Extracellular polysaccharides in twenty *Chlamydomonas* strains of the Mosonmagyaróvár Algal Culture Collection

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SUMMARY

Extracellular polysaccharides (EPS) are high-molecular-weight polymers of carbohydrates. Many microorganisms secrete extracellular polymeric substances during their life-cycle. Some species of the genus *Chlamydomonas* EHRENBERG nom. cons. (1833) and other green algae secrete EPS under specific conditions. For the first time, strains of the Mosonmagyaróvár Algal Culture Collection (MACC) were investigated for EPS production. In this study twenty *Chlamydomonas* strains were analyzed using the Phenol-Sulfuric Acid method (*DuBois et al.* 1956). Three strains produced more than 2 g/L and seven strains more than 1 g/L soluble EPS (sEPS). Strain MACC-398 was the highest sEPS producer (2763 mg/L) after 30 days of incubation. This study highlighted promising strains for application in soil conditioning.

Keywords: EPS, MACC, *Chlamydomonas*, green algae, soil algae, soil conditioning

INTRODUCTION

EPS

The term "EPS" was coined by *Sutherland* in 1972 to describe high-molecular-weight carbohydrate polymers produced by bacteria (*Costerton et al.* 1999). The formation of

biofilm or production of EPS supports the survival of bacteria and algae under extreme temperature, salinity, and nutrient availability (*Poli et al.* 2010). EPS perform a variety of functions and are involved in diverse biological processes, e.g. transport and transformation of organic matter, complexation of dissolved metals and biogeochemical cycling of elements. In addition, EPS are an important carbon source for different organisms in the food chain because they are rich in organic carbon. EPS are also involved in flocculation and the nature of EPS controls the floc formation rate, e.g. sulfates can generate flocs in the presence of deoxy sugars (*Zhou et al.* 1998, *Delattre et al.* 2016). Some of the useful properties include stabilizing, suspending, thickening, flocculating, encapsulating, emulsifying and water retention activities. Due to such properties, the polysaccharides have a wide range of industrial applications in textiles, pharmaceuticals, cosmetics and brewing (*Raza et al.* 2012). They are also an abundant source of structurally and compositionally diverse biopolymers which possess unique bioactivities for special high-value applications, specifically as antivirals, antitumor agents, antioxidants, anticoagulants and anti-inflammatories. Their superior rheological properties also make microalgal EPS particularly useful in mechanical engineering (e.g., biolubricants and drag-reducers) and in the food industry (e.g., thickener and preservatives, *Xiao and Zheng* 2016). Macroalgal polysaccharides, like agar, alginates and carrageenans, are economically the most important products from algae (*Pulz and Gross* 2004). Microbial exopolysaccharides (EPS) are also receiving increased attention because they are biodegradable, generally non-toxic and do not cause secondary pollution. Hence, there is great interest in screening algal strains for valuable compounds, including new polysaccharides that may compete with traditional polysaccharides. During the last decades selected MACC strains have been screened for their lipid productivity and fatty acid composition (*Ördög et al.* 2016), phytochemical content and pharmacological activities (*Aremu et al.* 2016), lipid, fatty acids and bioactivities *(Aremu et al.* 2015), and hormone profiles (*Stirk et al.* 2013), but no report about the EPS production of any MACC strains has been published till now. According to the available literature, *Chlamydomonas* species are promising subject to EPS related studies, thus the aim of the present study is to screen selected MACC *Chlamydomonas* strains for their EPS content.

Algal EPS for soil conditioning

Carbohydrates are common constituents and metabolic products of plants and living organisms. With respect to quantity and availability they are very important components of soil organic matter (*Safarik and Santruckova*1992). Algae are known to contribute to soil fertility through nitrogen fixation and to the stabilization of soils largely through the production of extracellularpolymers. Such polymers often occur as gelatinous sheaths or capsules enveloping the algal cells. The functional role may be to keep the cells glued into the uppermost layers of the soil, where they can continue to grow by photosynthesis or to be more resistant to lethal effects of desiccation than cells without capsules (*Barclay and Lewin* 1985). Besides, this material appears to promote aggregate formation, and may be important in ionic interactions with solid and liquid phases (*Martin* 1971). *Metting and Rayburn* (1983) reported empirical evidence in support of the use of microalgal conditioners. *Metting* (1986) also analysed the population dynamics of *Chlamydomonas sajao* R. A. LEWIN (1984) and its influence on soil aggregate stabilization in the field. *Kroen* (1984) studied the growth and polysaccharide production by *Chlamydomonas mexicana* R. A. LEWIN (1957) on soil as well.

Methods used for EPS determination

Delattre et al. (2016) differentiate four categories for quantitative analysis of EPS from microalgae. The first group summarises the global composition and includes methods such as total sugar (phenol sulphuric acid method), neutral sugar, uronic acid, polyphenol and protein measurement. These methods are cost-effective and reliable but attention should be drawn to other compounds, such as lipid, protein, and pigment, which can interfere with the analysis (*DuBois et al.* 1956). Today, the Phenol–Sulfuric Acid method of *DuBois et al.* (1956) is still considered the most reliable method for an accurate and less underestimated response of each monosaccharide in the sample. It has been extensively used in a wide range of fields (*Albalasmeh et al.* 2013). This method approximates a 'universal method' for determining all types of sugars, including carbohydrate residues of nucleic acids and other sugar derivatives (*Herbert et al.* 1971) and found to be best suited for total carbohydrate analysis because its molar absorptivities for most sugars fall within a short range (*Gerchakov and Hatcher* 1972).

Although the method detects almost all carbohydrates, the absorptivity of the different carbohydrates varies. Thus, unless a sample is known to contain only one carbohydrate, the results must be expressed arbitrarily in terms of one carbohydrate. This method was chosen for the present study as it is cost-effective.

The second group uses chromatographic methods such as HPLC (High-Performance Liquid Chromatography) and GC (Gas Chromatography) for estimating molar composition. They offer quick, automated and highly accurate methods to identify certain chemical components in a sample, but they can be costly, complex and do not work for all samples.

The third group requires strong chemical modification to carry out linkage analysis like NMR (Nuclear Magnetic Resonance spectroscopy). Its major advantage is that it is nondestructive in nature, quick and makes sample preparation easy. However, there are some disadvantages such as being expensive and having a limited wavelength range.

The last group is the full structural analysis which can be accomplished by modifications/degradations procedures and homo- and heteronuclear 2D NMR (Two-dimensional nuclear magnetic resonance spectroscopy). Detailed molecular structure can be expensive and time consuming but is a powerful method.

EPS in Chlamydomonas

Allard and Tazi (1993) studied two *Chlamydomonas* species by GC and according to their study, the EPS released by *Chlamydomonas augustae* SKUJA (1943) contained mainly arabinose, glucose and galactose at the beginning of growth, and glucose and glucuronic acid in the stationary phase. In sharp contrast, the EPS from *Chlamydomonas corrosa* PASCHER AND JAHODA (1928) was independent of growth status and contained arabinose and galactose as major sugars. *Lewin* (1956) examined eighteen unicellular or colonial green algae, mostly *Chlamydomonas* species by paper chromatographic method. In all species but one, galactose and arabinose were the main components of the polysaccharides. Glucose and xylose predominated in *Chlamydomonas ulvaensis* R.A. Lewin (1957). Associated sugar moieties included fucose, rhamnose, mannose, uronic acids and several unidentified components. *Jiang and Barber* (1975) determined the polysaccharide content from cell walls of *Chlamydomonas reinhardtii* using molecular sieve chromatography and they identified arabinose, mannose, galactose and

glucose in the sample. *Bafana* (2013) analyzed the antioxidant activity of EPS derived from *Chlamydomonas reinhardtii* P.A. DANGEARD (1888) by GC-MS. Chemical analysis showed the presence of galacturonic acid, ribose, arabinose, xylose, glucose, galactose and rhamnose sugars.

MATERIAL AND METHODS

Strains and growth conditions

Based on the characteristic of showing jelly-like units when grown in a Petri dish, twenty green algae strains were selected from the *Mosonmagyaróvár Algal Culture Collection (MACC),* Department of Plant Sciences, Faculty of Agricultural and Food Sciences (FAFS), Széchenyi István University. Twenty *Chlamydomonas* strains (*Table 1*) were cultivated as described earlier by Ördög (1982). Strains from stock cultures were inoculated into 500 mL Erlenmeyer flasks containing 250 mL modified Z8 medium (*Zehnder in Staub* 1961) at 24-26 °C under a light intensity of 130 µM m^{-2} s^{-1} provided by Lumoflor and cool white fluorescent tubes (14 h/10 h light/dark cycle). Cultures were aerated with 20 L h^{-1} (=1.33 L air L^{-1} nutrient medium per minute) sterile humidified air enriched with 1.5 % CO_2 during the light period. Harvesting was done at regular time intervals (every 5thday) until the 30th day. There were three replicates for each strain. Algal growth was measured by dry weight.

Biomass (dry weight) determination

Whatman GF/C glass fiber filters (5 cm diameter) were dried for 2 h at 105 °C and then cooled in a desiccator and weighed. After this, 10 mL algal sample was filtered. Each filterwas dried for 2 h at 105 °C again, cooled in desiccator, and weighed. The density of the suspension was calculated as g L^{-1} DW. The DW was used to construct growth curves. There were three replicates for each sample.

Table1. List of the tested strains including their taxonomic name (followed by taxonomic authority), origin and growth characteristics under laboratory conditions

MA CC	Taxonomic name	Origin	Growth characteristicsunder laboratory conditions
75	*Chlamydomonas subtilis* PRINGSHEIM (1930)	CCAO 249 (Trebon)	It sticks to the bottom of the flask and algae pieces float in it.
194	*Chlamydomonas gloeogama* KORSHIKOV IN PASCHER (1927)	Sunflower soil, Hungary	It does not stick to the flask but foams moderately. Homogeneous and very difficult to filter.
327	*Chlamydomonas sp.*	Soil, Brazil	It does not stick to the flask. Dark green and difficult to filter.
382	*Chlamydomonas sp.*	Soil, Brazil	Dark green. It does not stick to the wall of the flask. Slightly foaming. Easy to filter.
398	*Chlamydomonas sp.*	AL/G-23, Czech Republic	It foams a little bit. Homogeneous, does not stick to the flask. It is extremely difficult to filter, completely jelly.
402	*Chlamydomonas sp.*	Sunflower soil, Hungary	It does not stick to the flask. Dark green and easy to filter.
460	*Chlamydomonas sp.*	Puddle, Brazil	It is slightly foaming. Sticks moderately to the flask. It is difficult to filter. Dense syrup-like phase.
526	*Chlamydomonas sp.*	Soil, Brazil	It sticks to the bottom and the wall of the flask, as well. Pieces float in it. It has a yellowish green colour.
530	*Chlamydomonas sp.*	Soil, Brazil	Light green, slightly adheres to the wall of the flask and it is difficult to filter.
531	*Chlamydomonas sp.*	Soil, Brazil	Light green. It does not stick to the flask. It is difficult to filter.
544	*Chlamydomonas sp.*	Soil, Brazil	Light green, very difficult to filter.
549	*Chlamydomonas intermedia* CHODAT(1894)	Soil, Hungary	Dark green. It does not foam. Sticks to the flask and contains algae pieces clogged together. It is difficult to filter.
579	*Chlamydomonas sp.*	Soil, Hungary	Light green, does not foam and stick. Easy to filter.
674	*Chlamydomonas callunae* ETTL (1976)	Kiev, Ukraine	Light green, difficult to filter.
771	*Chlamydomonas sp.*	Soil, Brazil	Homogeneous, dark green. It does not stick to the flask. It does not foam. Filterability is moderate.
784	*Chlamydomonas reinhardtii* DANGEARD (1988)	Soil, Brazil	Homogeneous, light green. It adheres slightly to the flask. It is slightly foaming. Easy to filter.
788	*Chlamydomonas reinhardtii* DANGEARD (1988)	Soil, Brazil	Strongly adheres to the flask. It is difficult to filter. Light green.
806	*Chlamydomonas sp.*	Soil, Brazil	It sticks to the wall a little bit. Homogeneous, green. It strongly foams and difficult to filter.
825	*Chlamydomonas sp.*	Soil, Brazil	It sticks to the wall a little bit. Homogeneous, dark green. It strongly foams and difficult to filter.
835	*Chlamydomonas sp.*	Soil, Brazil	Dark green, very difficult to filter.

Extraction of EPS.

Carbohydrates were measured in two different fractions (*Figure 1*) using the phenol-sulphuric acid assay (*DuBois et al.* 1956). The differentiation of soluble and bound EPS was based on the *De Brouwer and Stal* method (2002).

Sampling was done at regular time intervals (every 5th day) until the 30th day. Soluble EPS was obtained by centrifuging 10 mL culture at 3500g for 15 min at room temperature. The cell free supernatant was transferred to a centrifuge tube containing 30 mL cold ethanol (75%), and the soluble EPS was allowed to precipitate overnight at –20 °C. After centrifugation (15 min at 3500g), the EPS pellet was dried under a flow of nitrogenand subsequently resuspended in 300 µL of distilled water (*Figure 1*). A volume of 200 µL was used for analysis of carbohydrate.

Bound EPS was extracted by resuspending the culture pellet in 2 mL distilled water. The cell suspension was thoroughly stirred and incubated for 1h at 30 °C. After centrifugation at 3500g for 15 min, the bound EPS was isolated from the supernatant and prepared for analysis as described above.
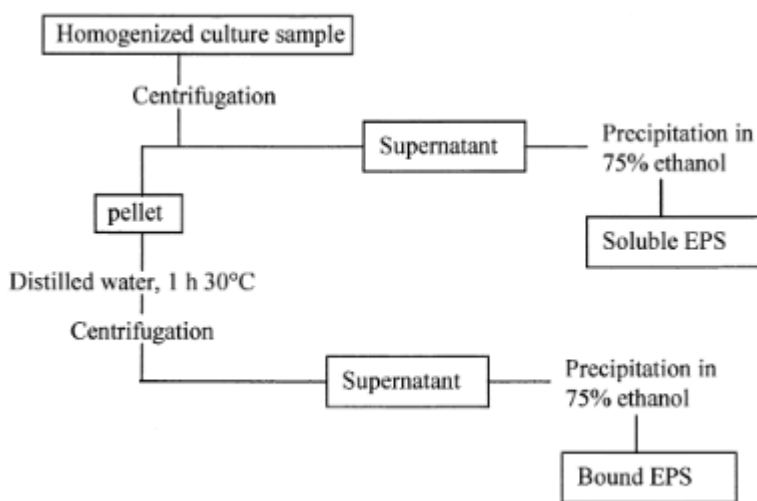

Figure 1. Flow chart of the fractionation of extracellular carbohydrate fractions by *DeBrouwer and Stal* (2002).

Carbohydrate estimation by phenol-sulphuric acid method

In this method, the concentrated sulfuric acid breaks down all kind of polysaccharides, oligosaccharides, and disaccharides to monosaccharides. Pentoses (5-carbon compounds) are then dehydrated to furfural, and hexoses (6-carbon compounds) to hydroxymethyl furfural. These compounds then react with phenol to produce a yellow-gold colour. Glucose is commonly used to create the standard curve and the absorption is measured at 485 nm (*DuBois et al.* 1956). The colour for this reaction is stable for several hours, and the accuracy of the method is within ±2% under optimum conditions (*Nielsen* 2010).

A 200 μg/mL stock of glucose was prepared and diluted to give five concentrations ranging from 0-200 μg/mL (*Moheimani et al.* 2013). Then 400 μL glucose solution was mixed with 400 μL 5% phenol and 2 ml conc. H_2SO_4. These solutions were kept at room temperature for 15-20 minutes. Then the absorbance was taken at 485 nm (Varian CARY 50 spectrophotometer). A standard curve of glucose was prepared using triplicate samples.

The various fractionated EPS extracts were dissolved in 1 mL autoclaved distilled water. New, sterile test tubes were taken for each sample. From the solution 400 μL was mixed with 400 μL 5% phenol and 2 ml conc. H_2SO_4. Then mixtures were kept at room temperature and finally the absorbance was measured at 485 nm. The carbohydrate was estimated as glucose equivalent by using the glucose standard curve. There were three replicates for each sample.

RESULTS AND DISCUSSION

a) Strain growth and biomass

Strains were grown for 30 days. Lag phase of strains typically lasted until day 5. This was followed by exponential growth with the stationary phasereached between 15 and 20 days. Death phase began on day 25. The most biomass was produced by strain MACC 460 (4840 mg/L) and the least by MACC 579 (576 mg/L) (*Table 2*). The majority of the MACC eukaryotic algae can produce 1 to 3 g/L dry matter under the experimental conditions (*Ördög* 2015). According to previous experiments, strains with a concentration of more than 3 g/L at the end of culture period were considered to be highly productive. Accordingly, of the 20 strains analysed, three strains (MACC 402,

771, 530) were highly productive and all were isolated from soil. Extremely high biomass (>4 g/L) was produced by 3 three strains (MACC 398, 460, 825), one of which was isolated from water. Seven strains were considered to be moderate producers as their biomass amount was above 2 g/L. There are other seven strains had biomass production under 2 g/L. Thirteen of the 20 strains were Brazilian microalgae.

b)   *sEPS and bEPS production of the strains*

According to their sEPS production, strains were divided into 3 groups: high (A), medium (B) and low (C) producers (*Table 2*). Selected strains from each group demonstrate the essential difference between the groups, the amount of sEPS and bEPS comparing with the biomass produced (*Figure 2-4*). *Figure 2* shows two high producers (MACC 398 and 460) whereas *Figure 3* demonstrates two medium producers (MACC 825 and 544) in light of the amount of sEPS and bEPS. With regard to the low producers, *Figure 4* illustrates two strains (MACC 530 and 75) with very low sEPS and bEPS results comparing them to the strains on the abovementioned two figures *(Figure 2 and 3)*. In addition, strains in Group A produced soluble extracellular polysaccharide above 2000 mg/L. This is a high value compared to other reports in the literature e.g. measured 1000 mg/L sEPS in *Chlorella vulgaris* BEYERINCK (1890) (De Angelis 2009); *Chlamydomonas mexicana* and *C. sajao* both produced approx. 600 mg/L sEPS (Barclay 1985); 632.6 mg/L EPS in *Chlamydomonas reinhardtii (Bafana 2013)*. *Chlamydomonas sp.* had 224 mg/L sEPS and 19 mg/L bEPS (Moore and Tischer 1964). Group B included 7 strains where the sEPS content ranged from 1000 to 2000 mg/L. The remaining 10 strains were placed in Group C because their sEPS content was under 1000 mg/L. Literature supports the fact that bEPS content is in connection with the production of sEPS, but the bEPS quantity is substantially less (*Moore and Tischer* 1964, *Yang et al.* 2009, *Borowitzka* 2016). This was also detectable in our experiments (*Table 2*). These are strain specific features that should be further investigated as they can play a significant role in differentiating strains as part of the so called polyphasic approach. It is also important that soluble EPS is continuously released into the medium. It is an important characteristic because EPS were found to be indistinguishable upon elution from ion-exchange and gel filtration columns (*Sijam et al.* 1983). In contrast, bound EPS is produced exclusively in the light and to a large

extent, degraded in the dark (*De Brouwer and Stal* 2002) which explains why its concentration is less than sEPS. In the present study, bEPS showed the highest values on the same days as sEPS.

c) *Relations between EPS production and growth characteristics*

Accumulation of biomass leads to an increased production rate of EPS. However, there is no direct correlation between the biomass concentration and the total content of the EPS (*Ni* 2013). All strains analysed in the present study produced EPS in different quantities (*Table 2*) regardless the amount of biomass produced. For instance, weaker biomass producing strains (e.g. MACC 531 – 1880 mg/L biomass and 1337 mg/L sEPS) could also reach the nearly 1400 mg/L sEPS production level as strains that produced significantly more biomass (e.g. MACC 402 – 3816 mg/L biomass and 1323 mg/L sEPS). In regard to adhesion (wall growth), based on previous studies (*De Philippis and Vincenzini* 1998, *van Rijssel et al.* 2000, *Pajdak-Stós et al.* 2001, *Thornton* 2002, *Mann and Wozniak* 2012) the presence of EPScontributes to the sticky nature, cell aggregation ability and adhesion to the wall of the Erlenmeyer flasks. Nine strains showed these characteristics (MACC 75, 460, 526, 530, 549, 784, 788, 806, 825). Most of these strains were in Group C which had lower biomass and EPS production ability. In case of high EPS and biomass producing strains, only MACC 460 showed wall growth. These data confirm that EPS production varies from strain to strain (*Streshinskaya et al.* 1967, *Takeda and Hirokawa* 1978), but hydrophobic cells of benthic species have greater strength of adhesion and attach to the glass faster than cells of planktonic species, which have a hydrophilic surface (*Ozkan and Berberogul* 2013).

Table 2. Summary of the biomass, soluble (sEPS) and bound (bEPS) extrapolysaccharides results grouped in three different categories

Categories	Strains	Biomass (mg/L - dry weight)	Day of testing (th)	sEPS (mg/L)	Day of testing (th)	bEPS (mg/L)	Day of testing (th)
A	MACC-398	4088	25	2763	30	1296	30
	MACC-460	4840	15	2701	25	822	25
	MACC-194	1452	20	2304	30	135	30
B	MACC-674	2197	15	1458	20	141	20
	MACC-835	2079	20	1395	30	248	30
	MACC-382	2103	20	1355	30	136	30
	MACC-531	1880	15	1337	30	121	30
	MACC-402	3816	25	1323	30	556	30
	MACC-327	2934	20	1177	30	520	30
	MACC-825	4803	30	1091	30	121	30
C	MACC-544	962	20	731	20	99	20
	MACC-788	2191	20	688	25	144	25
	MACC-784	1075	20	676	25	85	25
	MACC-549	1553	20	672	15	76	15
	MACC-806	1186	15	422	20	96	20
	MACC-771	3580	30	371	20	65	20
	MACC-526	2042	20	106	20	25	20
	MACC-530	3551	20	94	30	51	30
	MACC-75	2699	20	42	20	18	20
	MACC-579	576	10	22	15	4	15

(A= high; B=medium; and C= low sEPS producers). The highest values of the three parameters are presented and the day of testing. The strains are listed in decreasing order of the sEPS production.
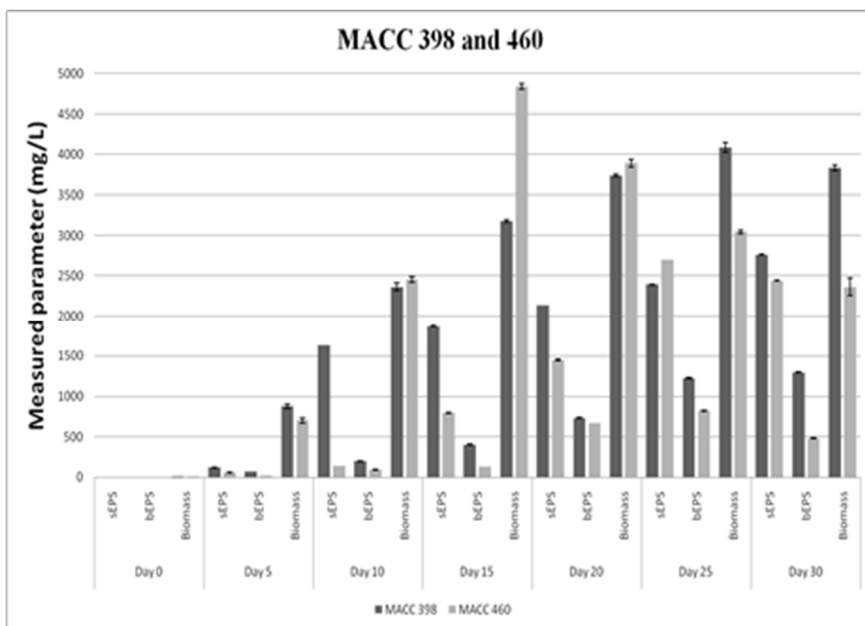
Figure 2. Biomass, soluble (sEPS) and bound (bEPS) production bar graphs of two high sEPS producing *Chlamydomonas* strains of the Mosonmagyaróvár Algal culture Collection (MACC), MACC 398 and 460.
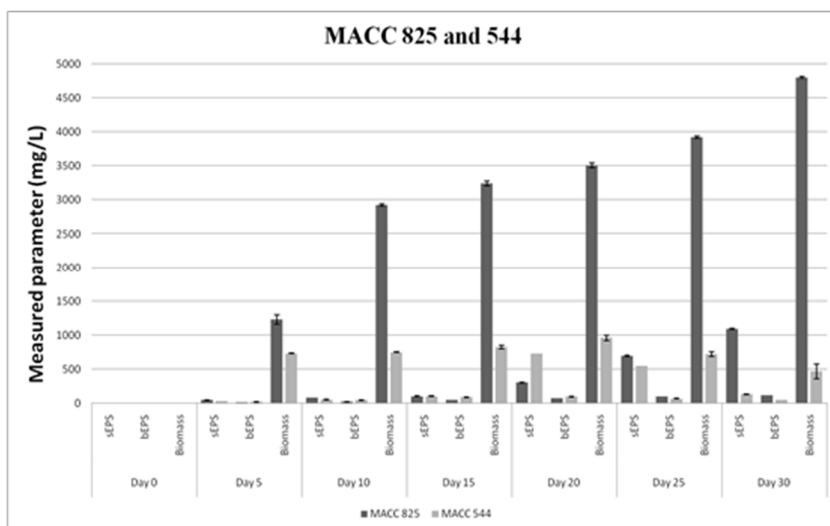

Figure 3. Biomass, soluble (sEPS) and bound (bEPS) production bar graphs of two moderate sEPS producing *Chlamydomonas* strains of the Mosonmagyaróvár Algal culture Collection (MACC), MACC 825 and 544
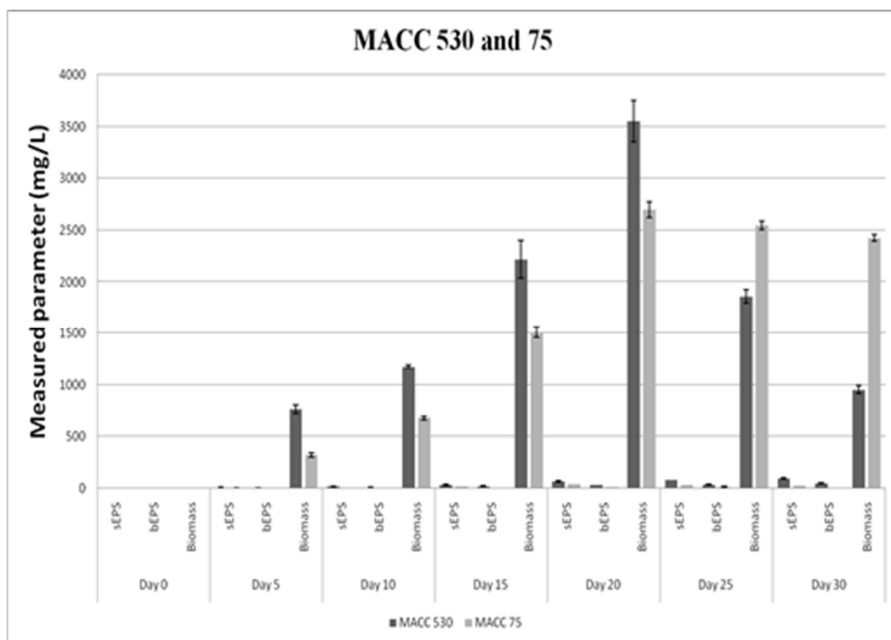
Figure 4. Biomass, soluble (sEPS) and bound (bEPS) production bar graphs of two low sEPS producing *Chlamydomonas* strains of the Mosonmagyaróvár Algal Culture Collection (MACC), MACC 530 and 75.

CONCLUSIONS

This is the first report of EPS production from MACC *Chlamydomonas* strains although there are few reports on this genus from other collections. Both sEPS and bEPS are useful by-products of microalgae. In the present study sEPS was always detected in higher concentrations than the bEPS. All strains produced EPS in different quantities regardless the amount of biomass produced. MACC 398 was the most productive sEPS and biomass producer among all tested strains. This work showed that the screened MACC *Chlamydomonas* strains were EPS producers. The potential use of these strains as source of biopolymers in the agriculture, food and medical industry may initiate further studies and field experiments. EPS production can also play a significant role in differentiating strains as part of the so called polyphasic approach which combines different methods such as morphology, cytology, ultrastructural, biochemical and molecular biological studies.

Extracelluláris poliszacharidok a Mosonmagyaróvári Algagyűjtemény húsz *Chlamydomonas* törzsében

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ÖSSZEFOGLALÁS

Az extracelluláris poliszacharidok (EPS) nagy molekulatömegű szénhidrát-polimerek. Számos mikroorganizmus bocsájt ki extracelluláris polimer anyagokat életciklusa alatt. A *Chlamydomonas* EHRENBERG nom. cons. (1833) nemzetség egyes fajai és más zöldalgák bizonyos körülmények között szintén képesek EPS-t kiválasztani. Az MACC (*Mosonmagyaróvári Algagyűjtemény*) törzseinek EPS termeléséről nem állnak rendelkezésre korábbi eredmények. Vizsgálatainkhoz az MACC 20 *Chlamydomonas* törzsét választottuk ki, amelyekből a kivont EPS mintát fenol-kénsavas módszerrel analizáltuk (*DuBois et al.* 1956). Hét törzs esetében több mint 1 g/L oldható EPS-tmértünk, három törzs tenyészetében pedig az oldható EPS mennyisége meghaladta a 2 g/L-t. A legtöbb EPS-t az MACC 398 törzs termelte (2763 mg/L) a 30 nap inkubálás során. Az eredményekkel igazoltuk, hogy az MACC *Chlamydomonas* törzsei között vannak értékes EPS-termelők, amelyek például talajkondicionáló készítmények alapanyagai lehetnek.

Kulcsszavak: extracelluláris poliszacharid (EPS), MACC, *Chlamydomonas* nemzetség, zöld algák, talaj algák, talajkondicionálás

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