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Characterization of exopolysaccharides from floccular and aerobic granular activated sludge as alginate-like-exoPS

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ABSTRACT

As a component of activated sludge (AS) extracellular polymeric substances, exopolysaccharides (exoPS) have been associated with contributing physically and chemically to the structural strength and coherence of both conventional (floccular) (CAS) and aerobic granular activated sludge (GAS). The study aims at characterization of exoPS extracted from CAS fed first with synthetic wastewater, then with brewery effluent and from GAS treating the latter. CAS and GAS exoPS were analyzed for their gel-forming capacity, morphology, and moisture content of the formed hydrogels, and chemical properties of the extracted exoPS by Fourier transform infrared (FTIR) spectroscopy. Commercially available sodium alginate was used as the reference polysaccharide for comparison. The extracted exoPS from both CAS and GAS formed hydrogels or gel-like structures in $CaCl_2$ solution, similar to $CaCl_2$ reactions of alginate. Environmental scanning electron microscopy images displayed similarities in the morphologies of hydrogels formed by the AS exoPS and alginate. The similarity between the two was further strengthened by FTIR spectroscopy which indicated similar functional groups in the chemical structure of both exoPS extracts and alginate. Moreover, characteristic peaks of the uronic acid residues, namely guluronic and mannuronic acids, were detected at 948 and 882 cm^{-1}, 964 and 879 cm^{-1}, 902 and 879 cm^{-1} for alginate, CAS-exoPS and GAS-exoPS, respectively. Results indicated that the exoPS extracted from the AS samples resembled alginate and likewise the functions of alginate; they seemingly contribute to the physical and chemical properties and structural strength and coherence of AS, especially to the compact and durable nature of excellently settling aerobic GAS.

Keywords: Aerobic granular activated sludge; Alginate; Extracellular polymeric substances; Exopolysaccharides; FTIR spectroscopy; Gelation; Hydrogel

1. Introduction

Activated sludge (AS) microflora secretes a highly hydrated gel matrix of extracellular polymeric substances (EPS) in which they are embedded. The EPS is mainly composed of exopolysaccharides (exoPS), extracellular proteins, some humic substances, nucleic acids, some non-cellular organic/inorganic materials, like metals and ions, etc. The importance of EPS includes the retention of exoenzymes near the

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surface of the cells and the building up of organic matter. Furthermore, EPS is known to influence the cell surface properties of AS; reducing the surface charge which in turn decreases the repulsive forces and increases the proximity between cells. As a result, there is a physicochemical bridging of the microbial cells leading to aggregation and/or immobilization [1,2]. The exoPS component of EPS is known to play a critical role in the aggregation of cells by acting as physicochemical bridges and forming net-works which promote formation of the 3-D structural matrix. This matrix enables association of more cells and provides structural strength and coherence to AS.

In addition to the procedure for extraction of EPS with cation exchange resin (CER-DOWEX) first introduced by Frølund et al. [3], several other extraction methods have been employed for isolation of EPS from AS samples [4,5]. EPS-associated exoPS have been mostly quantified by spectrophotometry using glucose as the reference carbohydrate (i.e. anthron method). Recently, a limited number of studies have concentrated on characterization of AS-exoPS to include exoPS-specific extraction methods and detailed chemical characterization. Lin et al. [6,7] conducted studies with those perspectives and concluded that the EPS-associated exoPS extracted from CAS and aerobic granular activated sludge (GAS) treating municipal wastewater resembled alginate; a sugar polymer naturally produced by i.e. brown seaweed, and thus named the polymer as alginate-like-exoPS (ALE). They reported that ALE from both samples formed hydrogels, albeit with different morphologies, when introduced into $CaCl_2$ [7]. Using another exoPS-enriching method, Seviour et al. [8] isolated EPS from GAS treating abattoir wastewater, characterized the polymer and identified it as a complex heteropolysaccharide [9], and named it as Granulan [10]. They also showed that the EPS extracted from GAS formed a strong gel at $pH < 9.0$ in sol–gel transition experiments [8].

Concurrently, this study focuses on explicit characterization of AS-associated exoPS extracted from conventional floccular (CAS) and aerobic GAS collected from a lab-scale bubble-column sequencing batch reactor (SBBR), which was fed with synthetic wastewater for about two months, then with brewery effluent for over four months. AS-associated exoPS were extracted by the method referred in Lin et al. [6,7]. Characterization was carried out by running gelation experiments in $CaCl_2$ solution, observation of gel-structures by environmental scanning electron microscopy (E-SEM), and performing Fourier transform infrared (FTIR) analyses. Total carbohydrates were also measured. All experiments were carried out both with extracted exoPS and with commercially available sodium alginate. Some EPS extracts obtained by the DOWEX extraction method [3,11] were also included in the experiments. Results obtained for exoPS/EPS extracts were compared to those obtained for alginate to evaluate possible similarities between those two as proposed by Lin et al. [6], and also to establish the relationship between chemical characteristics of AS-exoPS and their role in structural integrity and coherence of both floccular and granular AS.

2. Materials and methods

2.1. Biomass samples, extraction of EPS and exoPS

Biomass samples were collected from a lab-scale SBBR at different stages of reactor operation: the system was fed with synthetic wastewater including acetate as the only carbon source for about two months and then with brewery effluent for more than four months. The sludge was floccular (CAS) during the period of acetate feeding and remained so for about one month after changing the feed to brewery effluent. Then, a totally granular sludge was formed and maintained in the system.

Two different extraction methods were employed in this study: extraction with cation exchange resin (CER) and with Na_2CO_3 and heating. The first one was used to extract the EPS and the latter was used to extract specifically the exoPS. EPS extraction by CER (DOWEX; Fluka) was carried out according to the procedure outlined by Frølund et al. [3] with minor modifications [11]. ExoPS extraction with Na_2CO_3 was carried out as outlined by Lin et al. [6,7]. For the latter, thawed biomass samples were centrifuged at 9,000 rpm for 20 min and the initial supernatants were spared and stored at −20°C. Eighty milliliter of 0.2 M Na_2CO_3 was added to each sludge pellet and extraction was carried out at +80°C in a water bath. After 1 h of extraction, centrifugation was repeated to obtain the supernatants containing exoPS. Pellets were discarded and pH of each supernatant was adjusted to 2.0 using 0.1 M HCl. After another round of centrifugation, supernatants were discarded and pellets were washed with deionized water until a pH of 7.0 was reached. The pellets were then dissolved in 5 mL of 0.1 M NaOH and the exoPS in the supernatants were precipitated with 20 mL of cold absolute ethanol to obtain a final concentration of 80% (v:v). The samples were subjected to gentle shaking and allowed to precipitate overnight in fridge. Next day, the resultant solution was centrifuged at 9,000 rpm for 30 min to obtain the exoPS pellets, which were finally washed with cold absolute ethanol and then lyophilized.

2.2. Gel formation capacity, gel morphology, and moisture content

Gel formation capacity of the extracellular materials extracted from AS was tested by introducing the extracted exoPS or EPS into $CaCl_2$ solution and waiting overnight for crosslinking and gelation at room temperature. Next day, formed hydrogels were examined by E-SEM (FEI Quanta 600, USA) for their morphological properties. ExoPS extracted with Na_2CO_3 was in powder form, hence the freeze-dried samples were dissolved in deionized water to prepare aliquots at desired weight-to-volume values (w:v). Accordingly, 2 mL of 1, 0.2 or 0.1% (w:v) dissolved exoPS samples were added drop-wise into 1, 0.2, or 0.1% $CaCl_2$ solutions with the aid of a syringe and needle (Fig. 1). EPS extraction with DOWEX, on the other hand, yielded aqueous samples (EPS attained in extraction buffer); hence, those were introduced into $CaCl_2$ solution directly. Gelation experiments were also carried out with commercially available sodium alginate (low viscosity, A2158, Sigma) for comparison. Ruthenium red is reported to interact with and precipitate on –COO groups of the uronic acid residues forming alginate, thus has been used to stain ALE gels [6]. Accordingly, 0.1% (w:v) ruthenium red (Merck) was added (50–200 μL) to the samples to develop a dark pink color and ease in visualization of the formed hydrogels (Fig. 1).

Percentage moisture content of the hydrogels formed by the exoPS extracts and alginate were determined by the gravimetric method: samples were filtered through previously washed, dried, and weighed filter papers, then dried in an oven at +105˚C for 1 h, cooled in a desiccator and reweighed to determine the mass of water lost through drying [7,12].

Fig. 1. Preparation of the exoPS gels by dropping i.e. 0.2% exoPS into 0.2% $CaCl_2$ solution.

2.3. Total carbohydrate content (glucose equivalence)

Anthron method [13] was used to estimate the glucose equivalence of the extracted exoPS and the commercially available sodium alginate. D-(+)-glucose (Sigma) was used as the reference carbohydrate and spectrophotometric measurements were carried out at 578 nm (UV–vis Spectrophotometer, Lambda25, Perkin Elmer). All reactions were run in triplicate.

2.4. FTIR spectroscopy

The FTIR spectra of the lyophilized exoPS samples and sodium alginate were recorded in the 4,000–400 cm^{-1} wavenumber region (Spectrum100, Perkin Elmer). Data derivation was performed using spreadsheets.

3. Results and discussion

3.1. Extraction efficiency and yield

Yield of the exoPS-specific extraction method was described here as the ratio between the mass of the lyophilized exoPS extract to the mass of volatile suspended solids of the biomass sample (mg exoPS/g VSS), and the extraction efficiency as %mg exoPS/mg VSS. ExoPS-specific extraction efficiency was slightly higher for the GAS samples, compared to those for the CAS samples: for the CAS samples efficiency varied between 1.5 and 3.8% resulting in an average of 2.3% mg exoPS/mg VSS. For the GAS samples, efficiency was in the range of 2.2–6.5%, with an average of 4.3% mg exoPS/mg VSS. Average extraction efficiency for all samples was 3.5% and the average yield was 34.6 mg exoPS/g VSS. Yield value reported by Lin et al. [6] for extraction of ALE from their granular biomass sample was 160 mg ALE/g biomass (VSS ratio). Assuming a VSS/TSS ratio of 0.7, this corresponds to an efficiency of about 11%. Comparison of these values with those obtained in this study indicates that the exoPS-enriching extraction performed in this study was less efficient than that reported by Lin et al. [6]. Nonetheless, the method was successful in extracting the exoPS both from floccular and from granular sludge samples in this study. Moreover, efficiencies varied within a narrow range (1.5–6.5%), indicating the consistency in the extraction procedure.

3.2. Gel-forming capacity of exoPS/EPS

3.2.1. Gel formation with alginate and the effect of solution concentration

Alginate is a naturally produced (i.e. by brown seaweed, brown algae) sugar polymer, with repeating uronic acid residues, namely guluronic- and mannuronic-acid residues forming homo-monomeric [–GG–, MM–] and/or hetero-monomeric [–MG–] blocks randomly distributed inside the molecule. The GG blocks are suggested as providing the gel-forming capability; whereas the MM and MG blocks are described as providing flexibility to the polymer [6,7]. Fragments rich in GG blocks tend to aggregate together and when the GG blocks on two fragments are aligned, diamond-shaped holes are formed in between, which then act as sites for divalent cations, esp. Ca^{+2}, to interact with and cross-link the GG-blocks, forming an "egg-box"-like network, physicochemically bridging the individual polymer fragments, enhancing aggregation, and resulting in formation of gel structures in a solution including i.e. Ca^{+2} [6,7,14,15].

Previously, some researchers reported successful gelation results with sodium alginate where 2% alginate solution (w:v) was introduced into 2–3% $CaCl_2$ solution (w:v) [6,7,15]. To test whether it is possible to obtain alginate gels with less concentrated solutions, a series of different alginate solutions were prepared in this study and introduced to the corresponding $CaCl_2$ solutions (1:1 ratio). Results of those experiments are given in Fig. 2. As seen from the figure, best gelation results were obtained in the experiment with 2% alginate in 2% $CaCl_2$ (Fig. 2(a)), yet perfectly spherical and compact alginate hydrogel beads were also obtained in the experiments with 1% alginate in 1% $CaCl_2$ (Fig. 2(b)). Decreasing the concentrations by 10-folds yielded gel-like structures in the form of loose and diluted, yet still distinguishable, aggregates rather than spherical compact gel beads (Fig. 2(c) and (d)). No alginate gel was detectable in the experiment with 0.01% alginate in 0.01% $CaCl_2$ solution (Fig. 2(e)).

3.2.2. Gel formation with exoPS/EPS extracted from floccular and granular biomass

Gel-forming capacity of alginate due to its interaction with divalent cations is a property that has been employed in i.e. immobilization of cells and enzymes [16]. Recently, Lin and her co-workers reported that the exoPS they extracted from CAS and aerobic GAS treating municipal wastewater resembled alginate in terms of gel-forming properties and chemical structure, thus named the polymer as ALE, and concluded that ALE was comprised of the functional gel-forming exoPS, contributing to the compactness and strength of aerobic GAS [6,7].

In the current study, gel-forming capacity of the extracellular polymeric material secreted by AS was tested for the exoPS/EPS extracted from floccular (CAS) and aerobic GAS collected from the lab-scale SBBR at different stages of operation. As seen from Fig. 3, all gelation experiments with different combinations resulted in formation of gels and/or gel-like structures similar to the ones obtained with sodium alginate, seen in Fig. 2. ExoPS extracted by Na_2CO_3 from floccular biomass fed with acetate formed nearly spherical gel beads in $CaCl_2$ (1:1 ratio) (Fig. 3(a)). Gels formed by the exoPS extracted the same way from aerobic granular sludge fed with brewery wastewater were rather loose but still apparent (Fig. 3(e)). EPS extracted by DOWEX from floccular and granular sludge fed with brewery wastewater (days 64 and 109, respectively) also formed distinct gel-like structures in 2% $CaCl_2$; however, gel formed by CAS-EPS was rather dispersed compared to the compact and well-structured gel formed by GAS-EPS (Fig. 3(b) and (c), respectively). Gel formed by GAS-EPS in 0.2% $CaCl_2$ was rather loose (Fig. 3(d)).

As mentioned previously, Lin et al. [7] reported that the ALEs they extracted from floccular and granular sludge both formed hydrogels in 2% $CaCl_2$, yet the resulting gels had different morphologies: ALE from aerobic granules formed spherical beads, whereas that from floccular sludge had a fluffy and floccular appearance, both repeating morphologies of their origin. They concluded the difference in gel morphologies as pointing to the difference in characteristics of the two ALEs. In this current study, on the other hand, it was possible to produce self-standing gel beads with the exoPS extracted from floccular sludge fed with acetate (Fig. 3(a)). Considering the results reported by Lin et al. [7] and those obtained here, the differences among those observations are attributed to two factors: (i) the systems were fed with different types of wastewaters and (ii) the microbial communities in the systems were likely to be different; which both might have an impact on the chemistry and characteristics of the exoPS produced by the corresponding microflora. Despite those differences, this study showed that the exoPS produced by floccular sludge can also form self-standing hydrogels in $CaCl_2$, just like alginate and the exoPS produced by aerobic granular sludge.

3.3. Morphology (E-SEM) and moisture content of formed gels

E-SEM analysis showed that the hydrogels formed by sodium alginate were continuous and folding structures (Fig. 4(a), 500× magnification) with a smooth and significantly dense appearance (Fig. 4(b), 4,000×) indicating a strong cross-linking through Ca^{2+} in the reaction with $CaCl_2$. Similar to alginate hydrogels, those formed by exoPS extracted from floccular and aerobic granular sludge both displayed continuous and dense structures (Fig. 4(c) and (f), respectively), yet the resemblance between the gels formed by alginate and that by exoPS extracted from aerobic GAS was prominent (Fig. 4(b) and (f), respectively). Gel-like structures formed by the EPS extracted by DOWEX from floccular and aerobic granular sludge displayed a somewhat different morphology with patches and irregular surfaces rather than being smooth and compact (Fig. 4(d) and (e), respectively).

Hydrogels have the ability to swell and de-swell as described by Seviour et al. [12]. This is primarily due to crosslinking of polymers, especially the hydrophilic ones, creating a matrix within which water molecules are entrapped. This cross-linking property forming an encompassing polymer has also been associated especially with the maintenance of the shape and consistency of aerobic GAS and this is evident in the regular and smooth shape of GAS with clearly-defined outer surface [12]. Alginates are rich in hydrophilic polymers, consisting of more than 90% water and thus share similar properties with hydrogels. To determine whether the gels obtained in this study were also hydrogels, % moisture content values were measured at the end of the gelation experiments. Spherical gel beads formed by 2% alginate had a moisture content of 94%. Values for the gels formed by exoPS extracts were in the range of 93–98%, which were very close to the values reported by Seviour et al. [12] and Lin et al. [7]. Those results indicate that the gels formed by sodium alginate and by the exoPS extracted from CAS and GAS were in fact hydrogels.

3.4. FTIR spectroscopy and chemical features of exoPS extracted from floccular and granular sludge

FTIR spectroscopy results further established the similarities between the extracted exoPS and sodium alginate (Fig. 5): the spectra of exoPS extracted from AS samples collected at different stages of operation displayed peaks—assigned to characteristic functional groups—similar to those in alginate, as well as those reported by other researchers (Table 1). In the alginate's spectrum (Fig. 5(a)), a wide-ranging band of O–H stretching vibrations (A), and a weaker C–H stretching peak (B) of methyl groups [12], which are typical for polysaccharides, were observed above 3,000 cm^{-1} (max. at 3,258 cm^{-1}) and at 2,914 cm^{-1}, respectively (Fig. 5(a)). The display of a peak at 1,027 cm^{-1} is confirmation of the presence of carbohydrates indicated by peaks within the range of 1,000–1,200 cm^{-1} and being specific for the C–O–C and C–O vibrations (F) [17]. Results obtained for the exoPS extracted from CAS (Fig. 5(b)) and aerobic GAS (Fig. 5(c)) with regard to the bands/peaks typical for polysaccharides (A, B) and carbohydrates (F) were very similar to those obtained for alginate (Fig. 5(a), Table 1).

Leal et al. [18] addressed the peak at 1,035 cm^{-1} as the C–O stretching vibration of uronic acid (Table 1; F). As expected, a peak at a very similar wavenumber (1,027 cm^{-1}) was detected for alginate in this study. Corresponding peaks at the FTIR spectra of exoPS from CAS and aerobic GAS were also at a very similar wavenumber (1,046 cm^{-1}); hinting similarities with alginate and confirming the presence of uronic acid in those AS-associated exoPS (Fig. 5, Table 1). Moreover, peaks for the asymmetric (C) and symmetric (D) stretching vibrations of O–C–O groups, the latter addressed as being specific for uronic acid residues, were also observed at the spectra of alginate and the exoPS extracts at wavenumbers similar to those reported by Leal et al. [18] for brown seaweed pure cultures' alginic acids, as well as those reported by Lin et al. [6] for GAS-exoPS treating municipal wastewater (Table 1). One exception was that in alginate's spectrum, the peak for the O–C–O asymmetric stretching vibrations was at 1,597 cm^{-1}. This was attributed to using a particular type of commercially available sodium alginate (A2158, Sigma) having a particular chemistry and exhibiting probable slight differences with the exoPS compared, as also highlighted by Leal et al. [18].

For the commercially available alginate used in this study, signature peaks of the two monomers of alginate, namely guluronic and mannuronic acids were detected at 948 and 882 cm^{-1}, respectively. Signature peaks for mannuronic acid residues detected at the spectra of the exoPS extracts of both types of sludge samples were at a wavenumber (879 cm^{-1}) very close to that in alginate's spectrum. However, readings for guluronic acid residues were slightly different: for the floccular sludge-exoPS, the signature peak for guluronic acid residues was at 964 cm^{-1} and for the aerobic granular sludge-exoPS, it was at 902 cm^{-1} (Fig. 5, Table 1). Those might be considered as pointing to a slight difference between the alginate, the CAS-exoPS and the GAS-exoPS in terms of their guluronic acid residues, which might partly explain the differences observed in the gelation experiments with those polymers (Figs. 2–4).

The FTIR results also give more insight on the interactions between alginate/exoPS extracts and Ca^{2+}: as mentioned at Section 3.2.1, the GG blocks on different polymer chains of alginate form ionic crosslinks with each other through Ca^{2+}, forming "egg-box"-like

Table 1
Summary of the FTIR spectroscopy results obtained for the exoPS/EPS extracts in comparison with alginate and with some examples from the literature

References/Samples	Wavenumbers (cm^{-1}) and assigned functional groups (A–I)[*]	A	B	C	D	E	F[a]	H	I
Leal et al. [18][a]	Seaweed pure culture alginic acid	>3,000 peak at 3,428	2,927	1,616	1,415		1,125, 1,091, 1,035	949	888/820
Lin et al. [6][b,c]	GAS-exoPS (municipal ww)	>3,000	2,927	1,654	1,400	1,240	1,000–1,200	902[b]	820[c]
This study	Alginate (A2158, Sigma)	>3,000 peak at 3,258	2,914	1,597	1,407	1,294	1,027	948	882
This study	CAS-exoPS (day 49, acetate)	>3,000 peak at 3,273	2,973	1,643	1,429	1,237	1,046	964	879
This study	GAS-exoPS (day 199, brewery ww)	>3,000 peak at 3,273	2,943	1,643	1,429	1,237	1,046	902	879
This study	GAS-EPS (day 109, brewery ww) (DOWEX extract)	>3,000 peak at 3,324	–	1,635	–	–	–	–	–

[*] Assigned functional groups (A–I) A: O–H stretching vibrations, B: C–H stretching vibrations, C: O–C–O asymmetric stretching, D: C–OH deformation vibrations and/or O–C–O symmetric stretching vibrations, E: O-acetyl ester, F: C–O–C and/or C–O vibrations, H: C–O stretching vibrations of guluronic acid residues, I: C1–H deformation vibrations of mannuronic acid residues/characteristic peak for mannuronic acid

[a] Values reported by Leal et al. [18] for C–O stretching of pyranose rings, C–O and C–C stretching vibrations of pyranose rings, and C–O stretching vibration of uronic acid, respectively.

[b,c] Second derivative values reported by Lin et al. [6] for anomeric C–H deformation vibrations of L-guluronic acid residues and for C1–H deformation vibrations of mannuronic acid residues, respectively.

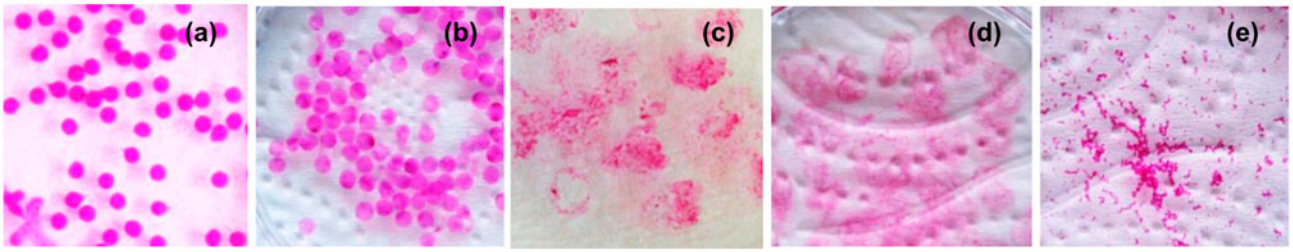

Fig. 2. Results of gelation experiments with sodium alginate in $CaCl_2$ solution (1:1 ratio) for (a) 2%, (b) 1%, (c) 0.2%, (d) 0.1%, and (e) 0.01% alginate solutions (w:v).

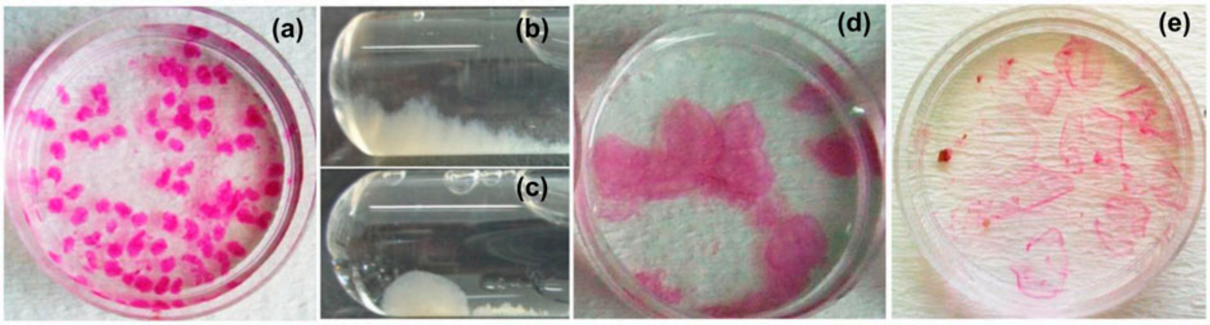

Fig. 3. Results of gelation experiments with exoPS/EPS extracts in $CaCl_2$ solution: (a) 0.2% CAS-exoPS (day 35, acetate feeding, Na_2CO_3 extraction) in 0.2% $CaCl_2$, (b) CAS-EPS (day 64, brewery ww, DOWEX extraction) in 2% $CaCl_2$, (c) GAS-EPS (day 109, brewery ww, DOWEX extraction) in 2% $CaCl_2$, (d) GAS-EPS (day 109, brewery ww, DOWEX extraction) in 0.2% $CaCl_2$, and (e) 1% GAS-exoPS (day 150, brewery ww, Na_2CO_3 extraction) in 1% $CaCl_2$.

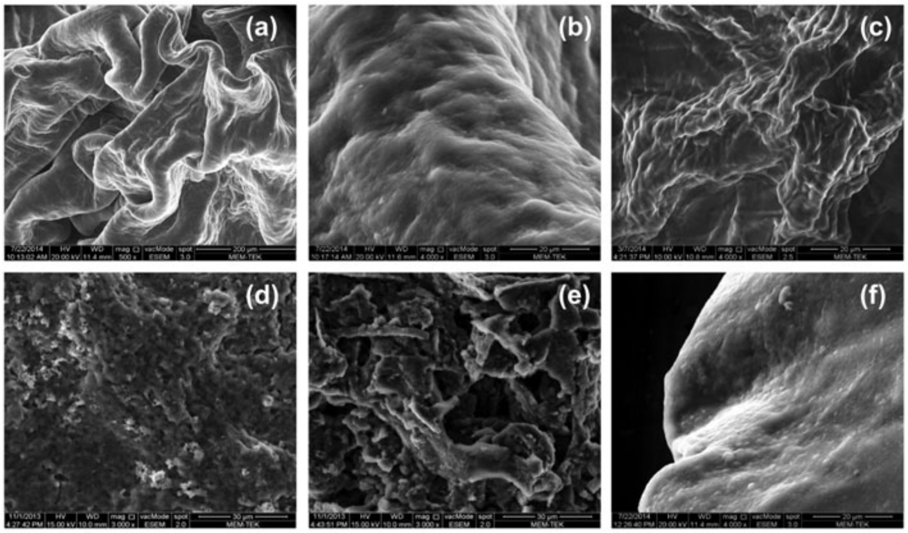

Fig. 4. Morphologies of the hydrogels displayed by E-SEM: results for (a) 1% alginate (500×), (b) 1% alginate (4,000×), (c) 0.2% CAS-exoPS (day 35) (4,000×), (d) CAS-EPS (day 64) (3,000×), (e) GAS-EPS (day 109) (3,000×), and (f) 1% GAS-exoPS (day 150) (4,000×).

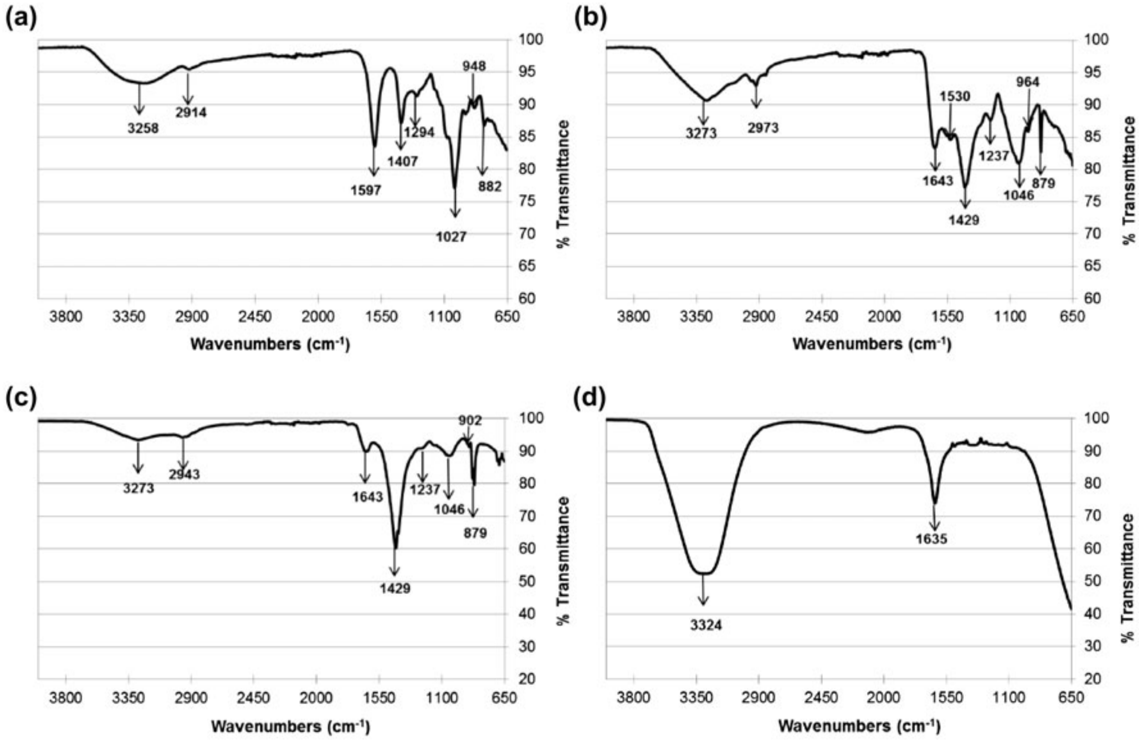

Fig. 5. FTIR spectra of (a) alginate, (b) CAS-exoPS (day 49), (c) GAS-exoPS (day 199), and (d) GAS-EPS (day 109) (DOWEX extract in aqueous form).

locked structures, resulting in formation of 3-D networks and then the hydrogels. One of the important groups contributing to this cross-linking through Ca^{+2} might be the carboxyl (O–C–O) groups, which were addressed by Bramhachari et al. [17] as possibly serving as binding sites for divalent cations. Hence, detection of peaks assigned to O–C–O groups, especially those at wavenumbers slightly above 1,400 cm^{-1} (Fig. 5, Table 1) indicating the noteworthy presence of the symmetric stretching vibrations (D) addressed as being specific for uronic acid residues, both in alginate and in the exoPS extracts were considered as further evidence confirming the similarities between those polymers originating from different sources (brown algae and AS, respectively) in terms of both hydrogel formation properties and chemical structures.

EPS extracted with DOWEX displayed a FTIR spectrum quite different from those attained for the lyophilized exoPS extracts obtained after extraction with Na_2CO_3. Only two bands were observed (Fig. 5(d)); (i) the broad stretching vibration of O–H above 3,000 cm^{-1} (max. at 3,324 cm^{-1})—which is a polysaccharide band—and (ii) the asymmetric stretching of carboxylate O–C–O vibrations at 1,635 cm^{-1}. Supernatant samples both from DOWEX and Na_2CO_3 extractions generated similar spectra (data not shown) with only two peaks at wavenumbers similar to those given above for the EPS extract obtained by DOWEX extraction method (Fig. 5(d)). The absence of other peaks in the FTIR spectra of those samples can clearly be attributed to the nature of those samples being aqueous rather than solid; a natural result of the DOWEX extraction method and a property interfering with FTIR spectroscopy.

Despite slight differences described above, the FTIR spectroscopy results obtained in this study in overall further confirmed the similarities between sodium alginate and the exoPS extracts. Based on the results from gelation experiments and FTIR analyses, and also similar to the ones reported by Lin et al. [6,7] for CAS and GAS treating municipal wastewater, it was concluded that the exoPS extracted from floccular and aerobic granular sludge fed with acetate and/or brewery effluent in fact resembled alginate in terms of chemical structure and gel-forming properties, thus were addressed as ALE, after Lin et al. [6].

3.5. Total carbohydrates content of the extracted exoPS

Lin et al. pointed out that using glucose as the standard for quantification of total carbohydrates might result in underestimation of carbohydrate content of sodium alginate by 62.6% when phenol-sulfuric acid assay was employed, and the values obtained for ALE by preferring alginate as the standard were three times higher than those obtained with glucose as the standard [6]. Concurrently, to determine the glucose equivalence of sodium alginate and % recovery of the anthron method used in this study for determination of total carbohydrate content, a dilution series of sodium alginate was prepared, subjected to anthron assay along with the reactor samples and quantified using the calibration curve prepared from glucose as the reference carbohydrate. For 2, 1, and 0.2% (w:v) alginate solutions, glucose concentrations were measured as 1,443, 736, and 151 mg glucose/L, respectively. Corresponding % recovery values were 7.2, 7.4, and 7.6%, with an average of 7.4% recovery for alginate in the anthron assay. Average glucose equivalence of alginate was determined as 73.8 mg glucose/g alginate.

Values obtained for 0.2% solutions (w:v) of the exoPS extracted by the Na_2CO_3 extraction method from the floccular (day 35) and aerobic granular sludge samples (day 199) were as follows: total carbohydrate content of CAS-exoPS was 75 mg glucose/L and that of GAS-exoPS was 156 mg glucose/L. Corresponding % recovery values were 3.7 and 7.8%, respectively, and the glucose equivalence were 37.5 mg glucose/g CAS-exoPS and 78 mg glucose/g GAS-exoPS. Those values clearly showed that using glucose as the reference carbohydrate in the anthron method for quantification of total carbohydrates in sodium alginate and in the exoPS extracted from the sludge samples result in underestimation of the actual carbohydrate content of those polymers, as also reported by Lin et al. [6] for the same/similar polymers when phenol-sulfuric acid assay is employed.

Those observations are considered as pointing to the bias that might be introduced by the methods used for total carbohydrate quantification, underlining the impact of the reference carbohydrate selected, and highlighting the importance of choosing a reference material to be more appropriate for AS applications, especially for determining the total carbohydrate content of EPS and/or exoPS extracts from floccular and granular sludge.

In addition to the exoPS extracts, carbohydrate contents of the initial supernatant samples collected prior to extraction by Na_2CO_3 method were also determined: values were 31 and 60 mg glucose/L for the supernatants from the floccular and granular sludge samples, respectively.

3.6. Potential technical uses and economic considerations of AS-associated biopolymers

The idea of approaching to WWTPs not only as sites providing everyday water treatment services, but also as industrial production sites with value-added products has been around for some time with successful applications like nutrient recovery from domestic and/or industrial waste streams or biogas/bioenergy harvesting from anaerobic waste treatment units. Another promising chapter in conversion of WWTPs into feasible and sustainable contributors of emerging bio-based economies would be production/harvesting of available biopolymers while continuing with the treatment services.

Among those biopolymers, PHAs (polyhydroxyalkanoates) have been at the center of interest for some time. Industrial production of PHAs requires feeding VFA-convertible valuable organics to the fermenters as raw materials; but now it might be possible and feasible to produce those bioplastics also from organic wastes in or out of WWTP upon harvesting functional active biomass with maximal PHA pools or with high potential of PHA accumulation, respectively [19,20]. Studies including optimization of operational conditions for maximum PHA yield, trials with different waste streams at different strengths or compositions, evaluation of the economic aspects of downstream applications like optimization of PHA extraction methods, thus overall feasibility of bioplastic production from WWT processes continue to accumulate, while real-world implementations have already been underway [19].

Another biopolymer of interest is the exoPS, and more specifically the ALE with chemical properties and thus potential uses similar to those of the commercially available agents: hydrogels, in general, are used as scaffolds for tissue engineering, vehicles for drug delivery, actuators for optics and fluidics, and model extracellular matrices for biological studies [21]. Technical and commercial uses of alginic acid and alginate, in particular, might be listed as being: enzyme carriers [16]; microencapsulation systems for drug delivery [22]; dye-thickeners in textile printing; thickeners, gelation agents, stabilizers, moisture barriers, etc. in food production; immobilizing agents entrapping biocatalysts; surface sizing and coating agents in paper industry; coating agents for welding rods; disintegrating agents in pharmaceutical tablets, cold-setting gels in dental impression compounds, swelling agents in diet foods; binders for fish feed in aquaculture sector; agents used in medical dressing, in controlled release of chemicals, etc. [23,24]. Former reports listed the market price of alginates in the range of US$ 2.5–7 per pound, varying according to desired grade [23,25]. Majority of the annual alginates market demand comes from three main sectors, namely textile printing sector, food industry, and medical applications and pharmaceuticals industry [24]. According to the 2013 market figures, food and beverage industry has the major share of the alginate and derivatives market. The largest and the fastest-growing markets are North American and Asia-Pacific regions, respectively, with corresponding market value of US$ 120.3 million in 2013 and a projected compound annual growth rate (CAGR) of 5.6% between 2014 and 2019. The estimated value of the global alginates market for 2019 is US$ 409.2 million, growing at a compound annual growth rate of 3.8% from 2014 to 2019 [26].

Forming hydrogels and upholding chemical properties resembling those of commercial alginate, AS-associated ALE are baring the potential technical uses similar to some of those given above. In addition, ALE extracted from adhesion/aggregation-based AS applications (i.e. biofilm systems or aerobic granular sludge systems, respectively) has recently been reported to offer the potential technical use as a biopolymer, i.e. for forming a water-resistant film on the surface of cellulose sheets, offering a similar result as commercial synthetic chemicals like alkenyl succinic [27]. Notwithstanding all these promising potential uses, ALE and/or EPS harvesting and purification feasibility data still need to accumulate to find an acceptable ground of technical applicability with economic reasoning. As a starting point, initial findings indicate that similar to the case for bioplastics (i.e. PHAs), it is possible to recover biopolymers like ALE as value-added products from AS in quantities and costs that may provide a sustainable alternative source to meet today's market demand at compatible prices [20].

4. Conclusion

Setting the promising potential technical and commercial uses of AS-associated ALE in reference to those of commercial alginates and the facts of the global mature alginate market, this study focused on assessing chemical and physical characteristics of AS exoPS/EPS extracted from floccular and aerobic granular sludge fed with acetate and/or brewery wastewater to evaluate possible similarities with alginate and to establish the relationship between those properties and the role of those sort of "*in situ* produced thickening agents" of AS, providing structural integrity and coherence to aggregation-based systems like conventional floccular and aerobic granular AS systems. The conclusive remarks are:

(1) Using 2% or 1% alginate solutions and keeping a 1:1 ratio of alginate:$CaCl_2$ yield the best gelation results for sodium alginate. That notwithstanding, even when keeping the 1:1 ratio, hydrogel formation is limited by the alginate concentration (i.e. 0.01%) below which gelation seems undetectable even for this commercially available polymer.

(2) Gelation experiments and investigation of the formed hydrogels are proved to be useful for determining the gel formation capability of the AS-exoPS and their similarities with alginate. Likewise, FTIR spectroscopy provides a good tool for approximation of the chemical structure of the exoPS extracts in comparison to that of alginate.

(3) Different extraction methods result in different types of extracts—lyophilized or aqueous, which then produce gels with different morphologies: better gelation results were obtained with lyophilized exoPS extracts attained by the Na_2CO_3 extraction method.

(4) % moisture content values indicate that the gels formed by the exoPS extracts were in fact hydrogels, like those formed by sodium alginate.

(5) Different from other works, this study showed that the exoPS produced by floccular sludge can form self-standing hydrogels in $CaCl_2$, just like alginate and the exoPS produced by aerobic granular sludge.

(6) Although the extraction efficiency was lower than that reported previously, the Na_2CO_3 extraction method was employed successfully for isolation of the exoPS from AS. Gelation reactions in $CaCl_2$, investigation of formed hydrogels, and FTIR spectroscopy all showed that the AS-associated exoPS resembled alginate in terms of their physical and chemical properties, and thus can be referred to as ALE, as proposed by Lin et al. [6].

(7) Similarities of the AS-exoPS with alginate signify the role of those extracellular polymers in floccular and aerobic granular sludge: seemingly, the gel-forming properties of the exoPS extracts, as well as the presence of the GG and MM blocks in those, the former known as the sites of crosslinking through divalent cations promoting aggregation and gelation of alginates, and the latter described as providing flexibility, confer onto their origin; the AS,

providing strength, compactness, and elasticity, and thus contributing to the structural strength and coherence of AS, especially to the compact and durable nature of the excellently settling aerobic granular biomass.

Future work for further characterization of the AS-associated exoPS will involve block fractionation to explore and compare the % distribution of the GG, MM, and MG blocks making up those extracellular polymers and commercially available alginates. NMR analyses might also be useful for further exploring the chemical structure of AS-exoPS and to see if those are complex heteropolysaccharides.

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