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Impact of biochemical properties on the gelation of EPS extracted from aerobic granules

Abdo Bou-Sarkis^{a,b}, Etienne Paul^b, Yolaine Bessiere^b, Nicolas Derlon^c, Elisabeth Girbal-Neuhauser^{a,*}

^a Université de Toulouse, UPS, LBAE, Laboratoire de Biotechnologies Agroalimentaire et Environnementale, URU 4565, Institut Universitaire de Technologie, 24 rue d'Embaquès, Auch 32000, France

^b Université de Toulouse; INSA, UPS, INP, TBI, 135 Avenue de Rangueil, Toulouse F-31077, France

^c EAWAG, Swiss Federal Institute of Aquatic Science and Technology, Department of Process Engineering, Dübendorf CH-8600, Switzerland

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ABSTRACT

Extracellular Polymeric Substances (EPS) extracted from aerobic granular sludge are a promising source of gel-forming biopolymers. Gaining insights on the charge distribution of these polymers was performed through a fractionation using anionic chromatography. A gelling factor (GF) based on the apparent gel volume and the reactivity of polymers to calcium was attributed to each eluted fraction. The GF values of the eluted anionic EPS correlated with their negative charge. A GF of 0.04 ± 0.03 is measured for molecules eluted at NaCl 0.25 M and a GF of 0.32 ± 0.02 for the molecules eluted at NaCl 0.75 M. However, upon reaching a very high negative charge (elution at NaCl 1 M), the gelation capacity decreases. The size distribution of gelling molecules was analyzed by Size Exclusion Chromatography. High Molecular Weight (MW) molecules are predominantly involved in gelation (between 65 % and 100 % of the high molecular weight fraction from each sample) while low MW molecules participation is relatively limited (lower than 40 % of the low molecular weight fraction from each sample). Moreover, infra-red spectroscopy analysis of the molecules before and after calcium addition showed that mostly proteins and polysaccharides contribute to the hydrogel formation. Colorimetric methods showed that around 60 % of the uronic acids present in the samples could form hydrogels whereas proteins' participation varied between 0 % and 100 % depending on the fraction.

Thus, modulating EPS extraction and purification methods to ensure the preservation of their molecular weight and the selection of appropriate negative charge could be a strategy for EPS valorization.

1. Introduction

Biopolymers produced from renewable sources are becoming more and more attractive since they fall within the circular economy concept. Currently, biopolymers are the focus of intensive research efforts with the goal of reaching a production less dependent on petrochemical industries. Microbial polymers are an important source of renewable biopolymers and include intracellular polymers (polyphosphate, glycogen...) and Extracellular Polymeric Substances (EPS) mainly composed of proteins and carbohydrates [1]. Lately, EPS have gained a lot of interest because of their biodegradability, and their easier recovery in comparison with petroleum-based polymers [1].

In the last ten years, aerobic granular sludge was recognized as a type of biofilm that proved to be efficient for the treatment of both industrial

and municipal wastewater [2,3]. EPS play many roles within aerobic granules such as settleability, nutrient sorption or diffusion. The subset of the EPS matrix that provides the physical structure of the granule is called structural EPS (sEPS). They are classically recovered after drastic granule solubilization and show the capacity of forming gels *in vitro* through ionic cross-linking with divalent cations [4-6,3]. Among all extraction methods, alkaline conditions resulted in the highest EPS extraction yields, using Na_2CO_3 or NaOH with a temperature varying from 4 to 80°C [4]. Acidic precipitation is then performed for the recovery of gel-forming EPS [4,7,2], but care must be taken when using a strong base coupled with a high temperature (above 50°C) because of chemical degradation of the polymers. That is why for valorization purposes, 0.2 M sodium carbonate used at 80°C offers a good yield of EPS extraction and good gel-forming capacities. Many applications have

* Corresponding author.

E-mail address: Elisabeth.neuhauser@iut-tlse3.fr (E. Girbal-Neuhauser).

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been reported in literature for the EPS that were extracted from granules. Indeed, they can be used as a coating material for paper packages to increase their impermeability [8], flame retarding properties have also been reported in literature [9]. In addition, their capacity to adsorb heavy metals was proven making EPS extracted from AGS a relevant biomaterial for bioremediation applications [10,11]. In addition, interesting applications in the agricultural sector were noted. Indeed, EPS can be used as valuable biomaterials to improve water holding capacity and nutrient retention of soils [12]. In 2022, two full-scale EPS recovery plants were established in the Netherlands, with an estimated recovery yield of 22.5% (18 kilos of EPS are extracted from 80 kg of granular sludge) [13]. One ton of extracted gel-forming EPS generates a final revenue of 1000–2000 € (after the extraction and refining costs). The total Dutch production for the year 2030 is expected to be at 85,000 tons of EPS thus generating between 85 and 170 million € [14]. Polysaccharides, proteins, humic substances, and DNA were all proven to be a part of the biochemical composition of sEPS [4,15,2]. In addition, the EPS matrix of aerobic granular sludge contains highly anionic polymers such as hyaluronic acid-like polymers and sulfated glycosaminoglycans (GAGs)-like polymers that were suspected as additional structural and gelifying components [16].

This wide diversity in macromolecules paves the way for the valorization of granular sludges through the extraction of many high added-value byproducts [6]. In particular, hydrogel forming polymers have elicited the most interest but the molecular properties governing the gel-formation of EPS are still unclear, and understanding them would help in orienting the valorization of EPS. Their capacity to retain water depends on the presence of hydrophilic functional groups within the polymeric backbone, mainly OH, COOH, NH₂, CONH, and HSO₃ groups [17]. Applications of hydrogels were described in the medical industry, food industry, agriculture, and water industry amongst others [12,18] that are dependent on the characteristics of the polymer being used as well as on the extrinsic factors (temperature, pH, crosslinker type and concentration). The molecular weight, chemical composition, charge density (sulfate/carboxylic groups), conformation (3D structure) related to either native or denaturated state and isoelectric point, all play important roles in the gelation process [19–21].

The effect of charge on gel formation can be seen clearly with the example of the three well-known types of carrageenan differing by their negative charge density due to the number of sulfates present on the disaccharide unit [22]. Shielding the electronegative repulsions is necessary for gelation to happen and hence the presence of excessive negative charges in carrageenan may cause repulsion and limits gelation [22–25]. That is why contrary to λ -carrageenan bearing 3 sulfates per disaccharide unit, κ - and ι -carrageenan bearing respectively 1 and 2 sulfates, are capable of forming ionically crosslinked hydrogels.

The molecular weight of gelling polymers can also drive their capacity to form hydrogels and modulate the final properties of the obtained hydrogels. Alginate is a linear polyuronic polysaccharide consisting of (1–4)-linked blocks of poly- β -D-mannuronic acid (M) and poly- α -L-guluronic acid (G) residues at different proportions and with different sequential occurrences. Many studies have reported the capacity of alginate to form hydrogels in the presence of an ionic cross-linker such as calcium and several results underlined that an increase in molecular weight is correlated with an increase in gel's strength [26, 27,17].

The gel-forming capacity of EPS has been mainly attributed to two types of polymers: (i) alginate-like exopolysaccharides (ALE) named that way due to their extraction method (sodium carbonate) and composition (rich in carboxyl groups attributed to uronic acids) that are similar to alginate [7] and (ii) granulan which is mainly composed by a complex heteropolysaccharide (rich in glucuronic acids, glucosamine, N-acetylgalactosamine, amongst others) extracted using sodium hydroxide [28]. However, recent studies have proved that the gel-forming sEPS extracted using sodium carbonate have a very complex biochemical composition and are made of diversified polymers including

polysaccharides, proteins, humic-like substances and DNA [4,15,2]. Examples of composite hydrogels obtained using polysaccharides and proteins are numerous and this combination allows the obtention of hydrogels with competitive characteristics. For example, a hydrogel with an enhanced water retention capacity was obtained through the association of β -lactoglobulin and xanthan gum [29,17,20].

Several studies have shown the capacity for sEPS to form hydrogels with different types of divalent cations other than calcium and in particular with copper and zinc [30,31]. Concerning the physico-chemical properties of sEPS, it was proven that sEPS extracted from aerobic granular sludge are a mix of molecules with different anionic properties and a wide distribution in molecular weight. Indeed, low, medium and high molecular weight polymers going up to several million Da were reported [4,32].

In order to understand the mechanisms implied in the gelation of sEPS, efforts must be made to fractionate the gel-forming EPS with the aims of deciphering which chemical groups participate in the gel formation. Especially, the combination of both molecular charge and size parameters was not examined yet, although controlling these aspects could drive EPS valorization.

This task will be achieved through EPS fractionation according to their net surface charge using a preparative liquid chromatography. The originality of this work lies in coupling two resolutive chromatographic techniques based on key characteristics which are the charge of EPS and their molecular weight. Moreover, an original and simple assay recently developed in the laboratory [33] was implemented for rapid evaluation of gelling capacity of the fractionated EPS. According to author knowledge, coupling gel-forming potential through a miniaturized gel-screening method with physico-chemical properties of EPS such as elemental composition, molecular weight and charge distribution has been rarely covered in literature.

These experiments will allow to deepen the knowledge about the key determinants that govern the gelation of sEPS extracted from granular sludge. Acquiring such knowledge helps to improve and guide the extraction of sEPS towards more adapted extraction methods. Moreover, understanding the biochemical functions that are implied in the gelation, as well as their molecular size and charge can orient the applications of sEPS towards new and improved applications.

2. Materials and methods

2.1. Biomaterial and sEPS extraction

Aerobic granular sludge was recovered from sequencing batch reactors (SBR) fed with 100%-VFA synthetic wastewater (equal COD in acetate and propionate) as described in Layer et al. [34]. The systems were operated at a constant volume of 12.9 L and SBR cycles consisted of the following phases: anaerobic plug-flow feeding (1.5 h), aerobic phase (4 h), settling (variable time), and excess sludge removal after settling (critical settling velocity of 1.7–5.1 m/h). The granules had a SVI₃₀ below 50 mL/g, and an SVI₃₀/SVI₁₀ around 1. The reactor was dominated by medium and large diameter granules ($d > 0.63$ mm) while flocs with a diameter lower than 0.25 mm represented a minor fraction. A detailed characterization of the influent composition as well as the physical properties of the granules used for the treatment of organic pollution, nitrogen and phosphorus is provided in Layer et al. [34].

The extraction protocol detailed in Bou-sarkis et al. [4] was used for the extraction of sEPS. Briefly, the extraction protocol consisted of rehydrating the freeze-dried granules for 30 minutes in 0.2 M sodium carbonate, followed by a homogenization step using an ultra-turrax and a heating phase at 80 °C for one hour. After centrifugation at 14,000 g for 15 minutes, the supernatant was recovered and an acidic precipitation step was done through the addition of HCl to reach pH 2 as described by Lin et al. [7]. The pellet obtained after the acidic precipitation step was solubilized in sodium hydroxide 0.1 M, dialyzed for 36 hours, and freeze-dried. The obtained powder was dissolved in 0.1 M

sodium-hydroxide and named sEPS extract. The AGS fed with 100 %-VFA synthetic wastewater (equal COD in acetate and propionate) was tested in this work and triplicates of every experiment were done. The extraction efficiency was calculated by determining the amount of Volatile solids (VS) in sEPS and dividing it by the initial amount of VS in the initial granules. After freeze-drying, the extraction efficiency was determined to be 342.3 ± 12.9 mgVS/gVS of initial granules.

$$MW \text{ Reactivity}(\%) = \frac{\text{Reactive area} * 100}{\text{Total area without calcium}} = \frac{(\text{Total area without calcium} - \text{total area with calcium}) * 100}{\text{Total area without calcium}} \quad (1)$$

2.2. Preparative liquid chromatography

2.2.1. Fractionation according to the net surface charge

The fractionation of sEPS was performed using an AKTA purifier system (Cytiva) equipped with an on-line spectrophotometer. The used column was the 6 mL Resource™ Q (Cytiva) filled with a cationic stationary phase grafted with quaternary ammonium allowing the separation of polymers according to their surface charge distribution. Equilibration of the column was done using Tris HCl 50 mM at a pH of 12 ± 0.02 , and elution was performed by sequential addition of NaCl 1 M, so to obtain five steps corresponding to various NaCl concentrations in the elution buffer. A solution of sEPS with a Volatil Solids (VS) content of 0.2 % (w/v) was injected (5 mL) and the flow was set to 1 mL/min during the initial step (0 M NaCl) to allow optimal adsorption of negatively charged polymers on the stationary phase. Then, the flow speed was increased to 2 mL/min for the following steps. Molecules were detected by monitoring the absorbance at 280 nm (mAU). The chromatograms were divided into five regions corresponding to the NaCl concentration of the elution buffer: Cat/Neut for Cationic/Neutral (0 M NaCl), LAC for low anionic charge (NaCl 0.25 M), MAC for medium anionic charge molecules (NaCl 0.5 M), HAC for high anionic charge molecules (NaCl 0.75 M) and VHAC for very high anionic charge (NaCl 1 M) molecules. The peak area (mAU x mL) was processed using the UNICORN 5.31 software. All the fractions were collected and dialyzed for 36 hours against distilled water using a dialysis membrane with a cutoff of 3500 Da (Spectrum labs). Afterwards, the dialyzed fractions were freeze-dried and used for further analysis.

An injection of 250 μ L of the Cat/Neut fraction, solubilized in NaOH 0.1 M to obtain a VS concentration of 0.4 % (w/v), was done in the cationic exchange chromatography HiScreen™ Cpto™ S Impact (Cytiva). The elution protocol and the used detection were the same as described for the anionic exchange chromatography.

2.2.2. Size exclusion chromatography

The fractions collected after anionic chromatography were solubilized in 0.1 M sodium hydroxide so to obtain solutions at a VS concentration of 1 % (w/v) and 200 μ L were mixed either with 200 μ L of calcium at 0.2 M or with 200 μ L of distilled water. After an hour, both samples (with and without calcium) were centrifuged 15 min at 14,000 g to collect the supernatant for further analysis with the size exclusion chromatography column SUPERDEX 200 (Cytiva). According to the manufacturer, this column separates molecules having apparent molecular weights (MW) between 10 and 600 kDa. The injection volume was set to 100 μ L and the mobile phase was Tris HCl 50 mM with 0.1 M NaCl, pH 12 ± 0.02 with a flow of 0.5 mL/min. According to their elution volume and apparent size, the peaks composing the chromatograms were divided into three regions: HMW for high molecular weights that were eluted first between 7.7 and 12 mL, MMW for medium molecular weights eluted from 12 to 17 mL and LMW for low molecular weights that were eluted lastly after 17 mL. Molecules were detected by monitoring the absorbance at 280 nm (mAU). The peak area (mAU x mL)

was processed using the UNICORN 5.31 software.

Comparison of the chromatograms obtained with or without calcium allowed to evaluate the % of reactivity to calcium in each MW category using Eq. (1):

2.3. Test for gelation capacity

The test previously described by Bou-Sarkis et al. [33] was used to compare the gelation capacity of the sEPS extract and collected fractions using solutions at final VS concentrations of 1 % (w/v). As described in supplementary data (Figure S1), 100 μ L of the tested sample at 2 % was mixed either with 100 μ L of calcium at 0.2 M or distilled water. After 1 hour, both samples (with and without calcium) were centrifuged and the volume of the supernatant was measured; therefore, the gel volume (Vg) was deduced and the gel volume percentage (%Vg) was calculated using Eq. (2):

$$\%Vg = \frac{(Vt - Vs) * 100}{Vt} \quad (2)$$

Where Vt is the initial total volume (μ L), and Vs the remaining liquid volume (μ L) after calcium addition (supernatant after centrifugation). Then, UV-Vis spectra between 220 and 500 nm were monitored for both samples (with and without calcium) to determine the percentage of reactivity (%R) of the sample through Eq. (3):

$$\%R = \frac{(Vt * AIS - Vs * ACaS) * 100}{Vt * AIS} \quad (3)$$

Where AIS is the cumulated area of the initial spectrum without calcium (AU x nm), ACaS is the cumulated area of the spectrum with calcium (AU x nm). Both factors, i.e. %Vg and %R were combined to obtain a gelling factor (GF) (Eq. (4)):

$$GF = \%R * \%Vg \quad (4)$$

2.4. Fourier Transform Infrared (FTIR) spectroscopy

Following the gelation procedure of the sEPS extract, gels, supernatants after gelation, as well as samples of the initial sEPS were freeze-dried, mixed at 3 % (w/w) with KBr and submitted to FTIR using a spectrometer (PerkinElmer) equipped with a Diffuse Reflectance Infrared Fourier Transform (DRIFT) module. The resolution was set to 1 cm^{-1} and a total of 32 scans were done on each sample. A mathematical model was applied on all the spectra using the Spectrum 65 software (Kubelka Munk) and the spectra were normalized according to the total surface of the peaks.

2.5. Biochemical composition

Triplicates of sEPS fractions collected from Resource Q were prepared in NaOH 0.1 M with a final VS concentration of 0.1 % (w/v) without or with 0.1 M calcium. After 1 hour, the samples were centrifuged for 15 min at 14,000 g. The supernatants were dialyzed against distilled water for 6 hours and then against NaOH 0.05 M for another 6 hours to remove residual calcium. The biochemical composition (neutral sugars, uronic-acid like sugars, proteins, humic acids, and DNA) of the dialyzed samples was characterized using different colorimetric

tests. All samples were assayed in triplicates in 96 well microplates, using the FLUOstar OPTIMA (BMG, Labtech) microplate reader. Comparison of the concentrations determined in the solutions with or without calcium allowed to evaluate the % of reactivity with calcium of each biochemical group (BG) using Eq. (5):

$$BG \text{ reactivity}(\%) = \frac{\text{Reactive BG} * 100}{\text{Concentration without calcium}} = \frac{(\text{Concentration without calcium} \left(\frac{\text{mg}}{\text{L}}\right) - \text{Concentration with calcium} \left(\frac{\text{mg}}{\text{L}}\right)) * 100}{\text{Concentration without calcium} \left(\frac{\text{mg}}{\text{L}}\right)} \quad (5)$$

2.5.1. Neutral and uronic-acid like sugars quantification

The Anthrone method used for the quantification of neutral and uronic acid-like sugars was adapted from Rondel et al. [35]. The standards used for neutral sugars and uronic acids are glucose (0–75 mg/L) and glucuronic acid (0–750 mg/L) respectively. The Anthrone reagent was prepared at 2 mg/mL in sulfuric acid 96 % (v/v) and 150 μL of this reagent were added to 75 μL of samples/standards in 96 well microplates. Incubation at 60 °C for 30 minutes followed by cooling at room temperature for 10 minutes was done. The Anthrone reagent gives chromogenic compounds depending on the nature of osidic monomers (neutral or uronic) and the absorbances were read at 544 and 620 nm. The slopes of the four equations linking the standard solutions with glucose or glucuronic acid at the two absorbances were calculated. Afterwards, the concentrations of the unknown samples can be calculated by solving a system of two equations as described in Rondel et al. [35].

2.5.2. Proteins and humic acid quantification

The modified Lowry assay was used for the quantification of proteins and humic acid-like substances according to Frølund et al. [36] and the used standards (0–500 mg/L) were respectively BSA (Sigma-Aldrich) and humic acid (Fluka-Aldrich). The measurement of absorbance was done at 750 nm, with or without CuSO_4 . With CuSO_4 both proteins and humic acids absorb, however when CuSO_4 is omitted, the color development is due mainly to humic substances and chromogenic amino acids, and BSA color development decreased to 20 %. The respective concentration of proteins and humic acid-like molecules can be calculated by using the equations described in Frølund et al. [36].

2.5.3. DNA quantification

The Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen) was used for DNA quantification. DNA from phage Lambda (100 $\mu\text{g}/\text{mL}$) was used for the DNA standard curve going from 0 to 2000 ng/mL. In a 96 well microplate, 100 μL of the reagent prepared according to the manufacturer were added to 100 μL of standards or samples. Fluorescence intensity was measured at an excitation emission wavelength pair of 485/520 nm.

2.6. Elemental and mineral analysis

The freeze-dried fractions collected during anionic chromatography were analyzed for their content of C, N, H and S using the flash2000 (ThermoFisher scientific) instrument and their oxygen content was analyzed using the NA2100 Protein (CE instruments) so to obtain the percentage of each element in the studied fractions. The samples were compared to Alginate with low viscosity from brown algae (Sigma-Aldrich), and BSA (Sigma-Aldrich). The samples were also analyzed for their trace elements using ICPOES and a particular interest was given to the following cationic elements: Na^+ , Ca^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , during this study.

3. Results and discussions

3.1. What is the FTIR chemical fingerprint of the sEPS involved in gelation?

A global biochemical fingerprint of complex samples can be obtained through the analysis of their FTIR spectra. sEPS from AGS were analysed by the FTIR technique to get a glimpse of the biochemical groups participating in the gelation. The initial sEPS sample before calcium addition and both the gelled pellet and the non-gelled supernatant were analyzed to understand which biochemical fractions do not participate in gelation (Fig. 1 and Table 1).

As seen in Table 1, the amide I and amide II region of proteins (1500–1700 cm^{-1}) were visible in the three samples. As reported by Campo et al. [13], the observed shifts of amide I and amide II peaks between samples could be attributed to cross-links between proteins and calcium. Moreover, it was discussed that the peaks in these regions could be attributed to amino-sugars and N-acetylated derivatives. The presence of glycosylated amyloids and sulphated glycosaminoglycans was also attributed to these regions and the possibility of their involvement in the gelation was previously elucidated for sEPS extracted from aerobic granular sludge [13]. Molecules belonging to the amide III region between 1200 and 1300 cm^{-1} were detected in the sEPS sample and within the gel pellet but not in the supernatant remaining after gelation. These results indicate that the extracted proteins participate extensively in the hydrogel formation. Such observation is in agreement with the model of the granular structure proposed by Caudan et al. [39]. In this model, anionic proteins may constitute an intercellular cement playing a role in the cohesion of the granule through cationic bridging with calcium.

Carboxylic groups and hydrocarbon-like molecules were also detected between 1400 and 1500 cm^{-1} in the initial sEPS extract and in the gel but peak shifts were noted between the FTIR profiles. These peaks can be attributed to the very diversified monosaccharides composing sEPS including glucosamine, galactosamine and seven other neutral sugars [40,41,28]. In addition, the detection of carboxyl groups in this region may be due to the presence of uronic acids [7]. Therefore, the presence of these groups in the gel pellet was expected; however, a small fraction remained in the supernatant after calcium addition. Finally, polysaccharides, extending at 1041 cm^{-1} in the sEPS extract [7], got transferred into the gel pellet (at 1046 cm^{-1}) after calcium addition, confirming the polysaccharidic nature of some gel-forming EPS.

Although FTIR showed that proteins and polysaccharides both interact with calcium and potentially participate in the formation of sEPS hydrogels, the FTIR signature of the sEPS gels is also compatible with the involvement of more complex composites such as glycoproteins or hetero-polysaccharides. Further purification of sEPS is needed in order to better identify the physico-chemical characteristics of the molecules that are the most implied in the gel formation.

3.2. What is the most favorable charge for EPS gelation?

3.2.1. Ionic fractionation

Structural EPS extracted from granules were separated according to their net surface charge using anionic exchange chromatography. As shown in Fig. 2, the sequential elution strategy allowed to effectively

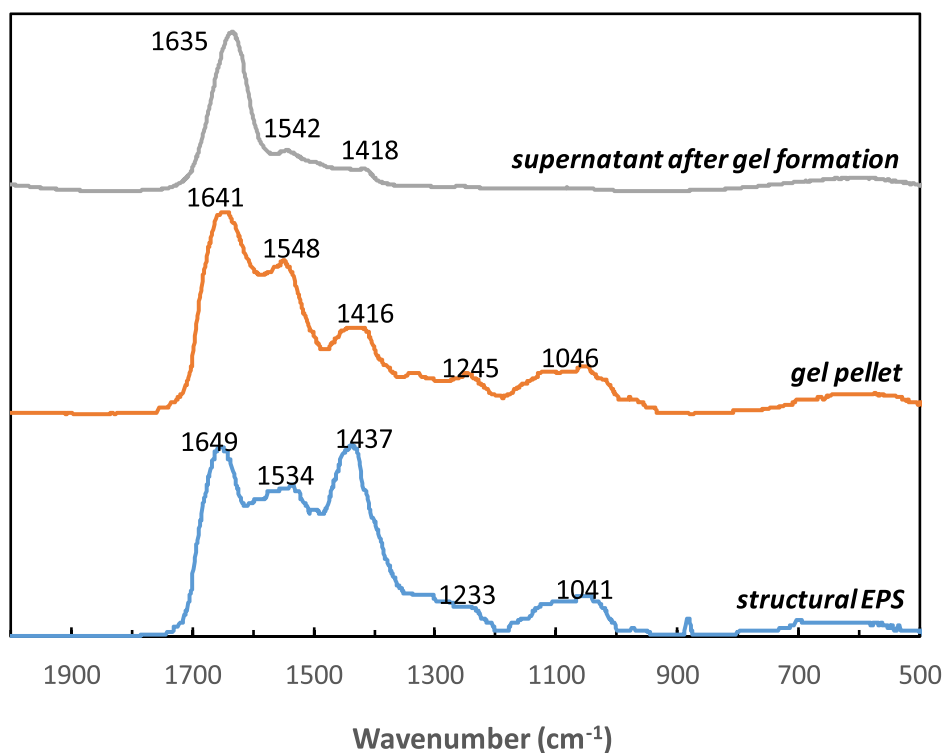


Fig. 1. FTIR spectra of the sEPS extract without calcium, the gel pellet and the supernatant after the addition of calcium.

Table 1

FTIR band assignments [13,37,38,7], with the observed peaks (Fig. 1) in the gel pellet, supernatant and structural EPS.

Wavenumber cm^{-1}	Assignment	Wavenumber (cm^{-1}) detected in the:		
		Structural EPS	Gel pellet	Supernatant
1600–1700	Amide I region of proteins	1649	1641	1635
1500–1600	Amide II region of proteins	1534	1548	1542
1400–1500	Carboxyl groups, hydrocarbon-like molecules	1437	1416	1418
1200–1300	Amide III region of proteins	1233	1245	-
1000–1200	Polysaccharides C-O-C, C-O	1041	1046	-

separate groups of molecules with a different negative surface charge thanks to the stepwise elution with increasing NaCl concentrations from 0 to 1 M. The eluted EPS were divided into five categories going from the cationic/neutral (Cat/Neut) that corresponds to unbound molecules to the very high anionic charged (VHAC) molecules that were eluted with the highest NaCl concentration. To further explore the charge profile in the Cat/Neut group, this fraction was injected in a cationic exchange chromatography column to determine if it contains cationic, neutral, or a combination of both molecules. The Cat/Neut fraction could not bind at all to the negatively charged column (supplementary data Figure S2) suggesting that it is composed of neutral polymers or tightly associated charged polymers forming neutral macromolecular complexes. Upon quantification of the peak areas, it can be noticed that most molecules present within the initial EPS extract (88.1 %) are negatively charged in the tested conditions. Medium (MAC) and very high anionic (VHAC) molecules appear as dominant in the charge profile with areas corresponding respectively to 31.4 and 30.8 % of the total Area (supplementary data Figure S3). However, this is not an absolute

quantification since the observed area are closely linked to the extinction coefficient value of the molecules at 280 nm. In agreement, EPS extracted from aerobic granules with a different multi-method protocol (mechanical and chemical) but fractionated with similar anionic exchange chromatography were also found as mainly negatively charged since approximately 75% could be adsorbed on a similar cationic resin [32].

Moreover, anionic exchange chromatography was also used for the enrichment of sulfated glycosaminoglycans which are highly negatively charged polymers allowing the presence of chondroitin- and heparan/heparin-like polymers to be elucidated in sEPS extract of aerobic granules [16].

3.2.2. Gelling capacities

The collected fractions were analyzed for their gelling capacity thanks to a simple assay recently developed in the laboratory [33] and results can be seen in Fig. 3. The unbound Cat/Neut group exhibited the highest GF at 0.37 ± 0.03 due to a very good reactivity to calcium since more than 80 % of the molecules were entangled in a gelled structure that represents 42 % of the total initial liquid volume.

Concerning the negatively charged polymers, the GF increases with charge from 0.04 ± 0.03 for LAC to 0.32 ± 0.02 for HAC group, indicating that the gelation capacity increases with the net negative charge. However, upon reaching a very high negative charge (VHAC group), the gelation capacity decreases abruptly and reaches 0.02 ± 0.01 . In the latter case, the high %R coupled to the low %Vg could be an indication of a precipitation instead of a gelation process [33]. As described for carrageenan hydrogels, the presence of very highly negatively charged polymers could hinder the hydrogel formation capacity due to an increased repulsion of polymers [24,25].

Upon comparison with the GF of the initial unfractionated sEPS (0.19 ± 0.02) some fractions exhibited a better gelling capacity up to 0.37 ± 0.03 for the Cat/Neut sample and 0.32 ± 0.02 for the HAC sample. This means that fractionation according to charge has allowed to concentrate gel forming polymers in the Cat/Neut fraction as well as in the HAC

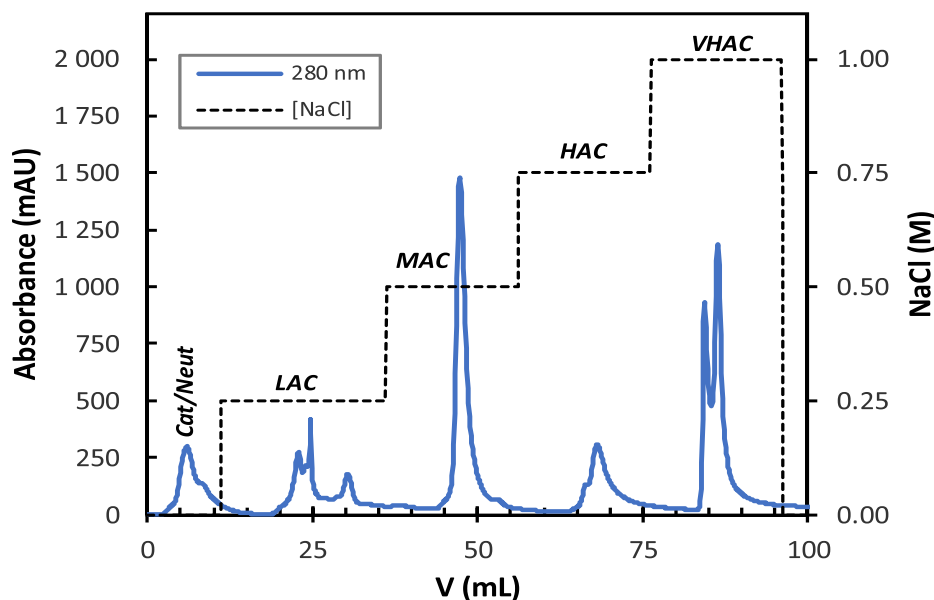


Fig. 2. Chromatogram showing the different fractions collected during structural EPS fractionation using anionic exchange chromatography. The shown chromatogram is obtained through the mean of 10 separate injections. Cat/Neut=cationic/neutral molecules, LAC= low anionic charge molecules, MAC= medium anionic charge molecules, HAC= high anionic charge molecules and VHAC= very high anionic charge molecules.

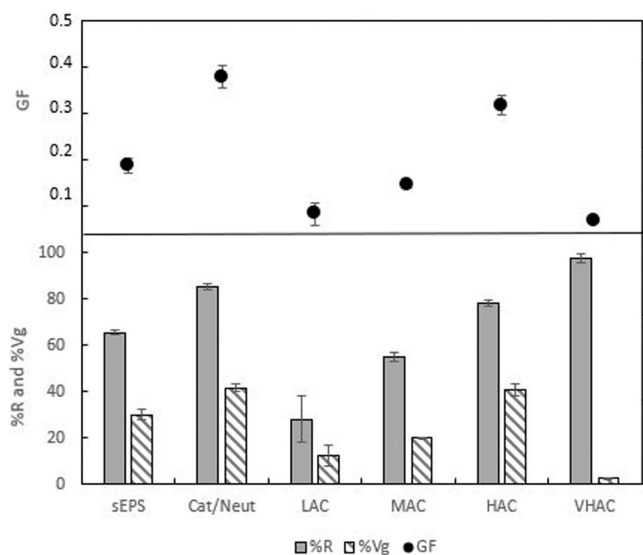


Fig. 3. The effect of surface charge on the capacity of gelation for EPS fractions: Cat/Neut=cationic/neutral molecules, LAC= low anionic charge molecules, MAC= medium anionic charge molecules and VHAC= very high anionic charge molecules. Triplicates of each measurement were done to obtain the %Vg and %R.

fraction that constitute the most favorable charges of EPS for the formation of ionic hydrogels.

In order to gain more insights on the composition of the fractionated sEPS, an elemental analysis was performed on each fraction collected after anionic exchange and on two reference polymers: the globular protein BSA and a low viscosity alginate (Table 2). These two reference polymers were chosen for the comparison with sEPS because they can represent the gelation mechanisms occurring during EPS gelation. Indeed, BSA is a protein that has the capacity to form hydrogels with divalent cations when subjected to a heating step [42]. sEPS were often compared to alginate because of some similarities in their biochemical

composition due to the fact that uronic acids have been identified in sEPS [7].

Interestingly, the Cat/Neut fraction has a S content equal to 2.6 % that is higher than that of the other groups. This suggests the presence of sulfate groups provided by other molecules than proteins since the model protein BSA, that contains a high ratio of 35 cysteine and 4 methionine residues upon 583 amino acids, exhibits a lower S content of 1.6 %. In fact, sulfation of anionic glycoconjugates is common in EPS extracted from granular biofilms [43].

The Cat/Neut molecules and the HAC polymers had low contents of nitrogen of 8.5 and 7.0 % respectively, whereas proteins are usually considered to have a N content ratio around 16 % [44]; this low nitrogen content could be attributed to the presence of aminosugars as it is the case for granulan, a component of sEPS which contains glucosamine and galactosamine [41]. However, sulfated glycoconjugates are considered as highly negative polymers and their presence in the Cat/Neut fraction could be possibly attributed to neutralization by cations. As seen in Table 2, the Cat/Neut showed the lowest content of Na^+ (10 $\mu\text{g}/\text{mg}$), and the highest content of Ca^{2+} (4 $\mu\text{g}/\text{mg}$) and a high Mg^{2+} (1 $\mu\text{g}/\text{mg}$) suggesting that the presence of divalent cations can give a global neutral charge to the polymers although they contain negatively charged residues. This can also be an indicator of the presence of granulan as a component of this fraction since its complex structure allows the formation of an anti-parallel double helix conformation stabilized by hydrogen bonds between amine and carboxylic groups and/or by divalent cation bridging (Table 2) and thus leading to globally neutral aggregates [41].

The fractionated groups and alginate have a quite different elemental composition. The sample having the closest content of C equivalent with alginate is the HAC sample, however similarly to other tested samples its content of oxygen is quite different from that of alginate. These observations reinforce the hypothesis that sulfate-containing molecules other than proteins are components of the sEPS.

3.3. Does the molecular weight of EPS affect their gelation capacity?

The molecular weight profiles of the fractions collected after anionic chromatography and of the supernatants remaining after their gelation

Table 2

Elemental analysis of the different fractions collected after anionic exchange chromatography, in comparison with alginate and BSA as well as the quantities of selected cations Na^+ , Ca^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} expressed in $\mu\text{g}/\text{mg}$ in each fraction.

Fractions (NaCl)	% N	% C	% H	% S	% O	C/N	Na^+	Ca^{2+}	Mg^{2+}	Cu^{2+}	Zn^{2+}
Cat/Neut (0)	8.5	40	5.7	2.6	23.9	4.7	10	4	1	0.04	0.3
LAC (0.25 M)	10.7	40.2	6.3	1.4	22.4	3.8	22.9	3.8	0.7	0.2	0.2
MAC (0.5 M)	12.1	47.2	7.1	0.7	24.3	3.9	19.9	1.6	0.3	0.04	0.03
HAC (0.75 M)	7	31.2	4.6	0.5	16.4	4.5	39.3	0.2	0.7	0.1	0.2
VHAC (1 M)	7.4	23.7	4.8	1.5	22.2	3.2	55.2	0.9	1.1	0.2	0.6
Alginate	-	33.1	4.8	0.06	43.2	-	-	-	-	-	-
BSA	15	49.9	7.1	1.6	27	3.3	-	-	-	-	-

Cat/Neut=cationic/neutral, LAC= low anionic charge, MAC= medium anionic charge, HAC= high anionic charge and VHAC= very high anionic charge molecules.

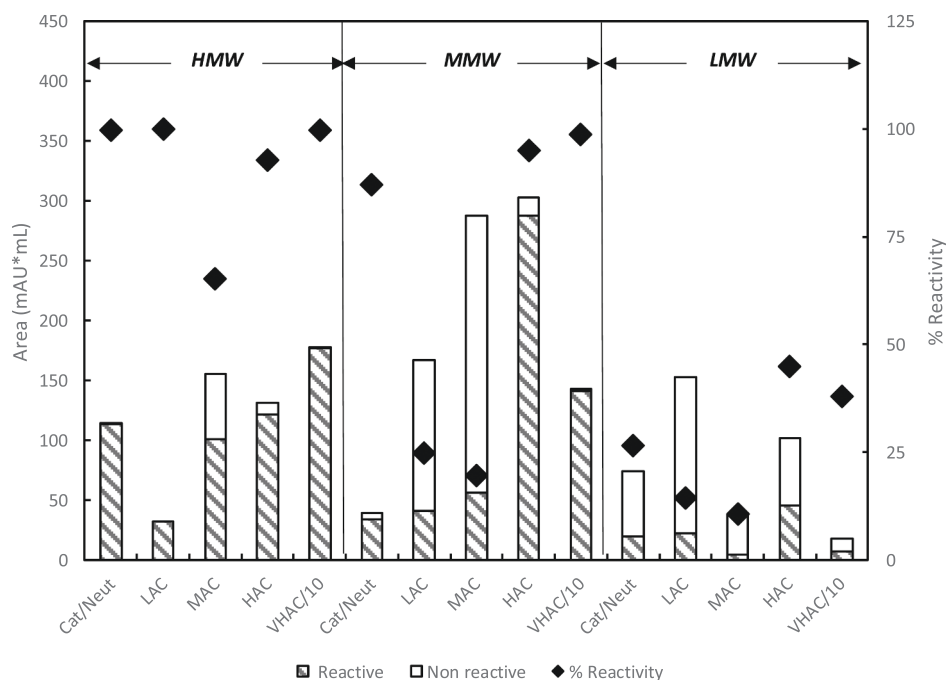


Fig. 4. Impact of the molecular weight on the gelation of sEPS eluted according to their surface charge during anionic exchange chromatography (Cat/Neut, LAC, MAC, HAC, VHAC). Non-reactive and reactive areas were calculated by comparison of the SEC chromatograms obtained for the initial and the remaining solution after gelation. The VHAC sample peak areas were divided by 10 for visual representation.

were analyzed by SEC. Depending on their elution volume that is correlated with their apparent molecular weight, the gelling polymers were divided into High, Medium or Low Molecular weights. The molecular weight distributions of the collected fractions along with their % MW Reactivity after calcium addition are presented in Fig. 4. This MW Reactivity corresponds to the proportion of molecules that have disappeared from the supernatant in each MW category due to their reaction with calcium; the remaining molecules which did not react with calcium constituted the non-reactive part. No matter what the net negative surface charge is, the HMW group always participates in the gelation with a percentage of MW Reactivity going from 65 % for the MAC fraction to values as high as 100 % for the LAC fraction (Fig. 4). On the contrary, participation of MMW molecules to the hydrogel formation is dependent on the net negative surface charge. Indeed, for the LAC and MAC, the % of MW Reactivity is below 25 % while for the fractions with high negative charge (HAC and VHAC), the participation of MMW is above 95 %. The Cat/Neut fraction has a percentage of MW Reactivity for MMW equal to 87 %, supporting the hypothesis that this fraction contains aggregates of charged polymers. When it comes to LMW molecules, their participation is always limited, reaching 27 %, 14 % and 11 % respectively for Cat/Neut, LAC and MAC and around 40 % for HAC and VHAC.

According to the reactive areas presented in Fig. 4, the reactive Cat/

Neut, MAC and VHAC fractions forming hydrogels are mostly formed by HMW molecules, whereas for the LAC and HAC hydrogels, it is the MMW molecules that mostly participate to the hydrogel. Thus, both HMW and MMW are convenient molecular characteristics for ionic-induced gelation.

Fernández Farrés and Norton [27] have compared alginate samples having the same guluronic acids content and observed that the formation of complexes with calcium is privileged for high molecular weight polymers because of their higher flexibility allowing the polymer to contract/shrink more freely. In addition, the molecular weight impacts the mechanical properties of the formed hydrogels; indeed, a positive correlation was found between the molecular weight and the gel's stiffness [26,45,17,46]. It was observed that the drastic alkaline and heating treatments used for sEPS extraction severely reduce their molecular size because of chemical degradation [4]; therefore attention must be paid to avoid any chemical hydrolysis of molecules which lowers their molecular weight and negatively affects their participation in the hydrogel network.

3.4. Which biochemical groups participate in EPS gelation?

The fractions collected after anionic chromatography as well as the supernatants remaining after their gelation were analyzed for their

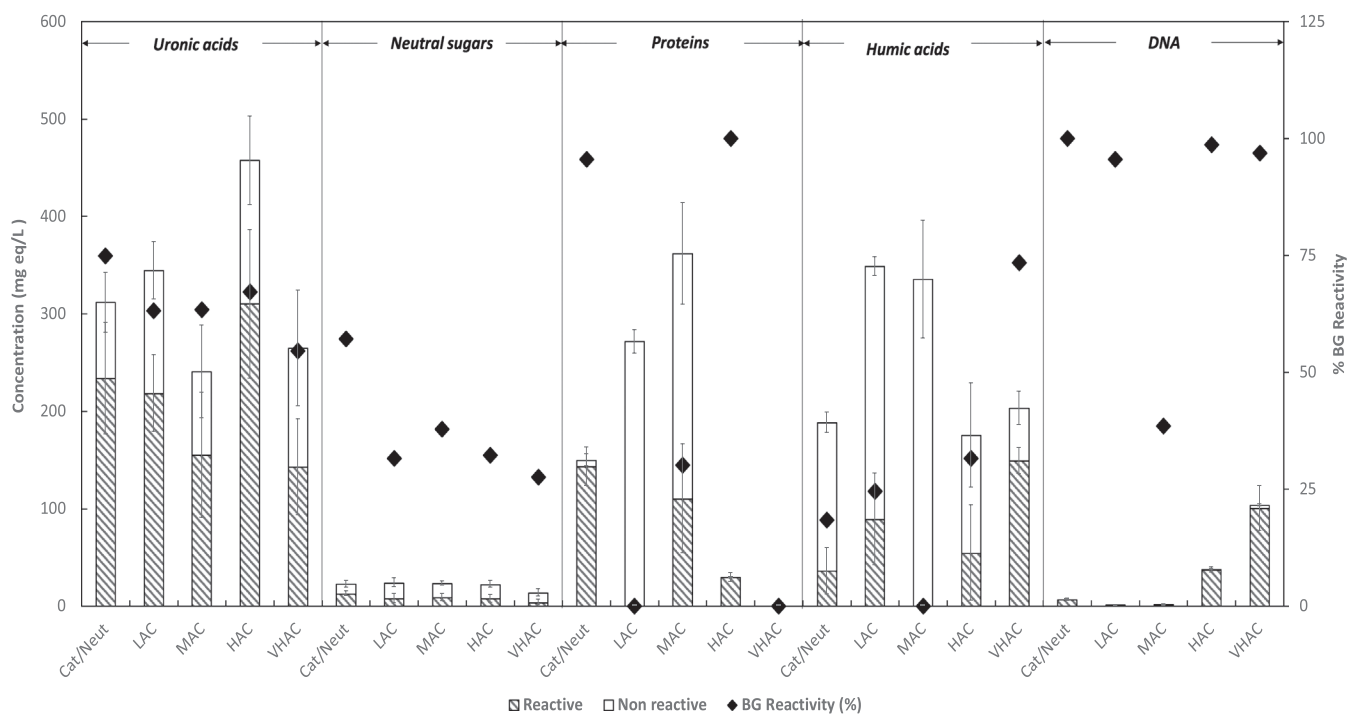


Fig. 5. Impact of polymers' biochemical nature on the gelation of sEPS eluted according to their surface charge during anionic exchange chromatography (Cat/Neut, LAC, MAC, HAC, VHAC). For biochemical tests three analytical assays were performed and for BG evaluation, three gels were analyzed to get the means and the associated standard errors.

biochemical diversity. The molecules assayed in the supernatants after gelation were considered as non-reactive, thus allowing to evaluate the reactive ones (Fig. 5).

As shown in Fig. 5, except the VHAC sample in which proteins were undetectable, all the samples exhibited the presence of all the tested biochemical groups. The VHAC gel appeared to be particularly rich in humic acid-like molecules and DNA compared to the other fractions. Globally, it can be seen that uronic acids are those that participate the most in the formation of the hydrogels in terms of quantity and their BG Reactivity, corresponding to the proportion of molecules that have disappeared from the supernatant due to their reaction with calcium, was around 60 % for the five fractions (Fig. 5). This data confirms the structural involvement of uronic acids in calcium induced EPS gelation observed by Schambeck et al. [2]. Neutral sugars are very little represented in the gel composition and their presence as reactive molecules with calcium can be explained by the complex sequence of some bacterial polysaccharides built with alternating neutral and anionic sugars which is the case of granulan [41].

Proteins participate in the formation of the hydrogels for three out of five fractions since they were undetectable in the VHAC fraction and were not quantitatively represented in the LAC hydrogel. Depending on the fractions, the participation of proteins in the hydrogel formation varied: Cat/Neut and HAC samples exhibit an almost total reactivity but the MAC fraction shows a reactivity of 30.1 %. The differences in reactivity observed for proteins are not really correlated with their global charge suggesting that other gelation mechanisms based on molecule/molecule interactions may have occurred such as hydrogen bonds and hydrophobic interactions [47,48].

Humic acid like substances also participate in the formation of the hydrogel and their reactivity is more important in the VHAC group (73.4 %) than in others fractions (< 35 %). It was proven elsewhere that humic substances tend to form aggregates with calcium instead of gels [49]. However, although they tend to form aggregates, they can influence the formed gel properties since Venezia et al. [50], showed that humic acids can modulate the gel properties of gelatin depending on their concentration in the medium.

Lastly, DNA is more present in the HAC and VHAC than in the other fractions and this is linked to the presence of phosphate groups that confer significant negative charges to these molecules. Interestingly, almost the totality of DNA molecules assayed in the initial solutions react with calcium, except for the MAC sample where only 40 % of the DNA molecules have disappeared from the liquid supernatant. DNA is a polyanionic polymer and DNA hydrogels have been reported in literature and can be obtained using various methods including interaction with calcium ions [33,51-53].

As a summary, the results presented in Fig. 5 showed that three major biochemical groups participate in the hydrogel formation of sEPS: uronic acids are the main contributor to hydrogel formation followed by proteins and finally DNA. Neutral sugars participate only slightly, and humic acid like molecules are suspected to form aggregates instead of hydrogels.

4. Conclusions

This study proposes an original approach based on simultaneous analysis of gel-forming potential and physico-chemical properties of sEPS and provides new results on the processes involved in ionic-induced gelation of the complex sEPS mixture extracted from aerobic granules:

- Polysaccharides and proteins, both interact with calcium, and thus are most likely implied in the gelation.
- An increase in the negative net surface charge increases the polymers capacity to form hydrogels since the gelling factor is 8 times higher for the HAC fractions (GF of 0.32) compared to the LAC fractions (GF of 0.04).
- High molecular weight polymers are the most reactive with calcium and overall more than 90 % of them participate effectively in the formation of hydrogels. The preservation of the native molecular weight during the extraction steps and/or the elimination of LMW, can be thus strategies used to enhance the gelation of EPS.

- The cationic/neutral fraction exhibits a 2.6 % S content and a 2 times higher gelation capacity than the initial unfractionated sEPS extract. This fraction can be isolated by anionic exchange chromatography. Therefore, a fractionation based on anionic exchange seems relevant to collect fractions enriched in gel-forming polymers such as the HAC and Cat/Neut fractions.

The presented results make it possible to definitively classify sEPS gels as mixed gels containing diversified polymers for which a high molecular weight and a negative surface charge are determining factors for gelation.

CRedit authorship contribution statement

Yolaine Bessiere: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Nicolas Derlon:** Writing – review & editing, Resources. **Elisabeth Girbal-Neuhausser:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Abdo Bou Sarkis:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Etienne Paul:** Writing – review & editing, Funding acquisition, Conceptualization.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jece.2024.113648](https://doi.org/10.1016/j.jece.2024.113648).

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