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Extracellular Polysaccharides and Biomass Production in Cyanobacteria Isolated from Stone Monuments in Dry Zones of Mexico

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Abstract: Microorganisms develop in the form of cellular clusters known as biofilms. Biofilm's microbial cells are embedded in a matrix of extracellular polymeric substances (EPS). Cyanobacteria are photosynthetic prokaryotes that produce considerable amounts of EPS. This characteristic allows cyanobacteria to develop on aerial mineral surfaces, exposed to the atmosphere under low humidity conditions. Cyanobacteria biofilms can grow on rock-monuments surface affecting the stability of this type of Cultural Heritage. In this work we studied production and composition of EPS in relation to cell growth. At the first stage, a biofilm-forming cyanobacterium was isolated from rock-monument surface. Growth and EPS production kinetics were assessed during 42 days. Polysaccharide composition was determined by GC-EIMS. The stationary phase occurred between day 28 and day 42, the biomass concentration during this phase was 1.28 g/L of culture medium. During the stationary phase the EPS production was the 10% of the dry weight of culture. The 30% of these polymers are polysaccharides mainly composed by D-galactose, D-mannose, and D-glucose. The production peaks of released exopolysaccharides were at days 15 and 42. The production peak of cell envelope exopolysaccharides was at day 28. It was observed that growth is not proportional to the EPS production (RESP and CEPS), therefore the EPS production could be dependent on cell density.

Keywords: Cyanobacteria, Extracellular polysaccharides, Subaerial biofilm, GC-EIMS analysis

INTRODUCTION

In Nature, microorganisms can develop in the form of complex communities and are usually embedded in a matrix of EPS. This form of development is known as biofilm[1,2]. Biofilms that develop on mineral surfaces and are continuously exposed to the atmosphere are called subaerial biofilms (SABs). A SAB is composed of autotrophic and heterotrophic microorganisms[3].

Autotrophs are responsible for the carbon and nitrogen input to biofilms[4] while the heterotrophs are responsible for nutrient cycling[5]. Cyanobacteria and microcolonial melanized fungi are the most abundant groups of autotrophic and heterotrophic microorganisms in the SABs, respectively[3].

The SABs development starts with the mineral-surface colonization, and occurs in the presence of suitable environmental conditions. Autotrophs are the colonizer group of mineral surfaces. Heterotrophs organisms appear when the sufficient amount of autotrophic biomass has been synthesized[4-6]. Successful establishment of a SAB depends on EPS matrix synthesis. EPS have the function of support and attachment material for microbial cells, also serve as medium where nutrients and cellular secretions flow and concentrate. EPS provide important characteristics to biofilms, since they form a gel that creates a microenvironment with relatively constant conditions. This allows the SABs to resist processes like quick drying, long periods of drought, temperature fluctuations and elevated amounts of solar radiation[3,4,7,8]. These biofilms are ecologically important, since they occupy niches not taken by other organisms. Furthermore, SABs act as the main sources of carbon and nitrogen in ecosystems without plants, because some of the microorganisms that comprise them have the capacity to fix CO_2 and atmospheric N_2. Likewise, it has been reported that SABs can function as reservoirs of microorganisms, and when the appropriate conditions arise they are able to spread and colonize other niches[6,9].

In addition to its biological importance, recently many studies have been directed to assess their impact on the deterioration of the Cultural Heritage, especially in relation to the conservation of stone monuments. Estimations has shown that between 20 and 30% of monuments deterioration is due to biological activity[10]. Microbial metabolic activity is the main cause of biological deterioration or biodeterioration. Development of SABs on rock monuments surfaces affects its appearance decreasing its aesthetic. Metabolites of SABs microorganisms can alter the structure and composition of rock[11]. For example, EPS can alter the structure of rock, due to its high hygroscopic capacity and the increment of SABs volume could cause rock fractures. In addition, these polymers have affinity for calcium ions, thus it could extract these ions from the rock[7,11].

Cyanobacteria are a group of prokaryotes that carry out oxygenic photosynthesis and their primary photosynthetic pigments are chlorophyll-A and phycobiliproteins[12]. The establishment and successful development of biofilms depend on cyanobacteria, since they produce most of the EPS in SABs[4,13]. It has been described the impact of certain factors on EPS production, such as salinity, temperature and light, among others[14]. However, the mechanism of regulation of exopolymers synthesis is still unknown. It has not been clearly established the relation between biomass production and EPS production and composition.

In order to obtain information about the relationship of cell density with EPS synthesis, in this work it was assessed the biomass production, and composition and production of EPS by a strain of cyanobacteria. The cyanobacterium strain selected for this study was isolated from a SAB of the *Complex-A* or *"Casa de los Trece Cielos"* in the archaeological zone of *"Cañada de la Virgen"*. This

zone is located 30 km south-west from the municipality of San Miguel de Allende, in the State of Guanajuato, in central Mexico. This area has a semidry-temperate climate[15, 16].

MATERIAL AND METHODS

Cyanobacteria isolation: Non-destructive technique of sterile adhesive tape was applied to collect samples[17]. The enrichment, isolation and purification of microorganisms was conducted with the methodologies described by Waterbury[12], Benson[18], Ferris[19] and Stanier[20]. The criteria for the selection of the cyanobacterium strain was the capacity to form biofilms in axenic culture.

Morphological identification: The strain was identified by its morphological characteristics under the microscope[12,21-24].

Growth conditions and kinetics: The strain was grown in BG-11 liquid medium[12] with continuous agitation of 150 rpm and photoperiod of 16 h light / 8 h dark at 25 °C. Growth was evaluated during 42 days. Samples were taken every 7 days and processed independently by triplicate for each point. Growth was determined based on the biomass dry weight, measured as g/L of culture medium.

EPS extraction and production kinetics: Release-exopolysaccharides (REPS) production was evaluated according to the methodology proposed by Volk *et al.*[25] For the cell envelope-exopolysaccharides (CEPS) extraction, four extraction-solutions were tested: a) deionized water (≥ 16 MΩ); b) 1.71 M NaCl solution, c) 0.90 mM Tween 20 (v/v) solution; and d) 1.71 M NaCl (w/v), 0.9 0mM Tween 20 (v/v) solution. The extraction method described by Plude & Parker[26] was used, and tested for each extraction solution. To determine the effectiveness of each extraction solution, the area of EPS adhered to the cells was evaluated by staining with alcian blue[27]. The kinetics were obtained using the dry weight values of RESP and CEPS for each point measured.

EPS acid hydrolysis: Acid hydrolysis of the extracted-EPS was carried out to determine its composition[28]. EPS were hydrolyzed with 4 M hydrochloric acid in a ratio 1:20 (w/v), respectively. The mixture was incubated at 110 °C. Optimal time of hydrolysis was determined with a hydrolysis kinetics and the released-monosaccharides concentration was quantified by GC-EIMS. Once hydrolyzed, the solution was neutralized with 4 M NaOH. Finally, the solution was dried and the powder was stored at -20 °C until analysis. A standard curve of D-glucose was used for the quantification, $y = 3E^{-07}x + 3.4716$ and $R^2 = 997$.

EPS-monosaccharidic composition: For the hydrolyzed-polysaccharides analysis we proceeded to prepare trimethyl silylane derivatives according to Ruiz-Matute[28] and Walford[29]. Analysis was performed on a gas chromatograph (Agilent Technologies 7890A) coupled to a selective mass detector with ionization by electron impact (Hewlett Packard 5973). The initial oven temperature was 150 °C and hold for 3 minutes, then applied a temperature ramp of 4 °C per minute until it reaches 280 °C and was hold for 25 minutes. The injector temperature was 250 °C. A Zebron ZB-1MS (60 m x 320 um x 1 um) capillary column was used. The flow of helium was 1 mL/min. 1 µL of sample was injected. Monosaccharide identification was obtained by comparing the mass spectrum and retention time of the components of each sample with data of the National Institute of Standards and Technology Database using the *NIST MS Search 2.0 software*, and of the analyses of trimethyl silane derivatives of standards of D-arabinose, D-glucose, D-fructose, D-fucose, D-galactose, D-mannose, D-ribose, D-xylose, L-rhamnose

and galacturonic acid. The total sugar content was determined by the anthrone method. The protein content was measured by the ninhydrin method.

RESULTS AND DISCUSSIONS

Morphological identification and growth kinetics of the cyanobacterium strain: The strain under study presents morphological characteristics that correspond to the genus *Nostoc* (Figure 1A). This genus grouped filamentous cyanobacteria that can develop akinetes and heterocysts. The filaments do not show true branching. Cells are spherical or barrel-shaped and their width varies between the 3 and 6 μm[12, 24].

Nostoc sp. exponential growth phase extends from day 15 to day 28, stationary growth phase begins and continues until day 42 (Figure 3). The carrying capacity was 1.28 (sd 0.10) g/L. Maximum specific growth rate was 0.40 $days^{-1}$ (sd 0.01). The kinetic parameters were determined by modelling data with the software *SciDAVis* (http://scidavis.sourceforge.net/). The axenicity of *Nostoc sp.* was verified by culturing the strain in LB media (solid and liquid) and cell growth was monitored microscopically. Staining with safranin was conducted to evaluate the presence of heterotrophic bacteria, however only was noted one type of cell morphology (Figure 1B).

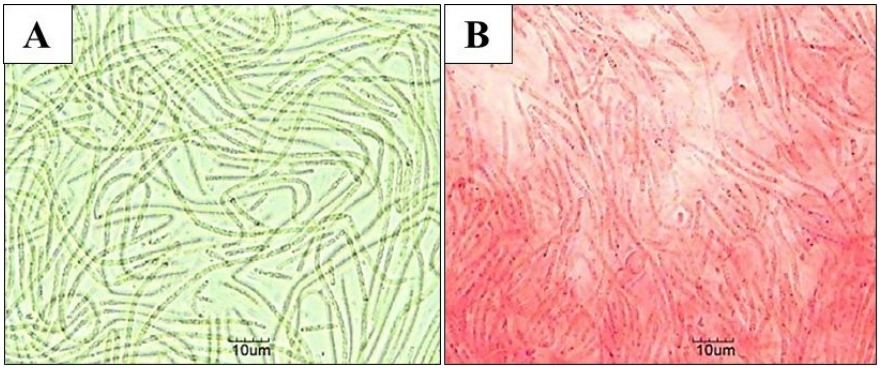

Fig.1: Optical microscopy of the isolated cianobacterial strain. A. Cellular filaments. B. Simple staining with safranin

EPS extraction: The most effective extraction solution of CEPS was the solution d (Table 1 and Figure 2). *Motic Images Plus 2.0* software was used to measure the CEPS-dyed area, alcian blue selectively binds to anionic polysaccharides, such as those of cyanobacteria[27]. Extraction percentages were calculated with respect to a sample without extraction treatment. We recommend perform tests for polymers-extraction of each strain to be studied, since due to their unique structural and compositional characteristics each solution can work different for each strain.

Table 1: Effectiveness of the CEPS extraction solutions

Extraction solution	Composition	Extraction percentage
a	Deionized water	2 (sd 0.4)
b	1.71 M NaCl	26 (sd 5.1)
c	0.9 mM Tween 20	57 (sd 3.9)
d	1.71 M NaCl, 0.9 mM Tween 20	85 (sd 4.2)

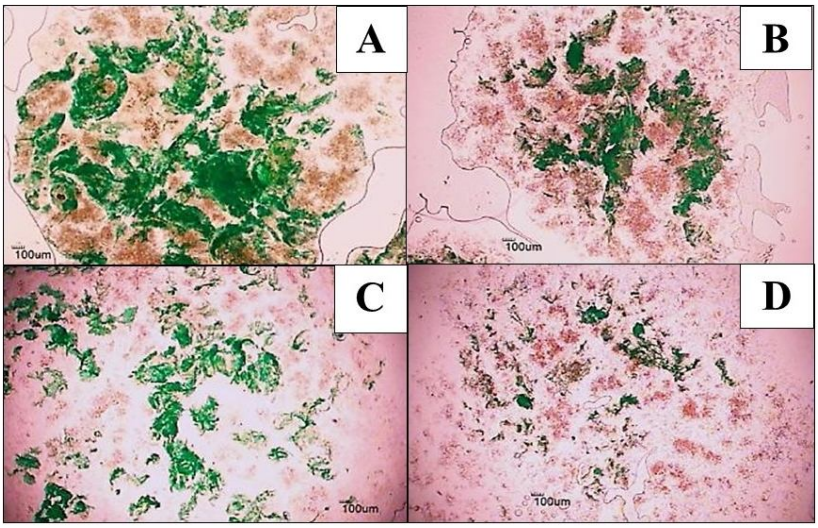

Fig.2: Evaluation of extraction solutions effectiveness by alcian blue staining. A. Deionized water, B. 10% NaCl solution, C. 0.01% Tween 20 solution, D. 10% NaCl, 0.01% Tween 20 solution. Blue-green area corresponds to stained polysaccharide and brown aggregates to cellular filaments. In D, cellular filaments appear scattered

EPS composition and production kinetics: Total cyanobacterium-mass production was calculated of the sum of the biomass (cell mass without EPS) and the CEPS and REPS. In the stationary phase, REPS and CEPS represented 10% of the total mass production. The 55% of REPS and 30% of CEPS were carbohydrates (measured as total sugar content). The protein content was 40% in REPS and 35% in CEPS. It has been reported that EPS represent between 50 and 90 % of the organic carbon of a biofilm[14]. However, De Philippis *et al*.[30] found cyanobacterium strains with a relatively low production of EPS (0.088-0.152 g/L). *Nostoc sp.* produced around 0.12 g/L in the stationary phase. It has been reported that the protein content in EPS generally is lower than 20%[14]. CEPS and REPS of this strain contain around 40% of protein. It has been reported that microbial EPS are composed mainly by polysaccharides, although they also contain considerable amounts of proteins and extracellular DNA[8].

Balanced growth is defined as cell growth where the macromolecular composition of microbial cells remains in the same proportion generation after generation. Balanced growth approaches to the exponential growth phase or to cell growth in a continuous culture. It is important to note that in the growth exponential phase the synthesis of development and reproduction metabolites occurs in constant relationship to biomass production. It is known that development-metabolites kinetics have the same behaviour as growth kinetics. Therefore, it is possible to estimate microbial growth by measuring the concentration of these metabolites, such as the protein content. The production kinetics of metabolites that are not directly related to the primary metabolism do not have a proportional distribution to the growth curve, so quantifying them is not useful for estimating microbial growth[31].

RESP production kinetics has a very variable slope with production peaks that correspond to days 15 and 42 of culture, while the production of CEPS has a peak at day 28. Production of CEPS was observable but not quantifiable (< 0.2 mg) for the days 7 and 15. Figure 4 shows the REPS and CEPS production kinetics.

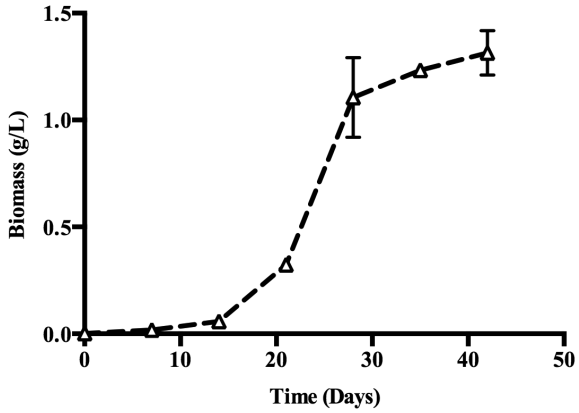

Fig.3: Growth curve of *Nostoc sp.* Exponential phase starts at day 15 and continues until day 28. Then, stationary phase starts.

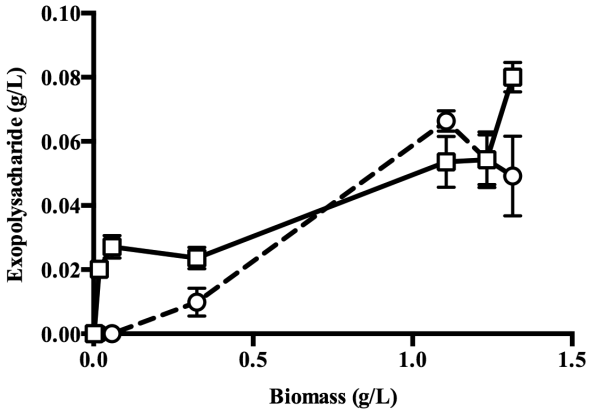

Fig.4: CEPS (- - o - -) and REPS (-□-) production by *Nostoc sp.*

If REPS and CEPS synthesis was regulated directly by primary metabolism it would be observed a trend proportional to the growth curve. Nevertheless, the growth and EPS production kinetics obtained in this work suggest that the regulation on the production of exopolysaccharides is by a different mechanism. Nonetheless, the production peaks of CEPS and REPS occur when a specific biomass is reached, which could mean that the synthesis of these polymers is regulated by a mechanism dependent on cell density. Something similar was reported by Zhu *et al.*[32] in some strains of the genus *Microcystis*. They observed that EPS production had a peak when the microorganism colonies reach a size of 150 µm. The variability of the EPS content - regarding the size of the colony - was attributed to the structural conformation of the polymer matrix, beyond a critical value of the EPS content. However, their data also could be explained by a phenomenon of cell density-dependent regulation, since the size of a colony is directly related to the number of cells within the colony. Usually, the bigger the colony, the bigger the cell content[31]. The effect on EPS content was observed in a specific range of size of colony, otherwise or seen, a specific cell density. Another similar case was observed by Zhai *et al.*[33] in a *Microcystis aeruginosa* strain, they reported that biofilm formation - which is closely related to the synthesis of EPS - is regulated

in a cell density dependent way, however their observations were only qualitative. In this paper we provide quantitative data that are consistent with previously published information.

It was observed that CEPS production on day 42 dramatically decreases while REPS production increases, this could be due to cell lysis that occurs at the end of microbial growth, since the cellular components solubilize in the culture medium[31].

The composition of the CEPS for each point of the production kinetics was constant, there were no differences in the proportion of monosaccharides (Figure 5). Similarly, it was not observed a change in the composition of the REPS (data not shown). Table 2 shows the CEPS and REPS monosaccharidic composition (data corresponds to the stationary phase of growth at day 28). The REPS had a higher proportion of D-glucose and galacturonic acid. They did not contain D-arabinose and amino-sugars, unlike the CEPS. These differences may relate to the function of each type of polymer. For example, a greater proportion of acid monosaccharides can make a more soluble polymer[14], which is consistent with the highest proportion of galacturonic acid in REPS. D-Glucose, D-mannose and D-galactose represent 90% of the monosaccharides in REPS and CEPS. The content of levoglucosan was used as a marker of thermal-degradation of monosaccharides during the acid hydrolysis, in all samples was detected under 2%. Figure 6 shows a chromatogram of CEPS at day 28, the main peaks correspond to most abundant monosaccharides.

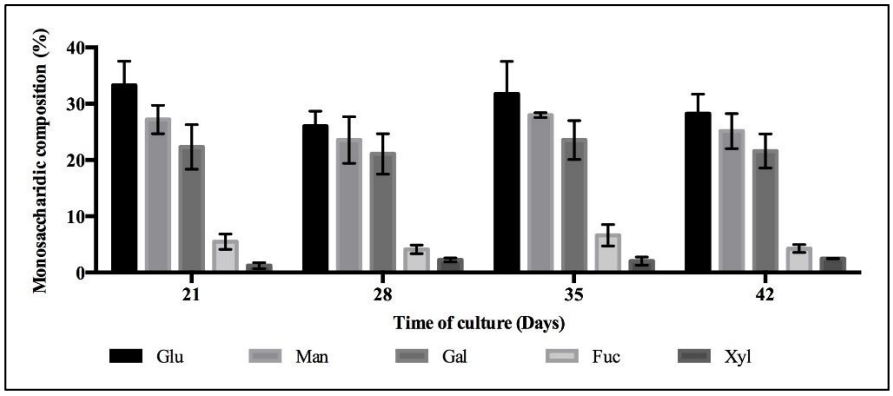

Fig.5: Composition of the CEPS during the culture period. Figure shows the five more abundant monosaccharides: D-glucose (Glu), D-mannose (Man), D-galactose (Gal), D-fucose (Fuc), D-xylose (Xyl). Composition is expressed as D-glucose-equivalents according the area of each component in the GC-EIMS chromatograms.

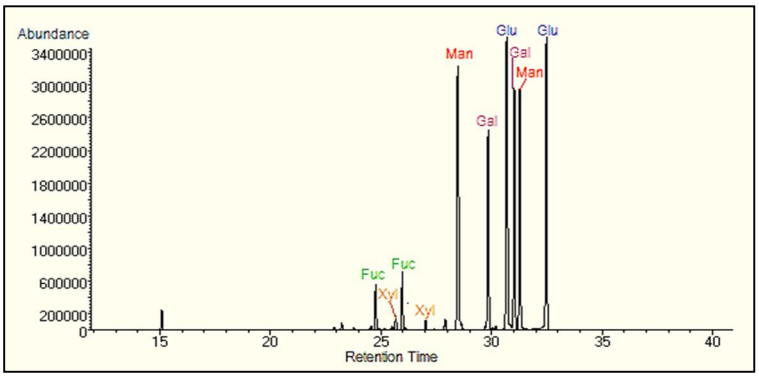

Fig.6: GC-EIMS chromatogram of CEPS at day 28. The main peaks correspond to the most abundant monosaccharides. D-glucose (Glu), D-mannose (Man), D-galactose (Gal), D-fucose (Fuc), D-xylose (Xyl)

Table 2: Monosaccharide composition of the CEPS and REPS

Monosaccharide	CEPS	REPS
	%	
D-Glucose	33.64	44.60
D-Mannose	29.91	15.34
D-Galactose	25.77	9.68
D-Fucose	5.28	5.67
D-Xylose	1.38	2.39
D-Arabinose	1.12	-
L-Rhamnose	0.50	8.46
Levoglucosan	0.10	1.39
Amino-Sugar	0.09	-
Galacturonic Acid	0.04	0.57
Unidentified	2.16	11.9

Unidentified components in the chromatograms of CEPS and REPS correspond to monosaccharides, since their mass spectrum have the characteristic m/z fragments of the trimethyl-silane derivatives of monosaccharides. Figure 7 shows a comparison between the mass spectra of a standard of D-glucose and an unidentified monosaccharide from our samples. The characteristic fragments of the monosaccharide trimethyl-silane derivatives have m/z = 204 and m/z = 217[29].

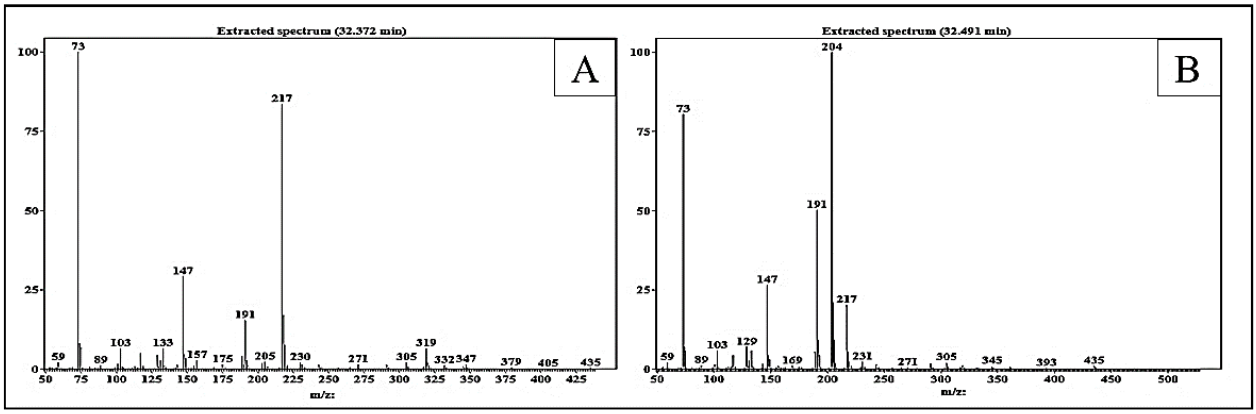

Fig. 7: Mass spectra of D-glucose (B) and of unidentified monosaccharide (A). The characteristic fragments of monosaccharide trimethyl silane derivatives have m/z = 204 and m/z = 217

CONCLUSIONS

An EPS producing cyanobacterium of the genus *Nostoc* was isolated from a SAB from a rock-monument surface. The extraction methodology of CEPS proposed in this work has a higher effectiveness than other methodologies before reported. This could be due that EPS of each strain has a unique composition and structure. Therefore, it is recommended the use of more than one extraction technique to find the most efficient for each specific case.

The exopolysaccharide production by *Nostoc sp.* is not proportional to the biomass production. EPS production peaks correspond to points within the exponential growth phase, at days 15, 28 and 42 of culture. Growth and EPS production kinetics obtained in this work suggest a type of cell density-dependent regulation, since during this phase the growth rate metabolic machinery and the nutrients uptake remains constants. Also, it was found that the composition of the REPS and CEPS is characteristic for each type of polymer and is constant throughout growth. D-glucose, D-mannose and D-galactose represent around 90% of monosaccharides in the REPS and CEPS.

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