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Structural characterization of exopolysaccharides obtained from *Porphyridium cruentum* exhausted culture medium

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ABSTRACT

Microalgae produce and secrete large quantities of polysaccharides into the culture medium that is discarded when biomass is separated. The main objective of this study was to add value to the exhausted culture medium (ECM) of *Porphyridium cruentum* through the recovery of extracellular polysaccharides (EPS) by different extracting and purifying methods for the future biotechnological applications. The ECM was submitted to precipitation with cold absolute ethanol, using ultrasound, dialysis, and trichloroacetic acid (TCA) as purification methods. The purification provided a lower yield, with higher carbohydrate content. The TCA purified sample presented up to twice as much total carbohydrates as the non-purified samples, mainly composed of xylose, galactose, and glucose. Scanning Electron Microscopy (SEM) images showed the smooth structure of *P. cruentum* EPS, in which TCA post-treatment and dialysis allowed obtaining larger and purest particles, being a good candidate for film making. Microalgal polymeric by-products are a sustainable source to recover valuable compounds, and the purification treatment proved to be an important step to valorize this material.

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1. Introduction

Exopolysaccharides (EPS) are secondary metabolites produced by plants, bacteria, fungi, seaweed, or microalgae in stressed conditions to support life survival (Mutmainnah et al., 2018, Delattre et al., 2016). They are extracellular polysaccharides therefore heteropolymers, consisting of a very complex structure and a promising natural bioactive compound. Thus, they possess an important biotechnological function in industrial applications such as thickeners, emulsifiers, and stabilizers, being some used also as reinforcing agents and film formers or in pharmaceutic and therapeutic industries due to their biological activity, as presented in Table 1 (Kraan, 2012; Raposo et al., 2014b).

Microalgae have been used as an important source of high-value compounds for a wide range of applications in food, animal feed, biofuel, pharmaceuticals, cosmetics, and nutraceuticals fields (Xiao and Zheng, 2016). As feedstocks, the usual focus of industrially cultivated microalgae is to produce fatty acids, pigments, and protein, whereas polysaccharides are considered to be a by-product. However, many microalgae produce and secrete large quantities the polysaccharides to the culture medium (about 0.5 g L^{-1} up to 20 g L^{-1}) (Markou and Nerantzis, 2013), which are lost when the biomass is recovered for commercialization (Delattre et al., 2016). It is estimated that to produce 1 kg of dry biomass is necessary to process between 200 mL and 2000 L of culture medium that is discarded to the environment (Chatsungnoen and Chisti, 2019).

Microalgal polysaccharides have the advantage of being a renewable material, eco-friendly, non-toxic, and biodegradable, as well as rapidly produced, and can be used as an important source of industrially polymeric material (Castellane et al., 2015). However, the recovery process is rather complicated as it requires several steps. The polarity of the extraction solvent (alcohol) and the temperature of precipitation will affect the yield of precipitation and its purity (Patel et al., 2013). Afterward, being produced in media with a high salt content an additional step to correct the salinity is needed (Delattre et al., 2016). Dialysis, membrane filtration, and/or trichloroacetic acid treatment are used to remove non-polysaccharidic molecules and increase the microalgal polysaccharides purity (Delattre et al., 2016). The literature lacks studies comparing different purification treatments for *P. cruentum* EPS focusing on feasibility and industrial application.

Porphyridium cruentum is a red microalga arousing commercial interest, classified as GRAS (Generally Recognized As Safe) by the Food Drug Administration (FDA) (Cherian et al., 2021). It has been deemed as a potential feedstock for the food, nutraceutical, pharmaceutical, and cosmetic industries as a source of compounds with nutritional and biological value (Bernaerts et al., 2018; Patel et al., 2013; Stengel and Connan, 2015). Its red pigment, phycoerythrin, has an estimated market size that will reach 6.3 million US $ by 2025 (Forecast, 2020). Interestingly, EPS, which are now seen as a by-product of pigment production recovered from the ECM, can be processed to a sustainable ingredient with a very promising outlook as well.

Many techniques for EPS extraction have been described in the literature, although the purification method is the procedure that most improves the properties and functions of the EPS (Xiao and Zheng, 2016). The main objective of this study is to compare the efficiency of different extracting and purifying procedures tested on an ECM of *P. cruentum* through the recovery of extracellular polysaccharides for future biotechnological applications.

2. Material and methods

2.1. Exhausted culture medium (ECM)

Porphyridium cruentum ECM was provided by the algal producer Necton, S.A. This rhodophyte was grown in a tubular photobioreactor placed outdoors and exposed to natural sunlight. Cultivation took place in December with pH set at 8.0 using CO_2 as a carbon source and nitrogen from the Phytobloom growth medium (Nutribloom Plus, Necton S.A.). ECM was collected at Necton´s harvesting point and underwent centrifugation at 12,298*g* for 20 min at 20 °C followed by freezing before proceeding to the precipitation step.

2.2. Exopolysaccharide extraction

EPS from the ECM was precipitated by different methods, as presented in Fig. 1. Firstly, an alcoholic extraction was performed by the addition of cold absolute ethanol in different volume ratios (1:1 and 1:2, v/v) (ECM: absolute ethanol),

Table 1 – Exopolysaccharides from microalgae and activity. (Xyl: xylose; Gal: galactose; Glc: glucose; Man: mannose; Ara: arabinose; Rha: rhamnose; Fuc: fucose; Rib: ribose; GlcA; galacturonic acid; GalA; glucuronic acid).

Strain	Monosaccharide composition	EPS Characteristic	Reference
Porphyridium cruentum	Xyl, Gal, Glc, GluA, GalA, Man. Ara, Rib, Rha, Fuc	Thickening agents	(Bernaerts et al., 2018)
Porphyridium cruentum	Glc, Ara, Man, Fuc, Xyl, Rha	Antiviral activity	(Raposo et al., 2014a)
Porphyridium cruentum	Xyl, Glc, Gal	Antioxidant	(Sun et al., 2009)
Chlorella pyrenoidosa	Gal, Ara, Glucosamine, Rha, Glc, Man, Fuc, Galactosamine, Rib, GalA	Antitumor	(Zhang et al., 2019)
Chlorococcum sp	Glc, Glucosamine, Man, Gal, Galactosamine, Ara, Rha, Fuc, Ribose, GalA	Antitumor	(Zhang et al., 2019)
Dunaliella tertiolecta	Glc	Bioethanol	(Geun Goo et al., 2013)
Grasiella sp.		Antioxidant	(Gongi et al., 2021)
Flintiella sanguinaria	Xyl, Gal, GlcA, Rha, Glc, Ara	Viscosity	(Gaignard et al., 2018)
Navicula sp	Glc, Gal, Rha, Xyl, Man	Gelation	(Fimbres-Olivarría et al., 2016)
Navicula sp.	Glc, Gal, Rha, Xyl, Man	Gelation	(Fimbres-Olivarría et al., 2016)
Scenedesmus sp.	Glc, Glucosamine, Man, Gal, Rha, Galactosamine, Fuc, Ara, Rib, GalA	Antitumor	(Zhang et al., 2019)

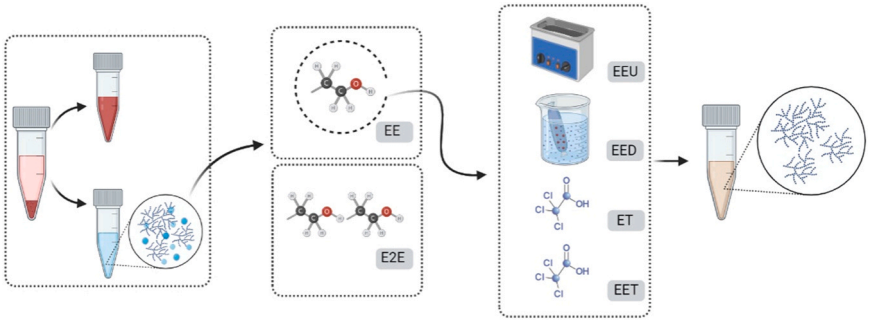

Fig. 1 – Flowchart of EPS precipitation. EE: ethanol extraction (1 vol); E2E: ethanol extraction (2 vol); EEU: ultrasound-assisted extraction; EED: dialysis; ET: trichloroacetic acid (pretreatment); EET: trichloroacetic acid (post-treatment).

samples EE and E2E, respectively, and stored at – 18 °C (overnight) to reduce the solubility of EPS followed by filtration of the precipitate. Next, the precipitate was washed twice with deionized water and recovered by centrifugation and stored again at - 18 °C before freeze-drying. In the preliminary tests, 1 vol of cold absolute ethanol was selected as the methodology to proceed with the purification (Fig. 1), as this extraction provided a higher carbohydrate content.

The extraction yield was determined in terms of extract yield and calculated according to Eq. 1:

$$\text{EPS yield}\,(\text{mgL}^{-1}) = \frac{\text{dry weight of EPS}\,(\text{g})}{\text{volume of ECM}\,(\text{L})} \times 1000 \quad (1)$$

2.3. Purification of EPS

The EPS from *P. cruentum* purification was tested by different methods as described below. The purification efficiency was calculated according to Eq. 2.

$$\text{Purification Efficiency}\,(\%) = \frac{\text{total carbohydrate}}{\text{yield of extraction}} \times 100 \quad (2)$$

2.3.1. Ultrasound-assisted extraction (EEU)

The ECM was mixed with cold absolute ethanol (1:1, v/v) (ECM: absolute ethanol) and ultrasonicated for 20 min (20 °C, 320 W) being the subsequent experimental steps the same as mentioned before (Section 2.2).

2.3.2. Dialysis (EED)

Precipitated EPS obtained by 1 vol of ethanol was dissolved in deionized water and dialyzed against distilled water under mild stirring using a 12–14 kDa cut-off membrane (Medicell) for two days and four renewals of water. The EPS were re-precipitated using ethanol.

2.3.3. Trichloroacetic acid

The trichloroacetic acid (TCA – BioChemica, Panreac Applichem) was added to ECM with a final concentration of 5% (v/v) to denature the proteins present in the aliquots. Samples were then homogenized at 37 °C for 40 min and centrifuged at 7871 × *g* and 10 °C for 10 min to remove any remaining protein. The supernatant was mixed with cold absolute ethanol (1:1, v/v) to EPS precipitation (Sample ET).

Another experiment was performed with EPS previously obtained by precipitation with 1 vol of ethanol. The crude EPS was dissolved in deionized water, treated with 5% (v/v) TCA, homogenized at 37 °C for 40 min, followed by centrifugation at 7871 × *g* and 10 °C for 10 min, to remove the remaining protein, and re-precipitated by alcoholic precipitation, as mentioned before (Sample EET).

2.4. Exopolysaccharide characterization

2.4.1. Biochemical composition

Total carbohydrate content of the extracts was determined using the phenol-sulfuric method (Dubois et al., 1956). A standard calibration curve of glucose (10–150 mg L^{-1}) was produced, and the results were expressed as milligrams of total carbohydrates per liter extract. Sulfated polysaccharide contents were determined using barium chloride-gelatin (20–1000 mg L^{-1}) method described by Dodgson and Price (1962). The protein content was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin (BSA) as a standard, with a linear range of 5–400 mg L^{-1}.

2.4.2. Structural characterization

2.4.2.1. *Monosaccharide analysis*. The monomer composition of EPS was determined by the method of alditol acetates (Selvendran et al., 1979). Briefly, 10 mg of samples were hydrolyzed with 200 µL of H_2SO_4 72% for 3 h at room temperature, following the addition of 4.4 mL of water to achieve H_2SO_4 1 M and kept at 100 °C for 2h30. The sugar mixture was reduced with $NaBH_4$ in aqueous ammonia at 30 °C for 1 h, neutralized with acetic acid and then acetylated by the addition of 1-methylimidazole and acetic anhydride at 30 °C for 30 min. Water was added to decompose the acetic anhydrate in excess and partitioned dichloromethane to separate the phases. After solvent evaporation under nitrogen flow, the alditol acetate was resuspended in 50 µL acetone and analyzed by gas chromatograph (Varian 3350) with FID detector (250 °C) equipped with DB-225 J&W capillary column (30 m x 0.25 mm diameter and 0.15 µm thicknesses). The temperature of the injector and column was set to 225 and 220 °C, respectively. The injection volume was 0.5 µL with a carrier flow set at 3 mL min^{-1}. As internal standard, 200 µL of 2-deoxyglucose were used.

2.4.2.2. *Fourier Transform Infrared (FTIR) Spectroscopy*. The morphologic properties of lyophilized EPS were analyzed by Fourier Transform Infrared Spectroscopy (Bruker, model Tensor 27, USA) in transmittance mode. The samples were mixed and pressed with KBr powder and scanned in a spectral range between 400 and 4000 cm^{-1}, with a resolution of 4 cm^{-1}. A 32-scan interferogram was collected. The results were obtained using the eFTIR software (Essential FTIR®, USA).

Table 2 – Biochemical composition of EPS extracted from *P. cruentum* ECM.

Sample[a]	Yield of extraction (mg L^{-a})	Total carbohydrates (mg L^{-a})	Sulfated carbohydrates (mg L^{-a})	Proteins (mg L^{-a})
EE	2573.7 ± 41.5^{ab}	118.8 ± 7.1^{bc}	146.3 ± 6.6^{b}	5.9 ± 0.8^{bc}
E2E	3539.0 ± 55.9^{a}	98.2 ± 5.8^{c}	118.0 ± 1.4^{c}	10.9 ± 0.4^{b}
EEU	2847.3 ± 385.0^{a}	128.5 ± 10.7^{bc}	127.7 ± 1.9^{c}	1.8 ± 0.8^{c}
EED	508.4 ± 58.3^{d}	150.4 ± 9.5^{b}	93.7 ± 2.8^{d}	27.4 ± 2.9^{a}
ET	1757.5 ± 430.4^{bc}	132.9 ± 10.0^{bc}	225.3 ± 1.4^{a}	8.3 ± 0.1^{b}
EET	1016.1 ± 118.6^{cd}	237.8 ± 19.2^{a}	102.7 ± 2.4^{d}	2.7 ± 0.4^{c}

[a] EE: ethanol extraction (1 vol); E2E: ethanol extraction (2 vol); EEU: ultrasound-assisted extraction; EED: dialysis; ET: trichloroacetic acid (pretreatment); EET: trichloroacetic acid (post-treatment).

2.4.2.3. *X-ray diffraction (XRD) analysis*. The X-ray diffraction was performed to obtain the structural information on the EPS from *P. cruentum*. An X-ray diffractometer (Malvern, PANalytical X'Pert Pro powder, Malvern Instruments Ltd., UK) was used with Cu Kα (λ = 1.5405 A°) and a radiation generator at 45 kV and 35 mA, with scans recorded from 10 to 79 (2θ). Data processing and analysis were carried out using OriginPro 2021 graphic software (OriginLab Corporation, USA).

The crystallinity index (CI_{xrd}) of the samples was calculated by the ratio of peaks of crystalline phases and total areas of crystalline and amorphous peaks according to Eq. 3.

$$\mathbf{CI_{xrd}} = \frac{\sum \mathbf{A_{crystal}}}{(\sum \mathbf{A_{crystal}} + \sum \mathbf{A_{amorphous}})} \quad (3)$$

2.4.2.4. *Scanning electron microscopy (SEM)*. The microscopic morphology analysis was studied by scanning electron microscopy (Hitachi, model S4100, Japan), equipped with X/EDS electron detectors (Bruker, USA), at an accelerating voltage of 30 kV. The samples were deposited directly on the carbon adhesive tape.

2.5. Statistical analysis

The experiments were processed at least in triplicate and results were expressed as mean ± standard error deviation. Significant differences of variance (ANOVA) were performed, using the Tukey HSD (honestly significant differences) test. The statistical analysis was performed using Statistica 7.0 software (Statsoft Inc., USA).

3. Results and discussion

3.1. Biochemical composition

The ECM biochemical composition is presented in Table 2. The highest and lowest yields were obtained from precipitation with two volumes of ethanol (E2E) and dialysis treatment, respectively (3539.0 and 508.4 mg L^{-1}). The highest carbohydrate concentration was obtained with a 5% trichloroacetic acid post-treatment (237.8 mg L^{-1}) with 102.7 mg L^{-1} of sulfated carbohydrates and 2.7 mg L^{-1} of protein (sample EET). Pre-purification with TCA allowed the extraction of more sulfated carbohydrates than other treatments, whereas dialysis yielded samples with the highest protein content.

The yield of EPS extracted from the ECM is highly dependent on the nutrients present in the growth medium, environmental conditions, such as seasonal variations of light and temperature, as well as extraction methods (Imjongjairak et al., 2016). Purification steps have been reported to have a direct effect on the yield, whose samples usually displayed lower yields (except for EEU), but higher carbohydrate content. The EED treatment allowed higher purification efficiency (Eq. 2), with 29.6% of the extracted material being carbohydrates. EET provides a similar efficiency with 23.4%, followed by ET (7.6%), and EEU (4.5%). The decreased yield obtained upon dialysis compared to ethanol extraction is similar to that reported by Patel et al. (2013) to dialyzed EPS from *P. cruentum* and is corroborated by Villay et al. (2013), who reported carbohydrates values between 133.4 and 380.9 mg L^{-1} for dialyzed EPS from *Rhodella violacea* when different growth conditions were applied.

Unlike dialysis, in which the purity increased with a concomitant decrease in yield, the addition of more ethanol to promote EPS precipitation increased the yield of extraction but decreased the total carbohydrate content. Similar results were reported by Patel et al. (2013) for EPS recovery from the same microalga and may be explained by the precipitation of salts and non-sugar compounds with low molecular weight.

As shown in Table 2, EPS recovery by EET was significantly higher in carbohydrates than in other treatments, with a similar result presented by Balti et al. (2018) to *P. cruentum* EPS obtained by membrane microfiltration. However, the yield decreased almost 2.5-fold compared to the simple extraction (EE) method used in this study. A study by (Rimada and Abraham, 2003) has reported a polysaccharide purification increase, whereas the reduction of EPS yield by up to 50% using the purification treatment with trichloroacetic acid is probably due to the EPS coprecipitation with protein removal.

Significant amounts of sulfated carbohydrates and a minor fraction of protein were observed in all samples. In a study with *Ulva lactuca* supernatant, El Azm et al. (2019) reported similar carbohydrates and sulfate contents. The sulfate content reported in tree brown algae was between 68 and 127 mg L^{-1} and was associated with its anticoagulant properties (Jayaraman et al., 2016). In another study, the sulfate carbohydrate content was associated with its antiviral activity (Gaikwad et al., 2020).

The protein content was higher with the dialysis treatment, being similar to that reported by (Raposo et al., 2014a) with the same treatment and lower than reported by Balti et al. (2018). The treatment with TCA (ETT) gave better purification results, which is in agreement with those reported by Chambi et al., (2021), where despite TCA performing the deproteinization, the EPS samples still contained some

Table 3 – Monosaccharide profile of EPS from *P. cruentum*.

Sample[a]	Rhamnose (%)	Fucose (%)	Xylose (%)	Mannose (%)	Galactose (%)	Glucose (%)
EE	0.45 ± 0.04^{b}	0.33 ± 0.07^{c}	99.22 ± 0.22^{c}	0	0	0
E2E	0.16 ± 0.01^{c}	0.29 ± 0.02^{c}	99.55 ± 0.02^{c}	0	0	0
EEU	0.25 ± 0.08^{c}	0.50 ± 0.06^{c}	99.16 ± 0.15^{c}	0.08 ± 0.02^{c}	0	0
EED	2.30 ± 0.02^{ab}	3.81 ± 0.04^{b}	86.41 ± 0.17^{b}	3.76 ± 0.19^{a}	2.37 ± 0.30^{b}	1.36 ± 0.01^{b}
ET	1.55 ± 0.29^{c}	0.34 ± 0.04^{c}	98.01 ± 0.28^{c}	0.07 ± 0.03^{c}	0.03 ± 0.01^{b}	0
EET	3.12 ± 0.22^{a}	5.38 ± 0.01^{a}	60.76 ± 2.43^{a}	2.82 ± 0.01^{b}	20.13 ± 2.03^{a}	7.79 ± 0.64^{a}

[a] EE: ethanol extraction (1 vol); E2E: ethanol extraction (2 vol); EEU: ultrasound-assisted extraction; EED: dialysis; ET: trichloroacetic acid (pretreatment); EET: trichloroacetic acid (post-treatment).

protein. The low amount of protein to EEU can be related to the protein denaturation by ultrasound treatment due to cavitation (Zhao et al., 2022).

The high total carbohydrate recovery and the low protein content proved the efficiency of the post-purification with TCA, providing a high purity EPS fraction. Several studies show similar behavior when a purification step is included for EPS extraction from different raw materials (Patel et al., 2013; Sharma et al., 2020; Ziadi et al., 2018).

3.2. Structural characterization of exopolysaccharide from *P. cruentum*

The monosaccharide profile is presented in Table 3, expressed in mass percentage. The EPS was composed mainly of xylose. Minor amounts of rhamnose and fucose were observed in EE and E2E samples, while purification with ultrasound and pretreatment with TCA presented traces of mannose and galactose.

The TCA post-treatment allowed for extracts with a more heterogeneous monosaccharide profile, with xylose, galactose, and glucose as main carbohydrates, with 60.76%, 20.13% and 7.79%, respectively. Minor amounts of fucose, rhamnose, and mannose were also found. Samples that underwent dialysis purification showed a similar monosaccharide composition, however with slight differences in their relative amounts.

The high amount of xylose in most of the samples can be related to their poor solubility, due to the low purified polymers. This behavior has been reported by some authors mainly due to the high salt contents and co-extractions of proteins and minerals (Bernaerts et al., 2018; Marcati et al., 2014; Patel et al., 2013).

Xylose, galactose, and glucose were the main constituents in EPS obtained by TCA post-treatment (EET). A similar sugar profile was previously reported for extracellular polysaccharides from *P. cruentum* (Balti et al., 2018; Bernaerts et al., 2018; Geresh et al., 2009; Percival and Foyle, 1979), where Bernaerts et al. (2018) identified xylose, galactose, and glucose as the main constituents of *P. cruentum* EPS, while Balti et al. (2018) identified galactose, xylose, glucose, and glucuronic acid. On the other hand, Patel et al. (2013) reported higher amounts of galactose than xylose, followed by glucose and glucuronic acid, and (Raposo et al., 2014a) previously described galactose, glucose, and arabinose in EPS from *P. cruentum*. One of the reasons for these differences in the literature could be the origin of supernatant and different cultivation process and media, as well as the extraction methodology and purification (Medina-Cabrera et al., 2020).

Other authors reported the carbohydrate composition of *Porphyridium* species (Daniel et al., 2022; Ferreira et al., 2021; Gloaguen et al., 2004; Medina-Cabrera et al., 2020). Ferreira et al. (2021) extracted EPS, from *P. purpureum* the monosaccharide analysis revealed mainly glucose, xylose, and galactose, while Medina-Cabrera et al. (2020) reported xylose, galactose, glucose, and glucuronic acid in *P. sordidum* and *P. purpureum*. Furthermore, it was revealed the main presence of xylose, galactose, and glucose, with a low amount of fucose, arabinose, and glucuronic acid in *P. marinum* EPS (Gargouch et al., 2021).

Based on Table 3, the main observation was the difference in carbohydrate composition of *P. cruentum* EPS. A wide range of extraction and purification methods can be used for EPS extraction leading to different results. Ziadi et al. (2018) studied the profile of monosaccharides by comparing two purification methods and identified differences in the composition. The ethanol precipitation method provided glucose and mannose monomers, while ultrafiltration yielded glucose, mannose, and rhamnose for EPS extracted from lactic acid bacteria.

These results could be related to the precipitation of higher molecular weight polysaccharides by ethanol treatment, while the dialysis can retain high and low molecular weight polymers (Ziadi et al., 2018). Moreover, TCA purification might have led to the precipitation of interferents and probable degradation of polysaccharides as a consequence of acid hydrolysis (Inbaraj and Chen, 2016; Nguyen et al., 2018).

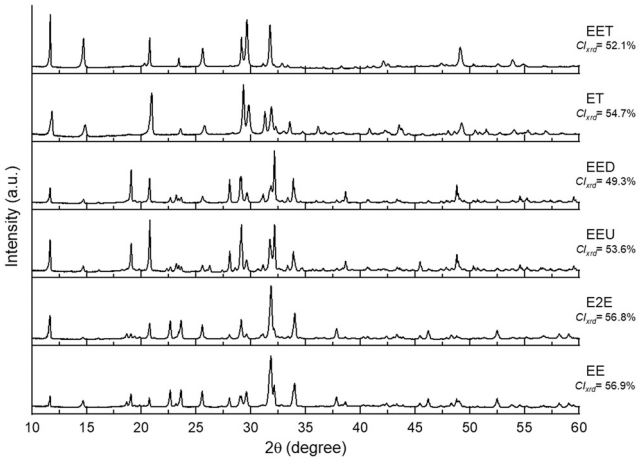

Fig. 2 – XRD profile of EPSs from *P. cruentum*. EE: ethanol extraction (1 vol); E2E: ethanol extraction (2 vol); EEU: ultrasound-assisted extraction; EED: dialysis; ET: trichloroacetic acid (pretreatment); EET: trichloroacetic acid (post-treatment).

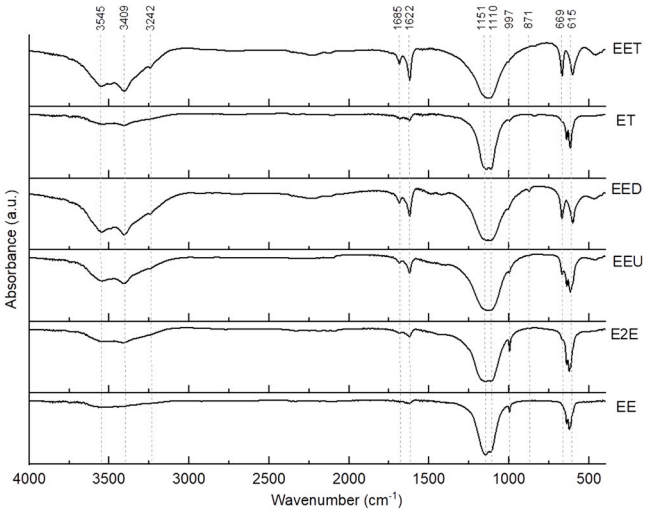

Fig. 3 – FTIR spectrum from 400 to 4000 cm^{-1} of EPS from *P. cruentum*. EE: ethanol extraction (1 vol); E2E: ethanol extraction (2 vol); EEU: ultrasound-assisted extraction; EED: dialysis; ET: trichloroacetic acid (pretreatment); EET: trichloroacetic acid (post-treatment).

Thus, a purification step, like dialysis and the use of trichloroacetic acid, could be suggested, allowing for the extraction of a more pure EPS, removing proteins, pigments, salts, and other contaminants (Delattre et al., 2016).

The X-ray pattern shows the crystallinity of the samples, as the several sharp thin diffraction peaks characterize the obtained patterns. The peaks can be observed in Fig. 2, centered around 11.6, 20.7, 29.1, and 31.8° with inter-planar spacing (D-spacing) of 7.59, 4.28, 3.07, and 2.81 A°, that were not changed after the purification processes. Peaks at 20.7, 29.1, and 31.8, with same D-spacing were also reported by Lian et al. (2017) and related to amylose and amylopectin present in sweet potato. The crystallinity index of EPS was similar between most of the samples, being lower in purified aliquots.

The X-ray analysis for *Porphyridium cruentum* EPS here given has not been previously reported. Lower crystallinity indices were reported by other studies evaluating EPS from microalgae, such as 32.76% for *Spirulina* sp. LEB18, 12% for *Dunaliella salina* and 15.2% for *Scenedesmus* sp. SB1 (Angelaalincy et al., 2017; de Jesus et al., 2019; Mishra et al., 2011). However, Gongi et al. (2021) and Wang et al. (2021) related the higher crystallinity of exopolysaccharides to more complex monosaccharide composition and a higher interaction between different compounds and non-linear structures.

The absorption band of the FTIR spectrum was assigned and is presented in Fig. 3. A major peak at 3545–3200 cm^{-1} was identified, indicating the hydroxyl group vibrational stretching (-OH) of the polysaccharides is more relevant in samples with purification, specially EED and EET. This absorption band is attributed to the carbohydrate ring that is responsible for the water solubility of EPS (Karbowiak et al., 2011). The polysaccharide solubility is dependent on its structure and can be interfered by purification (Kabir et al., 2022). Branched and charged polysaccharides are highly soluble whereas linear chains are mostly insoluble in water due to their high intermolecular affinity and can form crystalline or partial crystalline structures (Guo et al., 2017). These results are in accordance with Fig. 2, where a lower crystallinity index is related to noticeable -OH groups that characterize a more soluble sample. All samples present a strong IR absorbance near 1100 cm^{-1}, an important pattern used to characterize different polysaccharides.

The FTIR spectroscopy revealed that the strongest peaks in purified samples were in the range of 3000–3600 and 1000–1200 cm^{-1} and can be compared to results obtained in another study, which reported the presence of xylose, where OH-containing sugars contributed to an increase in the free hydroxyl absorption band (Liu et al., 2017).

Upon the purification step, significant peaks at 1685 and 1622 cm^{-1} were exhibited, except for the ET sample, indicating the presence of the carboxylic ester (C=O) or of the carboxylate anion (COO^-) forms, a characteristic IR absorption of polysaccharides (Delattre et al., 2016), as also reported by Singh et al. (2011) for seaweed EPS.

The pattern peak at 1100 cm^{-1} can be related to C-O stretching in the pyranoid ring and the glycosidic linkage C-O-C (Gómez-Ordóñez and Rupérez, 2011), also reported by Ziadi et al. (2018) for ethanol-precipitated EPS. Mandal et al. (2011), in a study with EPS from *Amphidinium carterae*, the strong absorption band in this range was attributed to the presence of sulfate groups such as S=O and C-O-S, which can provide an anionic nature to EPS. On the other hand, Gongi et al. (2021) assigned the peak at 1020 cm^{-1} to C-O, characteristic of uronic acids and acetyl ester linkage bonds in carbohydrates.

The characteristic peak at 870 cm^{-1} has been attributed to the α-glycosidic linkages between sugar monomers (Qin et al., 2018; Ziadi et al., 2018). The range near 600 cm^{-1} suggests a more alkylene C-H bond (Jayaraman et al., 2016).

The surface morphology is presented in Fig. 4. The treatment used can influence the average particle size and the particles agglomerates formed by smaller particles. In polysaccharides without purification, it can be observed aggregated particles with no defined shapes and more interferents (Fig. 4A, B), while the purification step that was added to process the polysaccharides enabled the obtainment of more regularly shaped (Fig. 4C), larger sized (Fig. 4D), longer particles (Fig. 4E, F).

Through the SEM data and considering the results already described, it is possible to suggest that *P. cruentum* has different types of EPS with different molecular weights. The conventional method of ethanol precipitation allows for the extraction of higher molecular weight fractions, and co-precipitation of interferents (Fig. 3A, B). However, the ultrasonication extraction presents a similar result, with fewer aggregates, possibly due to cavitation bubbles promoting the diffusion and osmotic processes and a rupture of other particles (Hou et al., 2016). Thus, when the purification method is used is possible to retain the purest polysaccharides. This result agrees with the monosaccharide composition, where fewer aggregate particles allow better carbohydrate identification.

The different treatments tested provide EPS with different morphologies that can affect the characteristics of the material to be produced, such as solubility as well as emulsive, thickening, gelling and bioplastic properties (Shehata et al., 2020). The SEM scan revealed a homogeneous matrix without porous structures in the particles, being a good candidate for film making, due to its structural integrity (Ahmed et al., 2013; Sajna et al., 2013).

The literature about the purification effect on microalgae EPS is very limited. Based on the results presented in this

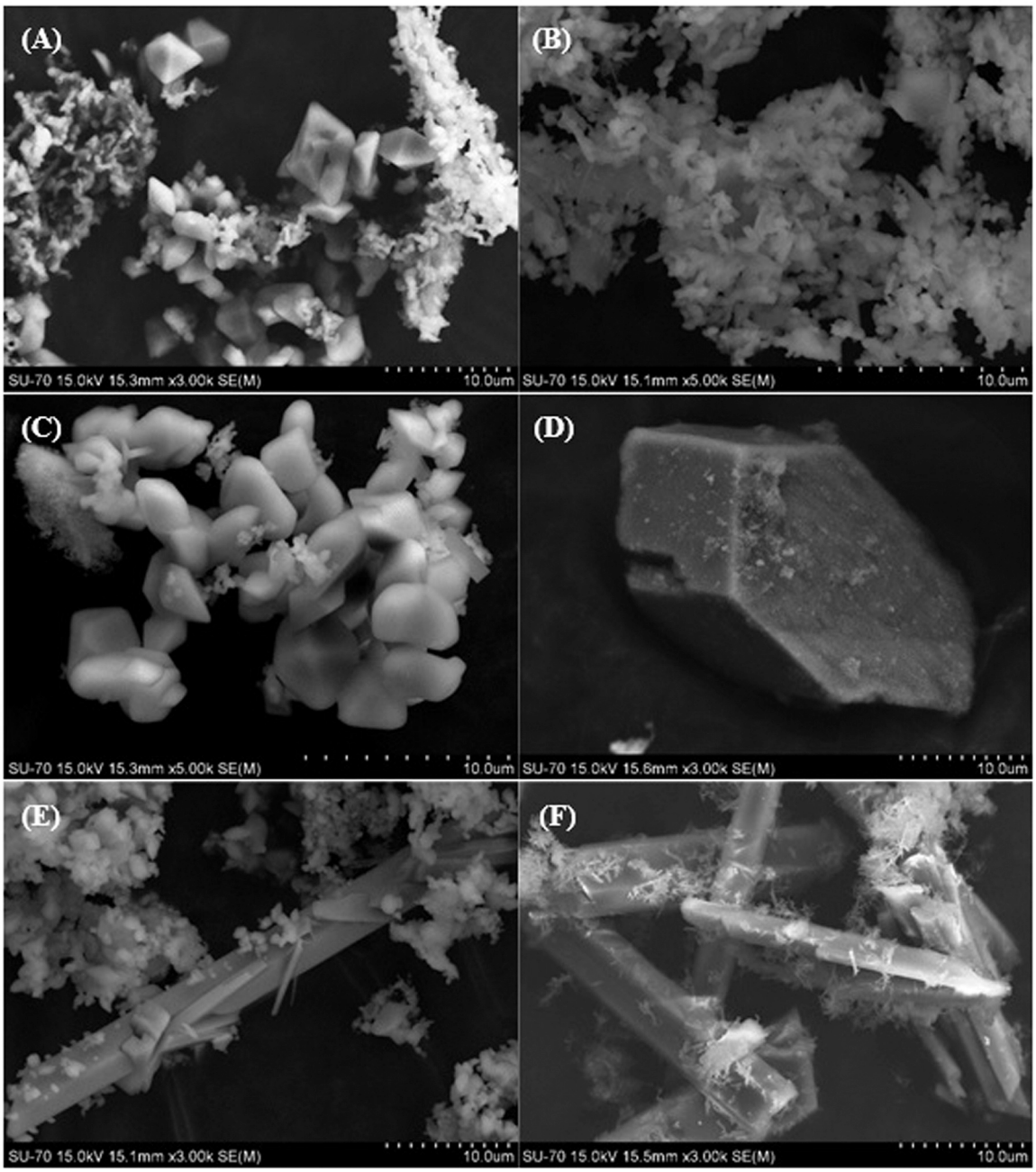

Fig. 4 – SEM images of EPS from *P. cruentum*. EE (A), E2E (B), EEU (C), EED (D), ET (E), EET (F). EE: ethanol extraction (1 vol); E2E: ethanol extraction (2 vol); EEU: ultrasound-assisted extraction; EED: dialysis; ET: trichloroacetic acid (pretreatment); EET: trichloroacetic acid (post-treatment).

study, it is possible to observe the influence of different treatments on the monosaccharide composition as well as on the microstructure of the polymers. However, the purification step possesses a challenge to obtain a pure material with low protein and salt content to allow the commercialization of these EPS. Based on the above, it is recommended at least two purification protocols after ethanol extraction to obtain a material with the required purity.

4. Conclusion

In this work, different methodologies were used to purify the EPSs recovered from *P. cruentum* ECM and the structure of the recovered material was analyzed. The polysaccharide extraction with one volume of ethanol allows the precipitation of more carbohydrate content, whereas larger amounts of ethanol were related to precipitation of more interferents. The purification treatment is a suitable method to purify exopolysaccharides, where trichloroacetic acid and dialysis are recommended to be used in the extraction of a polymer with low protein and salt content, and higher purification efficiency. *P. cruentum* EPS was found to be a renewable polymeric material suitable for producing heterogeneous monosaccharides, like xylose, galactose, and glucose. Conclusively, the current study showed that the different treatments affect the obtained EPS structure from *P. cruentum*, which displayed a smooth structure particle, strongly suggesting that the generated material could be applied to film making.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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