 10 min. The colour that developed was read at 525 nm. D-Glucuronic acid was used as standard solution for comparison (Filisetti-Cozzi & Carpita, 1991).

2.8. Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analysis

In order to observe how the sorption of metal ions on the cell surface would alter its morphology and to further confirm the identity of metal ions in the cell mass, SEM (scanning electron microscopy) and EDS (energy dispersive X-ray spectrometry) analysis were employed in this study. Cyanobacterial cells were also determined qualitatively by electron microscopic examination. After exposure to 10 ppm Cr(VI), Cd(II) and Cr(VI) + Cd(II), cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 h. After two washes with phosphate buffer, samples were dehydrated in a graded series of ethanol concentrations starting with 70% (v/v) followed by 80% (v/v), 90% (v/v), and finally 100% (v/v). The cells were dried in a critical-point dryer using CO_2 (Polaron, CPD 7501) and coated with gold (Polaron SC 502 sputter coater). The specimens were then examined with a Jeol JSM 6060 scanning electron microscope.

Energy dispersive X-ray spectroscopy (EDS) (OXFORD ISIS 300 EDS) was performed to detect Cr(VI) and Cd(II) and its compounds that had been adsorbed onto the cell surface or entrapped in the extracellular polysaccharides (EPSs). Operation conditions were 20 kV accelerating voltage, a 1 μm beam diameter and 20 nA beam current with spectral acquisition time of 100 s. Besides EDS analysis, the electron beam was spot over the selected area. The EDS analysis was carried out by the Faculty of Engineering, Marmara University.

2.9. Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR was used to analyze the chemical structure of the dry extracellular polysaccharide samples (exposed before–after chromium or cadmium). The FT-IR spectra of EPSs were measured on a Perkin-Elmer FT-IR spectrometer. Each spectrum was recorded between 4000 and 400 cm^{-1} with a 4 cm^{-1} resolution from KBr pellets.

2.10. Experimental design and statistical analysis

All experiments were done in triplicate and mean values are presented. Statistical analysis was performed on the data using SPSS 13.0 Bivariate Correlation Analysis. The Pearson rank order coefficient was determined for the comparison of metal tolerance between EPS production and also of metal removal by *Synechocystis* sp. BASO671. One-way ANOVA was used for detection effect of metal (Cr and Cd) concentrations on EPS production. Individual differences were detected by Dunnett and Tukey grouping tests. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. 16S rRNA-based identification of cyanobacterial isolates

The isolate was identified by amplification and sequencing of its 16S rRNA gene. The sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using BLAST (blastn) tool and corresponding sequences were downloaded. Using internal primers, the sequences belonging to the isolate BASO671 showed 98% similarity with *Synechocystis* sp. PCC 6803 (BA000022.2). On the basis of 16S rRNA gene sequence analysis the isolate is identified as *Synechocystis* sp.

3.2. Cr(VI) and Cd(II) toxicity and removal

While metalic trace element tolerance and biosorption has been extensively studied, there is lack of information on chromium and cadmium bioaccumulation and metabolism in cyanobacteria. The results of this paper also turn up evidence against the view of some authors that metal uptake depend mainly on surface-binding mechanisms rather than on metabolic activity. The EC_{50} values of Cr(VI) and Cd(II) on *Synechocystis* sp. BASO671 were 10.9 and 10.5 ppm, respectively.

Based on the EC_{50} values of the isolate, 10 ppm metal concentration was selected. *Synechocystis* sp. BASO671 was exposed to 10 ppm Cr(VI), 10 ppm Cd(II) and 10 ppm Cr(VI) + Cd(II) for 7 days in BG11 medium; and these showed differing abilities for Cr(VI) and Cd(II) removal (Fig. 1). One part of the total cadmium removed by the cells was accumulated intracellular and another part was adsorbed onto the cell surfaces for both isolates. *Synechocystis* sp. BASO671 removed more Cd(II) than Cr(VI). At the end of the 7th day, *Synechocystis* sp. BASO671 adsorbed 90% of 10 ppm Cd(II) onto the cell surfaces and not accumulated Cd(II) intracellular. However, *Synechocystis* sp. BASO671 accumulated 14% of 10 ppm Cr(VI) intracellular and not adsorbed onto the cell surfaces (Fig. 1A). Sabri, Sabri, and Sultan (2012) reported that some strains of cyanobacteria are reported to accumulate chromium intracellularly. The proportions of remaining Cd(II) in the medium were determined as 8% and the proportions of remaining Cr(VI) in the medium were determined as 85% for *Synechocystis* sp. BASO671. Cyanobacteria can be included among the potential sources of new polymers, several species being characterized by the presence of thick capsules surrounding the cells and by the ability to release polysaccharide material into culture medium. It is thought that loss of metal might be adsorbed on the EPS release into the medium. The fast binding of Cd(II) within the first minutes of contact – which is essentially followed by a plateau – suggests that the metal is removed via interactions with functional groups on the surface (Monteiro, Castro, & Malcata, 2009) have concluded likewise. On the other hand, *Synechocystis* sp. BASO671 was exposed to 10 ppm Cr(VI) + 10 ppm Cd(II) showed different metal removal abilities. *Synechocystis* sp. BASO671 evaluated with metal mixed prefered Cd(II) rather than Cr(VI) (Fig. 2). Metal removal behaviour of bacteria may change when exposed to metal mixed because of the competition on functional groups.

3.3. EPS production and effect of Cr(VI) and Cd(II) on EPS production

Hyperproduction of EPS in response to starvation, antiviral activity, thickening agent, and cosmetic industry for product formulations has been reported for cyanobacteria. On the other hand there are only a few reports about metallic trace element binding capabilities as one of the functions of cyanobacterial EPSs. And also, information about the effects of the metals on the physiology of cyanobacteria is still scarce particularly for situations of simultaneous exposure to more than one metal, which frequently occur in polluted environments (Burnat, Diestra, Esteve, & Sole, 2009). In this study we showed that the effect of different heavy metals on the isolate *Synechocystis* sp. BASO671 differed significantly in EPS production. The EPS production by the cyanobacterial isolate was assessed during their growth in batch culture at our previous study.

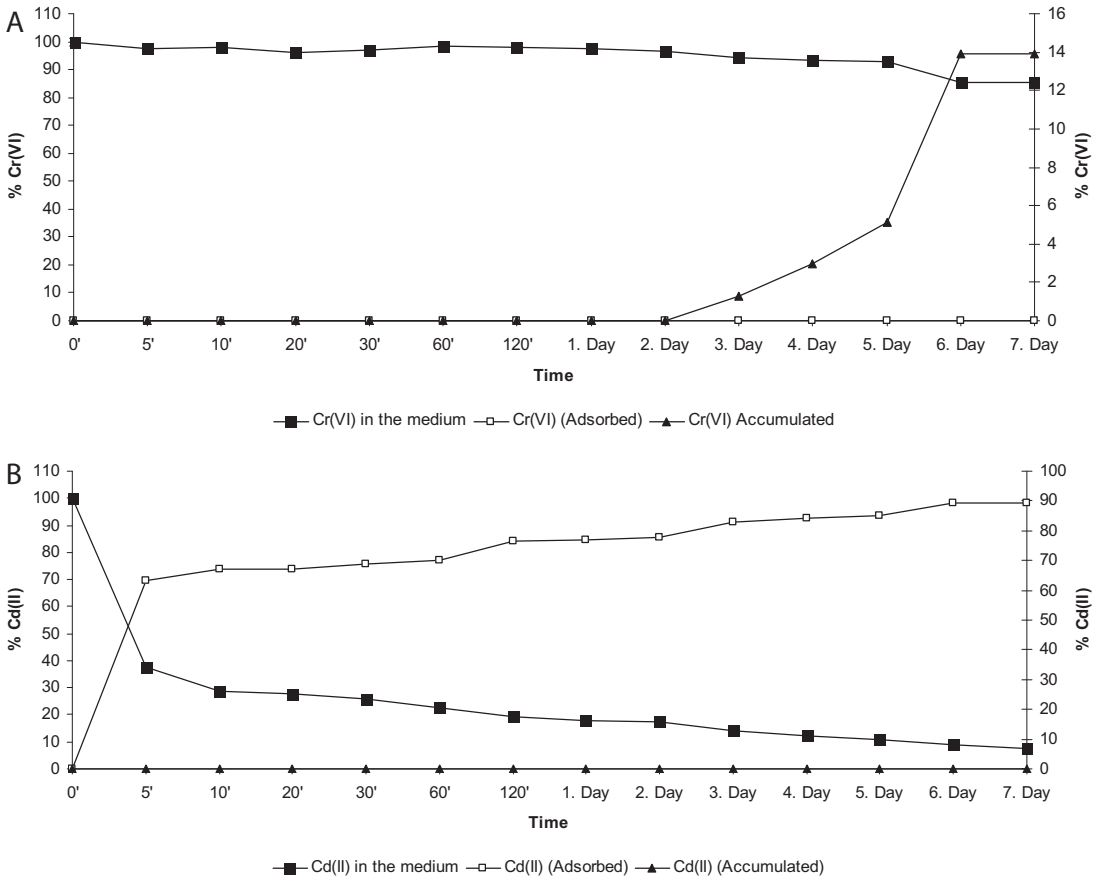

Fig. 1. Cr(VI) removal of *Synechocystis* sp. BASO671 (A), and Cd(II) removal of *Synechocystis* sp. BASO671 (B). *Y* axis (left): Cr(VI) or Cd(II) in the medium; *Y* axis (right): adsorbed and accumulated Cr(VI) or Cd(II).

As shown in Table 1, *Synechocystis* sp. BASO67l with Cd(II) produced higher amount of EPS than *Synechocystis* BASO67l with Cr(VI). *Synechocystis* sp. BASO67l with Cr(VI) and Cd(II) produced low amount of EPS when compared with control. In this study, a positive correlation was determined significantly between Cd(II) tolerance and EPS production ($p < 0.01$). Effect of Cr(VI), Cd(II) and Cr(VI) + Cd(II) on EPS production by isolate BASO671 was determined in the presence of 15 and 35 ppm Cr(VI), Cd(II) or Cr(VI) + Cd(II). Final biomass concentrations were equalled before determining EPS production. Experiments were repeated two times for each heavy metal. We observed a significant and regular increase in EPS production of *Synechocystis* sp. BASO67l both by Cr(VI), Cd(II) or Cr(VI) + Cd(II) at 35 ppm (Fig. 3). Many different environmental stresses have been previously reported to effect an increased production of extracellular carbohydrates in biofilms. On the other hand there are only a few reports about the effect of metals enhanced the production of EPS. Trivalent chromium resulted in a nearly 82% increase in extracellular carbohydrate in sulfate-reducing bacterial biofilms (Sharma, Kaushik, Somvir Bala, & Kamra, 2008). Mixed-species sulfate-reducing bacterial biofilms also exhibited an increase in extracellular carbohydrates when exposed to cadmium, possibly as a result of metal binding polymer production (White & Gadd, 1998). Also our previous studies confirmed the results of the present study (Ozturk & Aslim, 2008; Ozturk, Aslim, & Ugur, 2008).

3.4. EPS monomer characterization and uronic acid content

Cyanobacterial EPS (polysaccharidic in nature) present a set of unique biochemical properties that make them interesting from the biotechnological point of view. These complex exopolysaccharides are composed of at least 10 different monosaccharides and are characterized by the presence of pentoses (usually absent in polysaccharides of prokaryotic origin), as well as their anionic nature due to the presence of acidic sugars (glucuronic and/or galacturonic acids) and anionic organic (acetyl, pyruvil) and inorganic (phosphate and sulfate) substituents (Challouf et al., 2011).

Sugar monomer makeup of the EPSs produced by different isolates with and without exposure to 10 ppm Cr(VI), Cd(II) or Cd(II) + Cr(VI) was characterized and the quantified the monomer content by HPLC; the results are summarized in Table 1. The exposure of Cr(VI) and Cd(II) had an influence on the monomer composition of EPS belonging to *Synechocystis* sp. BASO671.

Table 1
The effect of Cr(VI), Cd(II) and Cr(VI) + Cd(II) on EPS values, uronic acid content and monomer composition of EPS belonging to *Synechocystis* sp. BASO671.

Isolate	EPS (mg L^{-1})	Treatment	Uronic acid (μg/mg)	Comparative monosaccharide composition of EPS[a] (%)				
				Glucose	Rhamnose	Xylose	Glucuronic acid	Galacturonic acid
BASO671	474 ± 5	Control	162 ± 3	92	6.60	1.26	0.06	0.08
	448 ± 2	Cr(VI)	121 ± 5	89.9	2.2	4.7	1.9	1.3
	453 ± 2	Cd(II)	186 ± 2	94.6	–	–	2.8	2.6
	326 ± 4	Cr(VI) + Cd(II)	199 ± 4	93	–	–	3.2	3.8

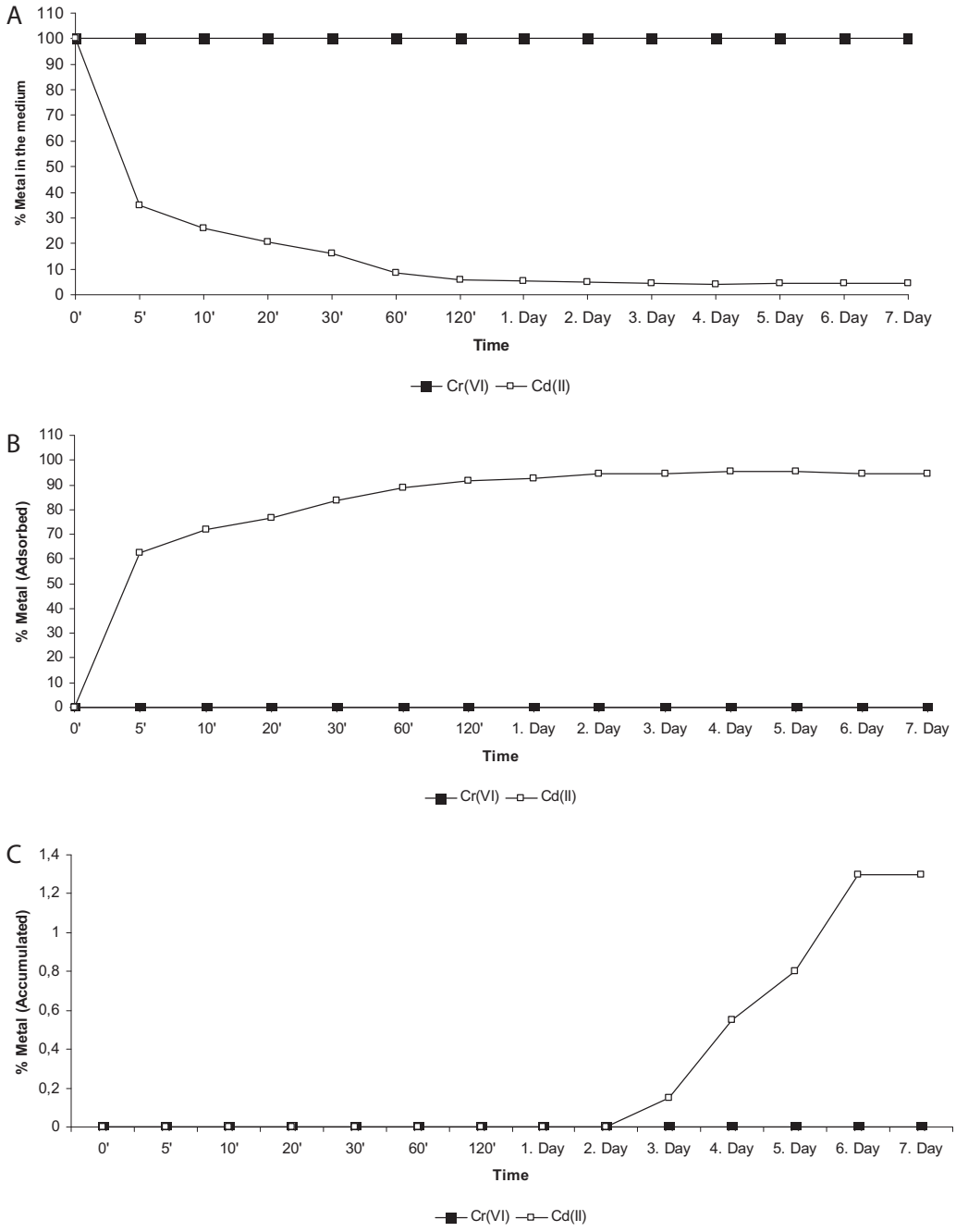

Fig. 2. 10 ppm Cr(VI) + 10 ppm Cd(II) removal of *Synechocystis* sp. BASO671. Cr(VI) + Cd(II) in the medium (A); Cr(VI) + Cd(II) adsorbed onto the cell surface (B); Cr(VI) and Cd(II) accumulated into the cell (C).

Following Cd(II) exposure, EPSs were composed mainly of glucose (94.6%) and small amounts of glucuronic acid (2.8%) and galacturonic acid (2.6%). Following Cr (VI) exposure, EPSs were composed mainly of glucose (89.9%) and small amounts of glucuronic acid (1.9%), galacturonic acid (1.3%), rhamnose (2.2%), xylose (4.7%) for *Synechocystis* sp. BASO671. On the other hand, metal mixed (Cr(VI) + Cd(II)) exposure had different influence on the monomer composition of EPS belonging to *Synechocystis* sp. BASO671. Cr(VI) + Cd(II) exposure, EPSs were composed mainly of glucose (93%) and small amounts of glucuronic acid (3.2%) and galacturonic acid (3.8%). Xylose and rhamnose were not detected in the EPSs produced by *Synechocystis* sp. BASO671 with or without Cd(II), and Cr(VI) + Cd(II) exposure. Also, mannose, galactose and arabinose were not detected in the EPSs produced by isolate for each treatment. In our previous study, we found differences in the monosaccharide composition and ratios of EPS in *Synechocystis* sp. exposed to Cr(VI) and Cd(II) (Ozturk, Aslim, & Suludere, 2009, 2010). Also, Priester et al. (2006) reported changes in the carbohydrate composition of *Pseudomonas putida* biofilms exposed to Cr. The change in EPS composition is specific to the cyanobacteria strain and may aid in the survivability of the organism. Differences in the monosaccharide composition and ratios of EPS may promote metalic trace element resistance in these microorganisms (Ozturk et al., 2010).

In the presence of more than one metal, the process of metal binding to the cyanobacterial EPS can be noninteractive, synergistic or competitive, depending on the metal ions and characteristics of the polysaccharide (Micheletti, Colica, Viti, Tamagnini, & De

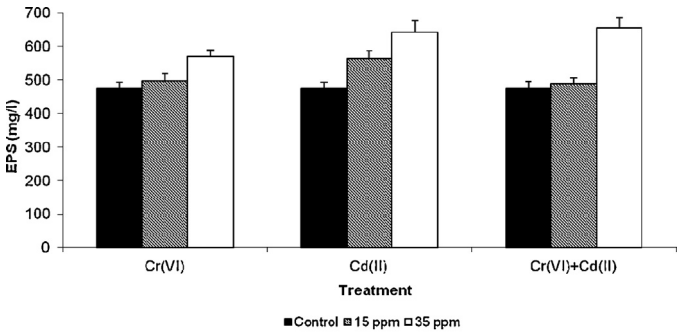

Fig. 3. Effect of Cr(VI), Cd(II) and Cr(VI) + Cd(II) concentrations (15 and 35 ppm) on EPS production of *Synechocystis* sp. BASO671. Data are presented as the means and standard deviation compiled from two replications of the experiment. Statistical analysis using Dunnett and Tukey grouping tests showed overall statistical significance ($p < 0.05$) as well as significant differences between 15 ppm and control, and 35 ppm and control.

Philippis, 2008). In the present study *Synechocystis* sp. BASO671 evaluated with metal mixed prefered Cd(II) rather than Cr(VI). This can result from (i) direct competition between the two metals for the EPS binding sites and/or (ii) modification of the EPS conformation by the already adsorbed metal ions, which will hinder the access of other ions to adjacent adsorption sites (Pereira et al., 2011).

Acidic polysaccharides are common in marine organisms, also reported from bacteria, cyanobacteria, and diatoms. Negatively charged cyanobacterial EPS containing uronic acids exhibited a high metal-complexing capacity. The presence of uronic acids and sulfates confers an overall negative charge and acidic property to the exopolymer and sulfated EPSs are of biotechnological importance (Mishra, Kavita, & Jha, 2011). In the present study uronic acid content of Cd(II) and Cr(VI) + Cd(II) treated cells was higher than control cells. On the other hand uronic acid content of Cr(VI) treated cells were lower than control cells. Because, Cr(VI) was only accumulated into the cells of *Synechocystis* sp. BASO671 exposed to Cr(VI). Also, percentage glucuronic acid and galacturonic acid content of EPSs belonging to Cd(II), Cr(VI) and Cr(VI) + Cd(II) treated cells was determined to be higher than that of control cells of *Synechocystis* sp. BASO671. It has been suggested that EPS produced by cyanobacteria could be useful in detoxification of metalic trace element, and removal of solid materials from water reservoirs (Bender & Phillips, 2004). However, despite their ubiquitous distribution, very little data regarding the physicochemical properties of cyanobacterial EPS exist (Trabelsi, M'sakni, Ouada, Bacha, & Roudesli, 2009). Such a deficiency in structural information has prevented a better understanding of their role in metalic trace element biosorption and possible applications.

3.5. SEM and EDS analyses

The SEM is used to observe the changes in the surface microstructures of the biomass resulting from surface modifications and cyanobacterial isolates under Cr(VI), Cd(II) and Cr(VI) + Cd(II) stress (see supplementary data in the online version of this article for SEM analysis). Supplementary data 1 shows the control cells of cyanobacterial isolates, supplementary data 2 shows the cells of the cyanobacterial isolates exposed to 10 ppm Cr(VI), supplementary data 3 exposed to 10 ppm Cd(II) and supplementary data 4 exposed to 10 ppm Cr(VI) + Cd(II). The differences in the surface morphology of the isolates after metal exposure are evident from supplementary data. The surface of the cells becomes rough after metal uptakes. Also, Sujatha et al. (2013) reported a significant difference on the surface morphology of *Trichoderma viride* after nickel biosorption. Two specific areas were selected for EDS analysis for each isolates exposed to metal. 'X' indicates the cell surface and 'Y' indicates the EPS-rich area (see supplementary data in the online version of this article for SEM analysis). EDS analysis of the cells showed that the EPS-rich area 'Y' of the isolates adsorbed more Cr(VI) or Cd(II) than cell surfaces of *Synechocystis* sp. BASO671 (Table 2). Isolates grown in a medium without metal (control) showed no EDS signals for Cd or Cr. The analysis confirmed the association of cadmium with the cell surface Demirel, Ustun, Aslim, and Suludere (2009) also reported similar results. In their study, *Synechocystis* sp. B35 which was exposed to 10 ppm Fe produced large quantities of EPS and most of the Fe was adsorbed onto the EPS (Murthy, Bali, & Sarangi, 2012). Also reported lead was mostly entrapped in the exopolysaccharides (EPS) in *Bacillus cereus*.

3.6. FT-IR analysis

The functional group is one of the keys to understand the mechanism of metal binding onto the *Synechocystis* sp. BASO671 EPS. To better understand the nature of the functional groups responsible for the biosorption process, the FTIR spectrum of the EPS is presented in supplementary data 5. Peaks appearing in the FT-IR transmission spectra of both isolate EPS were assigned to various groups and bonds in accordance with their respective wave numbers. The comparison of EPS in the controls with that in Cr(VI), Cd(II) and Cr(VI) + Cd(II) treated isolates shows that there is a shift in wave number of dominant peaks associated with the EPS of each metal treated isolates. The EPS displays a broad stretching intense peak at around 3400 cm^{-1} characteristic for hydroxyl and amino groups. The strong absorption peak at 2932–2934 cm^{-1} for algal biomass can be assigned to aromatic ring stretching (phenolic groups). The presence of a carboxylic acid is revealed by a band at 1653 and 1654 cm^{-1} (Pradhan, Singh, & Rai, 2007) which revealed the presence of —COOH groups (1600–1725 cm^{-1}) and —OH (2800–3600 cm^{-1}) groups, showing that the samples were exopolysaccharide. The peak at 1034–1028 cm^{-1} could be due to the involvement of the C—O bond of polysaccharides. The 1000–1125 range is characteristic of uronic acid, O-acetyl ester linkage bond (Morillo Perez et al., 2008). In the present study, the addition of metal markedly changed the shape of FT-IR spectra and metal removal might result in changes in the absorption frequencies of the various functional groups present in the EPSs of each

Table 2
EDS spectra of *Synechocystis* sp. BASO671 cells treated with or without 10 ppm Cr(VI), Cd(II) and Cr(VI) + Cd(II).

Isolate	Treatment	Area	Elements (%)								Total
			C	O	Na	Mg	K	Ca	Cr	Cd	
BASO671	Control	X	66.81	30.03	0.54	1.30	0.42	0.91	0.00	0.00	100.00
	Cr(VI)	X	72.22	23.74	0.50	0.79	0.31	1.87	0.57	0.00	100.00
		Y	76.71	18.60	0.52	0.83	0.60	1.93	0.81	0.00	100.00
	Cd(II)	X	50.36	38.59	3.00	1.49	0.33	2.96	0.00	3.27	100.00
		Y	43.14	41.46	3.22	1.79	0.46	3.78	0.00	6.15	100.00
	Cr(VI) + Cd(II)	X	42.85	34.93	5.94	1.79	0.37	6.13	0.00	8.00	100.00
		Y	50.08	31.30	5.02	1.42	0.40	2.41	0.00	9.37	100.00

isolates. Zhang, Pan, Zhao, and Mu (2011) also detected similar results at antimony treated *Synechocystis* sp.

4. Conclusions

Cr(VI), Cd(II) and Cr(VI) + Cd(II) removal of *Synechocystis* sp. was demonstrated comprehensively in terms of EPS production, monomer composition for the first time. Effect of metal on living cells was investigated by different parameters such as uronic acid content, SEM and EDS analysis.

Our results indicate that (1) metals were adsorbed on cell surface of BASO671 especially at Cd(II) and Cr(VI) + Cd(II) treatment; (2) exposure to 10 ppm of each metal affects the composition of EPS produced by BASO671; (3) EPS production of BASO671 was highly and significantly affected by elevated concentrations of each metals; (4) there is a correlation between uronic acid content of isolate and glucuronic acid–galactronic acid content of EPS; (5) metal adsorption on cell surface was proved by SEM and EDS analysis; (6) interactions between metal ions and functional groups on the cell surface were confirmed by FT-IR.

The relationship between proteins in EPS, whole cell proteins, metallothioneins, thiol groups, glutathions and the removal of Cr(VI), Cd(II) and Cr(VI) + Cd(II) by cyanobacteria is under further investigation.

Acknowledgement

Financial support was provided by Gazi University B.A.P. (05/2005-31).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.09.040.

References

Bender, J., & Phillips, P. (2004). Microbial mats for multiple applications in aquaculture and bioremediation. *Bioresource Technology*, *94*, 229–238.

Burnat, M., Diestra, E., Esteve, I., & Sole, A. (2009). In situ determination of the effects of lead and copper on cyanobacterial populations in microcosms. *PLoS ONE*, *4*, e6204.

Cérantola, S., Bounéry, J. D., Segonds, C., Marty, N., & Montrozier, H. (2000). Exopolysaccharide production by mucoid and non-mucoid strains of *Burkholderia cepacia*. *FEMS Microbiology Letters*, *185*, 243–246.

Challouf, R., Trabelsi, L., Ben Dhieb, R., El Abed, O., Yahia, A., Ghozzi, K., et al. (2011). Evaluation of cytotoxicity and biological activities in extracellular polysaccharides released by cyanobacterium *Arthrospira platensis*. *Brazilian Archives of Biology and Technology*, *54*, 831–838.

Demirel, S., Ustun, B., Aslim, B., & Suludere, Z. (2009). Toxicity and uptake of iron ions by *Synechocystis* sp. E35 isolated from Kucukcekmece Lagoon, Istanbul. *Journal of Hazardous Materials*, *171*, 710–716.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric methods for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Filisetti-Cozzi, T. M. C., & Carpita, C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

Finney, D. J. (1971). *Probit analysis*. Cambridge: Cambridge University Press.

Hirschberg, J., & Chamovitz, D. (1994). Carotenoids in cyanobacteria. In D. A. Bryant (Ed.), *The molecular biology of cyanobacteria* (pp. 559–579). Dordrecht: Kluwer.

Liu, H., & Fang, H. H. P. (2002). Characterization of electrostatic binding sites of extracellular polymers by linear programming analysis of titration data. *Biotechnology and Bioengineering*, *80*, 806–811.

Matsunaga, T., Takeyama, H., Nakao, T., & Yamazawa, A. (1999). Screening of marine microalgae for bioremediation of cadmium-polluted seawater. *Journal of Biotechnology*, *70*, 33–38.

McEldowney, S. (2000). The impact of surface attachment on cadmium accumulation by *Pseudomonas fluorescens* H2. *FEMS Microbiology Ecology*, *33*, 121–128.

Micheletti, E., Colica, G., Viti, C., Tamagnini, P., & De Philippis, R. (2008). Selectivity in the heavy metal removal by exopolysaccharide producing cyanobacteria. *Journal of Applied Microbiology*, *105*, 88–94.

Mishra, A., Kavita, K., & Jha, B. (2011). Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina*. *Carbohydrate Polymers*, *83*, 852–857.

Monteiro, C. M., Castro, P. M. L., & Malcata, F. X. (2009). Use of the microalga *Scenedesmus obliquus* to remove cadmium cations from aqueous solutions. *World Journal of Microbiology and Biotechnology*, *25*, 1573–1578.

Morillo Perez, J. A., 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*. *World Journal of Microbiology and Biotechnology*, *24*, 2699–2704.

Murthy, S., Bali, G., & Sarangi, S. K. (2012). Lead biosorption by a bacterium isolated from industrial effluents. *International Journal of Microbiology Research*, *4*(3), 196.

Nubel, U., Garcia-Pichel, F., & Muyzer, G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology*, *63*, 3327–3332.

Ozturk, S., & Aslim, B. (2008). Relationship between chromium(VI) resistance and extracellular polymeric substances (EPS) concentration by some cyanobacterial isolates. *Environmental Science and Pollution Research*, *15*, 478–480.

Ozturk, S., Aslim, B., & Suludere, Z. (2009). Evaluation of chromium(VI) removal behaviour by two isolates of *Synechocystis* sp. in terms of exopolysaccharide (EPS) production and monomer composition. *Bioresource Technology*, *100*, 5588–5593.

Ozturk, S., Aslim, B., & Suludere, Z. (2010). Cadmium(II) sequestration characteristics by two isolates of *Synechocystis* sp. in terms of exopolysaccharide (EPS) production and monomer composition. *Bioresource Technology*, *101*(24), 9742–9748.

Ozturk, S., Aslim, B., & Ugur, A. (2008). Chromium(VI) resistance and extracellular polysaccharide (EPS) synthesis by *Pseudomonas*, *Stenotrophomonas* and *Methylobacterium* strains. *ISIJ International*, *48*(11), 1654–1658.

Pereira, S., Micheletti, E., Zille, A., Santos, A., Moradas-Ferreira, P., Tamagnini, P., et al. (2011). Using extracellular polymeric substances (EPS)-producing cyanobacteria for the bioremediation of heavy metals: Do cations compete for the EPS functional groups and also accumulate inside the cell. *Microbiology*, *157*(2), 451–458.

Pradhan, S., Singh, S., & Rai, L. C. (2007). Characterization of various functional groups present in the capsule of Microcystis and study of their role in biosorption of Fe, Ni and Cr. *Bioresource Technology*, *98*, 595–601.

Priester, J. H., Olson, S. G., Webb, S. M., Neu, M. P., Hersman, L. E., & Holden, P. A. (2006). Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Applied Environmental Microbiology*, *72*, 1988–1996.

Sabri, A. N., Sabri, S., & Sultan, S. (2012). Metallurgical microscopy of bacterial biofilm representing Cr^{+6} conversion into Cr^{+3} by *Acienetobacter calcoaceticus*, *Staphylococcus aureus* and *Oscillatoria strain*. *Current Microscopy Contributions to Advances in Sciences and Technology*, *1*, 749–755.

Salehizadeh, H., & Shojaosadati, S. A. (2003). Removal of metal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. *Water Research*, *37*, 4231–4235.

Sharma, M., Kaushik, A., Somvir Bala, K., & Kamra, A. (2008). Sequestration of chromium by exopolysaccharides of Nostoc and Gloeocapsa from dilute aqueous solutions. *Journal of Hazardous Materials*, *157*, 315–318.

Sujatha, P., Kalarani, V., & Naresh Kumar, B. (2013). Effective biosorption of nickel(II) from aqueous solutions using *Trichoderma viride*. *Journal of Chemistry*, 7. http://dx.doi.org/10.1155/2013/716098. Article ID 716098.

Torino, M. I., Taranto, M. P., Sesma, F., & de Valdez, G. F. (2001). Heterofermentative pattern and exopolysaccharide production by *Lactobacillus helveticus* ATCC15807 in response to environmental pH. *Journal of Applied Microbiology*, *91*, 846–852.

Trabelsi, L., M'sakni, N. H., Ouada, H. B., Bacha, H., & Roudesli, S. (2009). Partial characterization of extracellular polysaccharides produced by cyanobacterium *Arthrospira platensis*. *Biotechnology and Bioprocess Engineering*, *14*, 27–31.

White, C., & Gadd, G. M. (1998). Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. *Microbiology*, *144*, 1407–1415.

Zhang, D., Pan, X., Zhao, L., & Mu, G. (2011). Biosorption of antimony (Sb) by the cyanobacterium *Synechocystis* sp. *Polish Journal of Environmental Studies*, *20*(5), 1353–1358.