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Characterization, structure, and function of extracellular polymeric substances (EPS) of microbial biofilm in biological wastewater treatment systems: a review

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ABSTRACT

A review concerning the definition, extraction, characterization, production, and functions of extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment reactors is given in this paper. EPS are a complex high-molecular-weight mixture of polymers excreted by microorganisms, produced from cell lysis and adsorbed organic matter from wastewater. The EPS fill and form the space between the biofilm constituents, e.g. prokaryotic and eukaryotic microorganisms which inhabit the aggregates. It seems that the EPS matrix may serve as a multipurpose functional element of microbial communities, including adhesion, structure, protection, recognition, and physiology. Due to the metabolic activity of the cells, gradients develop and create different habitats within small distances, allowing a wide variety of organisms to settle and grow in the aggregate. As many of these organisms produce their specific EPS, it is not surprising that an extremely wide variety of microbial aggregates result. All of them have in common their highly hydrated extracellular matrix which is the place the organisms shape their very own microhabitat in which they live. However, as EPS are very complex, the knowledge regarding EPS is far from complete and much work is still required to fully understand their precise roles in the biological treatment process.

Keywords: Extracellular polymeric substances (EPS); Extraction; Microbial aggregates; Sludge; Wastewater treatment; Biofilm

1. Introduction

In biological wastewater treatment systems, most of the microorganisms are present in the form of microbial aggregates, such as sludge flocs, biofilms, and granules. The presence of extracellular polymeric substances (EPS), a complex high-molecular-weight mixture of polymers, in pure cultures, activated sludge, granular sludge, and biofilms, has been confirmed and

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observed using various electron microscopy techniques. EPS have a significant influence on the physicochemical properties of microbial aggregates, including structure, surface charge, flocculation, settling properties, dewatering properties, and adsorption ability. EPS bind with cells through complex interactions to form a vast net-like structure with plenty of water that protects cells against dewatering [1] and the harm of toxic substances [2]. Part of EPS can serve as carbon or energy sources in conditions of nutrient shortage [2,3]. They also accelerate the formation of microbial aggregates through binding cells closely [4]. Thus, the in-depth study of EPS is a matter of great interest not only in terms of improving our comprehension of biological wastewater treatment, but in terms of also improving the efficiency of such treatment through the optimization of operational parameters.

EPS come from the natural secretions of bacteria, cell lysis, and hydrolysis products. The predominant components of EPS are carbohydrate, protein, lipids, nucleic acids, and various heteropolymers [5,6]. EPS composition determines many important properties of biofilm such as strength, elasticity, sorption capacity for adsorbents, etc. These properties are important for the behavior of biofilms in technical systems; for example, they can partly determine erosion and sloughing from biofilms, development of frictional resistance in pipes, adsorption of solids from the bulk liquid, and clogging of biofilters [7].

Our knowledge about the EPS is still limited. Recently, Nielsen et al. [7] presented a conceptual model, which included production and degradation of all-important organic constituents, including cell biomass and EPS components.

In pure cultures, it has usually been assumed that the bacteria do not degrade their own exopolysaccharides, but this has now been shown not to be true for several types of bacteria [8]. Our hypothesis is that EPS is biodegradable and mixed cultures will degrade their own EPS material when they are in a starved state. Cells may not necessarily degrade their EPS under a nutrient rich condition; otherwise, the biomass glycocalyx would never become established.

This review outlines some important discoveries with regard to the EPS extraction, spatial distribution of EPS in microbial aggregates, analytical techniques, EPS characteristics, and also the effect of EPS compositions on the functions of microbial aggregates.

2. Definition of EPS

Microbial EPS are biosynthetic polymers (biopolymers). EPS were defined as "EPS of biological origin that participate in the formation of microbial aggregates" by Geesey [9]. Another definition was given in a glossary to the report of the Dahlem Workshop on Structure and Function of Biofilms in Berlin 1988 by Wilderer and Characklis [10]. Here, EPS were defined as "organic polymers of microbial origin which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)."

The abbreviation "EPS" has been used for "extracellular polysaccharides," "exopolysaccharides," "exopolymers," and "EPS."

Polysaccharides have often been assumed to be the most abundant components of EPS in early biofilm research [11]. That may be the reason why the term "EPS" has frequently been used as an abbreviation for "extracellular polysaccharides" or "exopolysaccharides." However, proteins and nucleic acids [7,12–14] as well as amphiphilic compounds including phospholipids [15,16]; have also been shown to appear in significant amounts or even predominate in EPS preparations from activated sludges, sewer biofilms, trickling filter biofilms, and pure cultures of bacteria. In addition, some researchers described humic substances as components of EPS matrices of soil and water biofilms [7,17,18]. In the following, the abbreviation "EPS" is used for "EPS" as a more general and comprehensive term for different classes of organic macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids, and other polymeric compounds, which have been found to occur in the intercellular spaces of microbial aggregates. At present, other microbial biopolymers such as polyhydroxyalkanoates are not normally regarded as EPS, since they are typically intracellular components of microbial cells. To the authors' knowledge, their extracellular occurrence in biofilms has not yet been reported.

EPS are present both outside of cells and in the interior of microbial aggregates. Wingender et al. [1] used the abbreviation "EPS" as a more general and comprehensive term to represent different classes of macromolecules such as polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds presented in the interior of various microbial aggregates. Wingender et al. [19] proposed that all polymers outside the cell wall, which are not directly anchored to the outer membrane or murein-protein layer, should be EPS. This broad definition of EPS makes the results from the EPS studies unpredictable and controversial. Actually, the EPS are mainly the high-molecular-weight secretions from microorganisms, and the products of cellular lysis and hydrolysis of macromolecules. In addition, some organic matters from wastewater can also be adsorbed to the EPS matrix [19,20]. The EPS related with the microbial metabolism

may make more sense in microbial aggregate-related studies, and can affect various physicochemical characteristics of microbial aggregates.

However, their origins are not well known yet. Although the interaction between soluble EPS and cells is very weak, previous study showed that soluble EPS also have a crucial effect on the microbial activity and surface characteristics of sludge [21]. However, the study on the soluble EPS is limited, and the EPS mentioned in literature and this review without being specified are bound EPS. The structure of bound EPS is generally depicted by a two-layer model (Fig. 1) [22]. The inner layer consists of tightly bound EPS (TB-EPS), which has a certain shape and is bound tightly and stably with the cell surface. The outer layer, which consists of loosely bound EPS (LB-EPS), is a loose and dispersible slime layer without an obvious edge. The content of the LB-EPS in microbial aggregates is always less than that of the TB-EPS, and thus may have some influence on the characteristics of microbial aggregates [23,24].

3. Composition, secretion, and spatial arrangement of EPS

3.1. Composition and secretion of EPS

Carbohydrates and proteins are usually found to be the major components of EPS. Humic substances may also be a key component of the EPS in sludge in biological wastewater treatment reactors, accounting for approximately 20% of the total amount [13,25]. In addition, lipids, nucleic acids, uronic acids, and some inorganic components have also been found in EPS from various matrixes [13,14,26,27]. Their fractions in EPS depended strongly upon the extraction methods and the sludge origins.

The content and compositions of the EPS extracted from various microbial aggregates are reported to be heterogeneous [19]. The variation in the compositions of the extracted EPS is attributed to many factors, such as culture, growth phase, process parameter, bioreactor type, extraction method, and analytical tool used [22].

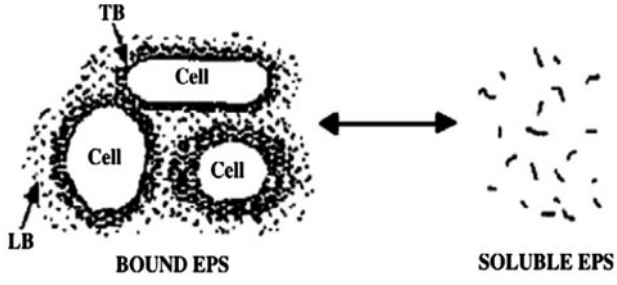

Fig. 1. Sketch of EPS structure by Nielsen and Jahn [22].

EPS are organic macromolecules that are formed by polymerization of similar or identical building blocks (Table 1), which may be arranged as repeating units within the polymer molecules such as, e.g. in many polysaccharides. EPS may contain non-polymeric substituents of low-molecular-weight which greatly alter their structure and physicochemical properties. Thus, extracellular polysaccharides often carry organic substituents such as acetyl, succinyl, or pyruvyl groups, or inorganic substituents such as sulfate. Proteins can be glycosylated with oligo saccharides to form glycoproteins or can be substituted with fatty acids to form lipoproteins.

By definition, EPS are located at or outside the cell surface independent of their origin. The extracellular localization of EPS and their composition may be the result of different processes: active secretion, shedding of cell surface material, cell lysis, and adsorption from the environment.

EPS may be actively secreted by living cells. Various specific pathways of biosynthesis and discrete export machineries involving the translocation of EPS across bacterial membranes to the cell surface or into the surrounding medium have been described for bacterial proteins and polysaccharides. Extracellular DNA can be produced by bacteria during growth; it is not known whether DNA is actively secreted or passively released due to increase in cell envelope permeability.

Another mechanism of release of extracellular polymers is the spontaneous liberation of integral cellular components such as lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria [29].

This may occur through formation of outer membrane-derived vesicles (blebs) which have been described as a common secretion mechanism in Gram-negative bacteria [30,31]. Surface blebbing occurs during normal growth and represents a process by which cellular macromolecules including periplasmic compounds and membrane components (nucleic acids, enzymes, LPS, phospholipids) are shed into the extracellular space in the form of membrane vesicles. Release of cellular material by this mechanism may be the result of metabolic turnover processes. Alternatively, membrane vesicles, into which hydrolytic enzymes are packaged (e.g. peptidoglycan hydrolases), may serve to degrade surrounding cells in the biofilm ("predatory vesicles"; [30,32], liberating nutrients for the vesicle-forming biofilm bacteria).

Table 1
General Composition of bacterial EPS; humic substances are included in the table, since they are sometimes considered as part of the EPS matrix by Nielsen et al., Jahn and Nielsen and Brown and Lester [7,17,28]

EPS	Principal components (subunits, precursors)	Main type of linkage between subunits	Structure of polymer backbone	Substituents (examples)
Polysaccharides	Monosaccharides Uronic acids Amino sugars	Glycosidic bonds	Linear, branched	organic: O-acetyl, N-acetyl, succinyl, pyruvyl inorganic: sulfate, phosphate
Proteins (polypeptides)	Amino acids	Peptide bonds	Linear	Oligosaccharides (glycoproteins), fatty acids (lipoproteins)
Nucleic acids	Nucleotides	Phosphodiester bonds	Linear	–
(Phospho)lipids	Fatty acids Glycerol Phosphate Ethanolamine Serine Choline Sugars	Ester bonds	Side-chains	–
Humic substances	Phenolic compounds	Ether bonds, C–C bonds	Cross-linked	–
	Simple sugars Amino acids	Peptide bonds		

Death and lysis of cells contribute to the release of cellular high-molecular-weight compounds into the medium and entrapment within the biofilm matrix.

Typically, intracellular organic polymers like poly-β-hydroxyalkanoates or glycogen as carbon and energy storage polymers or integral components of cell walls (e.g. peptidoglycan) and membranes (e.g. phospholipids, LPS) may thus become part of the EPS. As a consequence, the biofilm represents a "recycling yard" for intracellular components.

Finally, EPS which are shed from microbial aggregates can be adsorbed in other places. Thus, the sites of synthesis, release, and ultimate localization of EPS components are not necessarily identical.

3.2. Spatial arrangement of EPS

The spatial distribution of EPS is reported to be heterogeneous, and can be observed using confocal laser scanning microscopy (CLSM) or fluorescent microscopy after EPS stained by fluorescent dyes or lectins. EPS was concentrated in a sludge floc center [33] and that some polysaccharides were present around the network of filamentous fungi [34]. EPS are a major structural component of biofilms, and EPS were distributed in layers through the biofilm depth and their yield varied along the biofilm depth [35,36]. For anaerobic granular sludge, most of the EPS were distributed in the outer layer, and the remainder was distributed through the rest of the granules [37–39] reported that the EPS content in the inner layer of aerobic granular sludge was about four times greater than that in outer layer.

The distributions of various EPS components were also heterogeneous.

McSwain et al. [34] observed that the cells and carbohydrates were present in the outer layer of aerobic granular sludge, whereas most of the proteins were found in the inner layer. Chen et al. [40] found that in acetate-fed aerobic granules, protein and β-D-glucopyranose polysaccharides formed the core, whereas, the cells and α-D-glucopyranose polysaccharides accumulated in the granule outer layers. In the phenol-fed aerobic granules, proteins formed the core, and the cells and α- and β-D-glucopyranose polysaccharides were accumulated at an outer filamentous layer. These results indicate that the EPS distribution depends on the microbial aggregate types, structures, and origins.

4. Structure of EPS

EPS are not essential structures of bacteria in laboratory cultures, since loss of EPS does not impair

growth and viability of the cells. Under natural conditions, however, EPS production seems to be an important feature of survival, as most bacteria occur in microbial aggregates such as flocs and biofilms, whose structural and functional integrity is based essentially on the presence of an EPS matrix. Although EPS confer a macroscopically slimy appearance to bacterial masses in many environments and bacteria usually occur in an aggregated state where the cells are surrounded by EPS-containing material, the ubiquity and prime importance of EPS-containing structures has long been overlooked for several reasons. Traditionally, microbiologists used to study and to subculture individual bacterial strains in pure cultures using artificial growth media.

Under these *in vitro* conditions, bacterial isolates did not express EPS-containing structures or even lost their ability to produce EPS. It has been speculated that the energy-requiring process of EPS production does not confer any selective advantage to cells grown in the laboratory in contrast to the situation in competitive multispecies environments in nature and disease, where EPS functions seem to be essential for survival of bacterial populations [41]. Thus, extrapolating data from laboratory studies to EPS-dependent processes in natural and pathogenic environments gave results that did not reflect the actual mode of bacterial existence and activities under real conditions.

Furthermore, EPS were originally inadequately visualized by conventional light and electron microscopy [11]. Conventional optical microscopy of microbial aggregates showed that the pores between the cells seemed to be devoid of physical structures. Furthermore, due to its high water content (up to 99%) the glycocalyx collapsed under the dehydrating conditions of sample preparation for electron microscopy. Only stabilization of the glycocalyx by the use of antibodies or lectins and staining of EPS with dyes such as the commonly employed polyanion-specific ruthenium red allowed the visualization of fibrous material lying in the light-microscopically transparent space between the cells and gave an idea of the dimensions of EPS-containing structures around the cells [42,43].

It is assumed that the fibrils are colloidal aggregates of high-molecular-weight EPS molecules (predominantly acidic polysaccharides, but also proteins) representing the dominant physical bridging mechanism between cells and inorganic components within biofilms [44,45]. EPS fibrils display a wide variety of patterns in electron micrographs reflecting the different chemical compositions of EPS in mixed biofilm populations [41,46]. Examination of different environments by these techniques demonstrated the universal occurrence of EPS-containing structures of those bacteria which colonized, in the form of microcolonies or biofilms, surfaces of natural aquatic environments and technical water systems, the tissue surfaces of plants, animals, and man as well as surfaces of medical devices [11,41,44,46–49].

Only recently, CSLM in conjunction with fluorescent chemical probes enabled examination of the three-dimensional structure of fully hydrated and intact bacterial biofilms [42,48–50].

Application of this nondestructive technique changed the traditional view of the homogeneous biofilm as mainly maintained by researchers investigating biofilm reactors, but not shared by light microscopists (Marshall, personal communication; Szewzyk, personal communication). CSLM showed that within biofilms, bacteria grew as distinct matrix-enclosed micro colonies that were separated by less dense regions of the biofilm including water channels and pores [51]. The use of fluorescently labeled EPS-specific compounds such as carbohydrate-binding lectins or polyanionically charged dextran allowed study of the extent and spatial arrangements of EPS in natural biofilms and confirmed the heterogeneous distribution of EPS within the biofilm matrix [15,52,53].

5. Functions of EPS

After discovering the universal presence of EPS-containing structures, some general functions have been attributed to EPS such as the formation of a gel-like network keeping the biofilm bacteria together, the mediation of adherence of biofilms to surfaces, the involvement in the establishment of infections, and the protection of bacteria against noxious influences from the environment.

Meanwhile, extensive studies have resulted in a number of different and more detailed proposals for possible functions of EPS for biofilm bacteria, some of which are summarized in Table 2 and are discussed in several review articles.

In general, one of the most important functions of extracellular polysaccharides is supposed to be their role as fundamental structural elements of the EPS matrix determining the mechanical stability of biofilms, mediated by noncovalent interactions [54,55] either directly between the polysaccharide chains or indirectly via multivalent cation bridges.

More recent studies suggest that lectin-like proteins also contribute to the formation of the three-dimensional network of the biofilm matrix by cross-linking polysaccharides directly or indirectly through multivalent cations bridges [56]. Among activated sludge extracellular polymers, proteins predominated

Table 2
Selection of proposed effects due to microbial EPS

Function	Relevance
Adhesion to surfaces	Initial step in colonization of inert and tissue surface, accumulation of bacteria on nutrient-rich surfaces in oligotrophic environments
Aggregation of bacterial cells, formation of flocs and biofilms	Bridging between cells and inorganic particles trapped from the environment, immobilization of mixed bacterial populations, basis for development of high cell densities, generation of a medium for communication processes, cause for biofouling and biocorrosion events
Cell–cell recognition	Symbiotic relationships with plants or animals, initiation of pathogenic processes
Structural elements of biofilms	Mediation of mechanical stability of biofilms (frequently in conjunction with multivalent cations), determination of the shape of EPS structure (capsule, slime, and sheath)
Protective barrier	Resistance to nonspecific and specific host defenses (complement-mediated killing, phagocytosis, antibody response, free radical generation), resistance to certain biocides including disinfectants and antibiotics, protection of cyanobacterial nitrogenase from harmful effects of oxygen
Retention of water	Prevention of desiccation under water-deficient conditions
Sorption of exogenous organic compounds	Scavenging and accumulation of nutrients from the environment, sorption of xenobiotics (detoxification)
Sorption of inorganic ions	Accumulation of toxic metal ions (detoxification), promotion of polysaccharide gel formation, mineral formation
Enzymatic activities	Digestion of exogenous macromolecules for nutrient acquisition, release of biofilm cells by degradation of structural EPS of the biofilm
Interaction of polysaccharides with enzymes	Accumulation/retention and stabilization of secreted enzymes

and, on the basis of their relatively high content of negatively charged amino acids, they were supposed to be more involved than sugars in electrostatic bonds with multivalent cations, underlining their key role in the floc structure [14]. In addition, proteins have also been suggested to be involved in hydrophobic bonds within the EPS matrix [14]. However, the main function of extracellular proteins in biofilms is mostly seen in their role as enzymes performing the digestion of exogenous macromolecules and particulate material in the microenvironment of the immobilized cells. Thus, they provide low-molecular-weight nutrients which can readily be taken up and metabolized by the cells. Enzymes within the biofilm matrix may also be involved in the degradation of polysaccharide EPS causing the release of biofilm bacteria and the spreading of the organisms to new environments [8,57].

A function frequently attributed to EPS is their general protective effect on biofilm organisms against adverse abiotic and biotic influences from the environment (Table 2). As an example, it has frequently been observed that biofilm cells can tolerate significantly higher concentrations of certain biocides including disinfectants and antibiotics than planktonic populations [58,59].

The role of EPS components other than polysaccharides and proteins remains to be established. However, it is expected that EPS such as nucleic acids and lipids significantly influence the rheological properties and thus the stability of biofilms as can be deduced from basic laboratory studies on the properties of polymer mixtures. The colonization of surfaces may also be determined by EPS other than polysaccharides or proteins. Extracellular lipids from *Serratia marcescens* with surface-active properties (serrawettins) have been proposed to help bacteria in surface environments to overcome the strong surface tension of surrounding water, thus facilitating growth on solid surfaces [60]. In general, extracellular surface-active polymers with lipidic components seem to be involved in the interaction between bacteria and interfaces [15].

6. Approaches for studying microbial polysaccharides

Historically, microbial polysaccharides were studied for three reasons. First, polysaccharides represent a structural feature of the microbial cell; therefore, they were investigated for pure and basic research

interests. Second, polysaccharides were recognized as antigen determinants of the microbial cell surface; the knowledge of their structure was and still is of great importance in medical microbiology. Third, microbial polysaccharides were recognized as a source of polymers with unique properties. These applied aspects of polysaccharides were a reason to study their structure, properties, and production on the pilot and industrial scales.

All microbial polysaccharides were exclusively isolated by two methods.

After centrifugation of a liquid culture, they were either isolated from the cell-free culture supernatant by precipitation or they were recovered from the remaining cell pellet by various extraction methods. There have been very few attempts to study microbial polysaccharides with other approaches [15].

Generally, due to the pure culture philosophy in microbiology, the production, the isolation, and the preparation of EPS were done with pure cultures only.

A typical scheme of sampling, handling, and analysis of bound EPS in bioaggregates is as follows:

(1) Sampling and pretreatment: sampling of biological aggregates (e.g. activated sludge or biofilms) in environmental samples or sampling from bioreactors pretreatment often includes a washing step and homogenization of the sample; the samples might be stored before further handling.
(2) Extraction: the EPS components are extracted by an appropriate extraction procedure.
(3) Purification: in some cases the extracted EPS are purified before further analysis.
(4) Analysis: the EPS are usually analyzed for macromolecular composition (e.g. polysaccharides and proteins), and in some cases a more detailed investigation of the chemical composition or other characteristics is performed.

In the following sections, each of these steps will be described in more detail.

7. EPS extraction

Different isolation techniques were developed to recover EPS from pure cultures, mixed-species flocs, and biofilms from various environments, aiming to determine the quantitative composition of EPS, to elucidate the chemical structure of EPS, to examine the physicochemical properties of EPS, and to study biological functions of EPS [6,13,14,61,62].

For these studies, EPS were usually extracted and separated from the cells (e.g. by blending, sonication, cation exchange resin treatment, centrifugation, and membrane filtration techniques, alone or in combination), before the composition of the EPS was analyzed, mainly by colorimetric and chromatographic techniques.

When single EPS were studied in purified form, they were separated from other molecules, so that the native state of the EPS-containing structures was disrupted and interactions between different EPS were not considered. It has been observed that the frequent isolation process consisting of gravitational separation of EPS from cells of *Pseudomonas diminuta* with subsequent precipitation of polymers from the supernatant by addition of alcohol or acetone led to the disruption of the macromolecules (Schaule and Flemming, unpublished).

Research into EPS composition has been focused on the study of polysaccharides and also to some extent of proteins including enzymes. In microscopic studies, mainly polysaccharide-specific stains were employed to visualize EPS structures. Biochemical analyses of EPS were often restricted to the quantification of total carbohydrate, uronic acids, and proteins, whereas other polymeric constituents of the EPS such as DNA or lipids were only seldom studied.

7.1. Evaluation of EPS extraction methods

No universal extraction method exists for a quantitative extraction of bound EPS compounds from microorganisms growing in suspension or in aggregates. A number of different methods have been applied in studies on pure cultures or on undefined cultures, mainly related to activated sludge and biofilms. Only a few of the methods have been thoroughly evaluated to obtain an optimal extraction procedure with a high extraction efficiency without unwanted cell lysis and disruption of macromolecules. The extraction methods include various physical or chemical methods or combinations thereof. It is important to note that almost all methods rely on a certain water solubility of the components extracted, where the more hydrophobic compounds cannot be expected to be extracted.

An ideal EPS extraction method should be effective, cause minimal cell lysis, and not disrupt the EPS structure [13]. The extraction efficiency can be defined as the total amount of EPS extracted from the total organic matter, or the total amount of EPS extracted from the total EPS pool, for a given cell sample [22]. It should be noted that the EPS extraction efficiency differs significantly according to the extraction method used.

7.2. Selection of an appropriate extraction method

The extraction procedure must be selected for each case, considering the specific needs and constraints. In some studies, a certain fraction, e.g. the polysaccharides, are extracted for a more detailed chemical or structural analysis. In that case, lysis of the bacteria may not be a problem, if only the impurities can be removed before further analysis. In other cases, a quantitative extraction of all EPS is desired, if possible, and here it is of critical importance that no lysis takes place.

The best extraction method will depend on the type of interactions that keep the EPS components together in the matrix. The main forces involved in the binding of the polymers in the EPS matrix is van der Waal forces, electrostatic interactions, hydrogen bonds, hydrophobic interactions [63], and in some cases, covalent bonds as disulfide bonds in glycoproteins [64]. The dominating forces may be different from one EPS matrix to another, so various methods must often be tested.

Many chemical extraction methods rely on a breakage of the electrostatic interactions, thereby promoting an extraction of water soluble compounds. Less focus has been on the hydrophobic components, probably because it is difficult not to destroy the cells with the procedures.

It is particularly difficult to test the extraction performance in undefined cultures such as biofilms and flocs. As no universal extraction method exists, we recommend that extraction is only performed after running some comparative methods and initially optimizing and standardizing a selected extraction technique.

This can be done by varying the variable (extraction time, shear, temperature, or chemical) and by recovery of added standards. This is very important [13], because the EPS yield depended on the extraction time and shear rate, and moreover, the dependence was different for various EPS components. Furthermore, an evaluation of lysis and polymer disruption must be included.

7.2.1. Physical method

With the physical methods, a shear is applied to extract EPS by centrifugation, mixing, or shaking, sonication, or heat treatment (Tables 3 and 4). In general, the extraction/yield is lower when using a physical treatment than when using combined chemical and physical treatments (see below). The extent of lysis or disruption of macromolecules has been evaluated only in a few studies, but, particularly, heat treatment may cause significant lysis and disruption (see below).

Centrifugation is often used to separate the soluble (slime) EPS fraction from cell biomass [65,66]. The shear applied by centrifugation may only to a very limited extent extract bound EPS. High-speed centrifugation was proposed [28] as the most effective method for EPS extraction from *Klebsiella aerogenes*. However, centrifugation does not provide any significant extraction of bound EPS from microbial aggregates from natural systems, as the components are usually strongly bound [13,67]. Centrifugation is, however, almost always used after any extraction procedure to separate the extracted EPS from cells and other particles.

Shaking, stirring, or pumping the bacterial matrix through needles by using syringes to obtain a certain shear are other common physical methods used. A high shear level may be provided by sonication under defined conditions, which has been used to extract EPS from activated sludge and granular sludge. It should be noticed that it is essential to use defined and well-described shear conditions in order to get reproducible results. Also heat, either at 70–80˚C or boiling, typically for 1 h, has been used in several studies, sometimes combined with shear provided by shaking (Table 3).

7.2.2. Chemical methods

The chemical treatments include addition of various chemicals to the bacterial sample that can break different linkages in the EPS matrix, facilitating a release of EPS to the water (Tables 5 and 6).

Alkaline treatment by addition of NaOH causes many charged groups, such as carboxylic groups in proteins and polysaccharides, to be ionized because their isoelectric points are generally below pH 4–6. It results in a strong repulsion between EPS within the EPS gel and provides a higher water solubility of the compounds. Alkaline hydrolysis of many polymers may take place [89]. Also, the covalent disulfide bindings in glycoproteins may be broken at pH values above 9 [90], promoting an extraction of these compounds. Disulfide bindings can also be reduced by adding β-mercaptoethanol or other compounds [64]. The pH used in various studies using alkaline treatment varies from 9 to 13, or it is indicated as addition of 1–9 N NaOH (Table 5).

The repulsion among the components in the EPS matrix and the water solubility can also be increased by an exchange of divalent cations with monovalent ions. The divalent cations, mainly Ca^{2+} and Mg^{2+}, are very important for the crosslinking of charged compounds in the EPS matrix and by removing these, the

Table 3
Physical extraction methods for defined cultures (n.i. = not investigated)

Method	System	Intracellular leakage	Refs.
Low-speed centrifugation (5,000–10,000× *g*)	*Azospirillum brasilense*	n.i.	De Troch et al. [65]
	Pseudomonas putida/ fluorescens	n.i.	Conti et al. [68]
	Pseudomonas alcaligenes	n.i.	Titus et al. [69]
High-speed centrifugation (30,000–48,000× *g*)	*Klebsiella aerogenes*	Protein/DNA	Brown and Lester [28]
	Freshwater/marine isolates	n.i.	Kennedy and Sutherland [70]
	Different pure cultures	n.i.	Pavoni et al. [71]
	Pseudomonas aeruginosa	n.i.	Pavoni et al. and Buckmire [71,72]
High-speed centrifugation (113,000× *g*)	Marine *Pseudomonas* sp.	n.i.	Wrangstadh et al. [73]
Ultra Turrax (20,000 rpm, 60 s)	*Rhodopseudomonas capsulata*		Omar et al. [74]
Ultrasonication (10 min, 120 V, 18 W)	*Klebsiella aerogenes*	Protein/DNA	Brown and Lester [28]
Ultrasonication (10 min, 120 V, 18 W)/High-speed centrifugation	*Klebsiella aerogenes*	Protein/DNA Protein	Brown and Lester [28] Rudd et al. [75]
Boiling (30 min)	*Proteus vulgaris*	n.i.	Rahman et al. [76]
Steam (autoclave, 10 min)	*Klebsiella aerogenes*	Protein/DNA	Brown and Lester [28]

EPS matrix tends to fall apart. The divalent cations can be removed by using a resin, e.g. Dowex, or by using a complexing agent such as EDTA or EGTA.

Both methods have been used in biofilm systems, activated sludge and pure bacterial cultures (Table 3). There are some drawbacks in using EDTA. It can remove divalent cations from the cell wall, leading to destabilization of cell wall and release of components such as LPS [91] and possibly also contamination with cellular macromolecules. It may also interfere in the protein determination (see below).

Another way to carry out a cation exchange is by using a high concentration of NaCl. It has been used for the extraction of adhesive exopolymers from *Pseudomonas putida* and *Pseudomonas fluorescens* [92,93]. In some investigations, the combination of high pH and ion exchange has been applied by using NH_4OH and EDTA [94].

Enzymatic digestion may be used to destabilize bioaggregates and enhance extraction. A deflocculation enzyme, prepared from the fluid culture of a *Pseudomonas* sp. isolated from activated sludge, has been used to deflocculate cells before further extraction [95].

The more hydrophobic part of the EPS is usually not extracted by specific procedures. In some cases, however, specific hydrophobic components are extracted from pure cultures by use of detergents, e.g. to isolate capsular antigen from *Escherichia coli* [96,97] or to purify exopolysaccharides from *Klebsiella pneumoni* [98]. Very few studies are being published on the hydrophobic extracellular fraction in' environmental samples, probably because it is very difficult to assess whether cellular lysis takes place. Ethanol was used to extract lipids from activated sludge [99], but it is unknown to what extent cell lipids were extracted as well.

7.2.3. Combination of physical and chemical methods

Many of the chemical methods reported have been used without applying a defined shear during the extraction, e.g. by shaking a few times. However, it appears that the chemical extraction becomes more reproducible and effective when it is combined with defined shear. The shear is typically provided by heat, sonication, or stirring under defined conditions (Tables 7 and 8).

Alkaline treatment has been combined with heat treatment (70˚C) to extract capsular EPS from *Rhizobium trifolii* [104]. Ion exchange by a Dowex extraction has been used in combination with shear (stirring) to extract EPS from activated sludge and biofilms [13,62]. As ion exchange is controlled by diffusion, it is very important to standardize the experimental conditions such as shear, time, and temperature. NaCl,

Table 4
Physical extraction methods for undefined cultures (n.i. = not investigated)

Method	System	Intracellular leakage	Refs.
Passing through a syringe needle/PBS (phosphate-buffered saline) buffer	Anaerobic sludge	n.i.	de Beer et al. [37]
Filtration (0.4 μm)	Activated sludge	n.i.	Hejzlar and Chudoba [77]
Repeated homogenization with a hand homogenizer	Methanogenic granular sludge	DNA, protein, polysaccharide	Grotenhuis et al. [78]
Heating (70˚C, 1–8 h)	Anaerobic sludge	n.i.	Schmidt and Ahring [79]
Heating (80˚C, 1 h)	Activated sludge	n.i.	Horan and Eccles [80]
	Anaerobic/activated sludge	n.i.	Morgan et al. [81]
	Anaerobic/granular sludge	n.i.	Forster and Quarmby [82]
Boiling (1 h)	Activated sludge	n.i.	Beccari et al. [83]
Steaming treatment (autoclave, 10 min)	Activated sludge	Protein/DNA Protein/polysaccharide ratio	Brown and Lester [28] Karapanagiotis et al. [84]
Sonication (up to 60 W min^{-1})	Activated sludge	n.i.	King and Forster [85]
Sonication, 60 W, varying time	UASB granules	n.i.	Quarmby and Forster [86]
Sonication, 18 W, 120 V, 10 min	Activated sludge	Protein/DNA	Brown and Lester [28]
Sonication. 37 W, 60 s, centrifugation (20,000× *g*)	Activated sludge	DNA, cell count	Jorand et al. [87]
Sonication, two 15 s periods, 50 W, 20 KHz/centrifugation (33,000× *g*)	Activated sludge	n.i.	Urbain et al. [88]
Standard blender, 1 min/centrifugation (13,000× *g*)	Activated sludge	DNA	Gehr and Henry [61]

Table 5
Chemical extraction methods for defined cultures (n.i. = not investigated)

Method	System	Intracellular leakage	Refs.
NaOH (2 N), 2–3 h	*Escherichia coli*	n.i.	Sato and Ose [100]
EDTA (3.72 g L^{-1}),15 min stirring	Periphytic marine bacteria	n.i.	Labare et al. [101]
Zwittergent (0.1%) in 50 mmol L^{-1} citrate, pH 4.5, 42˚C, 30 min	*Klebsiella pneumoniae*	DNA	Domenico et al. [98]
Pyridine acetate (0.1 mol L^{-1}, pH 5.0), 1 h	*Escherichia coli*	n.i.	Pelkonen et al. [102]
NaCI (0.9%)	*Pseudomonas aeruginosa*	n.i.	May and Chakrabarty [103]
NaCI (0.5 mol L^{-1}), 60 min	*Pseudomonas* sp. NCMB 2021	n.i.	Christensen et al. [93]
NaCI (1.0 mol L^{-1})	*Pseudomonas puttda /Pseudomonas fluorescens*	n.i.	Read and Costerton [92]
Deflocculating enzyme! 0.5 N NaOH, stirring for 24 h	*Pseudomonas* sp.	n.i.	Tago and Aida [95]

Table 6
Chemical extraction methods for undefined cultures (n.i. = not investigated)

Method	System	Intracellular leakage	Refs.
K_2HP0_4 (0.4 mol L^{-1}), 15 min	Activated sludge	DNA	Gehr and Henry [61]
NaOH (1.5 mol L^{-1}, 2 h)	Digested activated sludge	Protein/polysaccharide ratio	Karapanagiotis et al. [84]
NaOH (2 N, 2–3 h)	Activated sludge	n.i.	Sato and Ose [95]
NaOH (2 N, 5 h)	Activated sludge	Protein, DNA	Brown and Lester [28]
Dowex (1:3 vol, 1 h)	Digested activated sludge	Protein/polysaccharide ratio	Karapanagiotis et al. [84]
Dowex (1:1 vol)/ formaldehyde (1%)	Activated sludge	n.i.	Rudd et al. [75]
Ethanolic extraction, 1–16 d	Activated sludge	n.i.	Forster and Clarke [100]
Phenol (45% (w/v)), 70℃, 15 min	Digested activated sludge	Protein/polysaccharide ratio	Karapanagiotis et al. [84]
Two-step extraction (1) 30 s homogenization with NH_40H (1N)/(2) EDT A (2% (w/v)), pH 10, 3 h, 4℃	Activated sludge	n.i.	Sato and Ose [95]

Table 7
Combined physical and chemical extraction methods for defined cultures. n.i. = not investigated; glucose6PDH = glucose-6-phosphate dehydrogenase

Method	System	Intracellular leakage	Refs.
Deionized water/blending (2 min)	*Sphaerotilus natans*	n.i.	Gaudy and Wolfe [107]
NaOH (pH 11.5)/heating (40℃, time unknown)	*Rhizobacteria*	n.i.	Hebbar et al. [66]
NaOH (1 N)/heating (70℃, 15 min)	*Rhizobium trifolii*	n.i.	Breedveld et al. [104]
Saline/sonication (60 W, 2 min)	*Staphylococcus epidermidis*	n.i.	Evans et al. [108]
EDTA (10 mmol L^{-1})/Waring blender (60 s)	Freshwater sediment bacterium	GluCQse6PDH	Platt et al. [12]
NaCl (0.1 mol L^{-1})/EDTA 0.01 moll-I, 30 min stirring	*Clostridium acetobutylicum*	n.i.	Junelles et al. [109]
Dowex (240 g g^{-1} organic carbon/shear (600 rpm, 1–2 h)	*Pseudomonas putida* (biofilm and suspended culture)	GluCQse6PDH	Jahn and Nielsen [62]
Ethanol/high-speed centrifugation (48,000× *g*)	Bacteroids *Bradyrhizobium japonicum*	n.i.	Streeter et al. [110]
Cetyltrimethylammonium bromide (1% (w/v)/NaCl (0.04 moll-I))/heating (50℃, 2 h)	*Rhodopseudomonas capsulata*	n.i.	Omar et al. [74]
Hexadecyltrimethylammonium bromide (0.1% (w/v))/$CaCI_2$ (l mol L^{-1}) precipitation	*Escherichia coli*	n.i.	Schmidt and Jann [97] Jann et al. [96]
NaCl (0.9%)/heating (50℃, 2 h)	*Rhodopseudomonas capsulata*	n.i.	Omar et al. [74]
K_2HP0_4 (0.02 mol L^{-1})/blending (2 min)	*Zoogloea*	n.i.	Farrah and Unz [111]

formaldehyde, and ultrasonication were used to extract EPS from anaerobic sludge [105].

The formaldehyde was added in order to tighten the cells to minimize cell lysis during the procedure. However, formaldehyde changes the properties of many EPS components and interferes in a possible later analysis of carbohydrates by the phenol–sulfuric acid method [106].

Table 8
Combined physical and chemical extraction methods for undefined cultures

Method	System	Intracellular leakage	Refs.
Dowex/shear (65–80 g g^{-1} VS, 900 rpm, 1–2 h)	*Activated sludge*	Glucose6PDH	Frølund et al. [13]
Dowex/shear (240 g g^{-1} organic matter, 600 rpm, 1–2 h)	Sewer biofilm	Glucose6PDH	Jahn and Nielsen [62]
NaCI (2.5% (w/v))/EDTA (100 mmol L^{-1}), for 15 min, Vortex mixing	Intertidal sediments	n.i.	Underwood et al. [106]
NaCI (8.5% (w/v))/formaldehyde (0.22% (v/v))/sonication (40 W for 3 min on ice) = "cold aqueous technique"	Anaerobic sludge	n.i.	Jia et al. [105]
Phenol extraction (20% (v/v)), 50°C, 45 min, intermittent sonication	Methanogenic granules	n.i.	Veiga et al. [112]

Notes: n.i. = not investigated; glucose6PDH = glucose-6-phosphate dehydrogenase; VS = volatile solids.

8. Analytical methods

8.1. Conventional chemical colorimetric analyses

The composition of the EPS matrix in biofilms and activated sludge is reported to be very complex, containing proteins, carbohydrates, nucleic acids, lipids, humic substances, etc. Conventional chemical colorimetric analyses can be used to quantify their contents in EPS [113]. Generally, the carbohydrate content is measured using the anthrone method or the phenol–sulfuric acid method. A comparison between the two methods for carbohydrates determination in EPS showed that the two methods yielded similar results, but that the coefficient of variation for the anthrone method was lower than that for the phenol–sulfuric method [13]. The protein content is measured using the Lowry method, the Bradford method, or the total N-content method. The Lowry method has a higher recovery than the Bradford method [13].

The total N-content method is more accurate, but the procedures are complex. Thus, the Lowry method is frequently applied for protein analyses in EPS characterization. As the phenolic functional groups of humic acids also react with the Lowry reagent, the appropriate correction is always needed (Table 8).

Humic substances are very complex, and there are fewer appropriate methods for measuring their content in EPS. Frølund et al. [25] proposed a modified Lowry method to determine the humic substance content by correcting the protein interference. The uronic acid content can be measured using the m-hydroxydiphenyl sulfuric acid method [114]. The DNA or nucleic acid content is measured using the DAPI fluorescence method [13], the UV absorbance method [115], or the diphenylamine method [116].

The UV absorbance method is easy to perform, but is readily interfered by proteins. The DAPI method for DNA estimation works well, but its procedures are complex. Therefore, the diphenylamine method could be used more conveniently and widely.

8.2. Innovative methods

The complex compositions make it difficult to analyze the conformation, structure, distribution, and functions of EPS. However, progress in analytical chemistry has led to the development of new instruments and techniques for the characterization of EPS, as listed in Table 9, which has generated a large amount of information about the structural and functional properties of EPS as well as their environmental behavior.

The EPS presented in microbial aggregates with amorphous-phase surrounding cells were directly observed using electronic microscopic technology [117]. Using the conventional scanning electron microscopy and transmission electron microscopy, the microbial aggregates should be fixed and dewatered first, which would change the original conformation of EPS. The environmental scanning electron microscopy [118], atomic force microscopy [119,120], and CLSM [36] could be used to observe the fully hydrated samples to obtain the original shapes and structures of EPS. After staining by various fluorescence probes, the spatial distributions of carbohydrates, proteins, and nucleic acids in EPS can also be obtained by CLSM [121].

The chromatography, mass spectrometry, and their combination could be used to qualitatively and quantitatively analyze the EPS compositions [14]. The spectroscopy, including X-ray photoelectron spectroscopy [122–124], Fourier transform infrared spectroscopy [123,125,126], three-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM)

Table 9
New analytical techniques used in EPS research

Analytical techniques	Descriptions	Refs.
Gas chromatography (GC) High-performance liquid chromatography (HPLC) Gas chromatography–mass spectrometry (GC–MS)	Qualitative and quantitative analyses of the monosaccharides and amino acids of EPS after hydrolysis	Dignac et al. and Ortega-Morales et al. [14,124]
Scanning electron microscopy (SEM) Transmission electron microscopy (TEM)	Observation of the microstructure and EPS of microbial aggregates after fixation and dewatering. Slices may be needed for the observation of the interior structure of microbial aggregates. Combining with energy dispersive X-ray spectrometer (EDX), the element composition and distribution of EPS can be obtained	Li and Ganczarczyk, Macleod et al. and Bura et al. [117,132,133]
Environmental scanning electron microscopy (ESEM)	Technique for observing fully hydratedsamples to obtain the original shapes and structure of EPS	Beech et al. and Surman et al. [118,134]
Atomic force microscopy (AFM)	Be used to observe the surface shape and structure of EPS in the bacteria cells. It can also be used to evaluate the adhesion force between cells and solid surface	Beech et al. and Li and Logan [118,120]
Confocal laser scanning microscopy (CLSM)	Be used to observe the structure of fully hydrated samples. The spatial distribution of EPS also can be obtained using various fluorescence probes. The total EPS content can be evaluated from the CLSM figures through the use of conversion factors	Zhang and Bishop, Staudt et al. and Kawaguchi and Decho [36,121,135]
Quartz crystal microbalance (QCM)	Be used to study the kinetics of EPS adsorption or cell adhesion onto material surface *in situ* and in real-time	Kwon et al. and Zhu et al. [136,138]
X-ray photoelectron spectroscopy (XPS)	Be used to study the surface functional groups of EPS, the interactions between EPS and metals, and the role of EPS in microbial adhesion to substrates	Dufrêne and Rouxhet and Ortega-Morales et al. [122,124]
Fourier transform infrared spectroscopy (FTIR)	Be used to study the functional groups of microbial aggregates and their associated EPS. The attenuated total reflection technique (FTIR/ATR) can be used to observe the microbial adhesion in solutions *in situ*	Omoike and Chorover, Allen et al. and Sheng and Yu [123,125,126]
Raman spectroscopy	Be used to study the chemical structure of EPS *in situ*	Wagner et al. [139]
Three-Dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM)	A sensitive and selective method that needs only very small samples and does not destroy the structure of the samples. Has recently been used to characterize EPS of various origins	Sheng and Yu and Esparza-Soto and Westerhoff [24,127]
Nuclear magnetic resonance (NMR)	Can be used to study the molecular structure of carbohydrates and proteins and the interaction between EPS and metals	Manca et al. and Lattner et al. [129,130]
High-performance size exclusion chromatography (HPSEC)	Can be used to characterize the molecular weight distribution of EPS and to evaluate extraction methods HPSEC combined with other techniques (e.g. FTIR) can be used to characterize EPS with different molecular weight fractions	Frølund et al. and Görner et al. [13,140]
Time-of-flight secondary-ion mass spectrometry (TOF-SIMS)	Be used to characterize the chemical compositions of EPS and the interaction of EPS and metals	Pradier et al. [141]

[127,128], and nuclear magnetic resonance [129,130] can be used to elucidate the functional groups and element compositions in EPS or microbial aggregates. EPS contain large quantities of aromatic structures and unsaturated fatty chains with various types of functional groups, which have fluorescence characteristics. As a rapid, selective, and sensitive technique, 3D-EEM fluorescence spectroscopy is useful for studying the physicochemical properties of EPS, as fluorescence characteristics are greatly related to their structure and functional groups in molecules.

Due to the high sensitivity, good selectivity, and non-destruction of samples, these spectroscopy techniques could also be used to characterize the adsorption pollutants to EPS from the changes of their functional groups in EPS [123,128,129]. For example, from the fluorescence quenching degree of EPS or UV–vis spectral changes before and after adsorption, the binding strength of pollutants onto EPS could be evaluated [21,131].

9. Conclusion

EPS are very important for microbial aggregates in biological wastewater treatment systems. However, there is still much to learn regarding the roles of EPS in the functions and characteristics of microbial aggregates. The following areas should be studied to gain a greater understanding of EPS:

(1) Development of EPS extraction methods. Extraction of EPS from microbial aggregates is the foundation to study the EPS characteristics and the roles of EPS played in bioreactors. As some of the fractions of EPS in microbial aggregates could not be extracted using the commonly used methods, new EPS extraction methods with a high efficiency should be pursued. Such methods should be mild to avoid the lysis of cells and the disruption of EPS.
(2) Establishment of EPS *in situ* analytical methods. The characteristics of EPS are expected to be changed after extraction, such as molecular structure and conformation. With the development of modern analytical techniques, it is possible to identify the EPS compounds using one or a combination of some *in situ* innovative analytical techniques. These methods should be able to analyze the hydrated samples without dewatering.
(3) Identifying the sub-fractions of EPS. In previous studies, little attention has been paid on the extraction, distribution, and characteristics of TB-EPS, LB-EPS, and soluble EPS, which have been proven to play different roles in microbial aggregates and biological wastewater treatment systems. Identification and elucidation of the origins, compositions, and characteristics of these sub-fractions of EPS could be useful to clarify the contradictions about EPS in previous studies. Also, their functions would be well evaluated.
(4) Elucidation of the key roles of EPS. In previous studies, the relationships between EPS and the functions of microbial aggregates (e.g. flocculation, settlement, dewatering, adsorption, etc.) are often controversial. The controversies on the roles of EPS might be attributed to their complicated compositions. Each component shows its own special effect and thus the overall effect might become unpredictable and case-dependent. This also causes some problems in manipulating the EPS content and improving the performances of microbial aggregates. Thus, it is essential to elucidate the roles of each major component in EPS and their subfractions, i.e. LB- and TB-EPS.
(5) Identification of the key factors influencing the production of EPS. As the origins of EPS are complex, many factors could influence the production of EPS. After the roles of EPS components become known, identification of such key factors would then be very useful to manipulate the EPS compositions and contents in microbial aggregates and thus to improve the functions of microbial aggregates, e.g. flocculation, settlement, and dewatering abilities.

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