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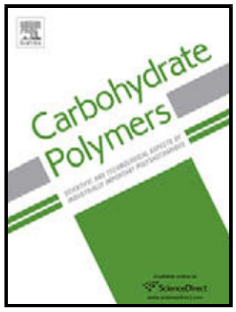

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Cyanoflan: a cyanobacterial sulfated carbohydrate polymer with emulsifying properties

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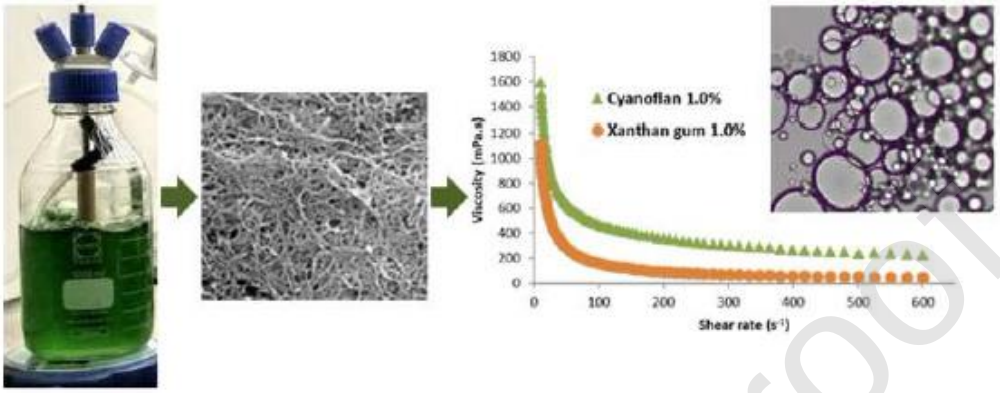
Graphical abstract



Highlights

- Cyanoflan a sulfated carbohydrate polymer from a marine cyanobacterium *Cyanothece* sp.
- Cyanoflan has a complex chemical structure with a high number of branching.
- Cyanoflan has high intrinsic viscosity and emulsifying activity in aqueous solutions.
- Cyanoflan is a promising emulsifying/thickening agent for industrial applications.

ABSTRACT

The extracellular polysaccharides produced by cyanobacteria have distinctive characteristics that make them promising for applications ranging from bioremediation to biomedicine. In this study, a sulfated polysaccharide produced by a marine cyanobacterial strain and named cyanoflan was characterized in terms of morphology, chemical composition, and rheological and emulsifying properties. Cyanoflan has a 71% carbohydrate content, with 11% of sulfated residues, while the protein account for 4% of dry weight. The glycosidic-substitution analysis revealed a highly branched complex chemical structure with a large number of sugar residues. The cyanoflan high molecular mass fractions (above 1 MDa) and entangled structure is consistent with its high apparent viscosity in aqueous solutions and high emulsifying activity. It showed to be a typical non-Newtonian fluid with pseudoplastic behavior. Altogether, these results confirm that cyanoflan is a versatile carbohydrate polymer that can be used in different biotechnological applications, such as emulsifying/thickening agent in food or cosmetic industries.

Abbreviations: Ara - Arabinose, Fuc - Fucose, Gal - Galactose, Glc - Glucose, Man - Mannose, Rha - Rhamnose, Xyl - Xylose

Keywords: Cyanobacteria, Cyanoflan, *Cyanothece*, Emulsification, Extracellular carbohydrate polymer

1. Introduction

Cyanobacterial extracellular polymeric substances (EPS) constitute a valuable alternative to the already commercially available polysaccharides of plant and algal origin, since these microorganisms have higher growth rates, minimal nutritional requirements and the carbohydrate polymers produced have very peculiar features, namely the high number of monomers, presence of sulphate groups, anionic character and amphiphilic behavior (Colica & De Philippis, 2014; Raposo, Morais, & Morais, 2015; Rossi & De Philippis, 2016). Cyanobacterial EPS can remain attached to the cell surface, as sheath, capsule or slime, or be released into the surrounding environment (released polysaccharides - RPS) (De Philippis & Vincenzini, 1998; Kehr & Dittmann, 2015; Pereira et al., 2009), being the released polymers particularly interesting to industrial applications due to the simplicity of the recovery process. Overall, the high structural complexity, the anionic amphiphilic nature and rheological behavior of the cyanobacterial EPS make them very promising for a variety of biotechnological applications (Bhunia et al., 2018; Raposo et al., 2015; Rossi & De Philippis, 2016). However, despite their highly acclaimed properties, the use of these EPS in industrial applications is still in its beginning compared to the use of polysaccharides from bacterial, algal or plant origin, such as alginate, xanthan gum, guar gum and dextran (Gomez d'Ayala, Malinconico, & Laurienzo, 2008; Moscovici, 2015; Rehm, 2010). This can be justified by the lack of systematic studies on the conditions that would allow yields optimization, and knowledge on the cyanobacterial EPS biosynthetic pathways that would allow its manipulation and the production of polymers with the desired characteristics (Delattre, Pierre, Laroche, & Michaud, 2016).

The marine unicellular cyanobacterium *Cyanothece* sp. CCY 0110 is among the most efficient cyanobacterial EPS producers and the majority of the produced carbohydrate polymer is released to the medium (RPS), expediting its isolation and purification (Mota et al., 2013). Moreover, the culture conditions that affects the production have been extensively studied and although the RPS production by this *Cyanothece´*s strain is mainly related to cell growth, high light intensities increase the yields. *Cyanothece*'s RPS is a complex macromolecule, composed by nine different monosaccharide residues including two uronic acids and sulfate groups. This polysaccharide contains also protein and is remarkably thermostable (Mota et al., 2013). Therefore, and as preliminary studies have shown, it has the versatility to be applied in different areas ranging from bioremediation to biomedicine; e.g. as a biosorbent for heavy metals bioremediation (Mota et al., 2016), as a vehicle for proteins or vitamins delivery (Estevinho et al., 2019; Leite et al., 2017; Mota, Tamagnini, Gales, Leite, & Pereira, 2018) and as anti-adhesive coatings (Costa et al., 2019). Moreover, the particular features of this carbohydrate polymer can be interesting for food and cosmetic industries, since similar polymers from other origins have been used as flocculants, thickening, hydrating, emulsifying and/or gelling agents, improving the product quality and texture. In addition, its use as stabilizer in paints and polishes, and in oil recovery can also be envisaged (Bhatnagar, Pareek, Ganguly, & Bhatnagar, 2012; Jindal, Singh, & Khattar, 2011; Khangembam, Tiwari, & Kalita, 2016). Being a natural polymer, it also present advantages over those of chemical synthetic origin, namely biodegradability, biocompatibility and low toxicity (Moscovici, 2015). However, commercial applicability largely depends on the physicochemical, emulsifying,

rheological and flocculating properties of the carbohydrate polymer (Han et al., 2014; Ozturk & Aslim, 2010). Therefore, the aim of this study was to further characterize *Cyanothece* sp. CCY 0110 released polysaccharide, here named "cyanoflan", in terms of glycosidic-linkage composition and surface morphology. In addition, the rheological and emulsifying properties of cyanoflan were evaluated to assess its potential in biotechnological applications, in particular for food and cosmetic industries.

2. Material and methods

2.1. Organism, culture conditions, and Cyanoflan isolation

The cyanobacterium *Cyanothece* sp. CCY 0110 (Culture Collection of Yerseke, The Netherlands, now available at Culture Collection of Algae and Protozoa - CCAP 1435/2) was grown in 1 L bioreactors (DWK Life Sciences, Germany) with ASNIII medium (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979), at 25 °C under a 16 h light (30 µE m^{-2} s^{-1}) / 8 h dark regimen with aeration (1.2 L min^{-1}).

Cells were grown until an optical density at 730 nm of approximately 3.5-4.5 and cyanoflan was isolated as previously described (Leite et al., 2017).

2.2. Determination of protein content

To determine the protein content, 5 mg of cyanoflan were dissolved in 1 mL of water, sonicated and successively diluted until a homogeneous solution was

obtained. The protein content was determined following the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.3. Carbohydrate composition

Neutral monosaccharides were determined as alditol acetates as previously described (Oliveira et al., 2017). Briefly, cyanoflan was hydrolyzed with 2 M H_2SO_4 at 120 °C for 1 h. Then the monosaccharides were reduced with sodium borohydride and acetylated by acetic anhydride using methylimidazole as a catalyst. 2-Deoxyglucose was used as internal standard. The alditol acetate derivatives were analyzed by gas chromatography with a 30 m capillary column DB-225 (J&W Scientific, Folsom, CA, USA), with internal diameter and film thickness of 0.25 mm and 0.15 µm, respectively, and using a flame ionization detector (GC-FID Clarus 400, Perkin Elmer, MA, USA).

To determine the uronic acids content, 3 mg of cyanoflan were hydrolyzed with 1 M H_2SO_4 at 100 °C for 1 h. Then, the uronic acids were determined according to a modification of the 3-phenylphenol colorimetric method as described previously (Coimbra, Delgadillo, Waldron, & Selvendran, 1996). A calibration curve was made with D-galacturonic acid (0-200 mg mL^{-1}). The analyses were performed in triplicate.

The sulfate content was determined using 5 mg of cyanoflan hydrolyzed with 2 mL of 2 M HCl for 5 h at 110 °C and the quantification was performed using the Sulfate Assay Kit (MAK 132, Sigma-Aldrich Co., MO, USA), according to the manufacturer's guidelines.

To determine the pyruvate groups, 2.5, 5 and 10 mg of cyanoflan were hydrolyzed with 2 mL of 1 M HCl for 3 h at 100 °C as described previously (Sloneker & Orentas, 1962). The quantification was performed using the Pyruvic Acid Assay Procedure (Megazyme, Ireland), according to the manufacturer's guidelines.

Five mg of cyanoflan were used to determine the acetate groups, starting by dissolution in 2.4 mL of water and sonication. The saponification occurred by the addition of 0.8 mL of 2 M NaOH, for 1 h at 25 °C, as described previously (Waldron & Selvendran, 1990), and the reaction was finished by the addition of 0.8 mL of 2 M HCl. Then the quantification was performed using the Acetic Acid Assay Procedure (Megazyme, Ireland), according to the manufacturer's guidelines.

2.4. Desulfation

Cyanoflan desulfation was performed as described previously (Oliveira et al., 2017). Briefly, 5 mg were dissolved in 1.8 mL of dimethyl sulfoxide. Then, 0.1 mL pyridine was added, followed by 6.5 mg of pyromellitic acid, 12 mg of NaF and 0.2 mL of pyridine. The solution was stirred at 120 °C for 3 h, cooled, poured into 1 mL of 3% of $NaHCO_3$ aqueous solution, dialyzed and freeze-dried. The procedure was repeated to assure the complete desulfation of the samples, as reported by Oliveira et al. (2017). Subsequently, the desulfated cyanoflan was submitted to methylation analysis.

Elemental analysis was performed in order to confirm the decrease of sulfur content in the desulfated cyanoflan, using a TruSpec Micro 630-200-200 (Leco

Corporation, MI, USA). The operating temperatures of the combustion furnace was 1075 °C and the afterburner temperature was 850 °C. The analyses were performed in triplicate.

2.5. Methylation analysis

Glycosidic-substitution analysis of cyanoflan was carried out by methylation as described previously (Ciucanu & Kerek, 1984; Oliveira et al., 2017). Briefly, 5 mg of native and desulfated cyanoflan were dissolved in 1 mL of anhydrous dimethyl sulfoxide and then powdered NaOH was added under nitrogen atmosphere. The methylation was performed with 80 µL of CH_3I during 20 min with stirring (steps repeated three times). Then 3 mL of $CHCl_3$:MeOH (1:1, v/v) was added and the solution was dialyzed against 50% EtOH. After dialysis, the solution was evaporated to dryness and sample was remethylated by repeating the procedure. The remethylated sample was hydrolyzed with 0.5 mL of 2 M TFA at 121 °C for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis (but using sodium borodeuteride instead of sodium borohydride). The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography-quadrupole mass spectrometry (GC-qMS, GC-2010 Plus, Shimadzu, Japan). The GC-qMS operating conditions were described previously by (Oliveira et al., 2017). The analyses were performed in triplicate.

2.6. Molecular mass analysis

The apparent molecular mass of cyanoflan was determined according to a previously reported method (Flores et al., 2019). The samples concentration was 3 mg mL^{-1} and as standards dextran (Sigma–Aldrich, USA) with different molecular weights (2000 kDa, 1100 kDa, 410 kDa, 150 kDa and 50 kDa) and sucrose (340 Da) were used.

2.7. Determination of zeta potential

The zeta potential (ζ) of 0.05% cyanoflan (w/v in ultrapure water) was determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm and a scattering angle of 13°. The measurements were performed at 25 °C in a polycarbonate folded capillary cell incorporated with gold plated electrodes (DTS1070C) using water as dispersion medium. The monomodal analysis model was used with the applied voltage and the number of subruns automatically defined by the Zetasizer software. The sample conductivity was measured and then the zeta potential values were automatically calculated by the software from the electrophoretic mobility (EPM) values, using the Henry equation with the Smoluchowski approximation (von Smoluchowski, 1903). Values are reported as average based on three individual measurements.

2.8. Surface morphology analyses

Cyanoflan was observed by several microscopy techniques. For negative staining transmission electron microscopy (TEM), 10 µL of *Cyanothece* culture

were mounted on Formvar/carbon film-coated mesh copper grid (Electron Microscopy Sciences, PA, USA) and left standing for 2 min. The excess of liquid was removed with filter paper and 10 µL of 1% phosphotungstic acid (w/v) in water, pH 7.0, were added to the grid and left standing for 10 s. Visualization was carried out on JEM-1400 (Jeol Ltd., Inc., MA, USA) at 80 kV. For scanning electron microscopy (SEM), the lyophilized cyanoflan was mounted on metal stubs using double-sided carbon tape and coated with a gold/palladium thin film for 100 s and 15 mA current by sputtering, using the SPI module sputter coater equipment (Structure Probe Inc., PA, USA). The analysis was performed using a high-resolution scanning electron microscope (JSM 6301F, Jeol Ltd., Japan), operating at 15 kV. For atomic force microscopy (AFM), the lyophilized cyanoflan was observed using a Multimode AFM and NanoScope IVa Controller (Veeco Instruments Inc., CA, USA) in tapping mode.

2.9. Rheological properties

To determine the rheology of cyanoflan, the viscosity of 0.1%, 0.5% and 1.0% (w/v) aqueous solutions was measured in a rheometer (Kinexus Pro, Malvern Instruments Ltd, UK) using a cone-plate system (upper geometry CP 4/40 SR0041 SS and lower geometry PLS55 C0127 SS). The measurements were performed by varying the shear rates (10-600 s^{-1}) at 25 °C (Peltier system). Aqueous solutions of 0.1%, 0.5% and 1.0% (w/v) of xanthan gum (molecular weight >2000 Kg mol^{-1}, MP Biomedicals, LLC., CA, USA) were used as standard. In addition, the viscosity of 0.1%, 0.5% and 1.0% (w/v) cyanoflan dissolved in 0.4 M NaCl solution was measured in the same conditions as

mentioned before, except that the range of shear rate was 0.01-1000 s^{-1}. All tests were performed in triplicate.

2.10. Emulsifying properties

To determine the emulsifying capacity of cyanoflan, the method described by Cooper and Goldenberg was employed (Cooper & Goldenberg, 1987). Briefly, in glass tubes with flat bottom and screw cap, 3 mL of *n*-hexane (95%, Romil Ltd, UK), liquid paraffin (Merck, Germany) or vegetable oil were added to 2 mL of 0.1%, 0.5% or 1.0% (w/v) cyanoflan aqueous solutions and stirred in a vortex for 2 min. After 24 h at room temperature, the emulsion index (E_{24}) was determined as %, using the Eq. (1).

$$E_{24} = \frac{h_e}{h_T} \times 100$$

where h_e is the height of the emulsion layer (mm) and h_T is the overall height of the mixture (mm). Aqueous solutions of 0.1%, 0.5% and 1.0% (w/v) of xanthan gum (MP Biomedicals, LLC., CA, USA) were used as standard. In addition, the emulsions were observed using an Olympus X31 light microscope (Olympus, Japan). All tests were performed in triplicate.

3. Results and Discussion

3.1. Chemical characterization

Cyanoflan, the extracellular polysaccharide released by the marine unicellular cyanobacterium *Cyanothece* sp. CCY 0110, revealed a carbohydrate content of

71%, determined as neutral sugars (56.8%) and uronic acids (13.7%), and being the sulfated residues 11% (Table 1). Protein account for approximately 4% of cyanoflan dry weight (w/w), and the remaining mass should correspond to inorganic compounds, as previously determined by thermogravimetric analysis (Mota et al., 2013).

Similar values of sulfate content, were previously detected in polysaccharides produced by different cyanobacteria isolated from Polynesian mats (Richert et al., 2005) or in *Nostoc carneum* (Hussein, Abou-ElWafa, Shaaban-Dessuuki, & Hassan, 2015), for example. The presence of sulfate groups, together with uronic acids, strongly contributes to the overall anionic character of these polysaccharides. The negative zeta potential values obtained confirmed the negative surface charge (Table 1). In addition, cyanoflan has low conductivity, as expected for a polysaccharide (i.e. highly resistive material) dissolved in ultrapure water (i.e. no salts present). By the methodology used, no pyruvate and only traces of acetate were detected in cyanoflan (Table 1). Ketal-linked pyruvyl groups were found in 14 out of the 15 polysaccharides produced by different *Cyanothece* strains, representing in average 1.4% of total carbohydrates content, while only nine of them contained O-acetyl groups, representing in average 1.2% (De Philippis, Margheri, Materassi, & Vincenzini, 1998). The protein content can be more variable in the polysaccharides of cyanobacterial origin, in the ones produced by *Nostoc* sp. and *Oscillatoria* sp. the amounts were similar to amount detected for *Cyanothece*, 4% and 3.4% of EPS dry weight (w/w), respectively (Parikh & Madamwar, 2006). Higher protein content was detected in EPS produced by *Anabaena* PC-1 (6.4% of EPS dry weight, w/w) or *Nostoc carneum* (10.1%) (Choi, Yoo, Oh, & Park, 1998; Hussein et al., 2015). While

lower values were detected for *Cyanothece* sp. ATCC 51142 EPS (1.8%) (Parikh & Madamwar, 2006). In contrast, in EPS from *Nostoc flagelliforme* proteins were reported to be completely absent (Han et al., 2014). However, one should bear in mind that these values can be influenced by the isolation procedure.

Table 1 General properties and composition of cyanoflan, the extracellular carbohydrate polymer released by *Cyanothece* sp. CCY 0110.

Properties/composition	
Color	White
Solubility	Water soluble
Zeta potential	- 22.7 ± 4.9 mV
Conductivity	18 μS/cm
Carbohydrate content	70.5 ± 2.4% [a]
Sulfate content	10.6 ± 1.6% [a]
Pyruvate content	Not detected
Acetate content	0.1 ± 0.01% [a]
Protein content	4.0 ± 0.3% [a]

[a] expressed in % of cyanoflan dry weight (w/w).

3.2. Glycosidic-composition of cyanoflan

Only a few number of structures have been proposed for cyanobacterial polymers, mainly due to their complexity (Pereira et al., 2009; Silipo et al., 2010). Cyanoflan revealed to be constituted by Man (20 mol%), Glc (20 mol%), uronic acids (18 mol%), Gal (10 mol%), Xyl (9 mol%), Rha (9 mol%), Fuc (8 mol%), and Ara (6 mol%). The type and quantity of the neutral monosaccharides

detected in cyanoflan by GC-FID were similar to the results previously obtained by ion-exchange chromatography (Mota et al., 2013) (Table S1). In order to identify the glycosidic linkages, cyanoflan was subjected to methylation analysis (Table 2). As carboxyl-reduction of the uronic acids was not performed, therefore the analysis only accounted for the neutral sugars. As methylation analysis also allows to determine the position of the sulfated groups, the samples were desulfated to enable the distinction between a possible branching point and a substitution by a sulfate ester. However, in contrast with the complete desulfation obtained for fucoidans using the same methodology (Oliveira et al., 2017), only a decrease of 45% in sulfate content was obtained, as confirmed by elemental analysis. This could explain the occurrence of 2,3,4,6-Man, 2,3,4,6-Glc, 2,3,4,6-Gal, 2,3,4-Fuc, and 2,3,5-Ara, not in accordance with the total percentage of terminally-linked residues (30%). If the contribution of these residues is not accounted as "branching points", the total percentage of branched residues is in accordance with the total percentage of terminally-linked residues. These results show that the glycosidic linkage composition of cyanoflan is highly branched, is composed of a large number of different sugar residues, with a high content of sulfated groups.

The main differences observed between native and desulfated samples were the decrease of 2,3,4-Fuc residues, with a concomitant increase of 4-Fuc, and the decrease of 3,4-Man and 4,6-Man, with an increase of 4-Man (Table 2), inferring the presence of sulfate groups on position 2 or/and 3 of fucose residues and in position 3 and 6 of mannose residues, respectively.

Table 2 Glycosidic composition (mol % of the total sugars) of native and desulfated cyanoflan.

Glycosidic substitution [a]	Native	Desulfated
3,5-Ara	3.5	3.4
2,5-Ara	2.8	2.3
2,3,5-Ara	0.9	1.5
Total Ara	***7.2***	***7.2***
t-Rha	5.7	6.1
3,4-Rha	2.4	3.0
2,3,4-Rha	3.4	-
Total Rha	***11.5***	***9.1***
t-Fuc	Tr [b]	Tr [b]
4-Fuc	5.3	6.6
2,3,4-Fuc	2.3	1.6
Total Fuc	***7.9***	***8.4***
t-Xyl	1.6	2.2
3-Xyl	2.4	2.3
4-Xyl	6.2	6.8
3,4-Xyl	1.8	2.0
Total Xyl	***12.0***	***13.3***
t-Gal	3.0	2.4
3,6-Gal	5.6	6.1
3,4,6-Gal	1.0	1.0
2,3,4,6-Gal	1.6	1.2
Total Gal	***11.1***	***10.7***
t-Glc	12.3	13.2
4-Glc	5.4	6.4
3,4-Glc	1.4	1.3
4,6-Glc	1.0	1.2
3,4,6-Glc	1.1	1.2
2,3,4,6-Glc	3.9	2.8
Total Glc	***25.1***	***26.2***
t-Man	4.6	6.4
4-Man	3.0	7.1
6-Man	4.4	4.2
3,4-Man	4.1	2.0
4,6-Man	3.1	1.6
3,4,6-Man	Tr [b]	0.8
2,3,4,6-Man	5.5	3.0
Total Man	***25.2***	***25.1***

[a] expressed in mol % of each sugar residue.
[b] Tr = traces < 0.5%

Previous studies revealed that EPS produced by different members of the *Cyanothece* genus have a very diverse monosaccharidic and linkage composition. It has been reported that the EPS from 15 different *Cyanothece* strains contain six to eight types of monosaccharides and one or two uronic acids (De Philippis et al., 1998). In addition, the EPS produced by *Cyanothece* sp. ATCC 51142 are composed by 2-methylglucose, mannuronic acid and sulfated mannose (Shah, Ray, Garg, & Madamwar, 2000), exhibiting a certain degree of similarity to cyanoflan that also contains sulfated mannose. In contrast, *Cyanothece* sp. 113 produces a α-D-1,6-glucan, a much less complex polysaccharide (Chi, Su, & Lu, 2007).

Concerning the molecular mass distribution of cyanoflan, five different fractions were detected (Fig. 1 & Fig. S1), a result that confirms the complexity of this polysaccharide. The number and size of molecular mass fractions can vary significantly depending on the cyanobacterial strain (Pereira et al., 2009). The polysaccharides produced by several strains of *Nostoc* have a single fraction (Han et al., 2014; Li et al., 2018), while the ones from *Synechocystis* sp. PCC 6803 and *Cyanospira capsulata* have several different fractions (Flores et al., 2019; Garozzo, Impallomeni, Spina, & Sturiale, 1998).

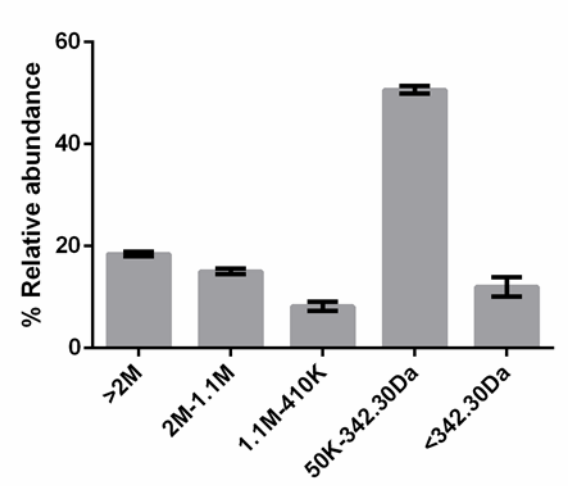
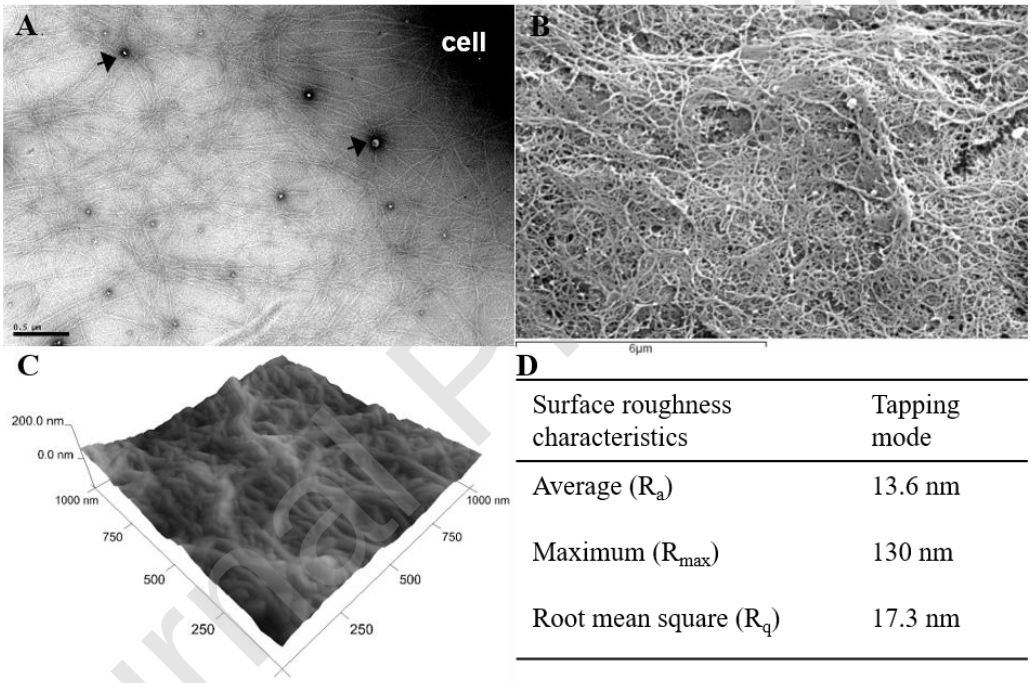


Fig. 1. Molecular mass distribution of cyanoflan, obtained by size exclusion chromatography. Data are means ± standard deviations (n = 3).

3.3. Surface morphology and topography

The morphology and topography of cyanoflan surface were studied by different microscopy techniques. Using negative staining, it was possible to observe the network of long fibers that constitute cyanoflan surrounding a *Cyanothece* cell (Fig. 2A). A similar pattern was previously observed for other strains of *Cyanothece* genus (Reddy, Soper, Tang, & Bradley, 1996). In addition, entrapped in the cyanoflan network, small spherical structures varying between 50 to 250 nm in diameter could be observed (Fig. 2A, arrows). These structures strongly resemble the extracellular vesicles reported for other cyanobacterial strains (Biller et al., 2014; Oliveira et al., 2015). The morphology of the intricate net of amorphous fibers formed by the lyophilized cyanoflan was observed by SEM (Fig. 2B), and its surface topography, analyzed by AFM (Fig. 2C). The images obtained revealed a very heterogeneous surface and allowed the characterization of the surface roughness of the carbohydrate chains (Fig. 2D). The topographic structure can vary significantly between polymers produced by different organisms, depending on the molecular mass and structural complexity.

For example, the height range of the EPS chains of the cyanobacterium *Nostoc* sp. FACHB 892 was around 10.14-18.25 nm (Ge, Xia, Zhou, Zhang, & Hu, 2014), whereas EPS produced by other microorganisms revealed more compact and porous structures and, consequently, a higher roughness. Two examples are the polymers produced by the dinoflagellate *Amphidinium carterae* with an average roughness (R_a) of 181 nm (Mandal, Singh, & Patel, 2011) and by two different strains of *Vibrio* spp. with a roughness root mean square (R_q) of 136 nm and 196 nm (Kavita, Mishra, & Jha, 2013).



Surface roughness characteristics	Tapping mode
Average (R_a)	13.6 nm
Maximum (R_{max})	130 nm
Root mean square (R_q)	17.3 nm

Fig. 2. Negative staining electron micrographs of *Cyanothece* sp. CCY 0110 culture (A), and lyophilized cyanoflan observed by SEM (B) and by AFM, revealing the 3 D surface organization (C) and the surface roughness characteristics (D). cell - *Cyanothece* cell, arrows - released vesicles. Scale bars = (A) 0.5 µm; (B) 6 µm; (C) 200 nm.

3.4. Rheological properties

To assess the rheological properties, the apparent viscosity of different cyanoflan aqueous solutions (0.1%, 0.5% and 1.0%, w/v) was measured over a

range of different shear rates and compared with the biopolymer xanthan gum (Fig. 3). Xanthan gum has great commercial significance, is composed by glucose, mannose and glucuronic acid, and has high molecular weight (2 MDa to 20 MDa) and water solubility (Garcia-Ochoa, Santos, Casas, & Gomez, 2000). The results revealed that cyanoflan viscosity started with values of 66.6, 922.1 and 1594.0 mPa.s, for 0.1%, 0.5% and 1.0% (w/v) solutions, respectively, at shear rate of 10 s^{-1} and reached 13.0, 102.7 and 229.5 mPa.s respectively, at shear rate of 600 s^{-1} (Fig. 3 A1). While the viscosity of xanthan gum started with values of 52.1, 499.9 and 1113.0 mPa.s, for 0.1%, 0.5% and 1.0% (w/v) solutions, respectively, at shear rate of 10 s^{-1} and reached 6.6, 21.2 and 43.9 mPa.s respectively, at shear rate of 600 s^{-1} (Fig. 3 A2). Therefore, the solutions of cyanoflan and xanthan gum showed a significantly decrease in viscosity with increasing shear rate, while viscosity increased with increasing polymer concentration (Fig. 3 B1 and B2). In addition, the shear stress tended to stabilize at low range of shear rates, demonstrating that both polymers exhibit a finite magnitude of yield stress. Moreover, at the initial shear rate of 10 s^{-1}, the cyanoflan solutions exhibit higher values of shear stress than the xanthan gum solutions, which suggest a higher viscosity of cyanoflan. On the other hand, the xanthan gum solutions exhibit smaller shear stress values at the end, suggesting a more plastic behavior.

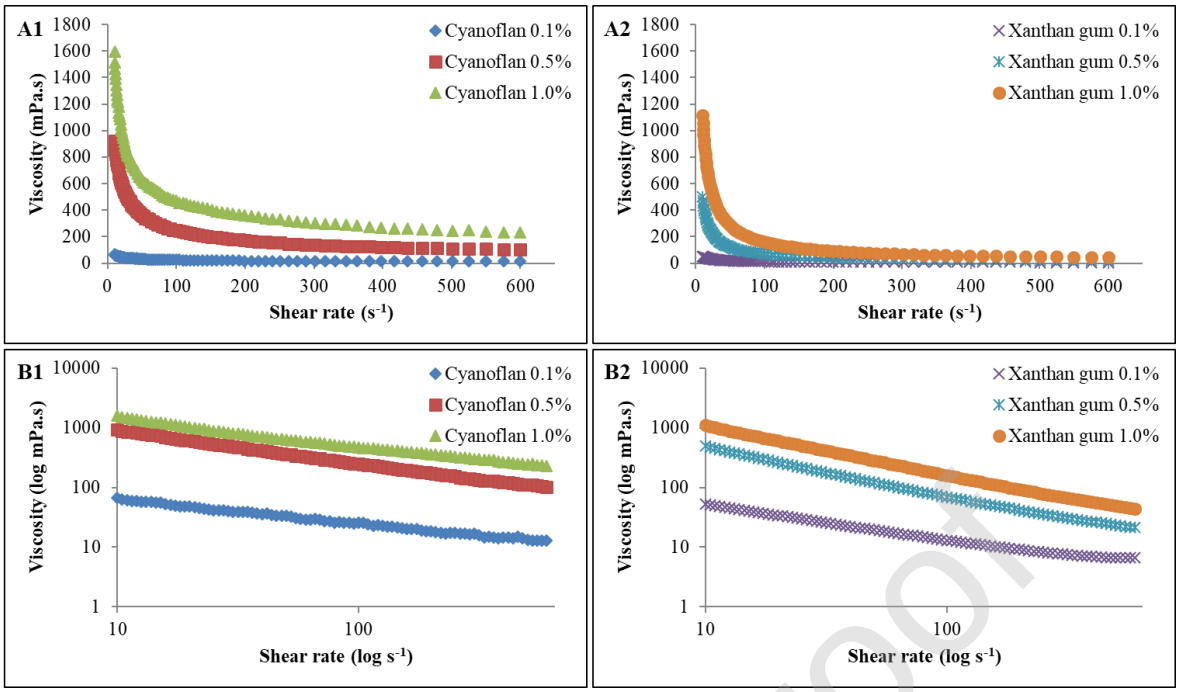


Fig. 3. Apparent viscosity, expressed in mPa.s, as a function of shear rate, expressed in s^{-1} (A) or as log-log plot (B), of aqueous solutions of cyanoflan at concentrations of 0.1% (dark blue diamond), 0.5% (red square) and 1.0% (green triangle) (w/v) (A1 and B1), or aqueous solutions of xanthan gum at concentrations of 0.1% (purple cross), 0.5% (light blue star) and 1.0% (orange circle) (w/v) (A2 and B2) (n = 3).

The rheological behavior observed for cyanoflan and xanthan gum is typical of non-Newtonian fluids with pseudoplastic behavior or shear thinning properties in aqueous solutions, i.e., decreasing apparent viscosity with increasing shear rate, which is attributed to the reversible disorientation and disentanglement of the macromolecular chains due to hydrodynamic forces generated during shear (Han et al., 2014). This rheological behavior was also observed in the presence of NaCl, used as counterion of the anionic groups that constitute the cyanoflan (Fig. S2). Similar rheological behavior has also been observed for several polymers produced by cyanobacterial strains (Bhatnagar et al., 2012; Han et al., 2014; Hussein et al., 2015; Khattar et al., 2010), including other *Cyanothece* strains (De Philippis et al., 1998), which can vary on polysaccharide concentration, temperature and pH. The pseudoplastic behavior

is important for industrial applications, in particular in food industry, for providing good sensory qualities, flavor release and suspending properties in the production of cake mixtures, salad dressings, sauces, puddings and dairy products for example, and in cosmetic industry, for the production of lotions, creams and gels (Jindal et al., 2011).

3.5. Emulsifying properties

In order to further study the emulsifying properties of cyanoflan, several emulsions were prepared with different concentrations of the *Cyanothece´*s polysaccharide and a hydrocarbon (*n*-hexane) or two distinct oils (liquid paraffin and vegetable oil), and the emulsification indexes (E_{24}) were determined (Table 3). All cyanoflan concentrations were able to form detectable emulsions in the presence of the solutions tested. Optical microscopy allowed to observe the emulsion droplets formation (Fig. 4, 1.0% (w/v) as example). In general, the results were similar regarding the droplets formed by the emulsions of cyanoflan or xanthan gum (≤ 200 μm), except the droplets formed with xanthan gum at 1.0% (w/v) and liquid paraffin that were more densely distributed (Fig. 4B2). Cyanoflan can also be considered a good emulsifier since the emulsification indexes were ≥ 50% (Table 3) and stable over a month (data not shown). The size of emulsion droplets is an important parameter to determine the physical stability, with smaller and more densely distributed droplets usually corresponding to emulsions with higher stability and higher emulsification indexes (Binks & Tyowua, 2016; De Philippis & Vincenzini, 1998; Shepherd, Rockey, Sutherland, & Roller, 1995).

Table 3 Emulsification index after 24 h of contact between different concentrations of cyanoflan or xanthan gum (0.1%, 0.5% and 1.0%, w/v) and a hydrocarbon (*n*-hexane) and two different oils (liquid paraffin or vegetable oil).

	Cyanoflan (%, w/v) [a]			Xanthan gum (%, w/v) [a]		
	0.1	0.5	1.0	0.1	0.5	1.0
n-hexane	47.6 ± 6.1	48.2 ± 1.5	74.1 ± 8.1	64.1 ± 2.4	57.9 ± 0.0	61.7 ± 2.9
Liquid paraffin	51.6 ± 1.4	64.9 ± 6.1	63.6 ± 0.0	67.4 ± 10.2	86.7 ± 11.5	95.2 ± 8.2
Vegetable oil	64.0 ± 1.8	73.2 ± 0.8	66.7 ± 2.6	68.0 ± 17.7	79.4 ± 15.3	60.3 ± 2.7

[a] Data are means ± standard deviations (n = 3).

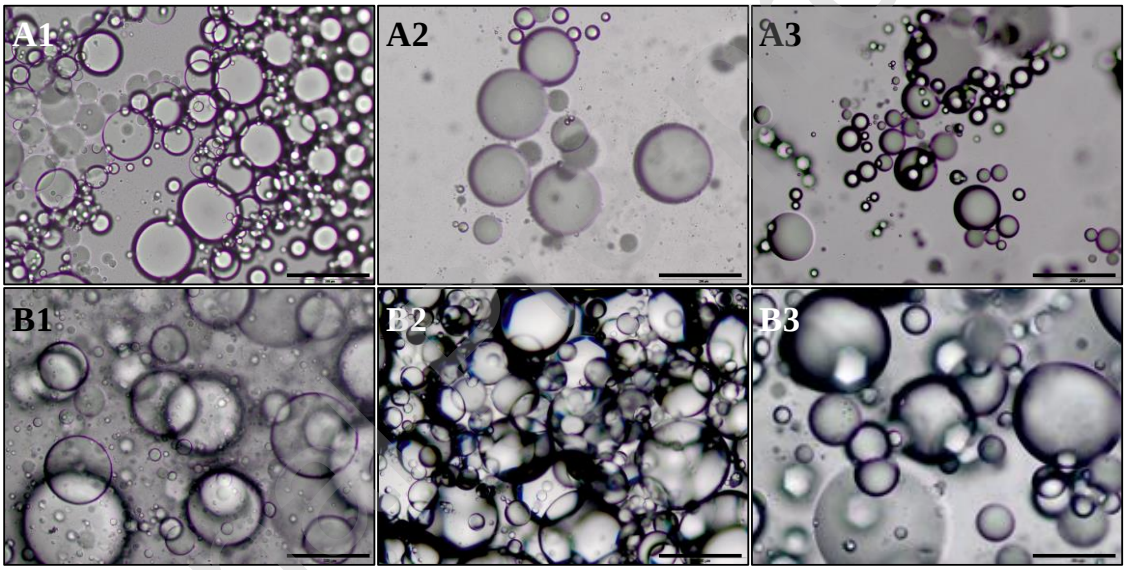


Fig. 4. Optical microscope images of emulsions prepared with 1.0% (w/v) cyanoflan (A) or xanthan gum (B) and *n*-hexane (A1 and B1), liquid paraffin (A2 and B2) or vegetable oil (A3 and B3). Scale bar = 200 μm.

The emulsions formed with different concentrations of EPS produced by *Nostoc flagelliforme* in the presence of *n*-hexane revealed a maximum of 27% of emulsification index with EPS at 0.45% (w/v) (Han et al., 2014). With this polysaccharide concentration, higher indexes were detected using other hydrocarbons and oils, as *n*-hexadecane, liquid paraffin or peanut oil, around 57%, 72% or 74% respectively, as well as small and densely distributed droplets

were observed. Similarly, an emulsion of 0.1% EPS produced by *Pseudomonas oleovorans* and *n*-hexadecane, allowed to obtain an emulsification index of 38%, similar to xanthan gum (41%), while with guar gum, carboxymethylcelullose and sodium alginate only negligible emulsifying properties were obtained for the conditions tested (Freitas et al., 2009). The best studied cyanobacterial carbohydrate polymer regarding the emulsifying properties is emulcyan, a patented extracellular sulfated heteropolysaccharide with high molecular weight and fatty acids and proteins in its composition, produced by the benthic filamentous cyanobacterium *Phormidium* J-l (Fattom & Shilo, 1984, 1985). This polymer can be used as emulsifier or flocculant to disperse hydrocarbons or oils in water or food preparations, in the secondary recovery of petroleum, and as a stain remover. A significant presence of pyruvate or ester-linked acetyl groups and deoxyhexoses (fucose and rhamnose) has been suggested to contribute to the polysaccharide emulsifying properties, since these molecules introduce a certain lipophilic character to the polymers that otherwise would be highly hydrophilic (De Philippis & Vincenzini, 1998). Bioemulsifiers are advantageous in comparison to chemical emulsifiers since they are biodegradable, less toxic and have activity under a wider variety of conditions (Banat, Makkar, & Cameotra, 2000).

4. Conclusions

The extracellular carbohydrate polymer produced by the marine unicellular cyanobacterium *Cyanothece* sp. CCY 0110, cyanoflan, has the complex structure of an anionic sulfated molecule composed of Man, Glc, uronic acids,

Gal, Rha, Xyl, Fuc and Ara. In aqueous solutions, cyanoflan exhibits high apparent viscosity typical of a non-Newtonian fluid with pseudoplastic behavior and high emulsifying activity, properties that are comparable to commercial polymer xanthan gum. Therefore, cyanoflan seems to be a complex, versatile and promising polysaccharide that can be useful in different industrial applications, namely as emulsifying/thickening agent. Altogether, the particular characteristics of cyanobacteria, such as their ability to sequester CO_2 and the minimal nutrient requirements, the easiness of cyanoflan isolation (since the majority is released to the culture medium) and its unique features, confer a significant advantage to the use of this carbohydrate polymer in biotechnological applications.

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