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Investigation of Exopolysaccharide Production Capacities of Cyanobacterial Strains Isolated from Lake Uluabat, Turkey

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ABSTRACT

Exopolysaccharides (EPS), important metabolites of microalgae and cyanobacteria, can be used as food additives, drug active substances, and in detergents, adhesives and waste water treatment processes. Cyanobacterial EPSs are divided into two groups: the first group includes EPSs which are associated with the cell membrane (capsular, sheath); while the EPSs in the second group are released into the culture medium. Cyanobacterial EPSs are heteropolymer structures consisting of monosaccharides, lipids, proteins and DNA. In this study, chemical characterization and metal binding properties of EPSs from 3 different cyanobacteria (*Cyanobium* sp., *Anabaena* sp. and *Limnothrix* sp.) were investigated. Total protein concentrations ranged between 98.8 mg g-1 and 171.6 mg g-1 and total carbohydrate concentrations ranged between 252.1 mg g-1 and 320.5 mg g-1 dry weight of the cyanobacterial EPS prepared in the study. Fourier-transform infrared spectroscopy (FTIR) analysis results suggested that EPS obtained from these cyanobacteria contained peptides, sulfate groups and uronic acids. Elemental compositions and metal (Cu+2, Cr+6 and Ni+2) adsorption properties of EPSs were determined via Scanning Electron Microscopy/Energy Dispersive Spectroscopy (SEM/EDS) analysis. According to elemental analysis EPS molecules mainly contained carbon (35.63%- 65.12%) and oxygen (21.39%- 40.27%).

K E Y W O R D S: Lake Uluabat, cyanobacterial exopolysaccharide, protein, carbohydrate, metal adsorption.

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1. Introduction

Cyanobacteria are prokaryotic organisms which generally live in freshwater and marine environments (Bhunia et al., 2018). Some species can produce large amounts of exopolysaccharides (EPS) (De Philippis et al., 2011). In stress conditions, these microorganisms secrete large amounts of EPSs, which protect them from adverse environmental conditions such as droughts (Angelis et al., 2012). Apart from their ecological significance for the producing cyanobacteria; cyanobacterial EPSs have potential biotechnological applications such as to be used as biopolymers (De Philippis et al., 1998). In spite of numerous sources of polysaccharides, the world market generally obtains these molecules from higher plants and algae (Donot et al., 2012). However, cyanobacteria may be sources of novel EPSs and culturing cyanobacteria for EPS production might be a more sustainable approach. For this reason, bioprospecting for new EPS producing cyanobacteria and characterization of their EPSs are ongoing research efforts (Garlapati et al., 2019).

Cyanobacterial EPSs can be classified into three types based on their structural properties: (1) Sheath, a thin external layer structure adjacent to the outer membrane. (2) Capsular or slime polysaccharide (CPS), more external unstructured zones. The capsular forms of polysaccharide strongly bond with the cell membrane, while the slime forms create a weak interactive bond with the membrane. (3) Released polysaccharide (RPS), a type of exopolysaccharide which can be released into the media by the microorganism (Li et al., 2001). These EPSs are usually composed of monosaccharides and some non-carbohydrate structures (such as pyruvate, succinate, phosphate, and acetate) (Nicolaus et al., 2010).

Exopolysaccharides may be in the form of homopolysaccharides or heteropolysaccharides. Homopolysaccharides are composed of a single type of monosaccharide, while heteropolysaccharides are composed of different types of monosaccharide structures (Paniagua-Michel et al., 2014). Cyanobacterial EPSs are generally heteropolysaccharides containing different neutral sugars like mannose, glucose, arabinose, fucose, rhamnose and xylose (Ohki et al., 2014). They generally show anionic character due to their uronic acid contents (Klock et al., 2007). Since EPSs are formed by molecules capable of adsorbing and retaining water, such as uronic acid, they are recommended for use in the cosmetic industry for this purpose (Morone et al., 2019). On the other hand, cyanobacterial EPSs have both hydrophobic and hydrophilic groups and because of these properties they can be used as bioflocculants (Khattar et al., 2010). Cyanobacterial EPSs have specific properties compared to other microbial polysaccharides: They (i) usually consist of 6-10 different monomers, (ii) exhibit high hydrophobic properties due to their peptidic moieties, deoxysugars and acetyl group contents, (iii) have anionic properties due to their sulfate groups and uronic acid contents (Mota et al., 2013).

Aims of this work were: (i) to investigate the EPS production potential of three cyanobacterial strains isolated from Lake Uluabat, Turkey (ii) to determine basic chemical and biochemical contents of these EPSs, and (iii) to investigate the metal binding properties and elemental compositions of isolated cyanobacterial EPSs with Scanning Electron Microscopy/Energy Dispersive Spectroscopy (SEM-EDS) analysis.

2. Materials and Methods

2.1. Cyanobacteria cultures

Three different cyanobacteria species were tested for their EPS production. These strains were isolated in 2015 and 2016 from Lake Uluabat, Turkey, using the isolation procedures described before (Yilmaz et al., 2018). Species identifications were performed according to Komarek and Anagnostidis (1989, 1998 and 2005). While *Limnothrix sp.* and *Cyanobium sp.* were cultured in BG-11 medium (Stanier et al., 1971), *Anabaena sp*. were cultured in BG-11 without a nitrogen source (BG-11-N). Strains were cultured at 25 °C and at 100 µE. $m^{-2}s^{-1}$ light intensity (12 h:12 h light:dark photoperiod) for about 5 months.

Exopolysaccharide isolation

Late stationary phase cyanobacteria cultures were harvested by centrifugation at 3900 ×*g* for 20 min. Supernatants were separated from cell pellets for released EPS isolations. One volume of culture medium was mixed with three volumes of absolute ethanol and then vortexed for 30 s. The mixture was kept overnight at 4 ℃ (Mandal et al., 2011). For the precipitation of EPS, samples were centrifuged at 3900 ×*g* for 20 min. The supernatants were discarded and the precipitated EPS samples were collected and freeze-dried in a TRP-6 freeze dryer (Teknosem, Turkey). Freeze-dried EPS samples were stored at -80 °C until analyses.

2.2. Chemical characterization of EPS samples

Functional group determinations of EPS samples were performed by Spectrum Two fourier transform infrared (FTIR) spectrometer (PerkinElmer, Massachusetts, U.S.A). FT-IR transmittance was measured in a spectral range between 400-4000 cm^{-1} with a data resolution of 4 cm^{-1} (Bhunia et al., 2018). Lowry method was used for total protein determinations of crude cyanobacterial EPS samples (Barbarino and Lourenço, 2005) where bovine serum albumin was used as the standard (Waterborg and Matthews, 1984). Phenol-sulfuric acid method was used for carbohydrate determination of EPS samples (Masuko et al., 2005), with D-glucose used as the standard. All measurements were performed with 3 repetitions.

2.3. Metal Binding, SEM and EDS analyses

To determine the metal binding properties of freeze-dried cyanobacterial EPS samples, aqueous solutions of EPS samples (7.5 mg mL^{-1}) were mixed with a stock metal solution including Cu^{+2}, Ni^{+2} and Cr^{+6} ions. Metal ions were dissolved in ultrapure Milli-Q water (Millipore, Germany) and the concentration of each metal ion in the stock solution was adjusted to 1 mg mL^{-1}. Then the stock solution was diluted 100-fold and 1.5 mL of the diluted metal solution was mixed with 1.5 mL of aqueous EPS solution (Sathiyanarayanan et al., 2016). EPS and metal solution mixtures were stirred at 200 rpm for 19 hours at room temperature on a PSU-20i multi–functional orbital shaker (Biosan, England). At the end of the incubation period, 3 volumes of ethanol were added onto 1 volume of EPS/metal mixture. The mixture was kept overnight at 4 °C. Then the mixture was centrifuged at 4200 ×*g* for 20 min; pellet was separated for freeze-drying and SEM analysis. Before SEM imaging, samples were coated with a thin Au-Pd layer (2-5 nm). Scanning electron microscopy (SEM) of freeze-dried EPS samples was performed using a Zeiss Gemini SEM 300 (Carl Zeiss, Oberkochen, Germany). Elemental composition of EPS samples and bound metals to EPSs were determined with EDS analysis using ZEISS/Gemini SEM 300 with a Bruker X-Flash 100 detector.

3. RESULTS

The functional groups of three different cyanobacterial EPSs were analyzed by fourier transform infrared spectroscopy (FT-IR) (Figure 1). The peak assignments observed in all three cyanobacterial EPSs were as follows: 3300 cm^{-1} was related to the vibration of the hydroxyl (O-H) groups of glucose moieties or amine groups (-NH) of some peptides or proteins (Mota et al., 2013; Parikh and Madamwar, 2006; Singh et al., 2016). The signal at 1640 cm^{-1} was due to the carboxylate (–COOH) group, formed in the presence of uronic acid (Richert et al., 2005). The signal at 1030-1080 cm^{-1} was due to the vibration of the C-H bond. Similar peaks were found in *Limnothrix sp.* and *Anabaena sp.* EPS samples in the range of 1380 cm^{-1} to 1540 cm^{-1} (Figure1A, 1B), while no peaks were observed in this region in the *Cyanobium sp.* EPS sample (Figure 1C). Peaks at 1380-1400 cm^{-1} were due to the vibration of the C-O bond and peaks at 1500-1540 cm^{-1} were due to the vibration of the C-C bond in aromatic compounds (Figure 1A, 1B). The signal at 1240 cm^{-1} was due to the sulphate group (Figure 1A, 1B), which was not observed in the *Cyanobium sp.* EPS sample (Yim et al., 2004) (Figure 1C). The signal at 610 cm^{-1} was due to the linkages between various monosaccharide units observed only in *Cyanobium* sp. EPS (Figure 1C) (Khattar et al., 2010).

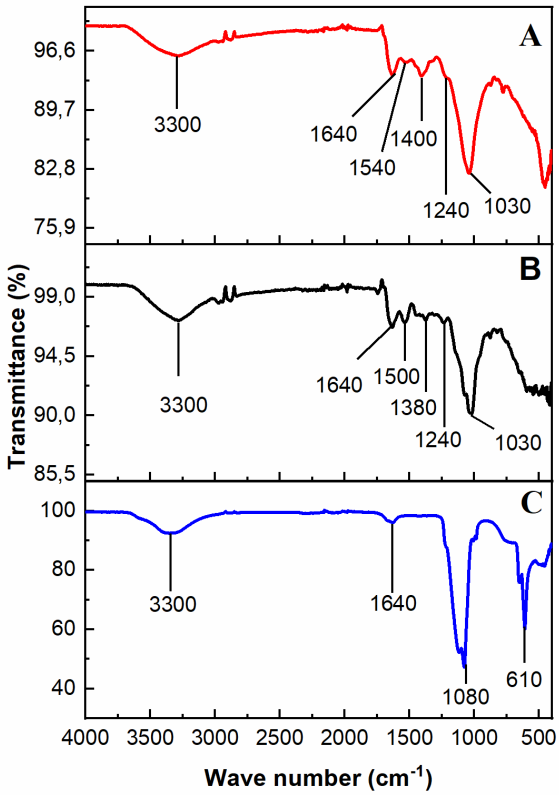

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Figure 1. FTIR spectra of cyanobacterial EPS samples. *Limnothrix sp.* EPS (A), *Anabaena sp.* EPS (B), *Cyanobium sp.* EPS (C)

Total protein contents of cyanobacterial EPS samples were between 98.8 mg g^{-1} dw and 171.6 mg g^{-1} dw. *Cyanobium sp.* EPS had the highest protein content with 171.6 ± 4.7 mg g^{-1} dw. *Anabaena sp.* EPS had the lowest protein content with 98.8 ± 17.5 mg g^{-1} dw (Table 1). Carbohydrate content of the EPS samples varied between 252.1 ± 51.8 mg g^{-1} dw (*Anabaena sp.)* and 320.5 ± 98.6 mg g^{-1} dw (*Limnothrix sp.*) (Table 1).

Table 1: Total protein and carbohydrate amounts of EPS samples. Dw, dry weight. Each value represents mean ± standard deviation (n=3).

Cyanobacteria	Protein (mg g^{-1} dw)	Carbohydrate (mg g^{-1} dw)
Limnothrix sp.	114.63 ± 37.9	252.1 ± 51.8
Cyanobium sp.	171.6 ± 4.7	284.8 ± 31.7
Anabaena sp.	98.8 ± 17.5	320.5 ± 98.6

Cyanobacterial EPS samples showed varying morphologies in SEM images (Figure 2). The elemental composition of EPS samples was demonstrated with SEM-EDS analysis (Figure 2, Table 2). Exopolysaccharides mainly consisted carbon and oxygen. Carbon content of EPS samples ranged between 35.92% and 65.12% (Table 2). Their oxygen content ranged between 21.39% and 40.27%. In addition, small amounts of sodium (Na), sulfur (S) and calcium (Ca) were detected in all three cyanobacterial EPS samples. Phosphorus (P) and potassium (K) were detected in EPS samples of *Limnothrix sp*. and *Anabaena sp.* Contrary to this situation, nitrogen (N) and fluorine (F) were not detected in *Limnothrix sp.* and *Anabaena sp.* EPS samples, but detected in *Cyanobium sp.* EPS. Silicon (Si) was detected in *Limnothrix sp.* and *Cyanobium sp.* EPS, while it was not detected in *Anabaena sp*. EPS. Chlorine (Cl) was detected only in *Anabaena sp*. EPS (Table 2). Chromium (Cr), nickel (Ni) and copper (Cu) detected in EPS samples of all cyanobacteria confirmed the binding of these metals onto the EPS surfaces (Figure 2, Table 2).

Table 2: Elemental compositions of cyanobacterial EPS samples obtained by EDS analysis. Wt% denotes percentage by weight. – denotes undetected elements.

Elements (Wt%)	*Limnothrix* sp. EPS	*Anabaena* sp. EPS	*Cyanobium* sp. EPS
C	35.92	65.12	35.63
O	36.07	21.39	40.27
Na	4.78	3.26	1.07
P	1.16	0.91	-
S	0.06	0.86	0.38
N	-	-	7.07
Si	19.69	-	13.3
K	0.53	0.85	-
Ca	1.18	3.92	1.01
F	-	-	0.52
Cl	-	2.53	-
Ni	0.12	0.12	0.26
Cu	0.33	0.56	0.28
Cr	0.15	0.46	0.2

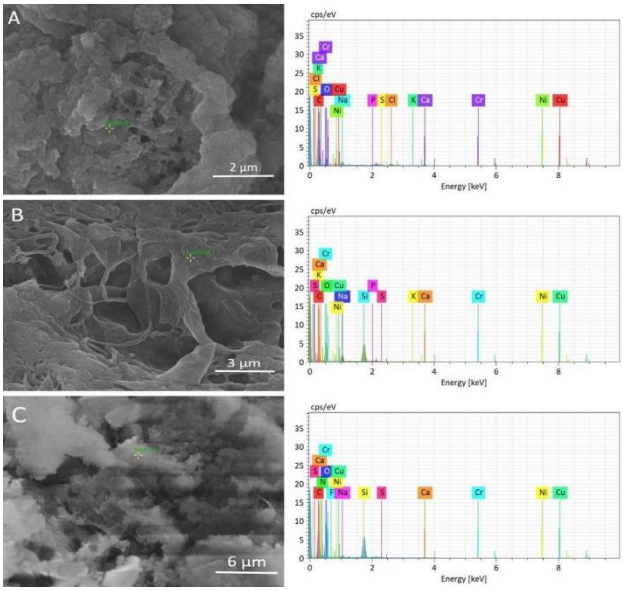

Figure 2. SEM images and EDS spectrum of EPS samples. *Limnothrix sp.* EPS (A), *Anabaena sp.* EPS (B), *Cyanobium sp.* EPS (C) keV: accelerating voltage range used for EDS analysis, kilo-electron-volt. cps/eV: counts per second per electron-volt.

4. Discussion

The amount of protein contained in EPS may affect the long-term stability of the biofilm structure formed by the microorganism and provides hydrophobicity and adhesive capacity to the polysaccharides (Kawaguchi and Decho, 2000). The protein content of EPS obtained from 3 cyanobacteria used in the study was slightly higher than the values in the literature (Kawaguchi and Decho, 2000; Angelis et al., 2012; Mota et al., 2013), which might be due to the extra purification stages used in other studies (Delattre et al., 2016; Klock et al., 2007; Mota et al., 2013).

In this study, the carbohydrate contents of the analyzed EPS samples were moderate according to the literature values (Tiwari et al., 2015; Kawaguchi and Decho, 2000; Angelis et al., 2012). Previous studies demonstrated that factors such as photoperiod, nutrient limitation, and the growth phase of harvested cells affected EPS production capacity in microorganisms (Khattar et al., 2010; Angelis et al., 2012; Arias et al., 2003; Singh et al., 2019). Although the current study did not aim to optimize EPS production in these 3 cyanobacteria species, future studies will focus on optimization of EPS production in the examined species.

FTIR analysis demonstrated the presence of sulfhydryl, phosphoryl, hydroxyl, carboxyl and amino functional groups in the EPS samples (Figure 1). Some absorption bands due to the aromatic compounds (1380-1540 cm^{-1}) and the sulphate group (1240 cm^{-1}) were not detected in EPS obtained from *Cyanobium sp.* Some researchers have determined the peptidyl moieties in the EPS by measuring the amount of N by elemental analysis (Khattar et al., 2010). EPS obtained from *Cyanobium sp.* showed higher protein content than other species with 171.6 mg g^{-1} of dw. Elemental analysis with SEM-EDS of EPS of *Cyanobium sp.* also showed the presence of 7.07% of N (Table 2). These results supported the presence of larger protein contamination in the EPS of *Cyanobium sp.* In general, FTIR analysis findings suggested that EPS obtained from *Limnothrix sp*., *Cyanobium sp.* and *Anabaena sp.* contained peptides, sulfate groups, and uronic acid.

EPS molecules mainly have an anionic character due to the presence of sulfate groups and uronic acids (Mota et al., 2016). Because of their anionic character EPS molecules can bind metal ions. The metal binding properties of EPS samples in this study were shown by detecting metal ions (Cu^{+2}, Cr^{+6}, Ni^{+2}) on the EPS surfaces with SEM-EDS. SEM-EDS analysis also demonstrated that EPS

molecules contain large amounts of carbon (35.63% - 65.12%) and oxygen (21.39% -40.27%) as previously reported (Kanamarlapudi et al., 2017). In addition to these major elements, some minor elements were also detected in EDS analysis such as Na, P, S, N, Si, K, Ca, F, and Cl. Some of these elements (i.e. Na, P, S, N, Cl, K) were higher than those reported in previous studies (Mota et al., 2016). The reason for the higher rate of these ions on the EPS surfaces could be explained by the fact that a pretreatment was not applied on EPS samples in this study. It was previously suggested that pretreatments with HCl or NaOH could remove ions such as Na and Ca bound on the polysaccharide surface. Pretreatments were also suggested to effect the metal binding capacity of EPSs by an ion exchange mechanism. Na^+ and H^+ ions change the cations coming from the culture medium, thus negatively charged binding sites of the polymer were discharged. Then in the metal binding process, Na^+ and H^+ ions were exchanged by the metal ions (Mota et al., 2016). In the SEM-EDS analysis of EPS samples in this study, Ni, Cu and Cr ions detected in the EPS samples showed that there were negatively charged groups on the surfaces of EPS molecules that bind with metal ions. Heavy metals are often present together in waste water, so the discovery of multiple metal adsorbing polymers is important. In future studies, the ability of these EPSs to adsorb to different metal combinations at different metal concentrations will be investigated.

5. Conclusion

Cyanobacterial EPSs are interesting microbial products that need further study of their structural and functional diversity. It is necessary to define the structural and functional properties of these polysaccharides to increase their usage potential in industry and biotechnology. For this purpose, studies on the detection of new cyanobacterial strains with high EPS production potential should be performed (Sun et al., 2012). For optimal EPS extraction with maximum efficiency, cell damage should be minimized and EPS structure should not be damaged (Frølund et al., 1996). The steps of obtaining and purification of EPS must be practical and easy to commercialize. In this study, chemical and biochemical characterizations of EPSs of three new cyanobacteria strains were performed. Results have shown that all 3 cyanobacteria can be used to obtain EPS. Especially due to the adsorption properties of metal ions, these EPSs can be used as bio-adsorbent in water treatment processes (Llamas et al., 2012).

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