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Evaluation of chromium(VI) removal behaviour by two isolates of *Synechocystis* sp. in terms of exopolysaccharide (EPS) production and monomer composition

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ABSTRACT

Chromium(VI) removal and its association with exopolysaccharide (EPS) production in cyanobacteria were investigated. *Synechocystis* sp. BASO670 produced higher EPS (548 mg L^{-1}) than *Synechocystis* sp. BASO672 (356 mg L^{-1}). While the EC_{50} of the Cr(VI) for *Synechocystis* sp. BASO670 and *Synechocystis* sp. BASO672 were determined as 11.5 mg L^{-1}, and 2.0 mg L^{-1}, respectively, there was no relation between Cr(VI) removal and EPS production. *Synechocystis* sp. BASO672, which has higher EPS value, removed (33%) more Cr(VI) than *Synechocystis* sp. BASO670. Monomer compositions of EPS of each of the isolates were determined differently. *Synechocystis* sp. BASO672 which removed higher Cr(VI), had higher values of uronic acid and glucuronic acid (192 μg/mg and 89%, respectively). Our results showed that EPS might play a role in Cr(VI) tolerance. Monomer composition, especially uronic acid and glucuronic acid content of EPS may have enhanced Cr(VI) removal.

1. Introduction

Hexavalent chromium, Cr(VI), in the forms of chromate and dichromate, is widely present in wastewaters from several industries such as pigment and dye production, leather tanning, electroplating, wood treatment, textile dyeing, and the steel industry. Industrial discharges containing Cr(VI) and improper disposal procedures have resulted in large-scale environmental pollution. Cr(VI) is harmful for flora and fauna of natural aquatic ecosystems, being highly toxic to all forms of life (Caravelli et al., 2008). Hexavalent chromium, has been classified as a human carcinogen (EPA, 1998). The WHO limit (WHO, 1993) for allowable concentration of Cr(VI) in drinking water is 0.05 mg L^{-1}. Thus, it is essential to reduce Cr(VI) concentrations from water/wastewater to acceptable levels.

The conventional methods of Cr(VI) removal in wastewaters comprise chemical reduction followed by chemical precipitation, ion exchange, and adsorption onto activated carbon. However, these methods are excessively energy consuming and utilize large amounts of reagents. Efficient and environmental friendly technologies, therefore, are needed to be developed to reduce the heavy metal contents in wastewaters to acceptable levels at economical costs (Saeed and Iqbal, 2003). The use of microbial biosorbents like bacteria, fungi, yeast, algae, and cyanobacteria (Anjana et al., 2007) for removal of toxic chromium from waste streams has emerged as an alternative to the existing methods as a result of the search for low cost-, innovative methods. It is suggested that cyanobacteria have some added advantages over other microorganisms because of their large surface areas, greater mucilage volume with high binding affinity, and simple nutrient requirements (Gupta and Rastogi, 2008).

Many microorganisms are capable of secreting high molecular-mass polymers, which 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 and Fang, 2002). These polysaccharides are believed to protect bacterial cells from desiccation, heavy metals or other environmental stresses, and to produce biofilms (Sutherland, 2001). In heavy metal pollution, bacterial exopolymers have become an alternative of interest as metal-binding agents in detoxification of contaminated waters (McEldowney, 2000).

Cyanobacteria, or blue–green algae, are naturally ubiquitous and serve as one of the biomaterials with high capacity for removing heavy metals from contaminated waters. They have been known since long as potential EPS producers. The presence of proteins, uronic acids, pyruvic acid, and *O*-methyl-, *O*-acetyl-, and sulfate groups emphasizes the complex nature of cyanobacterial EPS (Freire-Nordi et al., 2005) and act as a basic binding site for heavy metals. Therefore, they are vital organisms for the environment in terms of removing wastes from water (Ting et al., 1991).

The main objectives of this study were (i) to investigate the Cr(VI) tolerance and removal of *Synechocystis* sp. BASO670 and

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Synechocystis sp. BASO672, (ii) to determine the exopolysaccharide production by these two cyanobacterial isolates and (iii) to analyse monomer composition of EPS produced by both isolates. We also focused on the total amount of uronic acid and acidic sugars (galacturonic- and glucuronic acids), which play an important role in metal binding. Besides, this study was also designed to evaluate the cells of isolates exposed to Cr(VI) by the scanning electron microscope (SEM) method. The usefulness of the findings of the present study in examining the correlation between EPS production and Cr(VI) removal was also discussed.

2. Methods

2.1. Culture conditions and microorganisms

Isolates BASO670 and BASO672 were isolated from Mogan Lake (Ankara), and Bafa Lake (Aydin), Turkey, respectively. Isolation and purification were performed by dilution and plating of water samples. Isolates were grown in BG11 medium: [$NaNO_3$, 15; K_2HPO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.75; $CaCl_2 \cdot 2H_2O$, 0.36; citric acid, 0.06; iron(III) ammonium citrate, 0.06; Na_2-EDTA, 0.01; Na_2CO_3, 0.2 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; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 12.5 mg L^{-1}) pH: 6.8] (Rippka et al., 1979) at 25 °C with light/dark cycle of 12/12 h using an incubator shaker (MINITRON), for 20 days. The agitation of the incubator shaker was 100 rpm during incubation. The intensity of light employed during the light period was 3000 lux.

2.2. 16S rRNA-based identification of cyanobacterial isolates

Genomic DNA extraction was done with the DNeasy® Blood & Tissue Kit (Cat. No.: 69504, QIAGEN). Cyanobacterial 16S rRNA gene sequences were amplified using cyanobacteria-specific primers as previously described (Nubel et al., 1997; Thacker and Starnes, 2003): 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 by Nubel et al. (1997). Sizes of the amplified fragment for CYA106F-CYA781R and BACF-BACR primers were 640 bp and 629 bp, respectively. The sequencing process was performed by the REFGEN process (Ankara, Turkey), and the sequences obtained were searched against The Gen Bank DNA database using the blast function.

2.3. Cr(VI) toxicity

Solutions of different metal concentrations were prepared by dissolving $K_2Cr_2O_7$ salt (Merck) in distilled water to produce metal concentrations of 5, 15, and 35 ppm. Cr(VI) solutions were sterilized by filtration 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) resistance of cyanobacterial cultures was investigated by determining chlorophyll-a (Hirschberg and Chamovitz, 1994) every 48 h, for a period of 12 days. Also, flasks containing medium without Cr(VI) were inoculated in the same manner to serve as controls. Cr(VI) resistance was evaluated by comparison with the control. The main values and the standard deviation were calculated from the data obtained with duplicate trials.

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

2.4. Cr(VI) removal

The removal of chromium(VI) by *Synechocystis* sp. BASO670 and BASO672 was evaluated using a modified method described by Matsunaga et al. (1999). Isolates (OD_{664}, 2.5) were exposed to 10 ppm Cr(VI) for 7 days in BG11 medium at 25 °C with 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 Cr(VI) were assayed for 0–7 daytime intervals. Cr(VI) removal was determined as Cr(VI) in the medium, Cr(VI) adsorbed on the surfaces of the cells, and Cr(VI) accumulated in the cells. The concentration of Cr(VI) was measured by atomic absorption spectrophotometer (AA-6600, Shimadzu). The chromium removal rate (%) was calculated as follows: (amount of removed Cr)/(amount of initial Cr) × 100.

Samples were centrifuged (10,000 rpm) and residual Cr(VI) in the medium was determined from the supernatant. The pellet was further washed with 1 ml of 10 mM EDTA solution for desorption of Cr(VI) from the cell surfaces and centrifuged (10,000 rpm) once again. Cr(VI) adsorbed onto the cell surfaces was determined from this supernatant. The amount of intracellular accumulation of Cr(VI) was determined by measuring the Cr(VI) 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 characterisation of exopolysaccharide (EPS)

EPS was extracted by the modified procedure of Cérantola et al. (2000). After 20 days of cultivation, cells were harvested at room temperature by centrifugation at 10,000 rpm for 10 min. Supernatant was removed. Next, the pellet was dissolved in 1 mL deionised distilled water, and boiled for 15 min at 100 °C. The solution was then stored at room temperature for 10 min and after which 3 μL of 85% TCA was added. The mixture was then centrifuged at 10,000 rpm for 30 min. The supernatant, which contained EPS, was pooled and an equal volume of ethanol added. The mixture was stored at 4 °C overnight and centrifuged at 10,000 rpm for 30 min again. The precipitate was then washed two times with 96% ethanol and centrifuged at 10,000 rpm for 30 min. The final precipitate was dissolved in a 1 ml deionised distilled water and stored at −20 °C. Total carbohydrate contents of the EPS samples were determined by the method of Dubois et al. (1956) using glucose as a reference standard (Torino et al., 2001). The main values were calculated from the data obtained with duplicate trials.

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

2.6. 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 vortexed, and heated in a boiling water bath for 20 min. After cooling the solution 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-Glucronic acid was used as standard solution for comparison (Filisetti-Cozzi and Carpita, 1991).