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Isolation, purification, and metal-induced gelation of released polysaccharides from spent culture medium of *Arthrospira*

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ABSTRACT

Cyanobacteria release polysaccharides that accumulate in the culture medium. In this study, released polysaccharides were isolated from the spent culture medium of an *Arthrospira* farm with a carbohydrate concentration of 136 mg L^{-1}. The polysaccharides were concentrated 5.2 times by ultrafiltration using a 50 kDa polysulphone membrane and purified (desalting, protein removal). The polysaccharides were rich in uronic acid (159 mg g^{-1}), had a low sulfate content (2.3 mg g^{-1}), and contained 9 different monosaccharides, with 2-deoxy-D-galactose (27.9 %), fucose (22.7 %), glucose (22.0 %), and glucuronic acid (17.1 %) being dominant. A 1 % w/v polysaccharide concentrate formed stable gel beads in Al^{3+} and Fe^{3+} solutions. Rheological characterization of the gel revealed stable storage (G' = 38 Pa) and loss modulus (G" = 6 Pa) over a wide frequency range. This study demonstrates the feasibility to recover polysaccharides from *Arthrospira* spent culture medium and the potential to use these polysaccharides in food or biotechnological applications.

1. Introduction

Polysaccharides from macroalgae or seaweeds are extensively used as gelling agents and rheology modifiers in foods, medical applications, and cosmetics [1]. Commercially important polysaccharides isolated from macroalgae include alginate, agar, and carrageenan [1]. Compared to macroalgae, polysaccharides from microalgae and cyanobacteria have been less well-studied. Most work has focused on a few species, the most important being the microalgae *Porphyridium* [2]. Microalgal or cyanobacterial polysaccharides are often more complex in their composition compared to macroalgal polysaccharides and frequently contain between 5 and 12 different monosaccharides [3,4]. Research into these microalgal and cyanobacterial polysaccharides has shown that they may display rheological properties similar to those from macroalgae, such as the formation of hydrogels in the presence of di- or trivalent cations [5,6].

Of all microalgae and cyanobacteria, the cyanobacterium *Arthrospira* is cultivated on the largest scale worldwide, with an estimated annual global production of 70,000 tons per year [7]. Its leading position in the microalgae industry is due to the fact that cultures can be relatively easily maintained free of contamination, as *Arthrospira* is an extremophile prokaryote that grows in a highly alkaline medium and tolerates pH levels above 9. Moreover, it forms relatively large trichomes that can be readily harvested using simple screening. *Arthrospira* biomass is commercially used as a nutritional supplement and to produce the natural blue pigment phycocyanin. *Arthrospira* further accumulates the polysaccharide glycogen within its cell as an energy storage reserve, which is of interest to produce biofuels (e.g., bioethanol) or building blocks for the chemical industry (e.g., production of lactic acid by glucose fermentation). *Arthrospira* also accumulates more complex polysaccharides within its cells. For instance, the polysaccharide spirulan is isolated from *Arthrospira* biomass and is composed of rhamnose,

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3-*O*-methylrhamnose (acofriose), 2,3-di-*O*-methylrhamnose, 3-*O*-methylxylose, uronic acids, and sulfate groups. This polysaccharide has mainly been studied for its bioactivity and has been shown to possess anti-viral properties [8].

Microalgae and cyanobacteria often contain complex polysaccharides in the outer layers of the cell wall and release these polysaccharides in the culture medium [3]. Concentrations of polysaccharides in the culture medium often increase over time as the culture ages. In *Arthrospira*, Trabelsi et al. [9] reported that the concentration of released polysaccharides increased up to 210 mg L^{-1} after 21 days of cultivation. The concentration of released polysaccharides can become particularly high when the culture medium is repeatedly recycled after biomass harvesting, as harvesting removes the microalgal cells but not the polysaccharides from the culture medium. Total organic carbon increased from 30 to 914 mg L^{-1} in *Arthrospira* outdoor cultures over 4 weeks of cultivation during which the culture medium was recycled 4 times [10]. Similarly, Depraetere et al. [11] showed that the concentration of total carbohydrates in the culture medium of *Arthrospira* increased from 50 to 180 mg L^{-1} after 6 weeks, during which the culture medium was recycled 4 times. These polysaccharides that accumulate in the culture medium of *Arthrospira* are currently considered a waste product. In fact, they interfere with the production process as they slow down the filtration rate during harvesting, most likely due to an increase in the viscosity of the culture medium. Depraetere et al. [11] also provided evidence that the organic matter accumulated in the culture medium inhibited the growth of *Arthrospira*, resulting in a decrease in the productivity of the culture. Thus, removing these accumulated polysaccharides from the spent culture medium would be beneficial for the productivity of the culture, but may at the same time yield a novel material with interesting properties similar to polysaccharides from macroalgae.

Several studies have characterized polysaccharides that accumulate in the culture medium of *Arthrospira* [9,11–15]. All studies reported 5–7 different neutral monosaccharides as well as sugars containing negatively charged groups such as uronic acid and/or sulfated monosaccharides. However, while Depraetere et al. [11], Ahmed et al. [15], and Majdoub et al. [12] reported rhamnose as the dominant monosaccharide, Xia et al. [14] reported xylose, and Trabelsi et al. [9] reported xylose and galactose as the dominant monosaccharides. The content of uronic acid ranged from 30 % in the study of Majdoub et al. [12] to 12–15 % in the studies of Depraetere et al. [11] and Xia et al. [14]. The sulfate content ranged 0.5–2.4 % in Trabelsi et al. [9] and Chentir et al. [13] to 20 % in Majdoub et al. [12]. These studies show that the composition of polysaccharides produced by *Arthrospira* may be quite variable, most likely because they are influenced by culture conditions or culture age [3,16].

The culture medium of *Arthrospira* contains a high concentration of dissolved salts (about 18 g L^{-1} of sodium carbonate and bicarbonate). In addition, the spent culture medium not only contains polysaccharides but also proteins, with proteins representing 10–55 % of the total organic matter [9,11,15]. Therefore, desalting and deproteination are required to purify the polysaccharides. Chentir et al. [13] isolated polysaccharides from the spent culture medium of *Arthrospira* using ultrafiltration (5 kDa membrane) and removed salts by washing with pure water and proteins by means of a protease treatment. Santos de Jesus et al. [17] isolated polysaccharides by ethanol precipitation and removed salts by washing over a 12 kDa ultrafiltration membrane.

So far, only Chentir et al. [13] carried out a rheological analysis of these polysaccharides and concluded that they may display a gel-like behavior at high concentrations. For instance, 1 % w/v solution displayed a liquid-like behavior, while a gel-like behavior was observed at 5 % w/v. Because the polysaccharides contain anionic groups (carboxylic acids or sulfates), they may display gelation in the presence of cations, similar to other acidic polysaccharides such as alginate. While metal-induced gelation has not been reported for polysaccharides isolated from the spent culture medium of *Arthrospira*, gelation induced by trivalent cations was observed for polysaccharides isolated from biomass of the cyanobacteria *Nostoc* and *Microcystis* [5,6].

In this study, polysaccharides were isolated from a spent culture medium obtained from a commercial *Arthrospira* producer. The polysaccharides were concentrated using membrane ultrafiltration and purified to remove salts and proteins. An additional acidification step was included to remove any metals bound to anionic groups on the polysaccharide. The purified material was subsequently characterized in terms of carbohydrate and protein content, monosaccharide profile, uronic acid and sulfate groups, and metal content. Subsequently, the gelation behavior of the polysaccharide was evaluated in solutions of mono-, di- and trivalent cations by assessing the formation of bead-shaped gels as well as using the vial inversion test. These tests revealed that the polysaccharide displayed gelation in the presence of trivalent cations. Rheological analysis was further carried out on polysaccharides in solutions with and without Fe^{3+} present.

2. Materials and methods

2.1. Arthrospira cultivation

The spent culture medium of an *Arthrospira platensis* culture was obtained from a small 'Spirulina' producer (Agraqua, Belgium). The producer cultivated the strain *Arthrospira platensis* SAG 21.99 (*Arthrospira* hereafter) in Zarrouk's medium prepared in rainwater collected on-site. Cultivation was done in a small raceway pond (10 m^2, 20 cm depth) positioned in a greenhouse. The culture was continuously mixed using a paddle wheel. Natural sunlight was the sole source of irradiance. The pH of the culture medium was not controlled and ranged between 9 and 11. The culture was maintained as a semi-continuous culture and about 25 % of the biomass was harvested each time the optical density measured at 750 nm was >1 (corresponding to a biomass concentration of about 0.5 g L^{-1}). The biomass was harvested using a 20 μm nylon mesh, and the filtrate was immediately returned to the raceway pond after harvesting. The reduction in water level due to evaporation was compensated by the addition of rainwater. Concentrations of nutrients in the water level were measured monthly and maintained at a constant level by the addition of concentrated nutrient solutions. This continuous culture was maintained for 6 months during the summer half-year, between April and October 2019.

2.2. Polysaccharide isolation and purification

The spent culture medium was collected at the end of the summer growing season, at the beginning of October 2019. The spent culture medium was filtered once more over a 20 μm nylon mesh to ensure that all cells were removed and was subsequently stored frozen at −20 °C. Before further processing, the spent culture medium was thawed and centrifuged (6000 rpm for 10 min; Sigma 6-16 KS) to remove any remaining particulate matter. The spent culture medium was concentrated by ultrafiltration using a 50 kDa polysulfone ultrafiltration cartridge (UFP-50-C-4X2MA, Cytiva Life Sciences). Fifty kDa was selected as a cut-off size as released polysaccharides from *Arthrospira* as well as other cyanobacteria and microalgae typically have a molecular weight above 50 kDa, e.g. molecular weights of 199 kDa and 750 MDa have been reported for *Arthrospira* [12,17], 279 kDa for *Nostoc* [18], 1 MDa for *Cyanothece* [19], and 1.4 MDa for *Porphyridium* [2]. The polysaccharides were concentrated using ultrafiltration up to a point where the increase in viscosity prevented the pumping of the liquid. After concentrating, the concentrate was then washed with MiliQ water (conductivity 0.66 μS cm^{-1}) to remove dissolved salts until the conductivity of the filtrate was reduced to about 100 μS cm^{-1} (measured with Hach H1 40d). Proteins were hydrolyzed by adding the protease subtilisin A (protease from *Bacillus licheniformis*, Sigma-Aldrich P5380) [2]. The pH of the concentrate was adjusted to pH 8 (optimum for enzyme activity), 100 U protease per gram of protein was added, and the

concentrate was incubated for 24 h at 55 °C. After incubation, protein hydrolysates (peptides, amino acids) and the protease itself (27 kDa) were removed by ultrafiltration over the 50 kDa membrane. Subsequently, the pH of the concentrate was adjusted to 3 using 1 M HCl to promote protonation of the carboxylate groups and to release any cations bound to the polysaccharide. This acidification step is also used in the purification of alginic acid and sodium alginate [20]. The polysaccharides concentrate was washed with MiliQ water until the conductivity was reduced to 1 μS cm^{-1}. Only the first four of the seven washing volumes used acidified water at pH 3. The concentration of total carbohydrates and proteins (Section 2.3) was quantified at different steps in the purification process. The purified concentrate was stored at −80 °C and freeze-dried (Christ Alpha 1-4 LDplus).

2.3. Chemical analyses and characterization of the polysaccharide

2.3.1. General analyses

The freeze-dried material was dissolved in MilliQ water (1 % w/v) and further analyzed. The concentration of total carbohydrates in the polysaccharide concentrate was determined using the phenol-sulfuric acid method using glucose as standard [21]. Proteins were quantified by the Lowry assay using bovine serum albumin as standard [22]. Uronic acids were determined using sulfamate and m-hydroxy diphenyl as proposed by Filisetti-Cozzi et al. [23] using glucuronic acid as standard.

The sulfate content was determined in the hydrolyzed polysaccharide (1 M HCl, 100 °C for 5 h) by the $BaCl_2$-gelatin reagent using potassium sulfate as standard [24]. All glassware was pre-acid-washed (HNO_3) to eliminate possible contamination of the sample. The ash content was determined by incinerating the polysaccharide at 550 °C for 24 h and measuring the residual ash weight. The cation concentration was determined by dissolving the ash in 0.23 M HNO_3 solution and placed in a sonicator bath (Bio block, Fisher Scientific). The solution was then analyzed by ICP-MS (Agilent 7700×).

2.3.2. Monosaccharide profile

The monosaccharide profile was determined after hydrolyzing the polysaccharide in 2 M TFA for 2 h at 120 °C [25]. After hydrolysis, the samples were neutralized with two volumes of 1 M $NaHCO_3$ and centrifuged (13,000 rpm, 10 min) to remove any insoluble material. Monosaccharides were analyzed using high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS-5000$^+$ DC chromatography system. A Dionex CarboPac PA1 column (4 × 250 mm) with a guard column (4 × 50 mm) was used. The flow rate was set to 1 mL min^{-1}. The column was equilibrated with 18 mM NaOH for 8 min. The samples were isocratically eluted with 18 mM NaOH for 25 min for neutral monosaccharides elution followed by an isocratic elution of 32 mM NaOH in 325 mM sodium acetate for 10 min for uronic acids elution. The run was followed by a washing step with 250 mM NaOH for 5 min. The system was calibrated using standards of D-glucose, D-galactose, D-Fructose, D-xylose, D-mannose, L-rhamnose, L-fucose, L-arabinose, D-ribose, D-glucosamine, D-glucuronic acid, and D-galacturonic acid. The standard solutions (10 mM) were subjected to the same hydrolysis protocol as the polysaccharide concentrate. In the initial analysis, one large peak could not be matched with any of the standards listed above. The position between L-fucose and L-rhamnose pointed to the deoxy sugar 2-deoxy-D-galactose [26]. Therefore, the sample was run again and the unknown peak was matched with this monosaccharide standard.

2.3.3. Solid-state ^{13}C NMR spectroscopy

The solid-state ^{13}C-CP/MAS (Cross-Polarization/Magic Angle Spinning) NMR spectrum was recorded with a Bruker Avance 400 MHz spectrometer (9.4 T magnet) equipped with a 4 mm BL4 X/Y/H probe. The lyophilized polysaccharide sample (80 mg) was introduced into airtight ZrO_2 rotors. The aromatic signal of hexamethylbenzene was used to determine the Hartmann–Hahn condition for cross-polarization and to calibrate the carbon chemical shift scale (132.1 ppm) [27]. Magic angle spinning was performed at 12 kHz. The spectrum was acquired using the following parameters: a spectral width of 50 kHz, a 90° pulse length of 4.0 μs, a contact time for cross-polarization of 1.0 ms, an acquisition time of 20.0 ms, a recycle delay time of 2.5 s, and about 22,000 scans.

2.3.4. Size exclusion chromatography

Water-based size exclusion chromatography was done on a Thermo UHPLC 3000, with a quaternary pump, 600 μL autosampler, and VWD. The following columns were used: Shodex LB 806, LB 804, and LB 803. A flow rate of 0.5 mL/min of an aqueous buffer containing 0.1 M $NaNO_3$ was applied. The separation part was coupled to a multi-angle light scattering device (MALS) (Wyatt DAWN) with 18 angle light scattering, a ViscoStar viscosity detector, and a differential refractive index (dRI) Optilab dRI detector. No column calibration was performed as the use of light scattering allows determining the absolute molar mass of the polysaccharide; a specific refractive index increment (*dn/dc*) value of 0.142 was used. The polysaccharide samples (5 mg/mL) were dissolved in water containing 0.1 M $NaNO_3$ and shaken at 50 °C for 4 h. Subsequently, the samples were filtered over a 0.45 μm filter to remove residual particulates. Twenty-five microliters of the sample was injected into the system.

2.4. Cation-induced gelation

Anionic polysaccharides often display gelation in the presence of cations. To test whether specific cations induced gelation, droplets of a solution of the polysaccharide concentrate were dropped into a cation solution and the formation of stable bead-shaped gels was evaluated [28]. Droplets of a 1 % w/v polysaccharide solution (pH 6) were dropped into 100 mM aqueous solutions of monovalent (NaCl, KCl), divalent ($BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$), and trivalent ($AlCl_3$, $FeCl_3$, $CrCl_3$) cations. The formation of bead-shaped gels was recorded, and their stability was evaluated by manual shaking of the cation solution.

Further experiments were performed with Fe^{3+} as iron-polysaccharide gels have potential uses in biomedical applications e.g. in wound healing, tissue engineering, or drug delivery [6,29]. The influence of cation concentration and pH on gel formation was investigated using the vial inversion test [30]. A known volume of a 100 mM $FeCl_3$ solution was added to a 1 % w/v polysaccharide solution (Milli-Q water) and the pH of the mixture was adjusted to 6. The pH of the polysaccharide solutions was adjusted with 0.5 M NaOH and HCl solutions.

2.5. Rheological characterization

Further rheological characterization of the polysaccharide was carried out in the presence and absence of Fe^{3+}. A 1 % w/v solution of the polysaccharide was analyzed without and with the addition of 4 mM $FeCl_3$ with final pH of 6. The samples were prepared 24 h in advance and were stored in a cold room overnight before use. Rheological experiments were performed on an AR-G2 magnetic-levitation thrust bearing, controlled-stress rheometer (TA Instruments, United States). Flow-type experiments were performed with a 40 mm 2° steel cone upper geometry, 54 μm gap. All oscillatory measurements were performed with a 40 mm parallel steel plate upper geometry, 500 μm gap. The Peltier-heated stationary bottom plate of the AR-G2 rheometer was used to maintain a constant temperature of 25 °C ± 1 °C during the measurements unless stated otherwise. Samples were allowed to equilibrate at 25 °C for 1 min before performing the measurement. Experimental data were collected using the Rheology Advantage instrument's control software (TA Instruments, United States) and processed with Rheology Advantage data analysis software (TA Instruments, United States). All measurements were performed in triplicate.

A controlled shear rate experiment was performed to determine viscosity and flow behavior changes, e.g., Newtonian, shear-thinning, or shear-thickening. For that, samples were pre-sheared for 3 min at 0.001 s^{-1}, then equilibrated for 1 min. Measurements were performed with a logarithmic shear rate ramp (s^{-1}, 0.001–1000), consisting of 5 data points per decade. The power-law model $\eta = K\dot{\gamma}^{n-1}$ was applied to the flow curves, where η is the viscosity, K is the viscosity at the shear rate of 1 s^{-1}, and n *is* the power-law index [31].

The gel behavior of the polysaccharide was determined through the viscoelastic modulus. First, amplitude sweep measurements were performed to identify the linear viscoelastic (LVE) and high shear regions. These amplitude sweep measurements were measured with a logarithmically increasing oscillatory stress (Pa; 0.01–200 or 0.1–200, depending on sample viscosity) at a fixed frequency of 1 Hz; measurements were performed with 15 data points per decade. Later, frequency sweep measurements were performed logarithmically with increasing oscillatory frequency (Hz; 0.01–10) at constant oscillatory stress, chosen within the respective LVE region. The measurements were performed with 15 data points per decade.

The temperature dependency of the viscoelastic modulus was monitored through a temperature sweep (heating and cooling ramp). To avoid the sample drying out during the measurements, the sample area was closed off with a solvent trap filled with ultrapure water (Milli-Q, Merck, Germany). Measurements were performed at constant oscillatory stress, chosen within the respective LVE region, and at a constant 1 Hz oscillation frequency. The temperature was ramped continuously at a rate of 3 °C per minute (20 °C – 75 °C – 20 °C), with a sampling interval of 10 s.

Recovery of the viscoelastic modulus after high shear was also evaluated. Thixotropic behavior was analyzed using a three-step ('LVE' - 'high shear' – 'LVE') oscillation interval. In the first LVE step, the sample undergoes oscillatory stress within its respective LVE range for 10 min at 1 Hz. In the 'high shear' step, oscillatory stress, higher than the respective LVE region of the sample, is applied for 10 min at 1 Hz. During the third step (oscillatory stress within LVE, 10 min, 1 Hz), the structural recovery of the gel samples was then analyzed by plotting the percentage recovery of the storage (G') and loss (G") modulus relative to the plateau values of these respective values obtained during the first LVE step.

3. Results and discussion

3.1. Polysaccharide isolation and purification

The spent culture medium of *Arthrospira* had a carbohydrate concentration of 136 ± 14 mg L^{-1}. This concentration is relatively high compared to other studies that have reported carbohydrate concentration in *Arthrospira* culture medium (9 to 50 mg L^{-1}) [11,25]. This high concentration can be ascribed to the fact that the culture medium had been recycled for about 6 months, during which biomass was harvested but dissolved substances could accumulate in the culture medium.

In addition to carbohydrates, the spent culture medium also contained 163 ± 8 mg L^{-1} proteins. Trabelsi et al. [9] also reported high protein concentration relative to carbohydrates in the culture medium of *Arthrospira*.

The spent culture medium was concentrated by means of ultrafiltration using a membrane with a cut-off of 50 kDa. The volume of the concentrate was reduced from 4.1 to 0.8 L by ultrafiltration, corresponding to an up-concentration of 5.2 times. Further concentration was not possible because of a decrease in the filtration rate, presumably due to an increase in the viscosity of the liquid. In other studies, the spent culture medium of *Arthrospira* was concentrated 20 to 40 times, but this culture medium most likely contained lower initial concentrations of carbohydrates as the culture medium was obtained from 7 to 30 days old cultures [13,32].

The carbohydrate concentration increased from 136 ± 14 to 390 ± 28 mg L^{-1} during further concentrating, indicating that the membrane retained 55 % of the total carbohydrates (Table 1). At the same time, the protein concentration increased from 163 ± 8 to 418 ± 7 mg L^{-1}, indicating that the ultrafiltration membrane retained 49 % of the proteins. Low molecular weight carbohydrates and proteins <50 kDa were probably lost in the concentration step. Because the culture medium has a very high concentration of sodium (bi)carbonate salts (18 g L^{-1}), the concentrate was subsequently washed with several volumes of Milli-Q water to remove these dissolved salts (Fig. S1). This resulted in a decrease in the conductivity from 11,580 to 86 μS cm^{-1}. During this desalting step, an additional 10 % of the carbohydrates and 11 % of the proteins were lost from the concentrate. Proteins were subsequently hydrolyzed by a protease and peptides were removed by subsequent rinsing with Milli-Q water (Fig. S1). This reduced the protein concentration to 95 ± 1 mg L^{-1}, an 89 % reduction, and an increase in the carbohydrate to protein ratio from 1 to 2.6. Simultaneously, the concentrate was acidified to pH 3 to protonate the carboxylic groups and release bound cations. This further decreased the conductivity to 1.8 μS cm^{-1}.

In conclusion, we were able to recover polysaccharides from the spent medium of an *Arthrospira* culture medium using a 50 kDa polysulfone membrane. About 35 % of the initial carbohydrate concentration was recovered and the concentration of proteins and salts was reduced with 89 % and 99 %, respectively. Higher recoveries of *Arthrospira* carbohydrates (56–99 %) from culture medium have been previously reported [11,33]. In these studies, *Arthrospira* cultivation time was considerably shorter (15 to 50 days old culture) compared to our study (6 months old culture). It is possible that during this long cultivation time, environmental factors and bacterial activity may have an effect on the composition of the polysaccharide yielding higher low molecular weight carbohydrates (<50 kDa) that were lost in the concentration step. The recovery of polysaccharides from the spent medium also allows the recovery of water and nutrients that can be reused in the cultivation process. In fact, culture medium recycling is considered a strategy to reduce microalgae production costs as evaluated by techno-economic assessment [34]. By evaluating a similar carbohydrate concentration (117–245 mg L^{-1}) to that in our study, Koçer et al. [35] also concluded in their techno-economic assessment that the recovery of extracellular polysaccharides from the microalgae *Chlorella* and *Botryococcus* can be economically viable. Such assessment is yet necessary to determine the economic impact of the valorization of *Arthrospira* spent medium.

Ultrafiltration is a common technique applied among studies focused on the extracellular polysaccharides from *Arthrospira* [9,11–13,17]. In this study, we applied ultrafiltration not only to concentrate the polysaccharide but also to increase its purity (remove salts and protein hydrolysates). Besides ultrafiltration, ethanol precipitation has been also used to increase the purity of the polysaccharides from *Arthrospira* [13,17]. Membrane separation has been proven to be more selective and efficient in terms of the purity of polysaccharides compared to ethanol precipitation [36]. In addition, membrane separation, including ultrafiltration, is a mature technology applied in industry. In fact, a pilot-scale evaluation of extracellular polysaccharides from *Arthrospira* was reported by Li et al. [32] demonstrating the feasibility of this process. Zhao et al. [33] demonstrated that by finetuning membrane properties, such as hydrophilicity, higher retention of carbohydrates over proteins could be achieved. This could be further implemented to simplify the purification process of the polysaccharide. The concentrate obtained by membrane filtration needs to be dried for further use. In this study, we used freeze-drying, however, this is an expensive method if implemented on a large scale. There are alternative methods, such as spray and drum dryers that are commonly used during the processing of commercially available polysaccharides such as alginate, carrageenan, and xanthan [20,37] and could be also implemented on polysaccharides from *Arthrospira*.

Table 1
Changes in carbohydrate and protein concentration and conductivity during polysaccharide isolation and purification.

Ultrafiltration step	Carbohydrate mg L^{-1}	Yield %	Protein mg L^{-1}	Yield %	Conductivity μS cm^{-1}	Yield %
Initial spent culture	136 ± 14	100	163 ± 8	100	12,010	–
Concentration[a]	390 ± 28	55	418 ± 7	49	11,580[b]	100
Desalting	315 ± 1	45	320 ± 4	38	86[b]	1
Deproteinization	250 ± 5	35	95 ± 1	11	1.8[b]	0.02

[a] Spent medium was 5.2 times concentrated from its initial volume.
[b] Conductivity measured in filtrates.

3.2. Polysaccharide characterization

The concentrate was frozen and dewatered by freeze-drying, which yielded a dry material that was further characterized (Fig. S2). The material consisted of 407 ± 7 mg g^{-1} carbohydrates and 113 ± 1 mg g^{-1} of residual protein. This is similar to other studies, where carbohydrate concentration ranged from 400 to 700 mg g^{-1} [2,13,19]. Other studies reported similar residual protein concentrations, irrespective of whether polysaccharides were purified by alcohol precipitation or protease treatment in released polysaccharides isolated from *Arthrospira, Nostoc, Cyanothece,* and *Porphyridium* [6,13,19,38]. It has been suggested that these residual protein moieties are chemically connected to the carbohydrates and they may contribute to the viscosity of the polysaccharide material [4].

The polysaccharides had a high content of uronic acids (159 ± 12 mg g^{-1}) but a low content of sulfate groups (2.3 ± 0.4 mg g^{-1}). The uronic acids and sulfate content is known to be variable in released polysaccharides of *Arthrospira*, as reviewed by Phélippé et al. [16]. For instance, uronic acid content of 15 and 400 mg g^{-1} was reported by Ahmed et al. [15] and Filali Mouhim et al. [39], while sulfate concentrations ranging from 5 to 200 mg g^{-1} have been reported [9,12,13].

Despite the thorough washing with acidified water, the ash content of the polysaccharide was still 67 ± 12 mg g^{-1}. Phosphorus (4.7 mg g^{-1}) was the most abundant element in the ash, followed by calcium (3 mg g^{-1}), sodium (1.2 mg g^{-1}), magnesium (0.6 mg g^{-1}), and potassium (0.4 mg g^{-1}). Iron (68 μg g^{-1}) and zinc (44 μg g^{-1}) were also detected but at much lower concentrations. Compared to the culture medium in which sodium and potassium (315 and 17 mM) dominate over other cations (<0.3 mM), the polysaccharide seems to preferentially retain polyvalent cations.

Monosaccharide analysis showed that the dominant monosaccharides that could be identified using the initial set of standards were fucose, glucose, and glucuronic acid (Table 2, Fig. 1). Xylose, galactose, rhamnose, mannose, and glucosamine were present in minor amounts. In the initial analysis, an abundant monosaccharide was detected that eluted between fucose and rhamnose, but its identity did not match any of the standards that were initially used to calibrate our system. Its position in the chromatogram suggested it was a deoxy sugar. The unknown sugar was subsequently matched with a standard of 2-deoxy-D-galactose, which is known to elute between fucose and rhamnose. Hydrolysis of the polysaccharide followed by monosaccharide analysis yielded 483 ± 9 mg monosaccharides per g of polysaccharide sample, including neutral sugars and uronic acids.

Table 2
Monosaccharide profile of the polysaccharide concentrate.

Monosaccharide	% Relative composition
2-Deoxy-D-galactose	27.87 ± 1.42
Fucose	22.72 ± 0.53
Glucose	21.97 ± 0.53
Glucuronic acid	17.12 ± 0.51
Xylose	3.07 ± 0.60
Rhamnose	2.37 ± 0.08
Galactose	1.91 ± 0.09
Glucosamine	1.57 ± 0.13
Mannose	1.39 ± 0.43

Released polysaccharides from *Arthrospira* generally contain deoxy sugars and uronic acids, but apart from that the composition seems to be variable. For instance, Depraetere et al. [11], Ahmed et al. [15], and Majdoub et al. [12] reported rhamnose as the dominant monosaccharide in released polysaccharides of *Arthrospira*, while Trabelsi et al. [9] reported xylose and galactose, while Xia et al. [14] reported xylose as the dominant monosaccharide. The deoxy sugar 2-deoxy-D-galactose has not previously been reported from *Arthrospira* nor from other cyanobacteria, but other deoxy sugars such as rhamnose and fucose are commonly reported in extracellular polysaccharides from *Arthrospira* [9,12,15,16]. Deoxy sugars in general are known for their bioactivity [40]. The variability in monosaccharide composition of released polysaccharides from *Arthrospira* may result from differences in growing conditions among studies [16]. In addition, differences may be related to the age of the culture or the number of times the spent culture medium was recycled. Therefore, further work is needed to compare the polysaccharide material isolated in this study with polysaccharides isolated from the spent culture medium of other commercial *Arthrospira* producers to understand the variability of the properties of this material.

The ^{13}C-CP/MAS NMR spectrum of lyophilized extracellular polymeric material from *Arthrospira* is shown in Fig. 2. The broad resonance signals between 110 and 10 ppm point to a relatively high molecular weight polymeric material. The resonance signals in the 60–110 ppm range originate from the different carbons in the monomer units of the polysaccharide. The peak at 100.90 ppm corresponds to the anomeric carbons (C1) involved in glycosidic linkages (O—C—O functionalities). The intense, broad peak between 60 and 87 ppm (top at 72.46 ppm) covers the monomeric carbons C2, C3, C4, C5, and C6 bonded to oxygen in C—OH/C—OR moieties. The sharp signal at 174.07 ppm can most likely be attributed to carbonyl carbons (C═O) from carboxylic acid (COOH) functional groups, in agreement with the anionic nature of the polysaccharide. The sp^2 hybridized carbon (in aromatic rings and unsaturated C═C bonds) signals that are expected in the 115–165 ppm region were missing except for a small signal around 130.0 ppm. This points to a low protein content, which is to be expected given the deproteination. The peaks in the 15–40 ppm region are related to aliphatic hydrocarbons (CH_3, CH_2, and CH), suggesting that the polysaccharide may be linked to lipids. Lipopolysaccharides are indeed known to be common in cyanobacteria [41]. Derivatives containing, e.g., OCH_3, OR, OCH_2COOH, the CH_3 and CH_2 groups next to O can explain the 54–60 ppm signal.

Fig. 3 shows the results of the aqueous SEC analysis. At lower volumes (16–17 mL) a high molecular weight shoulder is visible in the molar mass and RI traces. At higher elution volumes (22–23 mL) a slight uprise in molecular weight is noticed indicating that some larger molecules have a delayed elution time. This delayed elution time might be caused by uronic acid groups interacting with the column. The UV trace shows the presence of UV-absorbing compounds that were too small to be detected by light scattering (Fig. S3). This was also observed in the SEC analysis of a polysaccharide of *Porphyridium* (Bernaerts et al. [2]). The mass recovery was 77 %, probably because some material was retained during the filtration step in sample preparation. The number average weight was Mn = 172.4 kDa, the weight average molecular

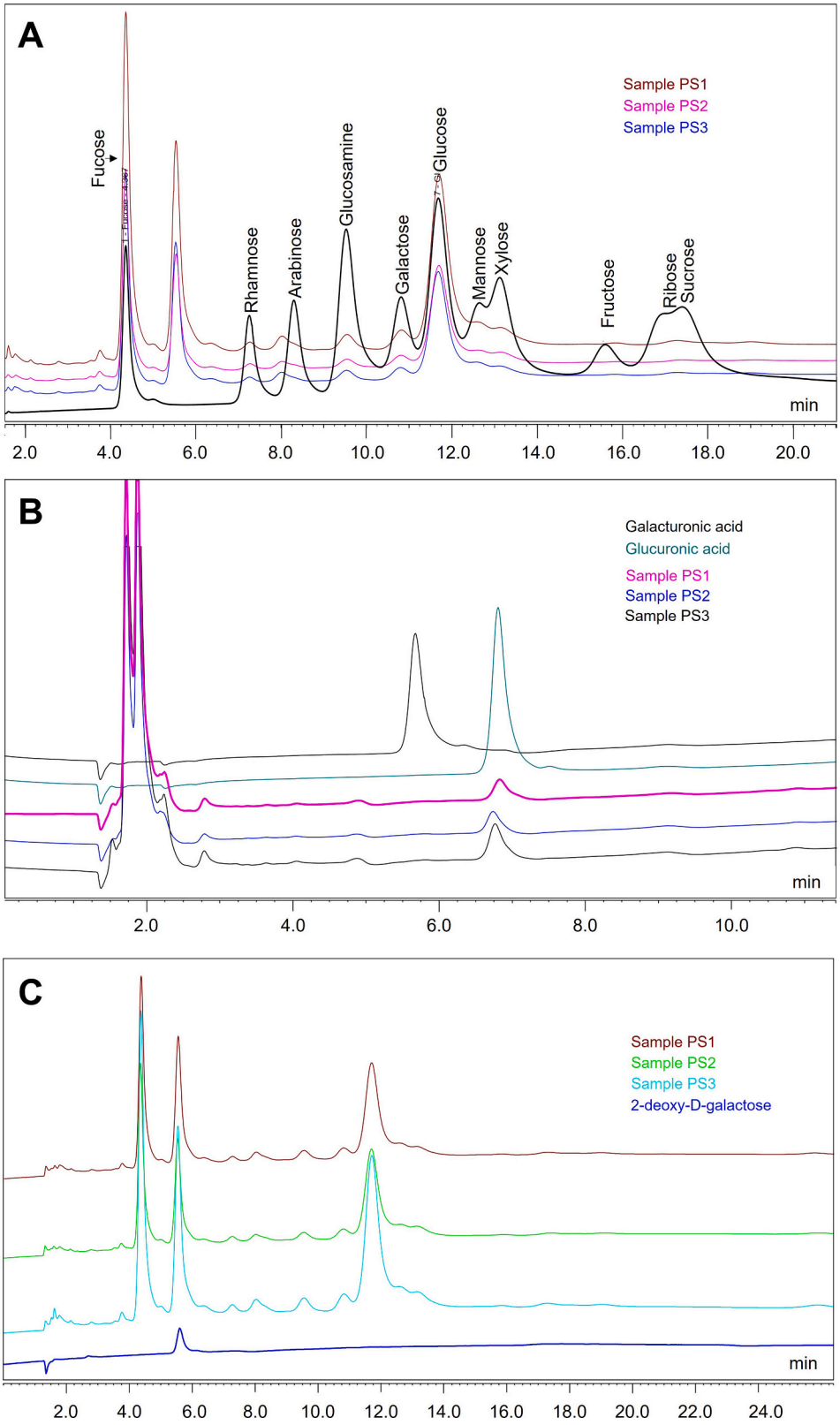

Fig. 1. HPAEC-PAD chromatogram of *Arthrospira* polysaccharides after TFA hydrolysis. A. Three replicate samples and the mixture of standards of neutral sugars. B. Three replicate samples and standards of uronic acids. C. Three replicate samples and standard of 2-deoxy-D-galactose.

weight, Mw, 463.5 kDa, and the *Z*-average molecular weight, Mz, 1383.9 kDa. This is in the expected range for polysaccharides from cyanobacteria including *Arthrospira* [4,17] and other microalgae (e.g. Bernaerts et al. [2]). The polysaccharide has a high polydispersity of Mw/Mn = 2.69. This points to a heterogenous composition, which is also in line with other polysaccharides isolated from *Arthrospira* (e.g. Santos de Jesus et al. [17]). To determine the chain conformation, the Mark-Houwink-Sakurada (MHS) plot (intrinsic viscosity vs. molar mass)

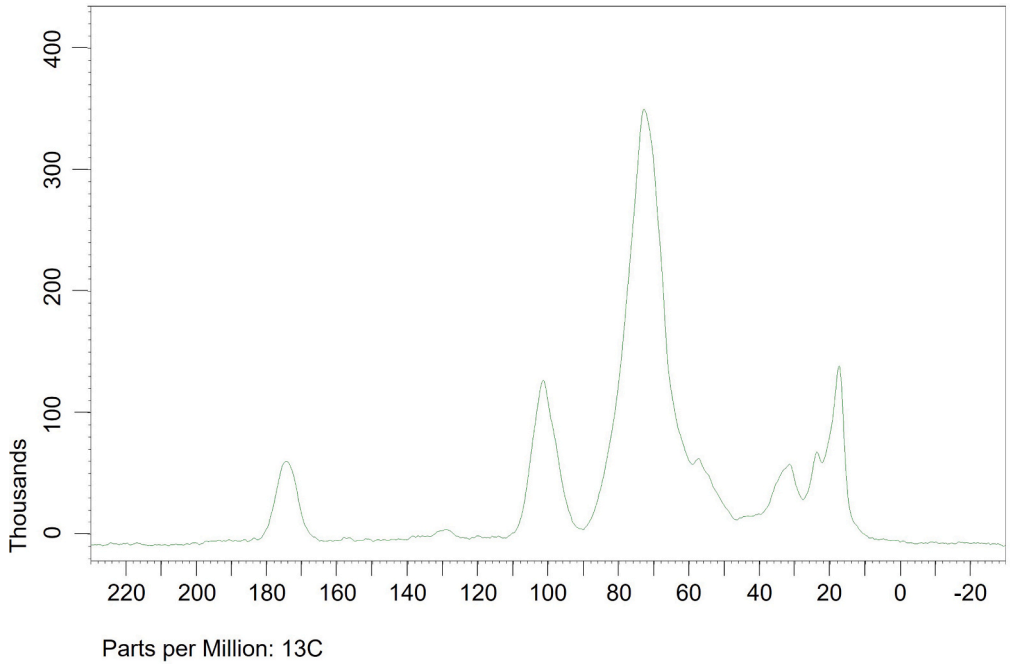

Fig. 2. ^{13}C-CP/MAS NMR spectrum of the purified polysaccharide isolated from spent *Arthrospira* culture medium.

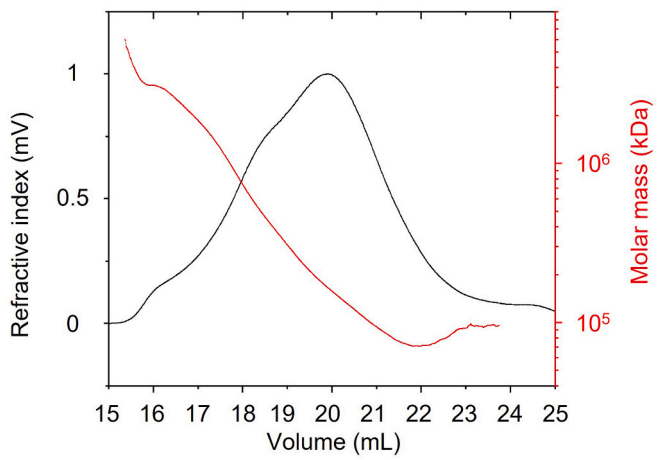

Fig. 3. Results of SEC analysis of the purified polysaccharide isolate from spent *Arthrospira* culture medium showing molar mass (red curve) and refractive index (black curve) versus elution volume. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the conformation plot (rms radius vs. molar mass) were analyzed. The slope derived from the MHS plot was 0.64 and the slope derived from the conformation plot was 0.5. As this slope is higher than 0.3, this suggests that the polymer behaved as a random coil and was not highly branched [42].

3.3. Interaction with cations

Commercially important polysaccharides from macroalgae such as alginate and carrageenan, and from bacteria such as xanthan are known to interact with cations to form gels. For instance, κ-carrageenan forms a gel in the presence of monovalent cations, while alginate and ι-carrageenan form a gel with divalent cations, and λ-carrageenan and xanthan form a gel in the presence of trivalent cations [1,43–45]. Gelation is assumed to result from the interaction of polyvalent cations with anionic carboxylate or sulfate groups. Because the material purified in this study had a relatively high content of carboxylate groups, we hypothesized that it might display gelation in the presence of cations. Gelation was evaluated by adding drops of a polysaccharide solution to a cation bath (100 mM) and monitoring the formation of bead-shaped gels (Fig. 4). Bead-shaped gels were obtained instantaneously by dropping the polysaccharide solution in the Al^{3+} and Fe^{3+} solutions. These bead-shaped gels remained intact after the vial was shaken by hand and were still intact after several weeks of storage at room temperature. In the case of Cr^{3+}, beads were formed, but they disintegrated after the vial was shaken by hand, forming a loose film. A different affinity of cations of the same valence has also been reported for alginate [46]. Although bead-shaped gels initially appeared in the divalent cation solutions, they disintegrated after the vial was shaken, forming a precipitate. In the monovalent cation solutions, no bead-shaped gels were formed, and the polysaccharide droplets simply dissolved.

In further experiments, Fe^{3+} was chosen as a trivalent cation since Fe^{3+} gels have shown potential in drug delivery, tissue engineering, and wound healing [6,29]. Vial inversion experiments were conducted to determine the optimal pH and Fe^{3+} concentration for gel formation (Figs. 5, S4). Gel formation was not observed at $FeCl_3$ concentrations of 1 to 3 mM but occurred at a concentration of 4 mM. No gelation was observed at pH < 5, possibly because of protonation of carboxylate groups at low pH which precludes interaction of cations with the polysaccharide; i.e., the pK_a of glucuronic acid is 3.3 [47]. When pH was increased above pH 6, the gel weakened, likely due to the precipitation of Fe^{3+} as hydroxides. Therefore, the optimal conditions for gel formation were 4 mM $FeCl_3$ and pH 6.

Gelation in the presence of cations is commonly reported for anionic polysaccharides and can be ascribed to cross-linking between polysaccharide chains due to the complexation of the metals with anionic groups. As the polysaccharide isolated has a high content of glucuronic acid but a low sulfate content, we assume that Al^{3+} and Fe^{3+} interact with negatively charged carboxylic acid groups on glucuronic acid. Few previous studies of polysaccharides produced by microalgae or cyanobacteria have evaluated their interaction with cations to form gels. Polysaccharides from the red microalgae *Porphyridium* have been widely studied, but these do not form gels in the presence of K^+, Na^+, and Ca^{2+} [2,38]. Nakagawa et al. [5] noted that bound polysaccharides isolated

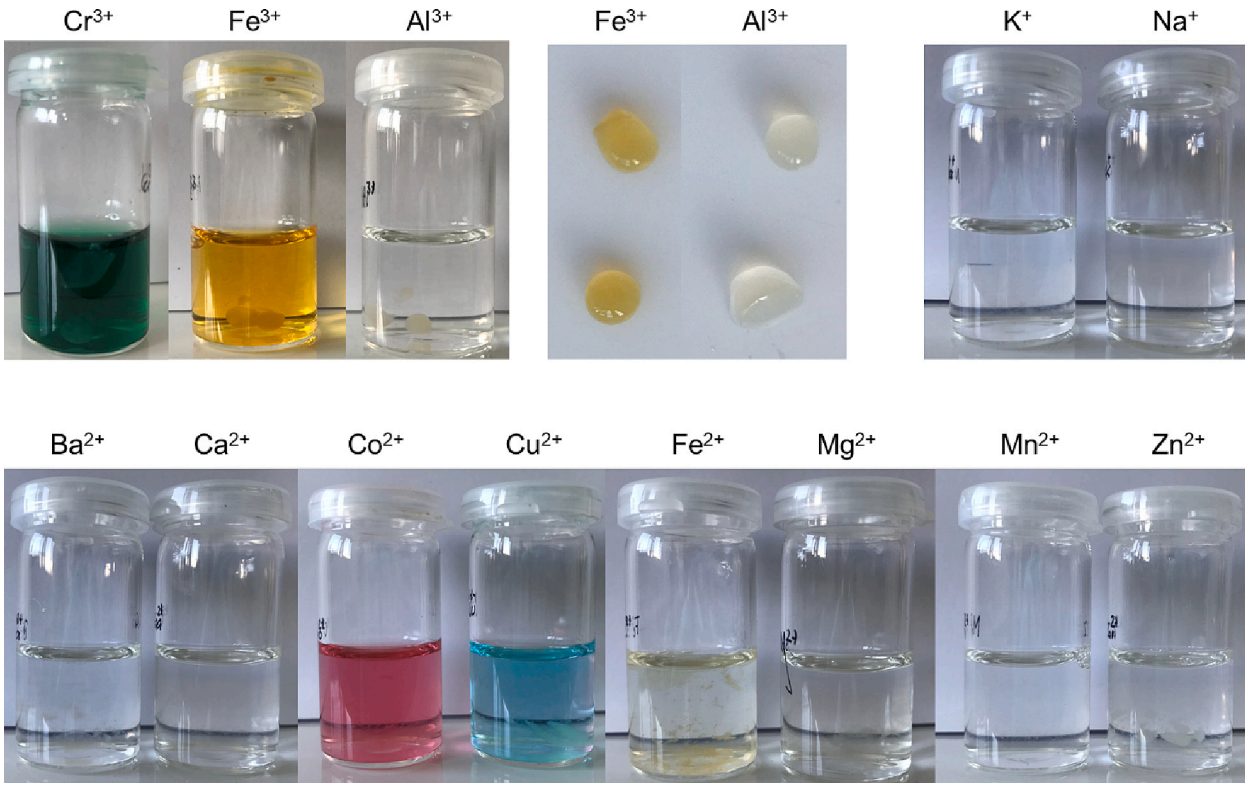

Fig. 4. Droplets of 1 % w/v polysaccharide solution (pH 6) in 100 mM cation solutions (5 mL) and Fe^{+3} and Al^{3+} bead-shaped gels out of the solution. Monovalent cations: NaCl, KCl; divalent cations: $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$; trivalent cations: $AlCl_3$, $FeCl_3$, $CrCl_3$.

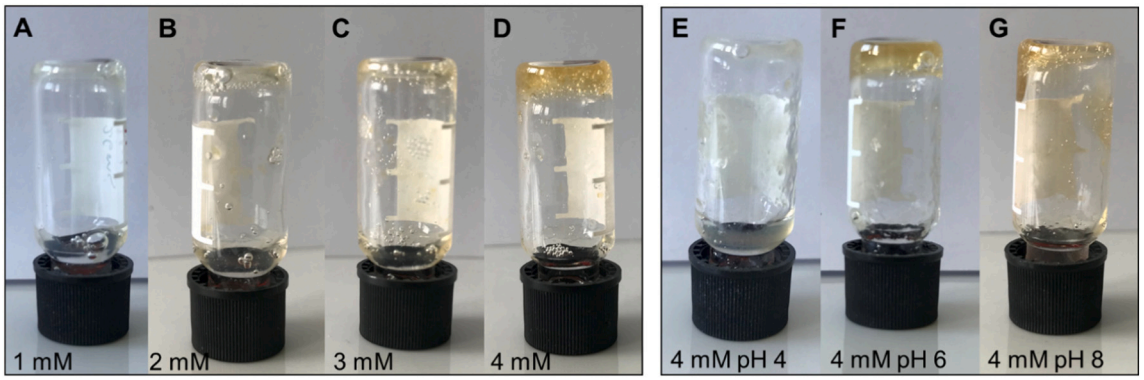

Fig. 5. The effect of Fe^{3+} concentration and pH on cation-induced gelation of 1 % w/v *Arthrospira* polysaccharide solutions through inversion tests. A–D: Addition of 1–4 mM Fe^{3+} to the polysaccharide (pH 6). E–G: the effect of pH on 4 mM Fe^{3+} polysaccharide solutions.

from the cyanobacterium *Microcystis* formed gels in the presence of Al^{3+} or Fe^{3+}. Also, gelation with Fe^{3+} was recently reported for a released polysaccharide isolated from *Nostoc* [6]. Like *Arthrospira*, polysaccharides from *Microcystis* and *Nostoc* contained 7–8 different neutral sugars, had a low sulfate content (<4.1 mg g^{-1}), and protein content of around 100 mg g^{-1}. Uronic acids comprised 16–28 % of the monosaccharides, similar to the 17 % in our polysaccharide concentrate isolated from the spent culture medium of *Arthrospira*. However, unlike *Arthrospira*, galacturonic acid and not glucuronic acid was the dominant uronic acid in the polysaccharides of *Microcystis* and *Nostoc*.

3.4. Rheological characterization

Rheological measurements were carried out to characterize the viscoelastic properties of a 1 % w/v polysaccharide concentrate solution in water and a 1 % w/v solution in the presence of Fe^{3+} (4 mM $FeCl_3$ at pH 6). Generally, polysaccharides form fibrils in solution [6,48], these fibrils are responsible for the hydrogel structure. These semiflexible networks were characterized by different rheological experiments, including flow curves, strain and frequency sweep, temperature sweep, and thixotropy.

The aqueous polysaccharide solution had a viscosity of 0.09 Pa·s at 10 s^{-1}. This is similar to the polysaccharide isolated from the spent culture medium of *Arthrospira* by Chentir et al. [13] (0.1 Pa·s at 10 s^{-1}). It is also comparable to 1 % w/v solutions of alginate polysaccharides isolated from macroalgae (0.07–0.13 at 10 s^{-1} [49,50]). The aqueous polysaccharide solution displayed a decrease in viscosity with shear, pointing to shear-thinning behavior (Fig. 6A). This was confirmed by applying a power-law model ($\eta = K\dot{\gamma}^{n-1}$), which yielded a flow index n < 1, which defines the steepness of the shear-thinning decay (Table 3, Eberhard et al. [31]). Shear-thinning behavior is commonly reported for polysaccharide solutions, including polysaccharides isolated from

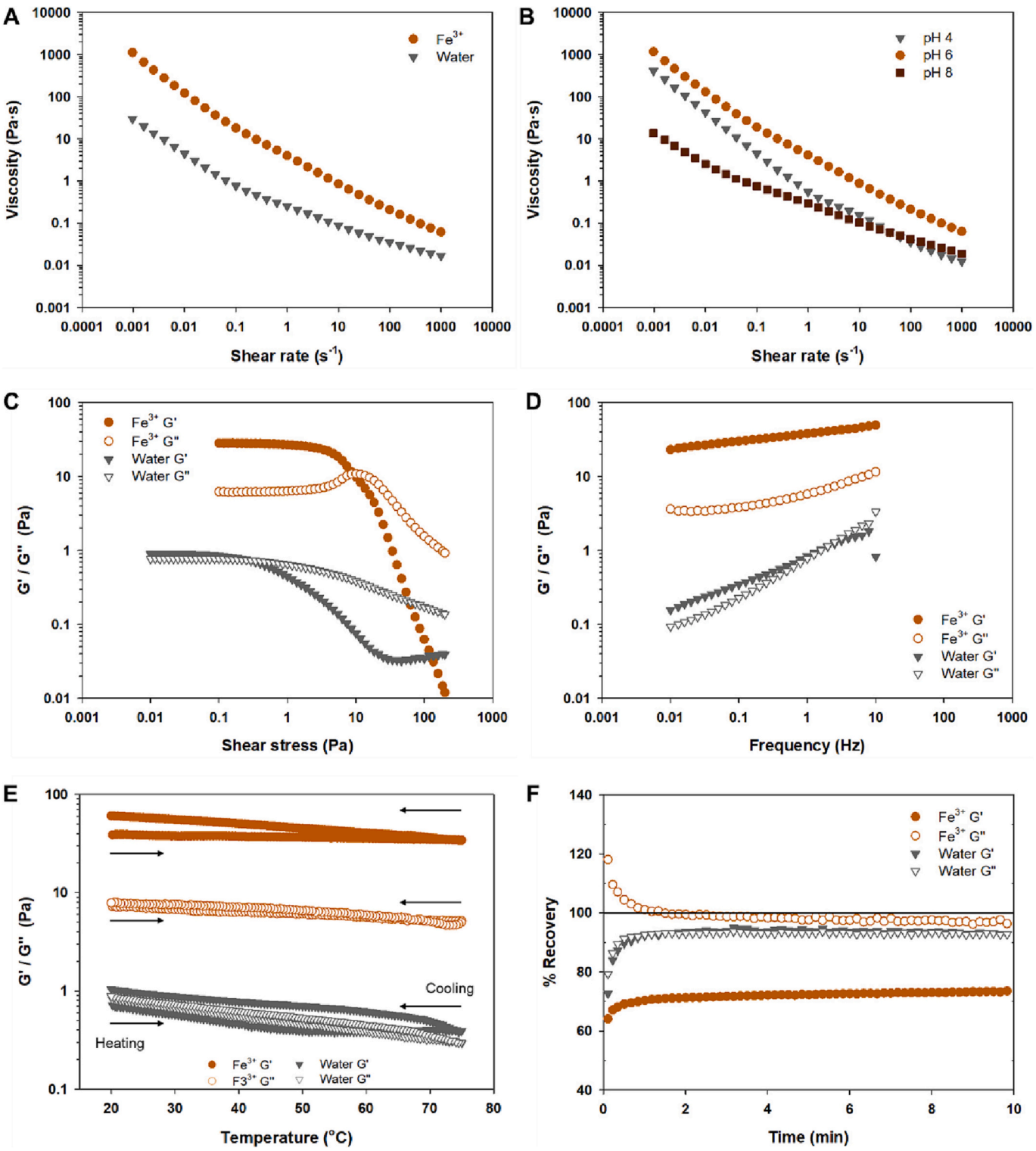

Fig. 6. (A) Flow curves of 1 % w/v polysaccharide solutions in water and Fe^{3+} at pH 6. (B) The effect of pH in the Fe^{3+} (4 mM) polysaccharide solution. Viscoelastic modulus analyzed by (C) amplitude sweep and (D) frequency sweep. Amplitude sweeps were performed at 1 Hz constant frequency. Frequency sweeps were performed at 0.025 Pa and 0.5 Pa for the water and Fe^{3+} polysaccharide solutions, i.e., the linear viscoelastic region (LVE) determined from the amplitude sweep. (E) Temperature sweep. Measurements were performed at 1 Hz constant frequency and 0.025 and 0.5 Pa for the water and Fe^{3+} polysaccharide solutions. (D) Thixotropy. Measurements were performed at 1 Hz constant frequency. Low shear was 0.05 Pa and 0.5 Pa for the water and Fe^{3+} polysaccharide solution, and high shear was 5 Pa and 75 Pa for the water and Fe^{3+} polysaccharide solution. All rheological measurements were performed in triplicate.

macroalgae [50,51], as well as microalgae and cyanobacteria [2,6,19,38]. Chentir et al. [13] also reported shear-thinning behavior for polysaccharide material isolated from *Arthrospira*.

Amplitude sweep and frequency sweep tests were carried out to determine the storage, G', and loss, G'', modulus (Fig. 6C and 6D). In the amplitude sweep the linear viscoelastic domain (LVE) is determined while keeping the frequency constant. This LVE represents the amplitude (or strain) range in which the structure of the sample is not

Table 3
Viscosity dependency to shear rate, power-law fitting model.

1 % w/v solution	Viscosity at 1 s^{-1} K	Index *n*	R^2
Water	0.27 ± 0.03	0.57 ± 0.01	0.993 ± 0.001
Fe^{3+}	4.38 ± 0.44	0.36 ± 0.02	0.998 ± 0.001

destroyed. For the polysaccharide solution without Fe^{3+}, the liquid behavior of the aqueous polysaccharide is predominant with G" > G'. Similar behavior was reported by Chentir et al. [13] for 1 % w/v *Arthrospira* released polysaccharides in an aqueous solution. In their work, only by increasing the concentration to 5 % w/v, the linear viscoelastic region was maintained at a high strain with a higher storage to loss modulus, indicating that the elastic behavior is becoming more dominant in comparison to the viscous behavior [13]. After adding the Fe^{3+} ions there was a clear change in the rheological behavior (Fig. 6A, Table 3). The viscosity became one order of magnitude higher (0.9 Pa s at 10 s^{-1}). The LVE was nearly constant until a shear stress of 4.6 Pa and after that, G' showed a marked decrease below 20 Pa. A crossover point occurred at 9 Pa where G" = G', indicating the point where the gel starts to flow and the liquid behavior becomes dominant (Fig. 6C). The decrease in G' seems to be linear indicating a homogenous structural breakdown. When observing the curve for the loss modulus, G" increases to 11 Pa, indicating a weak strain overshoot. This overshoot is characteristic of samples of biological sources as cells need to adapt against mechanical physical or chemical stress. This overshoot suggests that the polysaccharide backbone is highly extended and is able to form cross-links with Fe^{3+}. These cross-link points resist against the imposed deformation and hence G" increases. By applying an even higher deformation the cross-links are destroyed and G" decreases again [52].

The frequency sweep showed that both moduli were maintained over a wide frequency range, confirming the polysaccharide solution in Fe^{3+} as a gel/elastic solid (Fig. 6D). The storage G' modulus was higher than the loss G' modulus, e.g., G' of 38 Pa and G" of 6 Pa at 1 Hz which shows the predominant solid behavior of the formed gel. With higher frequency both moduli slightly increased, showing that the gel still maintained some liquid behavior. So far, no other studies have investigated the gelation of *Arthrospira* polysaccharides in the presence of cations. Also, no rheological measurements were reported for *Nostoc* and *Microcystis* gels formed in the presence of Fe^{3+} [5,6]. The properties of the *Arthrospira* polysaccharide gel are comparable to that of alginate gels formed in the presence of Ca^{2+}. For example, Cuomo et al. [53] reported that a 1 % w/v alginate gel in 6 mM Ca^{2+} displayed a viscosity of about 2 Pa·s and a storage and loss modulus of about 20 and 7 Pa, respectively. Ofori-Kwakye et al. [54] also reported a similar storage G' modulus of 19–21 Pa for a 1 % w/v alginate gel in 6 mM Ca^{2+}.

A temperature sweep test was carried out to study the stability of the polysaccharide solution in water and in Fe^{3+} at different temperatures (Fig. 6E). In the aqueous polysaccharide solution, a crossover point (G' = G") occurred at 60 °C, and a higher G' to G" was observed, a ratio that slightly increased during cooling. Proteins may form a gel during or after heating, as Chronakis [55] reported for a protein isolate from *Arthrospira* biomass. Protein moieties were 113 mg g^{-1} in *Arthrospira* polysaccharide concentrate and gelation of these proteins might explain the observed increase in the storage G' to the loss G" modulus in the polysaccharide with increasing temperature. Increases in the G' to G" modulus with temperature (80 °C) were also reported by Chentir et al. [13] for solutions of released polysaccharides from *Arthrospira* (1–5 % w/v in water) in which protein moieties were also present (51 mg g^{-1}). The increase in the storage modulus (G') upon heating may also occur in the Fe^{3+} solution. However, this effect is negligible compared to the different magnitudes between the moduli G' ≫ G".

Thixotropy was evaluated as the recovery of the viscoelastic modulus after high shear (Fig. 6F). The aqueous polysaccharide concentrate showed a fast recovery of about 90 and 94 % for the storage and loss modulus within 10 min. The polysaccharide solution in Fe^{3+} also showed thixotropic behavior (Fig. 6F), the loss modulus, G", showed a recovery close to 100 %, and the storage modulus, G', recovery reached 70 % within 10 min. The longer time to recovery may be due to the higher rigidity of the Fe^{3+} polysaccharide. Similarly, only a 50 % recovery of the storage G' modulus was reported for alginate in Ca^{2+} (6–10 mM) after 6 min [53].

The formation of a gel in the presence of Fe^{3+} is most likely due to the interaction of the cation with the carboxylic acid of glucuronic acid. These carboxylic acid groups (pK_a about 3–4) are anionic above pH 4 and non-charged below pH 4. Therefore, we would expect the gel to be stable at a high pH but unstable at pH 4 or lower. Indeed, the dependency of gel formation on the pH was confirmed by the flow curves shown in Fig. 6B. Higher viscosity was noted for the Fe^{3+} polysaccharide solution at pH 6 than at pH 4. Also, the viscosity of the Fe^{3+} polysaccharide solution at pH 6 was higher than at pH 8. This can be ascribed to the formation of hydroxide precipitates at alkaline pH, which reduces the free Fe^{3+} concentration and weakens the gel.

4. Conclusions

In this study, a released polysaccharide from *Arthrospira* spent medium was isolated and purified by ultrafiltration over a 50 kDa membrane. Dissolved salts were removed by washing, bound metals were removed by reducing pH, and proteins were removed by protease hydrolysis. Characterization of the polysaccharide showed an anionic character of the polysaccharide, mainly derived from the presence of glucuronic acid. Fucose, 2-deoxy-D-galactose, and glucose were the main neutral monosaccharides. The presence of deoxy sugars such as fucose and 2-deoxy-D-galactose warrants evaluation of the bioactivities of the polysaccharide. Interaction with cations showed that the polysaccharide displayed selective gelation in solutions of trivalent cations, but not in mono- or divalent cations. Rheological analyses showed that polysaccharide - Fe^{3+} gels had similar rheological properties as other well-studied polysaccharide gels such as alginate - Ca^{2+} gels. The spent culture medium is a waste product derived from *Arthrospira* cultivation and the removal of this polysaccharide material from the culture medium is beneficial for the productivity of the culture. Moreover, it yields a novel material with potentially high-value applications such as wound healing. Hydrogels create a hydrated environment that supports the healing process of wounds (e.g., diabetic, burn, infected wounds). In addition, hydrogels could be used as carriers in the topical administration of drugs. Thus, further evaluation of *Arthrospira* Fe^{3+} hydrogels is envisaged. In the microalgae industry, *Arthrospira* species are the most cultivated species compared to other microalgae and cyanobacteria, and thus the isolation of these polysaccharides could be directly implemented in the existing *Arthrospira* producing facilities.

CRediT authorship contribution statement

SPCB: conceptualization, methodology, investigation, data analysis, writing – original draft. DB: conceptualization, methodology, investigation, data analysis, writing – original draft. MAM: conceptualization, methodology, investigation, data analysis, writing – original draft. TS: conceptualization, methodology, investigation, data analysis, writing – original draft. SL: conceptualization, methodology, investigation, data analysis, writing – original draft. DV: conceptualization, data analysis, funding acquisition, supervision, writing – review & editing. PA: conceptualization, data analysis, funding acquisition, supervision, writing – review & editing. RS: conceptualization, methodology, data analysis, writing – review & editing. WT: conceptualization, data analysis, funding acquisition, supervision, writing – review & editing. WVDE: conceptualization, data analysis, funding acquisition, supervision, writing – review & editing. RW: conceptualization, data analysis, funding acquisition, supervision, writing – review & editing. KM: conceptualization, investigation, data analysis, funding acquisition,

supervision, writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103010.

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