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Effects of alkaline solvents and heating temperatures on the solubilization and degradation of gel-forming Extracellular Polymeric Substances extracted from aerobic granular sludge.

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Abstract

The effects of solvents and heating temperatures on the recovery and degradation of gel-forming EPS (Extracellular Polymeric Substances) extracted from aerobic granular sludge were investigated. Sodium hydroxide has allowed a better EPS solubilization compared to sodium carbonate (711.3±38 and 408.4±4 mg VS /g VS respectively) but the capacity to precipitate gel-forming EPS at acidic pH was higher in sodium carbonate (22% compared to 6% in sodium hydroxide at 80 °C). Increasing the temperature from 20 °C to 80 °C during EPS solubilization has multiplied the amount of solubilized matter by 4 in sodium carbonate and 2.2 in sodium hydroxide. Similar biochemical composition but different molecular weight profiles were observed for the two solubilized EPS extracts, evidencing partial degradation of the polymers in sodium hydroxide. Furthermore, coupling molecular weight analysis and precipitation yield measurement revealed that the lower precipitation of sodium hydroxide solubilized EPS is related to the size reduction due to degradation.

Altogether these results help to guide towards a unified protocol for the extraction of gel-forming EPS.

Keywords:

Extracellular polymeric substances (EPS), Aerobic granular sludge, alkaline solubilization, heating temperature, precipitation, gel-forming polymers.

1 Introduction

The use of aerobic granular sludge for the treatment of wastewater has elicited more interest over the past ten years. Granular sludges present many advantages over activated sludges among which a higher settling velocity *in fine* leading to a reduction in reactor size, energy consumption and environmental footprint. From an economical point of view, granular sludges are also interesting because they lower investment and installation costs [1,2]. Aerobic granular sludges are currently investigated as potential resources for the recovery of high value-added biomaterials to reduce the amount of wastes produced during water treatment processes. Extracellular polymeric substances (EPS) insuring the mechanical properties and stability of the granules [3] can provide interesting renewable materials for industrial applications [4–6]. The extracted material was shown to have amphiphilic properties due to the presence of a small fraction of lipids, allowing the use of EPS as a surface coating material for paper packages thus enhancing their water resistance [5]. Flame retarding properties [6] and heavy metal adsorption, particularly for Pb, Cd and Zn, have also been demonstrated for EPS extracted from aerobic granules [7] or anammox granules [8]. Among the existing applications, hydrogel-forming capacity has received the most attention. Indeed, EPS secreted by aerobic granular sludge can form hydrogels with ionic cross linkers such as calcium ions and precipitate at low pH [4]. Due to similar extraction methods and the presence of carboxyl groups, these structural EPS were later on named Alginate Like Exopolysaccharides (ALE) [4]. Extracted molecules value and their significance as alternatives to industrial products remain however dependent to the downstream granules biorefinery [9]. Indeed, due to the molecular diversity of the EPS extracted from aerobic granules, the precise composition and chemical nature of the structural EPS remain unknown. Recent works have confirmed this complex chemical composition including proteins, neutral sugars, uronic acids, phenolic compounds, hyaluronic acid-like and sulfated glycosaminoglycan-like polymers [10–12]. Extraction technologies have thus to be adapted to the diversity of the secreted molecules and a better understanding of their impacts on the recovered polymers (both in terms of quantities and properties) is needed to ensure the economic feasibility of this resource recycling. The lack of information on the chemical and physical identities of EPS greatly limits the potential to rationally propose relevant EPS extraction protocols. In addition, the extraction procedures have to be defined depending if the entire spectrum of the EPS is required for further comprehension of the diversity and biological significance of EPS or if specific functional EPS are targeted [13].

The aggregation of the EPS matrix is due to interactions between the polymers forming it; the main forces responsible of maintaining this structure are van der Waals forces, hydrogen bonds, electrostatic linkages, hydrophobic interactions and covalent bonds [14–16]. Solubilizing these polymers requires to break these interactions using extraction methods that can focus on one or multiple types of EPS bonds in the matrix. Considering stratified and dense aggregates such as fixed biofilms or granular sludges, harsh extraction methods are required to solubilize the extracellular matrix and recover EPS. Physical methods (sonication, heating, centrifugation…), chemical methods (alkaline extraction, chelators, Tween…) or a combination of both can be used to attain this objective of maximal extraction yield whether on biofilms [17] or granular sludges [18,19]. The extraction techniques are thus very heterogenous and there is no unified protocol. Understanding the factors influencing the extraction is therefore essential to propose relevant extraction protocols.

Alkaline conditions are widely used for the extraction of structural EPS showing gel-forming properties because such conditions allowed high extraction yields [20]. Due to the fact that EPS isoelectric points are generally below pH 6 [14], high pH conditions cause carboxylic groups in proteins and polysaccharides to be ionized which results in a strong repulsion between EPS and provides a higher water solubility of the compounds [21,22]. Different alkaline extraction procedures were tested previously which mainly relied on the use of sodium hydroxide or sodium carbonate at various concentrations [4,12,23,24]. High temperatures of 80 °C were used in combination with sodium carbonate while sodium hydroxide solubilization was generally performed at lower temperatures (4 °C) but no clear justification of the choice of such extraction conditions were provided by the authors [4,12,21,23]. Seviour et al. [24] suggested that granule solubilization could influence the properties and characteristics of the putative structural gel-forming agents extracted either with sodium hydroxide (as a complex hetero-polysaccharide named granulan) or with sodium carbonate (as uronic acids including ALE). Comparison between studies remains also difficult since the type of feeding may have a direct effect on the EPS extraction yield depending on the carbon sources and nutrient concentrations and an indirect effect via changes in microbial diversity: for example, some granular sludges are fed by municipal wastewater others are fed by synthetic wastewater, which influences the amount of EPS produced by the aerobic granules [12]. In addition, using the same type of biomaterial but changing the method of extraction gives different yields [20,23]. For instance, alkaline conditions are not efficient for structural EPS extraction from aerobic granular sludge enriched with ammonium-oxidizing bacteria [25] or with *Defluviicoccus* sp. [26]. Therefore, there is a need to understand what really influences the yield of extraction and the molecules selected by the extraction protocol.

The global solubilization efficiency cannot be considered as the only relevant parameter, particularly for the purpose of recovering polymers with specific functional properties. In the case of alginates from seaweed, a higher molecular weight was shown to give the compound a better gel formation kinetics and enhanced viscosities [27]. A molecular weight dependency of alginic acid has been proved to influence the gel strength since Young's modulus values were increased 5 times when the molecular weight of algal alginates increased from 60 to 400 kDa [28]. With the objective of recovering gelling biomaterials from granular sludges, preserving the entire native size of the polymers may be a key issue. Thus, attention must be drawn to the extraction conditions that could lead to depolymerization/alteration of the molecules forming the EPS matrix.

Two main objectives were set for this study. The first one was to compare the effect of chemicals and heating on the yields of solubilization and precipitation of gel-forming EPS extracted from granular sludge. Particular attention was paid for the adverse effect of the degradation/alteration of biopolymers on their recovery efficiency. The second objective was to evaluate the selectivity of the extraction methods on EPS in terms of molecular weight and biochemical composition of extracted molecules that are relevant parameters to drive aerobic granular sludge valorization towards gel-forming biomaterials.

2 Materials and methods

2.1 Biomaterial

EPS were extracted from aerobic granular sludges cultivated in 13 L column Sequencing Batch Reactors (SBR) fed with 100%-VFA synthetic wastewater composed of equal COD fractions of acetate and propionate [29]. All systems were operated at a constant volume and SBR cycles were as follows: 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 to 5.1 m/h). A detailed characterisation of the influent composition as well as the physical properties of the granules used for organic pollution, nitrogen and phosphorus removal is provided in Layer et al. [29,30].

2.2 EPS extraction

The extraction protocol was divided into two steps: (i) a solubilization step of polymers from aerobic granules using different solvents and (ii) a precipitation step of the solubilized polymers in the form of a gel pellet. As usually defined by many authors [12,20,31], the extracted material was named EPS for the sake of clarity, even if the presence of some intracellular contamination cannot be excluded. For EPS solubilization, a ratio of 0.2 g of freeze-dried granules to 32 mL of the chosen solvent at 0.2 M (urea, sodium carbonate, sodium hydroxide, and sodium chloride considered as a control) was kept for the rehydration of granules under stirring at 300 rpm for 30 minutes. Afterwards, the granule suspension was homogenized using the ultraturrax T25 (Janke & Kunkel from IKA-Labortechnik) at a speed of 13500 rpm for 3 cycles of 1 minute separated by 45 seconds of resting phases. Then the disrupted granules were heated for 60 min in a water bath at 80 °C. After centrifugation (22 000 g, 4 °C, 15 min) the supernatant was recovered (named S1) and the pellet (named P1) was analyzed for its content in volatile solids (VS). The extraction protocol was also performed at different temperatures (20 °C, 50 °C, 70 °C and 80 °C) with sodium hydroxide or sodium carbonate to determine the effect of the heating temperature on the yield and properties of extracted EPS.

As previously described for the acidic precipitation of ALE [4], the pH of the S1 extract was adjusted to 2 ± 0.02 with 1 M HCl, and after an incubation of 30 minutes in a water-ice bath, the precipitate was collected by centrifugation at 22 000 g, 4 °C for 15 minutes. The supernatant after acidic precipitation was recovered and named S’1. The precipitate was solubilized in 0.1 M sodium hydroxide then dialyzed for 36 hours against distilled water by using a dialysis membrane with a cutoff of 3500 Da (Spectrum labs). All the salts formed by the previous acidic precipitation and alkaline solubilization were eliminated thanks to this dialysis step, allowing direct evaluation of the recovered EPS by gravimetry after their lyophilization. The freeze-dried material (named P2) was finally resuspended in 0.1 M sodium hydroxide (named S2) for further analysis.

Efficiency of EPS solubilization was calculated by subtracting the VS amount remaining in the pellet P1 from the initial amount of VS present in the granules and dividing it by the initial amount of VS in the granules. EPS precipitation efficiency was evaluated by the dry mass of VS contained in the P2 lyophilizate. All the extraction protocols and yields evaluation were performed in triplicate and standardized by the initial amount of VS in the granules.

2.3 Size exclusion chromatography (SEC)

Chromatography analysis, to determine the apparent molecular weight of molecules, was performed using the AKTA purifier system (Cytiva) equipped with an on-line spectrophotometer.

The SUPERDEX 200 column was used to separate molecules between 10 and 600 kDa (Cytiva). The exclusion volume corresponding to the volume of liquid located outside the pores of the column is equal to 7.7 mL and the total inclusion volume corresponding to the volume of liquid included outside and inside the pores of the column is equal to 24 mL. The injection volume was set to 250 µL. The used mobile phase was Tris HCl

50 mM with 0.1 M NaCl, pH 9 ± 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=high molecular weights that were eluted first between 7.7 and 12 mL, MMW= medium molecular weights eluted from 12 to 17 ml and LMW= low molecular weights that were eluted lastly after 17 ml. The P2 samples were solubilized in 2 mL of 0.1 M sodium hydroxide and filtered through a regenerated cellulose membrane of 0.2 µm. Molecules were detected by monitoring the absorbance at 280 nm (AU). The peak area (AU×mL) was processed using the UNICORN 5.31 software.

For each molecular weight category, the percentage of precipitation was calculated by dividing the peak area in the S2 chromatogram with the peak area in the S1 chromatogram and multiplying by 100.

2.4 Asymmetrical Flow Field Flow Fractionation (AsFlFFF)

AsFlFFF was used to determine some molecular characteristics of the S2 polymers such as size and gyration radius. The setup was an Eclipse Dualtec separation system (Wyatt technology Europe) with a UV detector (Ultimate 3000, Thermo Scientific). Multi Angle Light Scattering (MALS) measurements were performed with a DAWN-HELEOS II (Wyatt technology Corporation) coupled to a differential Refractive Index (dRI) detector (Optilab rEX, Wyatt technology Corporation). Size distribution was determined by studying the dependency of the intensity of the scattered light as a function of the scattering angle with a laser source at a wavelength of 658 nm. Astra software package, version 7.1.2 was used to provide molecular weight (M_w) and gyration radius (R_g)

For the experiments, 50 µL solutions of acid precipitated EPS (S2), at 2 mg/mL in 0.1 M sodium hydroxide, were injected. Eluent for the AsFlFFF experiments was prepared from ultrapure water (Milli-Q), and consisted of 50 mM of Tris-HCl, 0.1 M NaCl, NaN_3 0,02% at a pH of 9. Elution mode was started with a crossflow rate of 3 mL/min for 10 min, and then reduced linearly for 10 min to a rate of 0.09 mL/min.

2.5 EPS biochemical composition

To evaluate the selectivity of the most efficient solvents, S1 and S2 extracts obtained with sodium hydroxide and sodium carbonate at 80 °C, were analyzed in triplicate for proteins, humic acid-like substances, neutral sugars, uronic acid-like and DNA components. All measurements were done in 96 well microplates, using the FLUOstar OPTIMA (BMG, Labtech) microplate reader.

2.5.1 Protein and humic acids quantification

The modified Lowry assay was used for the quantification of proteins and humic acid-like substance according to Frølund et al. [32] 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 $CuSO_4$. 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%. Therefore, concentration of proteins and humic acid-like molecules can be calculated by using the equations described in Frølund et al. [32].

2.5.2 Neutral and uronic acid-like sugars quantification

The Anthrone method was used for the quantification of both neutral and uronic acid-like sugars as adapted from Rondel et al. [33] with respectively glucose (0 to 75 mg/mL) and glucuronic acid (0 to 750 mg/mL) as standards. The Anthrone reagent was prepared with a 2 mg/mL concentration 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 absorbance was read at 544 nm and 620 nm. The slopes of the four equations linking the standard solutions with glucose or glucuronic acid concentrations 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. [33].

2.5.3 DNA quantification

The Quant-iT^{TM} PicoGreen dsDNA Assay Kit (Invitrogen) was used for the DNA quantification. Lambda DNA (100 µg/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/samples. Fluorescence intensity was measured at an excitation emission wavelength pair of 485/520 nm.

2.6 Young's modulus determination

In order to determine the gel stiffness a protocol was adapted from Felz et al. [34]. The dialyzed and freeze-dried material (named P2) was resuspended in 0.1 M sodium hydroxide at a final concentration of 8% w/v (mg VS / 100 mL). Hydrogels were then prepared via ionic cross-linking in the presence of calcium ions. The calcium ions (0.1 M $CaCl_2$) diffused into the EPS solution through a dialysis membrane (3.5 kDa MWCO, 36h dialysis). The Young's modulus (E, Pa) was selected as a sensitive parameter to analyze the polymers mechanical response. The mechanical tests were performed using a Mars III rheometer (Thermo Scientific) in a stress-controlled mode. The samples were subjected to consecutive compression-decompression cycles (n. 17) increasing the maximum deformation in the loading step up to 20%. The Young's modulus (E) was calculated from the linear regression of the stress (σ, Pa) – strain (ε, -) data in the linear elasticity domain of the material (i.e. $\sigma = E \cdot \varepsilon$). Each test was performed in triplicate and the Young's moduli were reported as average values ± standard deviations.

3 Results and discussion

3.1 Effect of chemical solvents on EPS solubilization and precipitation

3.1.1 Comparison of extraction efficiency between solvents

Results of the solubilization with the used solvents (urea, sodium carbonate, sodium hydroxide, and sodium chloride) followed by the precipitation step are shown in Figure 1. At 80 °C sodium hydroxide was the most efficient for solubilizing granules with 711.3 ± 38.0 mg VS solubilized/g VS followed by sodium carbonate with a solubilizing power diminished to 408.4 ± 4.0 mg VS solubilized/g VS as seen in Figure 1A. Urea and sodium chloride were clearly less efficient with a solubilization efficiency of 85.2 ± 30.0 mg VS solubilized/g VS and 146.7 ± 43.0 mg VS solubilized/g VS respectively. Those different solvents rely on contrasted mechanisms: alkaline solubilization, i.e. sodium carbonate or sodium hydroxide, is based on the increase of negative repulsive forces of the charged polymers while urea is known to facilitate the exposure of hydrophobic residues and to break hydrogen bonds [35,36]. The pH value is 11.30 ± 0.02 for sodium carbonate 0.2 M and 13.40 ± 0.02 for sodium hydroxide 0.2 M. In the case of proteins, a higher pH is likely to induce a higher negative charge of the chains containing acidic amino acids which will favor electrostatic repulsions between molecules. This repulsion contributes to the improvement of proteins solubility thanks to increased protein–water interactions [37]. Due to the ionization of carboxyl groups, some polysaccharides such as pectin or functional acidic polysaccharide extracted from okra pods, also show a better solubilization with the increase of pH [38,39].

Our results therefore suggest that the EPS matrix is composed of a majority of negatively charged polymers and that switching solvents used for extraction significantly affects the solubilization of the organic material extracted from aerobic granules. This is in agreement with results obtained in previous studies showing that alkaline treatments provide a higher solubilization yield in comparison with other physical or chemical methods [20].

After acidic precipitation an inversion in the final yield happened, leading to the recovery in P2 final samples of 44.3 ± 7.1 and 88.8 ± 11.5 mg VS precipitated/g VS of initial granules for sodium hydroxide and sodium carbonate respectively (Figure 1B). Materials recovered in S1 by sodium carbonate and sodium hydroxide can be precipitated by 21.7% and 6.2% respectively. Previous analysis of the zeta potential of EPS solubilized by sodium hydroxide from anammox granules, revealed that EPS precipitation should be done at a pH around 4 corresponding to a global neutral charge facilitating aggregation of polymers [31]. Indeed, biopolymers have minimal interactions with water at their isoelectric point, while molecule-molecule interactions are at their maximum promoting precipitation [37]. As described previously by many authors, a big amount of the solubilized EPS is lost after acidic precipitation similarly to what is observed in this study [12,31,40]. Therefore, initial solubilization should not be the only parameter considered to choose the extraction solvent and further investigation on the biochemical and molecular characteristics of the extracted molecules must be done in order to better understand this difference in precipitation yields between solvents. Based on the high yields of solubilization and precipitation, only sodium hydroxide and sodium carbonate will be investigated furthermore in this study.

3.1.2 Biochemical composition of extracted EPS

To evaluate possible selectivity of the two alkaline solvents on the recovery of a type of biopolymer (neutral or uronic acid-like polysaccharides, proteins or humic acid-like substances), S1 and S2 samples obtained using sodium hydroxide or sodium carbonate extraction at 80 °C were analyzed for their biochemical composition (Figure 2).

The solubilization of neutral sugar (Figure 2A) was slightly higher in S1 sodium carbonate compared to S1 sodium hydroxide (56.0 ± 4.9 mg/g VS compared to 40.1 ± 5.3 mg/g VS), solubilization of uronic acid-like molecules and proteins were approximately the same in both solvents. However, after precipitation (Figure 2B), 15.4% of the S1 uronic acid-like molecules solubilized in sodium carbonate precipitate (from 160.9 ± 18.5 to 24.8 ± 1.7 mg/g VS in S1 and S2 respectively) while only 5.8% precipitate for sodium hydroxide (from 182.2 ± 13.4 to 10.6 ± 1.7 mg/g VS in S1 and S2 respectively).

The same trend was observed for proteins: for sodium carbonate 33.7% of the proteins solubilized in S1 have precipitated (90.4 ± 14.2 mg/g VS in S1 to 30.5 ± 5.1 mg/g VS in S2) in comparison to only 4.6% of proteins solubilized in S1 sodium hydroxide (113.7 ± 40.5 mg/g VS in S1 to 5.2 ± 1.3 mg/g VS in S2) (Figure 2B).

Sodium carbonate solubilizes humic acid-like molecules less than sodium hydroxide (Figure 2A) but the molecules present in sodium carbonate are more precipitable and therefore the quantity of humic acid-like molecules in S2 is approximately the double (Figure 2B).

Altogether these results indicate that S1 sodium hydroxide and sodium carbonate samples have rather similar biochemical diversity and the inversion of yield observed from S1 to S2 after acidic precipitation cannot be attributed to the prevalence of a specific biochemical fraction. Precipitation requires molecules/molecules interactions that are favored by various polymer properties such as high molecular weight, low charge and the presence of apolar and hydrophobic zones [41]. Extreme alkaline pH used during solubilization may thus influence the precipitation ability of polymers because of denaturation or alteration of the polymer structure leading to modification of their molecular weight and/or spatial conformation.

As previously mentioned the molecular weight of molecules impacts precipitation. On one hand, polysaccharides are generally relatively stable to alkali; an exception is present for uronic sugars (for example alginate) that have a glycosidic linkage in a beta position in relation to a carbonyl group which makes them more sensitive to β-elimination and oxidative-reductive depolymerization [42]. As recently shown by Bai et al. [39] studying the influence of pH on the extraction and physico-chemical properties of galacturonic acid containing polysaccharides from okra pods, both the degree of esterification and the length of the polymers regularly decreased while increasing the pH from 2 to 12. On the other hand, proteins are known to be sensitive to degradation in alkaline conditions, as shown by Hou et al. [36]. The authors reported that when sodium hydroxide concentration was higher than 0.09 M, peptide bonds might be hydrolyzed and the size of the polypeptides reduced. To check if proteins are degraded in the conditions used in this study, BSA was submitted to both conditions and the resulting solutions were analyzed by SEC (Figure S1 of the supplementary material). While only a slight shift was observed for the peaks of BSA incubated in sodium carbonate, more than 80% of the peaks were found at lower elution volumes for BSA incubated in sodium hydroxide. Higher degradation in sodium hydroxide may thus explain the observed differences in precipitation yield between the two alkaline solvents. To support this, S1 SEC profiles were compared to SEC profiles of the remaining acidic supernatant S'1 after acid precipitation and it was observed that acidic precipitation is selective for HMW and MMW molecules whereas only a small fraction of LMW molecules is precipitated (Figure S2 of the supplementary material) reinforcing the hypothesis that molecular size influences the precipitation behavior of molecules.

Another factor influencing the precipitation is denaturation and the presence of apolar/hydrophobic zones. Among the effects induced by extreme pH, unfolding of proteins leads to substantial changes by the exposition of some hydrophobic areas that are buried at neutral pH. It can be supposed that denaturation occurred in both solvent but as described by Kristinsson and Hultin [43], different properties of the proteins were obtained after refolding. The differences in precipitation ability of proteins between sodium hydroxide and sodium carbonate solvents (4.6% and 33.7% respectively) suggest that not the same type of proteins is extracted with both solvents and/or that major changes happened to the structure of proteins in sodium hydroxide hindering their recovery in acidic conditions. A higher release of intracellular proteins during sodium hydroxide solubilization may be also hypothesized since DNA concentration (Figure 2) was approximately 2.5 times higher in S1 and 2 times higher in S2 (4.4 ± 1.2 and 1.2 ± 0.3 mg/g VS respectively in S1 and S2) compared to DNA in sodium carbonate (1.7 ± 0.1 and 0.7 ± 0.2 mg/g VS in S1 and S2 respectively). However, for the sodium hydroxide fractions, the obtained values are not correlated with a significant increase in the protein contents, eliminating thus a big contamination with intracellular proteins in sodium hydroxide in comparison with sodium carbonate. The presence of DNA may be attributed to both extracellular and intracellular origin.

The differences in gel forming polymers (S2) recovery and in particular in the precipitation efficiency observed for the two alkaline solvents cannot be thus related to their biochemical composition but rather to enhanced denaturation or degradation of the extracted polymers with sodium hydroxide. To confirm this, AsFlFFF fractionation was then performed in order to compare the molecular characteristics of the recovered gel forming polymers in each extraction condition.

3.1.3 Molecular characteristics of alkaline extracted EPS

The S2 samples obtained after acidic precipitation were analyzed by AsFlFFF coupled with MALS detection. This method provides a continuous separation of particles as a function of their diffusion coefficient, and allows determining parameters such as real molecular weight and gyration radius of the molecules.

The fractograms reveal molecular size heterogeneity within the samples with M_w increasing steadily with the elution time as expected for the normal mode of AsFlFFF operation (Figure 3A). Similar molecular size profiles were observed for sodium hydroxide and sodium carbonate extracted molecules eluted between 10 and 27 min. On the other hand, quite different profiles were noticed for the lastly eluted polymers (after 27 min): increasing molecular size reaching approximately 10 million Da was obtained for sodium carbonate extracted polymers while a pool of polymers of much smaller sizes (around 2 million Da) are eluted after sodium hydroxide extraction (Figure 3A). The radius of gyration was also determined for the lastly eluted polymers and it can be noticed that the gyration radius of polymers extracted in sodium carbonate can reach

277.2 ± 3.2 nm and is higher than that of polymers extracted in sodium hydroxide limited to 126.1 ± 2.2 nm (Figure 3B).

AsFlFFF analysis was used to focus on the HMW defined for elution time ranging from 27.2 min to 32 min (Table S1 of the supplementary material). For the HMW molecules eluted from sodium carbonate S2, the average value of Rg is 118.6 ±1.6 nm, M_w was $5.57\times10^6 \pm 3.34\times10^5$ Da with a number average molar mass M_n of $2.2\times10^6 \pm 4.6\times10^4$ Da leading to a polydispersity index (M_w/M_n) of 2.5. Lower values were found for sodium hydroxide S2 fraction with an average Rg of 61.7 ± 1.3 nm, M_w of $1.88\times10^6 \pm 3.70\times10^4$ Da and an average M_n of $1.67\times10^6 \pm 2.60\times10^4$ Da leading to a lower polydispersity index of 1.1. In other studies, the molecular weight of commercial alginates was found to vary on average between 115 000 and 321 700 Da and having slight fractions reaching approximately 1 million Da [44]. Another study carried out on ultra-high viscosity alginates found that the molar mass ranged from 330 000 to 2.9 million Da with gyration radius varying between 98 and 300 nm [45]. In the case of sodium carbonate solubilization, some of the particles eluted after 30 min had molecular weights approximately 3 times higher than those observed in the previously cited study and might be formed *in vitro* by the association of several polymers. Indeed, the precipitated EPS have a very complex biochemical composition, interactions between different types of molecules could be elicited during acidic precipitation as for example proteins with polysaccharides which are preponderant components of the precipitated EPS. Although the molecules eluted after 30 minutes for sodium carbonate have a higher M_w, they have similar R_g to that observed in the study of Storz et al. [45], implying a more compact structure.

Altogether, these data confirm that initial solubilization conditions influence the physico-chemical properties of the recovered precipitated EPS and that molecules recovered after sodium carbonate solubilization have a higher molecular weight and gyration radius in comparison with those extracted with sodium hydroxide. At this stage of the study, an adverse effect of the degradation/alteration of biopolymers on their recovery efficiency can be reasonably suspected since the final yield of EPS is higher for the extraction with sodium carbonate. The last aspect of this study has consisted of generating more or less denatured EPS during the solubilization step and studying the consequences on the molecular size and capacity of EPS for acidic precipitation.

3.2 Effect of heating temperature on EPS solubilization and precipitation

3.2.1 Comparison of extraction efficiency at different temperatures

Temperature was selected as a relevant parameter to modulate the degree of EPS denaturation. Moreover, this parameter is important for the economic feasibility of future fractionation/biorefinery processes of granular sludges leading to the valorization of biopolymers.

Considering the solubilization step performed at temperatures ranging from 20 °C to 80 °C, the amount of organic matter solubilized with sodium hydroxide remains systematically higher (Figure 4A). The heating has allowed to increase the amount of solubilized matter by a factor of 4 in sodium carbonate and 2.2 in sodium hydroxide. This better solubilization is explained by the fact that heating causes molecules to vibrate more quickly, which improves their solubility in the solvent [22]. In addition, the energy given during heating causes the cleavage of some low energy bonds which are an important part of the EPS matrix [46].

The inversion of solubilization and precipitation efficiency between the two solvents is also confirmed even at low temperatures where the yield for final EPS recovery is always higher in sodium carbonate compared to sodium hydroxide (Figure 4B). Moreover, it can be noticed that for both solvents, the temperature increase during the initial solubilization step has a similar influence on the precipitation efficiency (Figure S3 of the supplementary material).

Altogether, these data indicate that the temperature affects the solubilization of EPS from granules while it does not affect significantly their precipitation ability in acidic conditions. They also confirm that the choice of the solvent and associated pH values are relevant parameters since for all the tested temperatures, the yield for final EPS recovery is always higher in sodium carbonate compared to sodium hydroxide (Figure 4B).

3.2.2 Size distribution of alkaline extracted EPS

In order to better understand such low precipitation ability for sodium hydroxide extracted molecules, the molecular weight distribution of the S1 (solubilized) and S2 (precipitated) molecules were explored by size exclusion chromatography.

The EPS solubilized in S1 samples were divided into three classes of High, Medium and Low Molecular Weight molecules based on the integration of SEC peaks after various solubilization steps performed at temperatures ranging from 20 °C to 80 °C (SEC chromatograms provided in Figure S4 of the supplementary material). It can be noticed that the temperature increase has impacted the solubilization of all categories of polymers in both solvents (Figure 5A). However, the proportion of LMW polymers per g VS of initial granules remains much higher in sodium hydroxide compared to sodium carbonate, suggesting gradual degradation of the solubilized molecules.

After precipitation, the recovered polymers in S2 samples showed various molecular weight distributions depending on the temperature and the solvent used for solubilization (Figure 5B and Figure S4 of the supplementary material). At low temperatures (20 °C, 50 °C), LMW polymers are the major components of S2 sodium carbonate but at high temperatures (70 °C, 80 °C), the molecular weight distribution is in favor of high and medium sized polymers. The amounts of HMW reach their maximum at 80 °C and are in higher proportions than those observed with sodium hydroxide at similar temperatures.

The adverse effect of the degradation of biopolymers on their precipitation ability was further investigated studying the efficiency of precipitation for each category of polymers (Figure 6). After sodium hydroxide solubilization, acidic precipitation efficiency is correlated to the size of molecules since the percentages of molecules precipitated from S1 are always higher for HMW compared to MMW and LMW and this is true for all the tested temperatures (Figure 6A). This selectivity of precipitation towards high molecular weight polymers is less pronounced after sodium carbonate solubilization and varies with temperature: at 50 °C, LMW and MMW are precipitated with similar efficiency, at 70 °C, MMW are more efficiently precipitated than HMW and at 80 °C, the best precipitation is observed for HMW with a precipitation percentage of 34.7% (Figure 6B). In such sodium carbonate conditions, the precipitation ability of HMW and MMW molecules appears positively correlated with an increase in temperature between 50 °C and 80 °C while this is not the case for the lowest temperatures between 20 °C and 50 °C. This suggest that partial denaturation or alteration of the high and medium sized polymers by thermal treatments may be favorable for their precipitation. Interestingly, the positive influence of thermal action on the precipitation ability of HMW and MMW solubilized with sodium hydroxide is less obvious and this may be related to the fact that the polymers were previously drastically altered or denatured during the initial step.

This SEC analysis of solubilized and precipitated polymers after increasing extraction temperatures reinforces the hypothesis that we have greater signs of degradation in sodium hydroxide than in sodium carbonate. In addition, the loss of LMW molecules that represents a majority of the compounds extracted with sodium hydroxide, and the low precipitation ability of MMW (below 10%) explain the inversion of yields between the solubilization and acidic precipitation observed in Figure 1.

Since it was proven previously that acidic precipitated EPS have a gel forming capacity through ionic cross-linking with calcium ions [34], the S2 sodium hydroxide sample obtained at 70 °C and the S2 sodium carbonate at 80 °C were analyzed for their gelling capacity in the presence of divalent calcium.

Using similar amounts of acidic precipitated EPS, the gel formed with S2 sodium carbonate has a Young’s modulus of 10.40 ± 0.75 kPa slightly higher than that of sodium hydroxide which is equivalent to 8.82 ± 0.85 kPa. These values are very close and indicate that both alkaline solvents used at high temperature, allow the recovery of gel-forming EPS. The HMW/MMW and LMW distributions were respectively: 48.7% / 41.9% / 9.4% for sodium carbonate and 48.4% / 27.4% / 24.2% for sodium hydroxide. The equivalent percentage in HMW could explain their similar gelation behavior. This also indicates that despite some EPS degradation occurring during the solubilization step, the HMW molecules recovered by acidic precipitation show a gel-forming capacity, whether they have been extracted with sodium hydroxide or sodium carbonate. However, the study performed in this paper shows that temperature and pH conditions have to be carefully managed in order to find optimal conditions avoiding degradation and reduction of the polymer molecular weight since this property appears to be very impacting on the final EPS recovery.

4 Conclusion

- Alkaline chemicals coupled with heating provide efficient solubilization of aerobic granular sludges.
- Acidic precipitation of the solubilized EPS is influenced by their molecular weight distribution (HMW molecules are the most precipitable) but not by their biochemical composition.
- For the recovery of gel-forming EPS by acidic precipitation, care must be taken when using a strong base as sodium hydroxide coupled with a high temperature (above 50 °C) in regards to the degradation of the biopolymers.
- For valorization purposes, 0.2 M sodium carbonate used at 80 °C offers a good yield of acid precipitated EPS and good gel-forming capacities.

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Captions:

Figure 1: Effect of the initial solvent on the efficiency of EPS solubilization (A) and EPS precipitation (B). *Efficiency is expressed in mg of recovered VS in S1 (A) or mg of precipitated VS in P2 (B) and referred to the initial amount of VS in granules. Extractions and acidic precipitations were performed in triplicate.*

Figure 2: Biochemical composition of S1 samples containing EPS solubilized in alkaline solvents (A) and of S2 samples containing EPS recovered by acidic precipitation (B). *Neutral sugars and uronic acid-like molecules quantification were done using glucose and glucuronic acid as equivalents, respectively. Protein and humic acid-like molecules quantification were done using BSA and humic acid as equivalents respectively. DNA quantification was done using Lambda DNA as equivalent and values were multiplied by 20 for representation in the graphs. Analytical (3 assays) and biological (3 extractions) replicates were performed.*

Figure 3: Molar mass (A) and gyration radius (B) distribution of the S2 precipitated EPS after initial solubilization in sodium hydroxide or sodium carbonate. *The data was obtained using AsFlFFF coupled with a MALS detector. Light scattering signals (LS) were represented for each tested sample.*

Figure 4: Effect of temperature on the efficiency of EPS solubilization (A) and EPS precipitation (B). *The yields are expressed in mg of recovered VS in S1 (A) or mg of precipitated VS in P2 (B) and referred to the initial amount of VS in granules. Extractions and acidic precipitations were performed in triplicate.*

Figure 5: Size distribution of the S1 solubilized EPS (A) and of the S2 precipitated EPS (B) in regard to the temperature used for the initial alkaline solubilization. *The analysis was done using a Superdex 200 SEC column. The molecules were detected at 280 nm (AU: absorbance unit) and peak areas were integrated (AU×mL) and referred to the initial amount of VS in granules.*

Figure 6: Percentage of precipitated polymers by molecular weight category using SEC for sodium hydroxide (A) and sodium carbonate (B). *Molecules were detected at 280 nm. For each molecular weight category, the percentage of precipitated polymers was evaluated by comparison of the peak areas in S1 and S2.*

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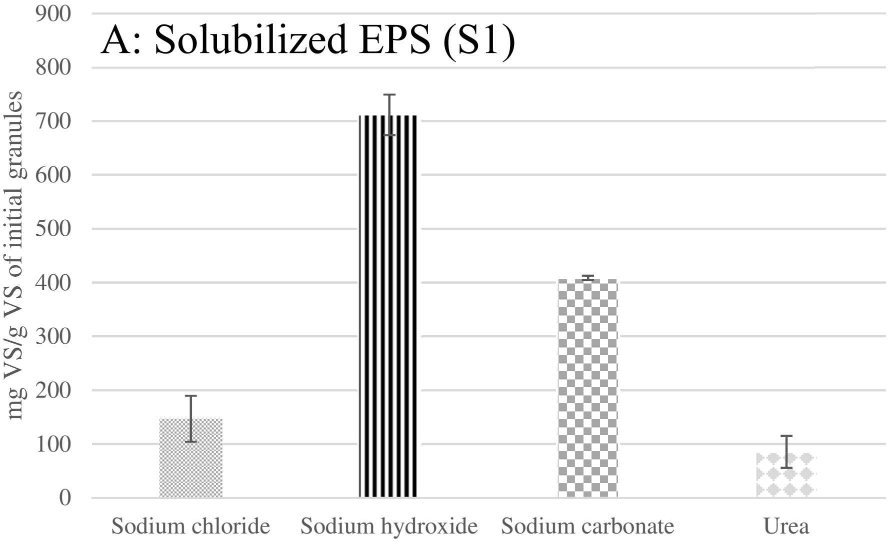
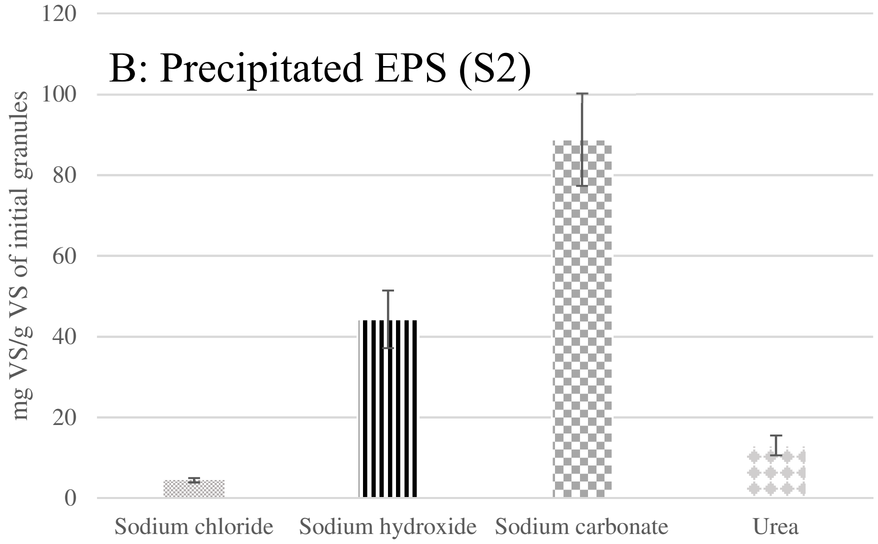
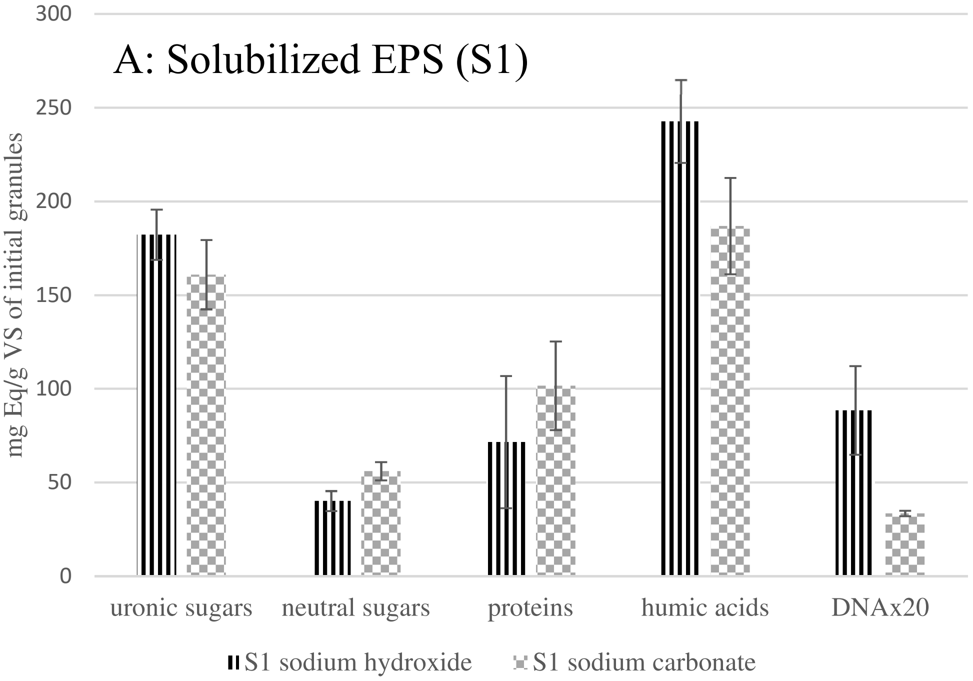
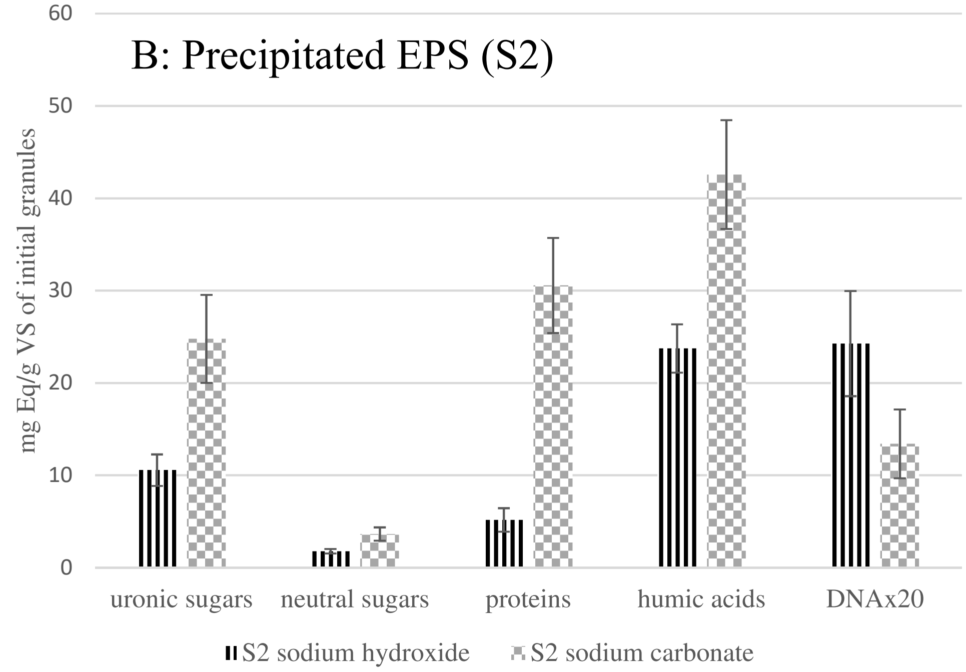
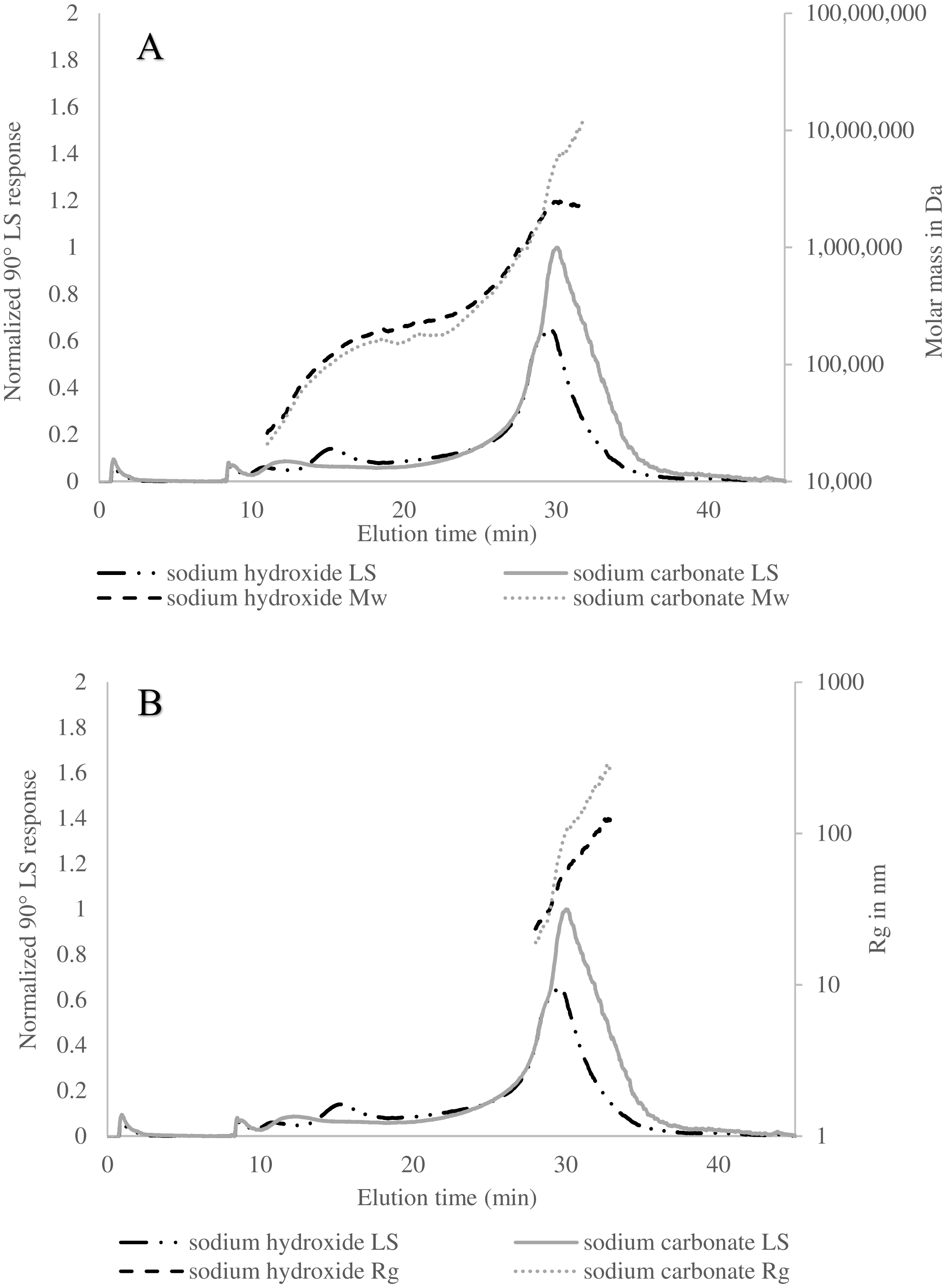
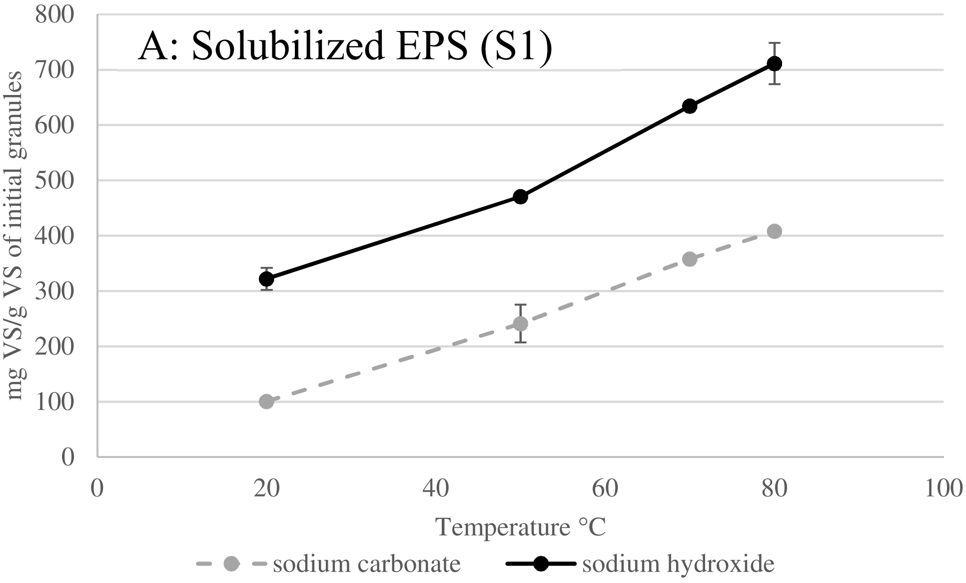

A: Solubilized EPS (S1)
mg VS/g VS of initial granules
900
800
700
600
500
400
300
200
100
0
Sodium chloride
Sodium hydroxide
Sodium carbonate
Urea

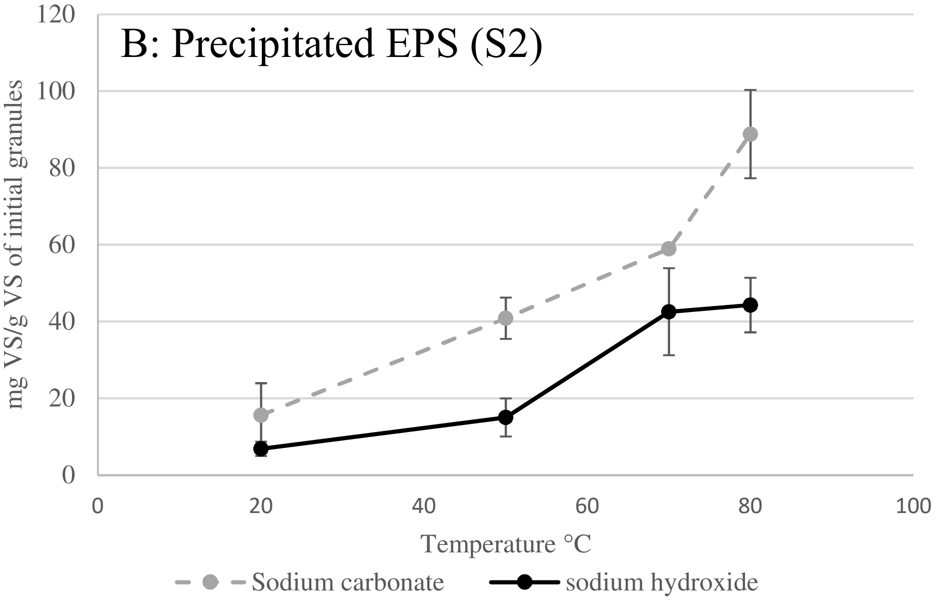

B: Precipitated EPS (S2)
mg VS/g VS of initial granules
120
100
80
60
40
20
0
Sodium chloride
Sodium hydroxide
Sodium carbonate
Urea

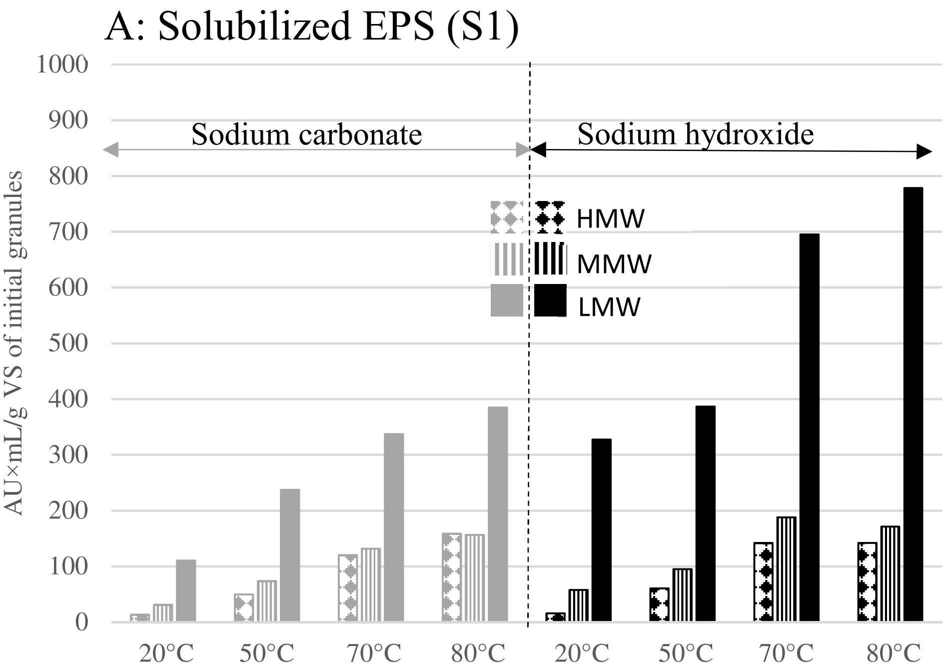

A: Solubilized EPS (S1)


B: Precipitated EPS (S2)


A
Normalized 90° LS response
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Molar mass in Da
100,000,000
10,000,000
1,000,000
100,000
10,000
0
10
20
30
40
Elution time (min)
sodium hydroxide LS
sodium carbonate LS
sodium hydroxide Mw
sodium carbonate Mw
B
Normalized 90° LS response
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Rg in nm
1000
100
10
1
0
10
20
30
40
Elution time (min)
sodium hydroxide LS
sodium carbonate LS
sodium hydroxide Rg
sodium carbonate Rg


A: Solubilized EPS (S1)
mg VS/g VS of initial granules
0
100
200
300
400
500
600
700
800
0
20
40
60
80
100
Temperature °C
sodium carbonate
sodium hydroxide


B: Precipitated EPS (S2)
mg VS/g VS of initial granules
0
20
40
60
80
100
120
0
20
40
60
80
100
Temperature °C
Sodium carbonate
sodium hydroxide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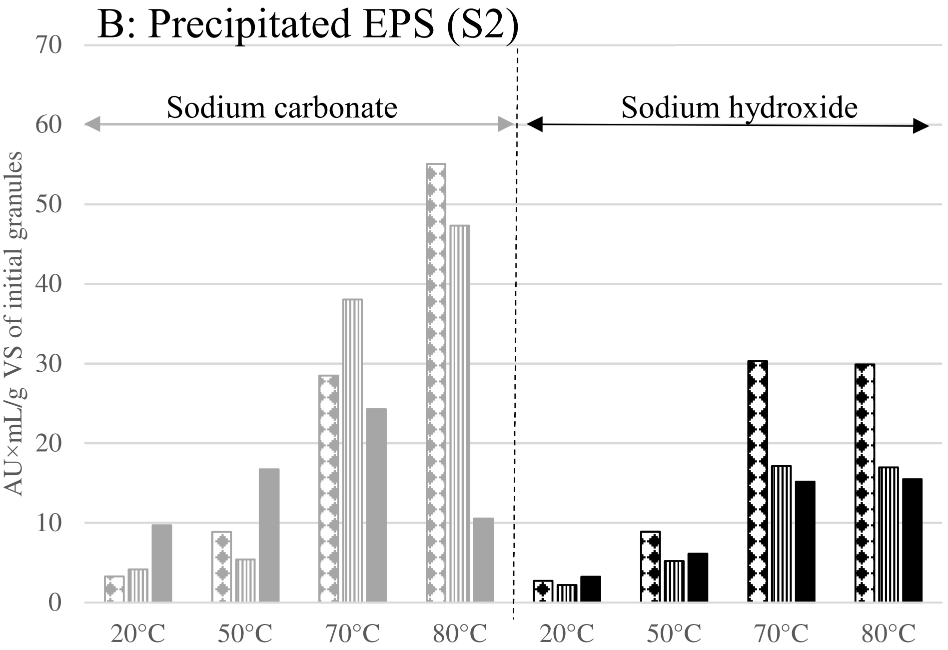
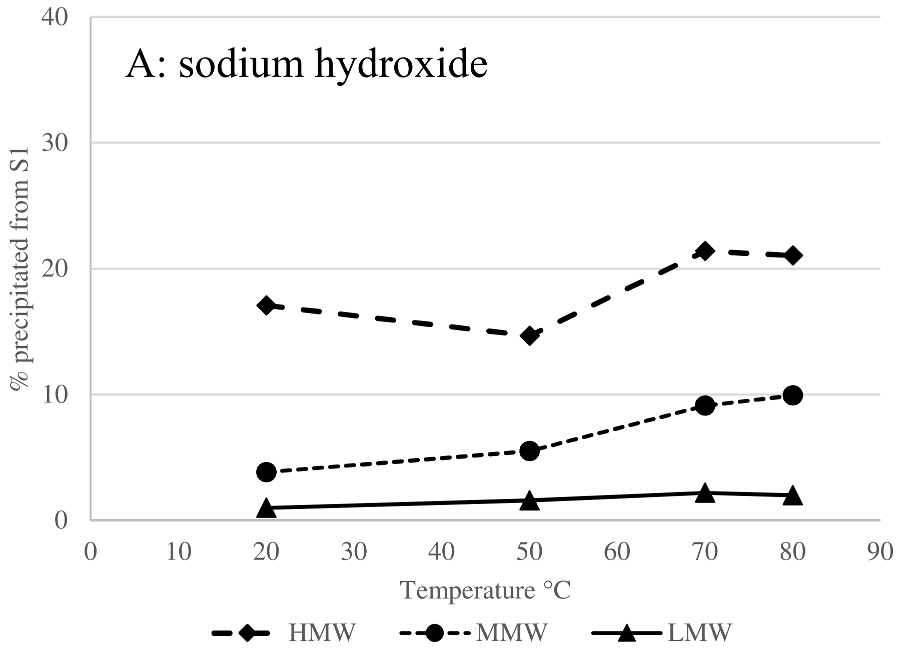
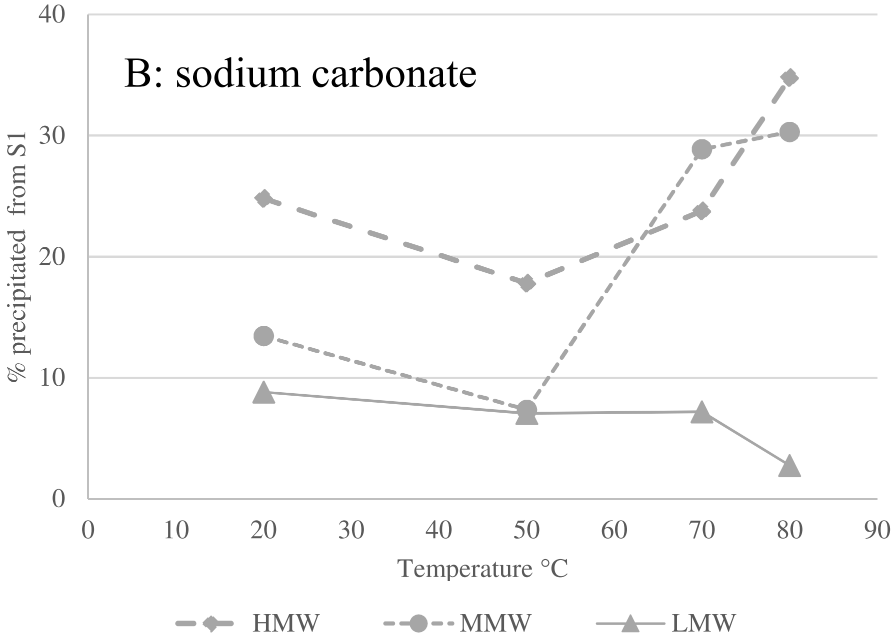

B: Precipitated EPS (S2)


A: sodium hydroxide
% precipitated from S1
0
10
20
30
40
0 10 20 30 40 50 60 70 80 90
Temperature °C
HMW
MMW
LMW


B: sodium carbonate
% precipitated from S1
0
10
20
30
40
0 10 20 30 40 50 60 70 80 90
Temperature °C
HMW
MMW
LMW