Evaluation of EPS extraction protocols from anaerobic sludge for gel-based proteomic studies

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ABSTRACT

Despite the importance of anaerobic sludge extracellular polymeric substances (EPSs), their characterization is limited to information regarding their chemical classes and molecular size. This work explores the possibility of using proteomic techniques to study the proteins present in this matrix. Thus, this paper compares eight EPS extraction methods regarding extraction yield, protein/carbohydrate ratio, size distribution profile and suitability to sodium dodecyl sulfate–polyacrylamide gel electrophoresis analyses. Despite the differences found in quantification and size exclusion chromatography assays, the band profile found for all methods was very similar. Considering the band pattern, extraction time and background level, heating method followed by ammonium sulfate precipitation proved to be the most appropriate method for gel-based analyses of anaerobic sludge EPS proteins.

Key words | anaerobic digestion, EPS proteins, EPS extraction methods, proteomics

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INTRODUCTION

The success of anaerobic wastewater treatment depends, among other factors, on the metabolites exchange among the micro-organisms. To achieve this, the microbial community produces biomacromolecules simultaneously to its growth, which are responsible for maintaining the proximity and physical union of cells. These molecules, known as extracellular polymeric substances (EPSs), constitute a matrix comprising a complex mixture of biopolymers (Zhou *et al.* 2012). This matrix, besides providing physical union and cooperation among different species of micro-organisms that coexist in the bioreactors, performs the functions of adhesion to surfaces, accumulation of water and nutrients, aggregation of microbial cells in granules or biofilms, adsorption of exogenous organic compounds and is an important factor in sludge digestibility (Laspidou & Rittmann 2002; Flemming & Wingender 2010).

While the EPS functions are well established, its characterization is limited, in most works, to the definition and quantification of the main chemical classes of its constituents. Among them, there are lipids, nucleic acids, humic acids, inorganic matter, humic substances and – as main polymers – carbohydrates and proteins, provided that the protein/carbohydrate ratio (P/C) of EPS is usually higher than one (Park *et al.* 2008; D'Abzac *et al.* 2010).

Several studies have focused on the analysis of the main proteins that constitute the complex mixture of EPSs, especially regarding its participation in the processes of biomass flocculation and membrane fouling, conditions in which proteins represent a large part of EPSs (Aquino *et al.* 2006; Park *et al.* 2008; Huang *et al.* 2012). Up to now, most papers found in the literature classify EPS based on size exclusion chromatography (SEC) and infrared and excitation/emission spectroscopies techniques. The study of molecular size can be an indicator of a compounds solubility and biodegradability, while the other cited techniques allow one to infer the main EPS chemical groups (Tian *et al.* 2011). However, in order to name these molecules, the use of techniques which are able to identify them more accurately is necessary.

The presence of proteins among EPSs raises the possibility of using proteomic techniques for identifying them. Similar to what occurs for other biological systems, information derived from proteomic analyses may identify the main extracellular matrix proteins as well as their producers, micro-organisms (Siggins *et al.* 2012). However, information about EPS proteomes on natural communities is still incipient, and to our knowledge there are no studies about EPS proteomes of anaerobic sludge.

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One of the main reasons for the scarcity of studies in this area is the difficulty in obtaining an extraction method that combines high efficiency and absence of cellular lysis. Moreover, different extraction methods could release distinct EPS fractions, including different proteins, as already demonstrated for activated sludge samples (Park *et al.* 2008).

Despite the development of 'gel free' proteomic techniques (called second generation proteomics), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the first step of classical proteomics approaches, is still an invaluable tool to access sample integrity and differences among protein extraction methods. Furthermore, the previous SDS-PAGE separation reduces the level of sample interference and serves as a pretreatment step before protein identification by liquid chromatography–tandem mass spectrometry. Thus, in this work, eight methods for EPS extraction from anaerobic sludge were assessed regarding the following parameters: (i) amount of extracted polymers (carbohydrates and proteins); (ii) molecular weight distribution profile of the extracts; and (iii) suitability to SDS-PAGE analysis.

MATERIAL AND METHODS

Sample source

The anaerobic mixed culture was collected from an anaerobic granular sludge original from a pilot scale upflow anaerobic sludge blanket reactor treating domestic wastewater. The culture was incubated in the laboratory for 1 year in a sealed flask in glucose medium. Aliquots of this culture were used for this study.

EPS extraction

EPS extraction was carried out using eight different methods. Previously to each extraction, 100 mL (2.2 g of volatile suspended solids (VSS)) of sludge were centrifuged (5,800 ***g*** for 25 min) and, with the exception of cation-exchange resin (CER) and sodium sulfide extractions, were vortexed in 100 mL of 0.05% (m/v) NaCl solution. After each extraction, samples were centrifuged and supernatant was collected.

Centrifugation (D'Abzac *et al.* 2010) – centrifugation was chosen as a control method for cell lysis, and carried out as described above. Only the second centrifugation supernatant was collected.

Heating (Li & Yang 2007) – 0.05% NaCl solution, previously heated at 70 °C, were added to the sludge. Samples were vortexed for 1 min and kept in a water bath at 70 °C for 30 min.

Formaldehyde (D´Abzac *et al.* 2010) – 600 μL of 37% formaldehyde was added to sludge. Samples were incubated at 4 °C for 1 h, with periodic manual stirring every 30 min.

EDTA (D´Abzac *et al.* 2010) – 100 mL of 2% (m/v) pH 7.5 EDTA solution were added to resuspended sludge. The flasks were incubated at 4 °C for 3 h, with periodic manual stirring every 60 minutes.

Sodium hydroxide (Park *et al.* 2008) – the pH of resuspended sludge was adjusted to 10 with a 1 mol/L NaOH solution. Flasks were sealed, purged with gaseous nitrogen for 15 min and stirred in a magnetic stirrer, for 1 h on ice.

Formaldehyde plus heating – formaldehyde extraction was used first, followed by heating extraction.

Formaldehyde plus sodium hydroxide – formaldehyde extraction was used first, followed by sodium hydroxide extraction.

CER (Frølund *et al.* 1996) – CER (Dowex® Marathon® C, Na^+-Form, Sigma-Aldrich) was washed in phosphate buffered saline solution (PBS – 2 mmol/L KH_2PO_4, 6 mmol/L Na_2PO_4 and 10 mmol/L NaCl, pH 7.8) by stirring in a magnetic stirrer for 1 h, on ice and in the dark. Washed resin (65 g/g VSS) was added to sludge resuspended in 100 mL of PBS. Extraction flasks were stirred for 1 h, on ice and in the dark.

Sodium sulfide (Nielsen & Keiding 1998) – sludge was resuspended in a 6 mmol/L Na2S (pH 7.5) solution. Extraction flasks were sealed, purged with gaseous nitrogen for 15 min and extraction mixture was stirred, with a magnetic stirrer, for 6 h, on ice.

Protein precipitation

An amount of 18.04 g of ammonium sulfate (Sigma) was added to 30 mL of supernatant (90% ammonium sulfate saturation) and the tubes were incubated on ice for 16 h. The precipitation was concluded by centrifugation at 20,000 ***g*** for 30 min (Sorvall 5C centrifuge). To remove the salt, the samples were resuspended and dialyzed against deionized water for 72 h at 4 °C, in 3.5 kDa membranes (Thermo). Samples were concentrated by freeze-drying (Liotop L101, Liobras).

EPS quantification

Carbohydrates were measured by the Dubois method (D'Abzac *et al.* 2010) and proteins were quantified by the

Lowry derived Frølund method (Frolund *et al.* 1996), which considers the interference of humic substances in protein quantification. Measurements were made in triplicate and polymer concentration was normalized by the amount of biomass (VSS) used and the results expressed as mg EPS/g VSS.

SEC of EPS extracts

SEC was carried out in a HPLC system (Shimadzu 20 A) equipped with a Biosep SEC-S 2000 (300 × 7.8 mm) column and an UV/VIS SPD-20A (Shimadzu) detector set at the wavelength of 254 nm. One-hundred millilitres of each extraction were injected and separation was carried out for 25 min, at 25 °C, using as mobile phase a 0.1 mol/L (pH 6.8) phosphate buffer at 1.0 mL/min isocratic flow. Uridine (0.25 kDa), ovalbumin (44 kDa), alcohol dehydrogenase (150 kDa) and thyroglobulin (669 kDa) were used as standards.

SDS-PAGE electrophoresis

SDS-PAGE was performed according to the Laemmli method. Ten millilitres of precipitated and freeze-dried extractions were mixed with 2× SDS-PAGE sample buffer (Tris–HCl 125 mmol/L, SDS 4% (m/v), glycerol 20%, DTT 200 mmol/L), heated for 5 min at 95 °C and applied in a polyacrylamide gel (4% stacking gel and 12% separation gel). Electrophoresis was carried out at 20 mA for approximately 1 hour, and gel was stained with Coomassie R-250.

Statistical analysis

The amount of EPS (proteins or carbohydrates) obtained by each method was compared using one-way analysis of variance (ANOVA) and Tukey-test. Mean values were considered to be significantly different for $\alpha < 0.05$.

RESULTS AND DISCUSSION

Evaluation of EPS extractions yields

As can be seen in Figure 1, each extraction method solubilized distinctive quantities of polymers. Despite some authors taking into consideration only the quantitative variations when choosing an extraction method (Frølund *et al.* 1996; Domínguez *et al.* 2010), it is important to highlight that the choice of a unique method could not provide representative information of the whole EPS polymer matrix (Park & Novak 2007).

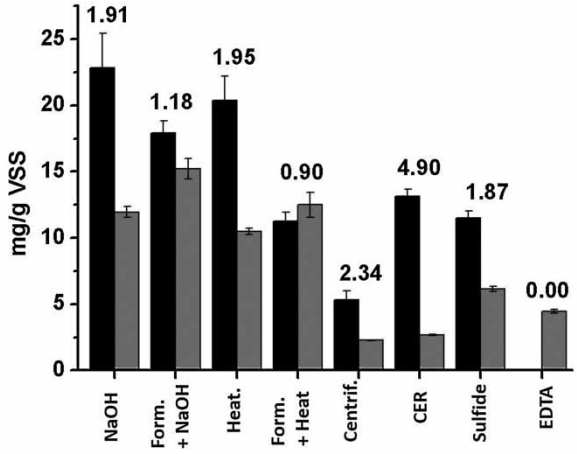

Figure 1 | Concentration of proteins (black bars) and carbohydrates (gray bars) for each extraction method. The numbers above the bars represent P/C ratio. For proteins, all extractions presented significant differences among them, except for NaOH vs. Heating, Formaldehyde plus NaOH vs. Heating, Formaldehyde plus Heating vs. CER, Formaldehyde plus Heating vs. Sulfide and CER vs. Sulfide. For carbohydrates, extractions presented significant differences among them, except for NaOH vs. Formaldehyde plus Heating and Centrifugation vs. CER.

In this study, the methods that solubilized the largest amount of total polymers were based on sodium hydroxide and heating methods. The pH increase caused by the addition of NaOH, besides deprotonating carboxylic groups and increasing the repulsion between cells and EPS, increases the solubility of aluminum in water (Park & Novak 2007; Sheng *et al.* 2010). As was shown by Park & Novak (2007), this metal helps to structure the extracellular polymeric matrix. Therefore, the combination of these two mechanisms might explain the high efficiency displayed by the alkaline method.

Some authors show that the addition of formaldehyde to anaerobic sludge can solubilize sludge exopolymers (Zhang *et al.* 1999). Interestingly, the previous treatment of samples with formaldehyde reduced the total yield of NaOH extraction, decreasing the protein content and increasing the amount of carbohydrates. This behavior was also observed for heating extraction. Formaldehyde, due to its fixation capacity, may have caused a protective action over the cellular membrane of micro-organisms, thereby reducing the lysis caused by the extraction procedure (Liu & Fang 2002). Thus, it is possible that complexation with formaldehyde led part of the polymers to be less susceptible to the action of other extraction agents (Fox *et al.* 1985).

Even though the use of formaldehyde is common for EPS extraction protocols, it is important to point out that this compound can cause cross-linking with biopolymers

(Fox *et al.* 1985). This must be taken into account in mass spectrometry analysis of proteins, because it may alter the mass of some peptides and, thus, preclude or invalidate protein identification. Regarding the low concentration used in our work, the occurrence of cross-linking cannot be discharged, since there are no reports in the literature about the minimum formaldehyde concentration that will not cause this phenomenon.

Two of the tested methods, CER and EDTA, are based on the destabilization of EPS through the removal of matrix-stabilizer cations. While the CER chelates preferably $Mg^{2+}$and Ca^{2+} ions, EDTA may also chelate Fe^{2+} or Fe^{3+} ions besides Mg^{2+} and Ca^{2+} (Park & Novak 2007). Despite this, the CER showed better yields, especially regarding the amount of proteins released. Possibly, this is due to the fact that the resin substitutes the divalent cations for Na^{+} ions. High concentrations of Na^{+} ions are related to the reduction of total EPS amount in flocs, with a consequent increase of soluble polymers in membrane reactors (Arabi & Nakhla 2009). The interference of EDTA in protein quantification by Lowry reaction cannot be discharged either, since this chelating agent can efficiently bind with the copper ions and decrease its affinity with proteins, thereby decreasing the intensity of color development, on which the measurement is based.

Another method that relies on the removal of stabilizing cations employs sodium sulfide (Na_2S). According to Nielsen & Keiding (1998), in assays with activated sludge, the addition of sodium sulfide removed both Fe^{2+} and Fe^{3+} ions, through the formation of FeS, which resulted in floc disintegration. In our work, the addition of Na_2S solubilized approximately the same amount of polymers obtained from CER extraction (Figure 1). According to Park & Novak (2007), the polymers extracted by each one of these two methods can be considered different fractions of EPS, since they are solubilized due to the removal of different matrix-stabilizers cations. Therefore, considering the sum of these fractions, the data depicted in Figure 1 shows that a large part of the anaerobic granule EPS was stabilized by the interaction with divalent and trivalent cations.

Among the physical extraction methods, heating was more efficient than centrifugation. This difference was expected, since polymer release due to the centrifugal force is small, making this technique less aggressive (Adav & Lee 2011). The enhancement of molecular movement generated by the temperature increase (Sheng *et al.* 2010), conversely, released a greater amount of exopolymers, compared to the most efficient chemical method (NaOH).

In this work, the protein/carbohydrate ratio was, generally, except for the formaldehyde plus heating and EDTA methods, higher than 1.0. The prevalence of proteins and peptides over other EPS polymers (e.g. carbohydrates) was already demonstrated in other papers with mixed cultures, contrary to what was observed for pure cultures (Flemming & Wingender 2010).

EPS molecular weight profile

After quantification, the molecular weight distribution of the extracted compounds was analyzed through SEC. In this type of separation, molecules size can be estimated by their elution time. The smaller the molecule, the larger the volume of the column to which the molecule has access and, consequently, the longer is its retention time.

The chromatograms of extracted EPS are shown in Figure 2. As can be seen, the extracted compounds may be grouped into two main regions: larger than 669 kDa and smaller than 44 kDa. The elution time of the latter is very close to that of uridine standard (0.25 kDa). This bimodal distribution has already been demonstrated for activated sludge EPS extracted by different methods (Adav & Lee 2011). According to previous works (D´Abzac *et al.* 2010), the data presented here suggest that the EPSs are formed both by complexes of high molecular weight and by smaller compounds and/or degradation products, independently of the interactions which are broken in every method and the type of polymers released.

For all methods, except for formaldehyde plus NaOH and centrifugation, there were relatively intense peaks in the chromatogram region, which corresponded to the smaller compounds (close to the retention time of 10 minutes). Interestingly, a high intensity peak in this region was obtained even for EDTA extraction, the method which rendered lower efficiency. When confronting these data with the protein quantification results for EDTA extraction, it can be supposed that humic acids are responsible for the occurrence of this peak, since they show an average size of 10 kDa and absorb UV light at the wavelength used in the assay (Peuravuori & Pihlaja 1997). Therefore, due to high concentration of humic acids and the correction performed with the Frølund method, they might have underestimated the concentration of proteins in this extract. Conversely, complexes such as EDTA/Fe^{3+} may have contributed to the intensification of such peaks, since they show maximum absorption in the wavelength of 258 nm (Bhattacharya & Kundu 1971). Also noted is the occurrence of an even more intense peak, with retention time close to

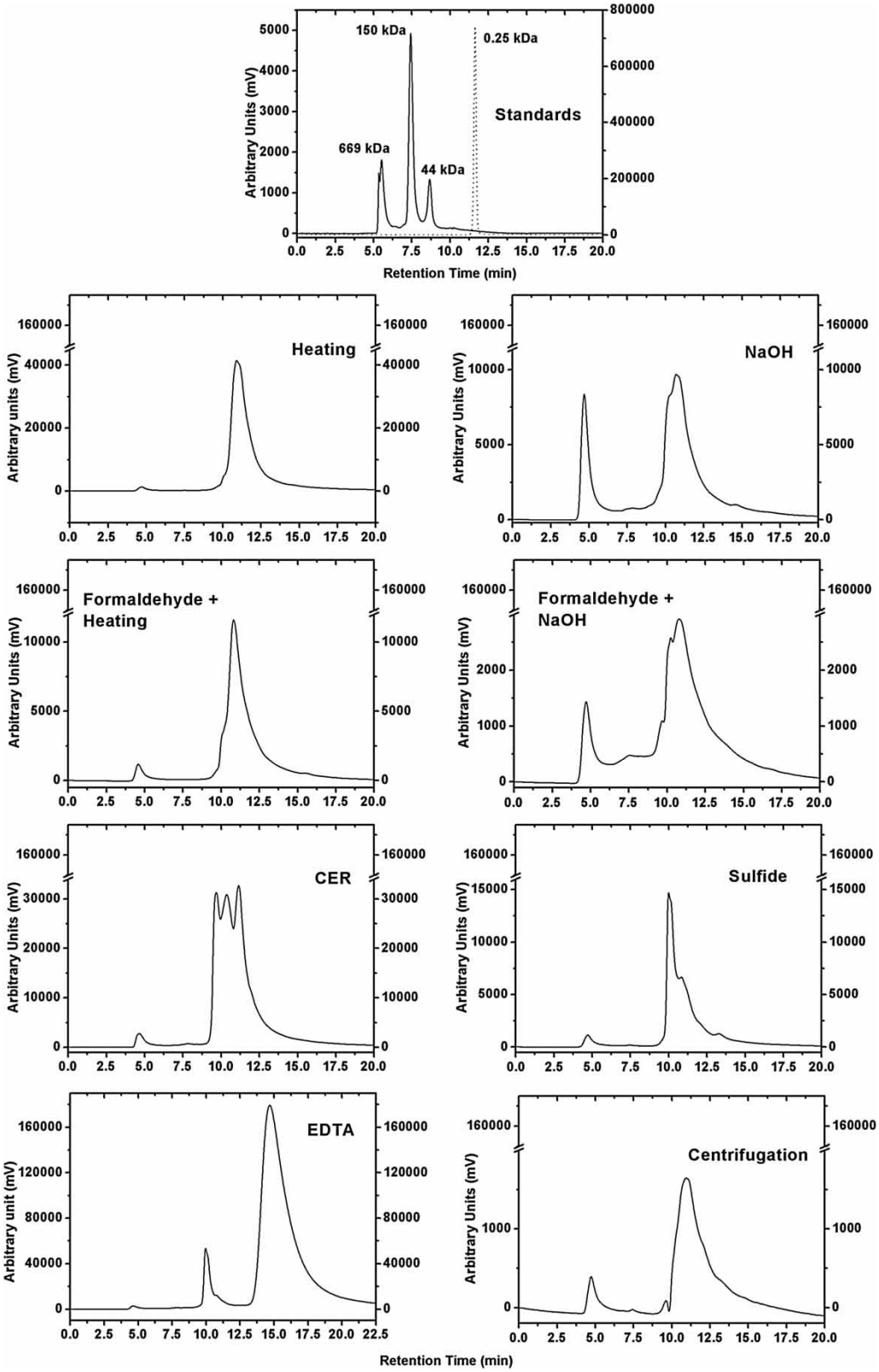

Figure 2 | EPS and standard chromatograms (669 kDa- thyroglobulin; 150 kDa- alcohol dehydrogenase; 44 kDa- ovalbumin; 0.25 kDa- uridine).

15 min, only for EDTA extraction. As EDTA-metallic ion complexes can interact with small molecules (like amino acids) the occurrence of this peak suggests the release of a large amount of monomers when using this method (Arena *et al.* 1985).

For compounds larger than 669 kDa, the NaOH extraction method showed the most intense peaks. According to Garcia Becerra *et al.* (2010), these peaks correspond to assembled structures of large molecular weight.

Similar to what occurred in the quantification assays, the addition of formaldehyde before the NaOH and heating extraction reduced the intensity of SEC peaks. Furthermore, the peak that corresponds to low molecular weight compounds ($t_r = 11$ min) for heating extraction, was the one with the most expressive decrease. This fact corroborates the hypothesis that the complexes formed between these polymers and formaldehyde are more difficult to extract, which decreases the performance of such extractions, especially for smaller compounds.

Profile of extracted EPS proteins

To analyze the protein profile of the EPS extracts and verify the method's suitability to classical proteomics approaches, the extracts were applied to SDS-PAGE gels after sample concentration. The freeze-drying technique was chosen as the first method to reduce the extract volume. However, the procedure turned out to be inefficient to concentrate EPS extracted proteins, since no bands were observed in an SDS-PAGE gel with these samples (not shown). Considering freeze-drying characteristics, protein degradation would not be likely to explain such a result. A more plausible reason would be that, at the end of freeze-drying, the sample's volume might not have been reduced enough due to the great amount of organic and inorganic matter present in the extracts.

Therefore, the proteins were concentrated through ammonium sulfate precipitation. According to Park *et al.* (2008), while working with the extraction of activated sludge EPS, among other precipitation methods – including acetone or trichloroacetic acid – the use of $(NH_4)_2SO_4$ yielded the best results for protein purification. To our samples, this procedure, followed by dialysis and freeze-drying, allowed the samples to be resuspended in much smaller volumes and bands were visualized for all extracts (Figure 3).

Notwithstanding the variations in extraction efficiency and the chromatogram profiles presented previously, there are no visual differences in the amount of proteins among

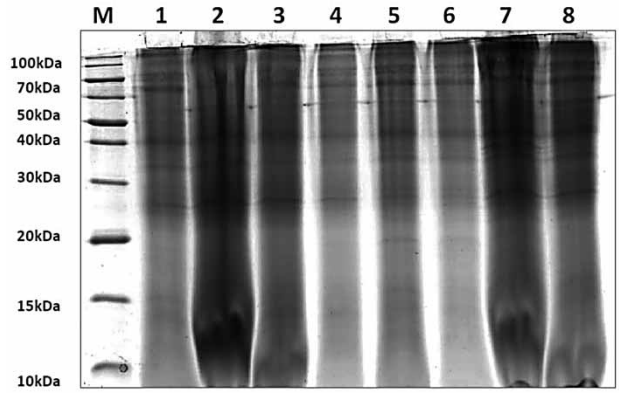

Figure 3 | SDS-PAGE separation of ammonium sulfate precipitated EPS extracts. M- molecular weight marker; 1- centrifugation; 2- CER; 3- NaOH; 4- formaldehyde + NaOH; 5- heating; 6- formaldehyde + heating; 7- EDTA; 8- Na_2S.

the methods. Moreover, many bands are common to all extracts, including the centrifugation method used as control, which, according to other studies, would not cause cell lysis (Sheng *et al.* 2010). Therefore, the proteins visualized in Figure 3 can be considered, indeed, constituents of the extracellular matrix. With these remarks, it is supposed that even for more severe and efficient extraction methods, such as NaOH or heating extraction, the damage caused to anaerobic granular sludge cells was minimal. Conversely, our data show that, for studies on the EPS proteome of anaerobic sludge, less aggressive methods can be used. This would avoid possible modifications in amino acid side chains caused by chemical agents, such as the ones that occur when formaldehyde is used (Fox *et al.* 1985). Such modifications, due to alteration of molecular weight of amino acid residues, may hinder the posterior protein identification by mass spectrometry.

Mostly, the precipitated proteins display high molecular weight and are above 20 kDa, many of them exhibit molecular weight of approximately 100 kDa. Curiously, these results differ from those found by SEC, in which high intensity peaks are not noted in this region. This discrepancy can be explained by the fact that in SEC analyses, the polymers are in a conformation which is closer to their natural state. Conversely, the treatment of samples (addition of a reducing agent (dithiothreitol) and a detergent (sodium dodecyl sulfate), combined with heating), which precedes its application on SDS-PAGE gels causes breaking of interactions and complete protein denaturation. This difference was also shown by Park *et al.* (2008) while assessing the size of EPS molecules through ultrafiltration fractioning. These authors found a high percentage of proteins larger than 500 kDa and smaller than 3 kDa, while the analyses

by SDS-PAGE revealed the occurrence of proteins with intermediate sizes.

The absence of low molecular weight proteins can be explained as a bias of ammonium sulfate precipitation, since larger or more hydrophobic proteins are readily denatured with the salt addition, and they are more likely to interact and become insoluble. Another explanation would be the removal of humic substances formed after the salt addition. As such substances interact with SDS-PAGE dyes, such a phase was excluded from the precipitation with the intention of diminishing the sample background.

According to Bastida *et al.* (2009), among the difficulties found in the study of natural community proteomes, the main one would be the development of protocols that conciliate the elimination of interference with the obtaining of representative samples of the extracellular proteome. As mentioned before there are small differences in the EPS proteins profile extracted through the tested protocols, in such a way that the main difference between the methods lies in the SDS-PAGE background level for each method. Thus, it may be suggested that extraction by heating is the most appropriate for gel-based proteomic analyses of anaerobic sludge EPS, due to the low level of background displayed.

CONCLUSION

The methods tested in this paper displayed great differences regarding the amount of extracted polymers, and the most efficient were NaOH and heating extraction. Such quantitative differences were also noted in SEC. However, the molecular weight distribution profile of the extracts was highly similar, with the exception of the occurrence of low molecular weight EDTA extracted compounds. Surprisingly, these differences were not reflected in the SDS-PAGE analysis, since a set of similar bands was found for all extracts, even for the methods with low protein extraction efficiency (e.g. EDTA). Furthermore, the P/C ratio found through quantitative methods could not be used to infer anything about the protein purity of the extracts, as was seen for CER extraction, which presented the higher P/C ratio (4.89) and, even so, high background in SDS-PAGE. This work suggests the application of heating extraction, followed by ammonium sulfate precipitation for the extraction of EPS proteins from anaerobic sludge aiming at gel-based proteomic analyses, due to low background and shorter time of extraction/precipitation in comparison to the other methods tested in this study.

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