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The use of a new mobile phase, with no multivalent cation binding properties, to differentiate extracellular polymeric substances (EPS), by size exclusion chromatography (SEC), from biomass used for wastewater treatment

Maud Villain[a,b], Stéphane Simon[a], Isabelle Bourven[a], Gilles Guibaud[a,*]

[a] Groupement de Recherche Eau Sol Environnement (EA 4330), Université de Limoges, Faculté des Sciences et Techniques, 123 Av. Albert Thomas, 87 060 Limoges, France
[b] Laboratoire en Procédés Propres et Environnement (LPPE-CNRS-UMR6181), Université Paul Cézanne d'Aix Marseille, Europôle de l'Arbois, BP 80, Bat. Laennec, Hall C, 13 545 Aix en Provence Cedex 04, France

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ABSTRACT

The fingerprints of extracellular polymeric substances (EPS) extracted from different types of biomass used for wastewater treatment (*i.e.*, activated sludge, filamentous activated sludge, anaerobic granular sludge, anaerobic flocculated sludge) were studied by size exclusion chromatography (SEC) with Amersham Biosciences Superdex 200 10/300 GL column with a theoretical resolving range of 10–600 kDa. A new mobile phase, which does not display binding properties for multivalent cations, was previously optimized. This mobile phase contained 75 mM Hepes buffer at pH 7 with 15% acetonitrile (v/v) and was selected to minimize ionic and hydrophobic interactions between the molecules that make up the EPS and the column packing.

When EPS extracted from similar sludges is analyzed using different mobile phases, the number of chromatographic peaks obtained is quite similar, and differences are mainly observed in the relative absorbance of the chromatographic peaks. However, very different chromatograms (number and relative absorbance of chromatographic peaks) are obtained for EPS extracted from different types of sludges. Furthermore, when dysfunctions, such as filamentous bulking in the activated sludge, occur in a bioreactor, they also induce strong variations in chromatographic profiles.

1. Introduction

Extracellular polymeric substances (EPS) represent an important part of bacterial aggregates (*e.g.*, biofilm, activated sludge, granular sludge) because the bacteria are dispersed within the EPS matrix. EPS have two origins: a bacterial origin from secretions or products of cell lysis and an abiotic origin from the adsorption of molecules found in the effluent. EPS are composed primarily of proteins, polysaccharides and humic-like substances but also, to a lesser extent, contain uronic acids, lipids and nucleic acids [1]. The EPS composition varies with the origin of the wastewater [2] and with the bacterial community [3,4]. EPS play structural and protector roles within microbial aggregates. EPS also affect the porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability of biofilms [3–5].

EPS have to be extracted to study their composition and their properties. There is no standard extraction method for EPS; however physical and chemical protocols are available in the literature [6,7]. These techniques are rarely criticized, but some of them can modify the organic composition and the physico-chemical properties of the EPS [8,9]. Common colorimetric methods to determine the biochemical composition of the EPS are insufficient in demonstrating the effect of the extraction method or in differentiating EPS according to their origin (*i.e.*, type of biofilm, process used, treated effluent). Moreover, colorimetric methods are unreliable in determining EPS biochemical composition [10]. Therefore, other approaches, such as size exclusion chromatography (SEC) [11] or asymmetrical flow field-flow fractionation [12], are required to better characterize EPS, so that a chromatogram, or "fingerprint" of the EPS can be obtained. Several studies have demonstrated that the fingerprint of an EPS corresponds to the sludge from which the EPS was extracted [11,13–15]. Comte et al. [14] and Simon et al. [15] showed that the EPS fingerprint from activated sludge or anaerobic granular sludge, respectively, can be affected by the extraction method used. Garnier et al. [13] demonstrated that the EPS fingerprint is drastically modified (increase of EPS with smaller molecular sizes) during sludge settling crises.

* Corresponding author at: GRESE, Université de Limoges, Faculté des Sciences et Techniques, 123 Av. Albert Thomas, 87 060 Limoges, France. Tel.: +33 5 55 45 74 28; fax: +33 5 55 45 72 03.
E-mail address: gilles.guibaud@unilim.fr (G. Guibaud).

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SEC is often used to obtain polymer size distributions and to determine molecular weight (MW) [16,17]. For EPS from activated sludge, Görner et al. [18] showed that the molecular weights of proteins vary over a wide range, from 45 to 670 kDa. However, polysaccharides have molecular weights of less than 1 kDa. Nevertheless, several studies highlight the presence of ionic and/or hydrophobic interactions between some EPS molecules and the column packing [11,14,15,18]. The presence of such interactions induces errors in determining the molecular weight of these molecules using synthetic polymers. Görner et al. [18] and Garnier et al. [13] preferred using proteins or polysaccharides, rather than synthetic polymers, as a molecular weight standard. These authors assumed that protein and polysaccharide standards interacted with column packing in a similar way to EPS molecules, which minimized errors in determining the molecular weight of the EPS molecules.

Mobile phases used in SEC often contain phosphate ions as a pH buffer [13–15,18]. However, phosphate ions are strong ligands for multivalent cations, which may affect the structure of EPS molecules. Therefore, the first aim of this work was to optimize a new mobile phase with a pH buffer that does not display binding properties for multivalent cations. Secondly, SEC was used with this new mobile phase to determine the fingerprints of EPS extracted from different types of biomass used for wastewater treatment (*i.e.*, activated sludge, filamentous activated sludge, anaerobic granular sludge, anaerobic flocculated sludge).

2. Methods

2.1. EPS from activated, anaerobic granular and anaerobic sludges

The EPS used in this study have already been characterized in other works [8,19,20]. Table 1 summarizes the origins of the sludges used to extract EPS, as well as the main characteristics of the EPS.

Five activated sludges were selected. Three have a floc shape (AS-As, AS-Ls and AS-Aa) and come from different wastewater treatment plants (WWTP) with functioning aeration tanks. The sludge AS-Ba was sampled from a 20,000 inhabitant equivalent WWTP with a dysfunctioning aeration tank. The influent contained an equal amount of domestic and industrial (dairy and paper mill) wastewater. The main observable characteristic of the AS-Ba sludge was a filamentous bulking with small filamentous bacteria. The final activated sludge (AS-APa) also presented a filamentous bulking but with long filamentous bacteria, and it came from a lab scale pilot plant supplied with a synthetic effluent (Viandox) [21]. This study also focused on four different anaerobic sludges used for methane production. Three types with a granular shape (GS-er, GS-em and GS-ne) originated from upward-flow anaerobic sludge blanket (UASB) or Expanded Granular Sludge Bed (EGSB) bioreactors, and another flocculant-type sludge containing very small granules (ANS-re) was sampled from an anaerobic digester with recirculation.

The EPS from AS-As and AS-Ls were extracted according to the method developed by Comte et al. [8], which uses cation-exchange resin coupled with ultracentrifugation. For EPS extracted from the activated sludges (AS-Aa, AS-Ba, AS-La and AS-APa), ultrasound and centrifugation were applied [19]. All of the EPS from anaerobic granular sludges and from ANS-re were extracted with cation-exchange resin coupled with ultracentrifugation [20].

The EPS were mainly composed of proteins, polysaccharides and humic-like substances and contained a lower amount of nucleic acids, indicating the absence of an abnormal rate of cell lysis during EPS extraction. The uronic acid content varied over a wide range from one EPS to another. EPS extracted from activated sludges displayed higher protein, polysaccharide, humic-like substances and uronic acid contents than EPS extracted from anaerobic sludges with a granular or flocculated aspect. Overall, the mineral fractions of EPS from activated sludges were smaller than anaerobic granular or flocculated sludges.

2.2. Size exclusion chromatography (SEC)

2.2.1. Chromatographic apparatus

To obtain the EPS chromatographic fingerprints, a Merck Hitachi LA Chrom Chromatograph was equipped with a L7200 autosampler, a L7100 quaternary pump, a L7000 interface and a L7455 diode array UV detector. An Amersham Biosciences column, the Superdex 200 10/300 GL, was used. For this column, the manufacturer reported a resolving range from 10 to 600 kDa. The total permeation volume of the column was determined to be 23 mL using a solution of 0.1 g L^{-1} sodium azide. For all experiments, a mobile phase flow rate of 0.4 mL min^{-1} was applied, which corresponded to an operating pressure of 15 MPa.

Table 1
Origin and main characteristics of EPS.

Sludge type	Activated sludges					Anaerobic granular sludges			Anaerobic flocculated sludge
Name of EPS	AS-As[a]	AS-Ls[a]	AS-Aa[b]	AS-Ba[b]	AS-APa[b]	GS-er[c]	GS-em[c]	GS-ne[c]	ANS-re[c]
Reactor type	WWTP 4000 eqh	WWTP 285 000 eqh	WWTP 4000 eqh	WWTP 20 000 eqh	Laboratory pilot	UASB	UASB	EGSB	Anaerobic digester
Influent	95% d 5%i	90% d 10%i	95% d 5%i	50% d 50%i	Synthetic	Paper mill	SO_4^{2-}/ethanol	Distillery	Brandy vinasse
Remark on sludge	–	–	–	Bulking with small filaments	Bulking with long filaments	–	–	–	Very mineral sludge (48% of DW)
Composition of EPS (mg g^{-1} DW)									
Proteins	301 ± 12	322 ± 13	261	261	293	93 ± 2	49 ± 11	143 ± 10	63 ± 2
Humic-like substances	107 ± 5	129 ± 6	241	245	275	109 ± 17	42 ± 8	61 ± 14	71 ± 6
Polysaccharides	132 ± 10	126 ± 10	199	142	187	76 ± 24	67 ± 1	11 ± 3	14 ± 1
Uronic acids	47 ± 2	54 ± 3	377	184	267	6 ± 1	14 ± 1	3 ± 1	8 ± 1
Nucleic acids	24 ± 2	16 ± 2	76	6	44	7 ± 1	5 ± 1	6 ± 1	2 ± 1
Proteins/polysaccharides	2.3	2.6	1.3	1.4	1.6	1.2	0.7	13.0	4.5
Mineral fraction (%DW)	30 ± 4	28 ± 4	21	29	31	52 ± 3	57 ± 4	65 ± 2	76 ± 4
Organic fraction (%DW)	70 ± 4	72 ± 4	79	71	69	48 ± 3	43 ± 4	55 ± 2	24 ± 4
Total organic carbon	135 ± 7	173 ± 9	298	272	526	160 ± 3	103 ± 6	50 ± 1	28 ± 2

WWTP: wastewater treatment plant; DW: dry weight; d: domestic; i: industrial.
[a] Comte et al. [8].
[b] Guibaud et al. [19].
[c] D'abzac et al. [20].