Extracellular polymeric substances (EPS) of microbial aggregates 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. They are a major component in microbial aggregates for keeping them together in a three-dimensional matrix. Their characteristics (e.g., adsorption abilities, biodegradability and hydrophilicity/hydrophobicity) and the contents of the main components (e.g., carbohydrates, proteins, humic substances and nucleic acids) in EPS are found to crucially affect the properties of microbial aggregates, such as mass transfer, surface characteristics, adsorption ability, stability, the formation of microbial aggregates etc. 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.

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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 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 (Wingender et al., 1999) and the harm of toxic substances (Sutherland, 2001a). Part of EPS can serve as carbon or energy sources in conditions of nutrient shortage (Sutherland, 2001a; Zhang and Bishop, 2003). Bound EPS are closely bound with cells, while soluble EPS are weakly bound with cells or dissolved into the solution. Generally, these two types of EPS can be separated by centrifugation, with those remaining in the supernatant being soluble EPS and those forming microbial pellets being bound EPS. 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 (Sheng and Yu, 2007). 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) (Nielsen and Jahn, 1999). 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 (Li and Yang, 2007; Sheng et al., 2006a).

1.2. Composition 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 (Frolund et al., 1995, 1996). In addition, lipids, nucleic acids, uronic acids and some inorganic components have also been found in EPS from various matrices (Frolund et al., 1996; Dignac et al., 1998; D’Abzac et al., 2010a, b). 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 (Wingender et al., 1999). 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 (Nielsen and Jahn, 1999).

1.3. Spatial distribution 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 fluorescent dyes or...
lectins. EPS was concentrated in a sludge floc center (de Beer et al., 1996) and that some polysaccharides were present around the network of filamentous fungi (McSwain et al., 2005). 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 (Zhang et al., 1998; Zhang and Bishop, 2001). For an anaerobic granular sludge, most of the EPS were distributed in the outer layer, and the remainder was distributed through the rest of the granules (de Beer et al., 1996; Zhang and Fang, 2004). Wang et al. (2005) 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. (2005) 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. (2007) 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.

2. EPS extraction

2.1. Evaluation of EPS extraction methods

An ideal EPS extraction method should be effective, cause minimal cell lysis, and not disrupt the EPS structure (Frolund et al., 1996). 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 (Nielsen and Jahn, 1999). It should be noted that the EPS extraction efficiency differs significantly according to the extraction method used.

Cell lysis might occur when EPS are extracted. However, the extent of cell lysis during extraction is difficult to evaluate. Some studies have taken the protein or nucleic acid content of EPS as an indicator of cell lysis (Brown and Lester, 1980), but it is recognized that the EPS matrix usually contains a large amount of proteins and a little nucleic acids, and thus neither macromolecule is an accurate indicator. However, as the nucleic acid content in EPS is usually low, a high level of nucleic acids after EPS extraction indicates severe cell lysis. Adenosine triphosphate and intracellular enzymes, such as glucose-6-phosphate dehydrogenase, have been used as intracellular markers (Frolund et al., 1996). The cell count coupled with microscopy methods, and the live/dead cells count or staining methods has also been used to evaluate cell lysis. This is actually based on cell wall integrity, and hence the cell wall interruption means intracellular content release. However, this can only be used to evaluate whether cells are destroyed, rather than to assess whether intracellular material has leaked out. Sheng et al. (2005a) proposed a UV–visible spectrometry method to evaluate cell lysis by measuring the release of intracellular compounds, and applied it to compare EPS extraction methods for photosynthetic bacteria.

The disruption of macromolecules can take place during EPS extraction, with boiling and alkaline treatments being reported to cause particularly severe disruption. NaOH has been found to cause a change in the polymer composition at pH > 9, and at high pH values, the disulfide bindings in glycoproteins are broken and uronic acids are degraded. The changes in the macromolecular composition of the extracted EPS can also be evaluated using gel permeation chromatography or high-pressure size exclusion chromatography (Frolund et al., 1996).

2.2. Selection of an appropriate extraction method

A typical EPS extraction procedure is as follows (Nielsen and Jahn, 1999): (1) pretreatment, including sampling, storing, washing, and homogenizing. This step allows the microbial cells to be dispersed. For EPS extraction from biofilm and microbial granules, homogenizing is always needed. (2) EPS extraction from the samples after the pretreatment. (3) Purification for further analysis. In these steps, the second step is the important one. EPS components should be extracted using an appropriate extraction procedure. For soluble EPS, centrifugation is always used, whereas for bound EPS many different extraction methods have been developed, and new methods are still coming to light (Nielsen and Jahn, 1999). These extraction methods can be classified as physical methods, chemical methods, and a combination of physical and chemical methods, as listed in Table 1. The physical extraction methods usually utilize the external forces, which are created by ultrasonic, centrifugation, or heating, to encourage the EPS to detach from cells and dissolve in solution. The chemical extraction methods involve in adding chemical compounds to disrupt the binding interactions between the EPS and the cells in order to accelerate the dissolution of the EPS. In general, the extraction efficiencies of the physical extraction methods are lower than those of the chemical extraction methods. Furthermore, different methods have various extraction efficiencies for these components, which results in various compositions in the EPS (Liu and Fang, 2002a; Sheng et al., 2005a; D’Abzac et al., 2010b).

To study the compositions and functions of the LB-EPS and TB-EPS in a sludge sample, the two fractions of the bound EPS may be extracted separately. As the LB-EPS bound with cell loosely, a mild method (e.g., high-rate shear, heating at low temperatures, or high speed centrifugation) should be chosen to avoid the inclusion of the TB-EPS. Subsequently, a harsh method (e.g., heating at high temperatures, sonication or chemical extraction methods) should be applied for the TB-EPS extraction. Li and Yang (2007) modified a heating extraction method which included a mild step and a harsh step for extracting the LB-EPS and TB-EPS from activated sludge subsequently. The cell lysis was not significant after such extraction process. The high speed centrifugation and ultrasonication were also used to extract the LB-EPS and TB-EPS from sludge (Ramesh et al., 2007).

As has been highlighted, a number of methods have been developed and applied to extract EPS from pure or undefined cultures. The extraction efficiencies of chemical methods, such as cation exchange resin (CER) (Frolund et al., 1996), EDTA (Sheng et al., 2005a), and the HCHO/NaOH methods (Liu and Fang, 2002a), are always higher than those of physical methods, but the application of chemicals brings about certain problems either in the extraction process itself or in the subsequent EPS analysis. Alkaline treatment can cause severe cell lysis and the disruption of macromolecules. The EDTA method has a high extraction efficiency and causes a low degree of cell lysis. However, the residual EDTA would contaminate the EPS extraction (Comte et al., 2006b), and then interfere with protein determination in the Lowry method. The dialysis is always needed for EDTA extraction method. The dose of HCHO in the HCHO/NaOH extraction method changes the EPS characteristics and causes substantial interference in the determination of carbohydrates. EPS extraction using an enzyme for hydrolysis is a mild and effective method, but the extraction efficiency is not very high for activated sludge (Sesay et al., 2006). Because of its high efficiency and low cell lysis, the CER method has become the most widely accepted EPS extraction method, largely because the resin can be removed readily, which means that pollution by chemical reagents is avoided and subsequent analysis is easier.

It should be mentioned that none of these methods can extract all the EPS entirely from microbial aggregates. The extraction strategies for the extractable EPS fractions in activated sludge using various cation-associated extraction methods were evaluated. Their results
show that the CER method is highly selective for the Ca- and Mg-bound EPS, whereas that the sulfide extraction method is selective for the Fe-bound EPS. On the other hand, the alkaline extraction is found to be less specific than these two methods, but it is more effective to extract the Al-bound EPS (Park and Novak, 2007). As no universal extraction method is available for the quantitative extraction of EPS from microbial aggregates, a method must be selected and optimized for each case, taking into consideration the sample characteristics. Several EPS extraction methods must be compared and the most appropriate method should be chosen carefully. A combined extraction was necessary to target different EPS fractions and that repeated extractions were always needed to obtain a high extraction efficiency. The cell lysis should be evaluated carefully for the combined and repeated extractions.

### 3. Analytical methods

#### 3.1. 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 (Raunkjær et al., 1994). 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 (Frolund et al., 1996). 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 (Frolund et al., 1996). 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.

Humic substances are very complex, and there are fewer appropriate methods for measuring their content in EPS. Frolund et al. (1995) proposed a modified Lowry method to determine the humic substance content by correcting the protein interference. The trichromatic content can be measured using the m-hydroxydiphenyl sulfuric acid method (Blumenkranz and Asboe-Hansen, 1973). The DNA or nucleic acid content is measured using the DAPI fluorescence method (Frolund et al., 1996), the UV absorbance method (Sheng et al., 2005a), or the diphenylamine method (Liu and Fang, 2002a). 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.

### Table 1

Methods for the extraction of EPS from various microbial aggregates.

<table>
<thead>
<tr>
<th>Method</th>
<th>System</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical methods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonication</td>
<td>UASB granule</td>
<td>The EPS part from the matrix under the impulsive pressure that is created by the sonication.</td>
<td>Quarmby and Forster (1995)</td>
</tr>
<tr>
<td>Sonication/centrifugation</td>
<td>Activated sludge</td>
<td>The EPS dissolve to solution under the impulsive pressure created by the sonication and centrifugal force.</td>
<td>Dignac et al. (1998)</td>
</tr>
<tr>
<td>High speed centrifugation</td>
<td>Anaerobic sludge</td>
<td>The EPS detach from cell surface and dissolve to solution under the centrifugal force.</td>
<td>Jorand et al. (1995)</td>
</tr>
<tr>
<td>Heating</td>
<td>Activated sludge</td>
<td>The molecular movement is enhanced that accelerates the EPS dissolution.</td>
<td>Fang and Jia (1996)</td>
</tr>
<tr>
<td>Chemical methods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline treatment</td>
<td>R. acidophila</td>
<td>Alkaline treatment by the addition of NaOH causes the groups, such as the carboxylic groups, to be ionized, resulting in a strong repulsion between the EPS and the cells, and thus makes the EPS dissolve in water.</td>
<td>Sheng et al. (2005a)</td>
</tr>
<tr>
<td>Sulfide</td>
<td>Activated sludge</td>
<td>Addition of sulfide to sludge could remove Fe by formation of FeS, resulting in a significant disintegration of the sludge floc structure.</td>
<td>Park and Novak (2007)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Anaerobic sludge</td>
<td>Divalent cations are very important for the crosslinking of charged compounds in the EPS matrix, and thus the removal of these cations using EDTA causes the EPS matrix to fall apart.</td>
<td>Fang and Jia (1996)</td>
</tr>
<tr>
<td>CER</td>
<td>Biofilms</td>
<td>CER removes the divalent cations, thus causing the EPS to fall apart.</td>
<td>Sheng et al. (2005a)</td>
</tr>
<tr>
<td>NaCl</td>
<td>P. aeruginosa</td>
<td>Cation exchange is promoted by using a high concentration of NaCl.</td>
<td>Bhaskar and Bhise (2006)</td>
</tr>
<tr>
<td>Acidic treatment</td>
<td>R. acidophila</td>
<td>Improves the repulsive force and disrupts the interaction between EPS and cells, causing the EPS to fall away from the cell surface.</td>
<td>Sheng et al. (2005a)</td>
</tr>
<tr>
<td>NH₄OH/EDTA</td>
<td>Activated sludge</td>
<td>This method combines the pH adjustment and ion exchange methods to improve the extraction efficiency.</td>
<td>Sato and Ose (1984)</td>
</tr>
<tr>
<td>Ethanol extraction</td>
<td>Activated sludge</td>
<td>Using a strong alkali such as NH₄OH reduces cell lysis. Denatures the EPS and reduces the binding force between EPS and cells.</td>
<td>Forster and Clarke (1983)</td>
</tr>
<tr>
<td>HCHO/NaOH</td>
<td>Activated sludge</td>
<td>The addition of HCHO reduces the cell lysis that is caused by the addition of NaOH.</td>
<td>Liu and Fang (2002a)</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Activated sludge</td>
<td>As glutaraldehyde has the ability to fix cells and denaturize EPS, it can also be used to extract EPS. Crown ether is used to combine divalent metals and disrupt the binding interaction between EPS and cells.</td>
<td>Azeredo et al. (1998)</td>
</tr>
<tr>
<td>Crown ether</td>
<td>Biofilms and activated sludge</td>
<td>The carbohydrate and protein-hydrolyzing enzymes were used to disrupt the structure of sludge and dissolve the EPS.</td>
<td>Wuertz et al. (2001)</td>
</tr>
<tr>
<td>Enzymatic extraction</td>
<td>Activated sludge</td>
<td></td>
<td>Sesay et al. (2006)</td>
</tr>
</tbody>
</table>
3.2. 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 2, 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 (Li and Ganczarczyk, 1990). Using the conventional scanning electron microscopy (SEM) and transmission electron microscopy (TEM), the microbial aggregates should be fixed and dried to the first, which would change the original conformation of EPS. The environmental scanning electron microscopy (ESEM) (Beech et al., 1996), atomic force microscopy (AFM) (van der Aa and Dufrene, 2002; Li and Logan, 2004) and CLSM (Zhang and Fang, 2001) 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 (Staudt et al., 2004).

The chromatography, mass spectrometry and their combination could be used to qualitatively and quantitatively analyze the EPS compositions (Dignac et al., 1998). The spectroscopy, including X-ray photoelectron spectroscopy (XPS) (Dufrene and Rouxhet, 1996; Omoike and Chorover, 2004; Ortega-Morales et al., 2007), Fourier transform infrared spectroscopy (FTIR) (Allen et al., 2004; Omoike and Chorover, 2004; Sheng et al., 2006b), 3-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM) (Esparza-Soto and Westerhoff, 2001; Sheng and Yu, 2006a), and nuclear magnetic resonance (NMR) (Manca et al., 1996; Lattner et al., 2003) 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 (Manca et al., 1996; Omoike and

Table 2
New analytical techniques used in EPS research.

<table>
<thead>
<tr>
<th>Analytical techniques</th>
<th>Descriptions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas chromatography (GC)</td>
<td>Qualitative and quantitative analyses of the monosaccharides and amino acids of EPS after hydrolysis.</td>
<td>Dignac et al. (1998) and Ortega-Morales et al. (2007)</td>
</tr>
<tr>
<td>High-performance liquid chromatography (HPLC)</td>
<td></td>
<td></td>
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<tr>
<td>Gas chromatography–mass spectrometry (GC–MS)</td>
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<tr>
<td>Scanning electron microscopy (SEM)</td>
<td>Observation of the microstructure and EPS of microbial aggregates after fixation and dewatering. Slices may need 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.</td>
<td>Li and Ganczarczyk (1990), Macleod et al. (1995) and Bura et al. (1998)</td>
</tr>
<tr>
<td>Transmission electron microscopy (TEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Environmental scanning electron microscopy (ESEM)</td>
<td>Technique for observing fully hydrated samples to obtain the original shapes and structure of EPS. 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.</td>
<td>Beech et al. (1996) and Surman et al. (1996)</td>
</tr>
<tr>
<td>Atomic force microscopy (AFM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confocal laser scanning microscopy (CLSM)</td>
<td>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.</td>
<td>Zhang and Fang (2001), Kawaguchi and Decho (2002), and Staudt et al. (2004)</td>
</tr>
<tr>
<td>Quartz crystal microbalance (QCM)</td>
<td>Be used to study the kinetics of EPS adsorption or cell adhesion onto material surface in situ and in real-time. 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.</td>
<td>Kwon et al. (2006), Long et al. (2009), and Zhu et al. (2009)</td>
</tr>
<tr>
<td>X-ray photoelectron spectroscopy (XPS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fourier transform infrared spectroscopy (FTIR)</td>
<td>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.</td>
<td>Allen et al. (2004), Omoike and Chorover (2004), and Sheng et al. (2006b)</td>
</tr>
<tr>
<td>Raman spectroscopy</td>
<td>Be used to study the chemical structure of EPS in situ. 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.</td>
<td>Wagner et al. (2009), Esparza-Soto and Westerhoff (2001) and Sheng and Yu (2006a)</td>
</tr>
<tr>
<td>3-Dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear magnetic resonance (NMR)</td>
<td>Can be used to study the molecular structure of carbohydrates and proteins and the interaction between EPS and metals.</td>
<td>Manca et al. (1996) and Lattner et al. (2003)</td>
</tr>
<tr>
<td>High-performance size exclusion chromatography (HPSEC)</td>
<td>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.</td>
<td>Frolund et al. (1996), Gorner et al. (2003), and Garnier et al. (2005)</td>
</tr>
<tr>
<td>Time-of-flight secondary-ion mass spectrometry (TOF-SIMS)</td>
<td>Be used to characterize the chemical compositions of EPS and the interaction of EPS and metals.</td>
<td>Pradier et al. (2005)</td>
</tr>
</tbody>
</table>
4. Characteristics of EPS

4.1. Adsorption characteristics of EPS

The EPS in microbial aggregates have many sites for the adsorption of metals and organic matters, such as aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates (Flemming and Leis, 2002). This reveals the potential roles of EPS in heavy metal sorption to bacterial cells and transporting in environments (Toner et al., 2005; Guine et al., 2006; Hu et al., 2007).

The presence of many functional groups in EPS, such as carboxyl, phosphoric, sulphydryl, phenolic and hydroxyl groups, can complex with heavy metals (Liu and Fang, 2002b; Joshi and Juwarkar, 2009; Ha et al., 2010). Based on the estimated numbers of the available carboxyl and hydroxyl groups, the EPS are regarded to have a very high binding capacity (Flemming and Leis, 2002; Guibaud et al., 2003; 2006). Proteins, carbohydrates and nucleic acids in EPS all have the abilities to complex with heavy metals (Dignac et al., 1998; Priester et al., 2006; Zhang et al., 2006). The binding capability and strength of the bonds between EPS and heavy metals were known to be high, and the adsorption obeyed the Langmuir or Freundlich equations (Bhaskar and Bhosle, 2006; Moon et al., 2006; Zhang et al., 2006). Furthermore, the soluble EPS might have a greater adsorptive ability for heavy metals than the bound EPS from sludge (Comte et al., 2006a).

The binding between EPS and divalent cations, such as Ca$^{2+}$ and Mg$^{2+}$, is one of the main intermolecular interactions in maintaining the microbial aggregate structure (Mayer et al., 1999). In the adsorption of heavy metal onto activated sludge, Ca$^{2+}$ and Mg$^{2+}$ were found to release into the solution simultaneously, indicating that the ion exchange mechanism was involved (Yuncu et al., 2006).

For instance, the EPS extracted using the CER method were aerobic biodegradable, while the EPS extracted using the sulfide method were anaerobic biodegradable. The small molecular substances that are produced as a result of EPS degradation can be used as carbon and energy sources for cell growth in conditions of nutrient shortage. EPS degradation can also result in the deflocculation of sludge flocs. The non-degradable portion of EPS may flow with the effluent from reactors and deteriorate the quality of the effluent.

4. Hydrophilicity/hydrophobicity of EPS

The EPS in microbial aggregates have many charged groups (e.g., carboxyl, phosphoric, sulphydryl, phenolic and hydroxyl groups) and apolar groups (e.g., aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates) (Flemming and Leis, 2002). The formation of hydrophobic areas in EPS would be beneficial for organic pollutant adsorption (Spaeth et al., 1998). The presence of hydrophilic and hydrophobic groups in EPS molecules indicates that EPS are amphoteric.

The relative ratio of these two groups is related to the composition of EPS. Jorand et al. (1998) used XAD resin to separate the hydrophilic and hydrophobic EPS fractions and found that approximately 7% were hydrophobic and mainly comprised proteins, whereas the hydrophilic fraction mainly consisted of carbohydrates. The analysis of monosaccharide and amino acid contents in EPS after hydrolysis revealed that approximately 25% of the amino acids were negatively charged and approximately 24% were hydrophobic (Dignac et al., 1998). The hydrophilicity/hydrophobicity of EPS is likely to significantly influence the hydrophobicity of microbial aggregates and their formation in bioreactors (Liu and Fang, 2003). It also demonstrates the importance of the EPS as the sorption sites for organic pollutants (Flemming and Leis, 2002).

5. Factors influencing EPS production

5.1. Substrate type

Substrate type has a substantial effect on the microbial communities in sludge, and the microbial metabolism, thus influences the production of EPS. Li and Yang (2007) reported that the sludge fed with glucose had more EPS production than that fed with acetate. Spaeth (2002, 2003) examined the EPS productions of the sludge from continuous stirred tank reactors treating various types of wastewaters. They found that under steady-state conditions the protein content was higher in the EPS from the sludge treating winery and municipal wastewaters than that treating pulp-paper, textile and petrochemical wastewaters. Sheng et al. (2006c) compared the EPS production from a photosynthetic bacterial strain using various substrates, and found that a bacterium would produce more EPS using benzoate as the substrate than using acetate, propionate and butyrate. This suggests that bacteria would excrete more EPS under unfavorable conditions.

5.2. Nutrient content

EPS can be degraded by bacteria as carbon and energy sources when there is a substrate shortage. Nutrient levels have a significant effect on EPS production and composition. The EPS content of the sludge increased with an increase in food to microorganism ratio (Jang et al., 2007). EPS production could be promoted when the phosphor was in short supply (Liu et al., 2006). Bura et al. (1998) and Hoa et al. (2003) also found that the carbohydrate content in EPS extracted from activated sludge increases when phosphorus is in short supply. Durmaz and Sanin (2001) found EPS in activated sludge to be rich in proteins but low in carbohydrates at a carbon to nitrogen ratio of 5, but as the carbon to nitrogen ratio increased to 40, the amount of protein decreased sharply whereas the amount of...
carbohydrates increased. Other researchers have found that activated sludge growing on wastewater with a low carbon to nitrogen ratio tends to produce EPS with a high proteins/carbohydrates ratio (Bura et al., 1998; Liu and Fang, 2003).

5.3. Growth phase

Jia et al. (1996) investigated the effects of cultivation time on EPS production in activated sludge, and found the EPS content to be closely related to the bacterial growth phase. During the exponential growth phase, the EPS content increased with cultivation time, but during the stationary phase it decreased with increasing cultivation time. In contrast, the EPS content from a photosynthetic bacterial strain decreased with cultivation time during the exponential growth phase, but remained almost unchanged during the stationary phase (Sheng et al., 2006b; Sheng and Yu, 2006b).

Solid retention time (SRT) also has a considerable effect on the production of EPS, but the results reported in literature are somewhat contradictory. Many researchers have found that the EPS in various microbial aggregates increases with an increasing SRT, which implies that bacteria produce more EPS in endogenous conditions. Sesay et al. (2006) found that an increase in SRT had a significant and positive correlation with the total quantity of EPS in activated sludge as well as the contents of proteins and carbohydrates in EPS. The ratio of proteins to carbohydrates also increased from 1.5 to 2.5 with an increase in SRT from 4 to 20 days. However, some researchers had suggested that EPS is independent of SRT. Liao et al. (2001) found that the EPS content did not change significantly with a longer SRT, and the protein/carbohydrate ratio increased as the SRT was increased from 4 to 12 days, but remained unchanged as the SRT was further increased from 12 to 16 days. Li and Yang (2007) reported that the TB-EPS of activated sludge had no relationship with the SRT, but that the LB-EPS decreased with an increasing SRT.

5.4. External conditions

As EPS are bound with cells mainly through ion bridging with multivalent metals, metal concentration may also influence the EPS content. Turakhia and Characklis (1989) and Sheng et al. (2006c) reported that the Ca\(^{2+}\) concentration had no effect on the EPS, whereas Higgins and Novak (1997) found that the protein content in the sludge EPS increased at higher Ca\(^{2+}\) or Mg\(^{2+}\) concentrations, and that higher Na concentrations led to a lower protein content. With the increasing concentration of Fe fed to sludge, the EPS characteristics and components could also be altered (Li, 2005).

In the presence of toxic substances such as heavy metals, microbial cells in activated sludge and biofilms produced more EPS to protect themselves from the harsh environment (Fang et al., 2002; Aquino and Stuckey, 2004; Priester et al., 2006). Furthermore, under toxic conditions the increase of the protein content far exceeded that of other components in EPS. As the concentration of toxic substances went over a threshold, its effect on the promotion of EPS production became less significant (Sheng et al., 2005b). However, some toxic substances, e.g., bismuth dimercaptopropanol, can also inhibit the production of EPS. At a level just below the minimum inhibitory concentration, the production of EPS of Brevundimonas diminuta could be significantly reduced. Total carbohydrates and proteins decreased by approximately 95% over a 5-day period after being exposed to bismuth dimercaptopropanol at a level near the minimum inhibitory concentration (Badireddy et al., 2008).

The shear rate of reactors can also influence the composition of EPS. An increase in shear rate or aeration intensity in SBRs could increase the EPS content in sludge (Adav et al., 2007). Research also found that the carbohydrate contents in EPS extracted from activated sludge increased with increasing air flow rate in a sequence batch reactor (SBR), whereas the protein contents remained almost unchanged at various air flow rates (Shin et al., 2001), which indicated that shear may stimulate bacteria to produce more carbohydrates. Ramasamy and Zhang (2005) found that a sudden increase in shear rate led to an increased carbohydrate content in EPS, but after a period of cultivation the carbohydrate content returned to the original level. The EPS in sludge also could be released by hydrodynamic or mechanic shear force, which would lead to an increase in the content of soluble EPS (Aquino and Stuckey, 2006; Sheng et al., 2006a).

The aerobic or anaerobic conditions also can influence the production of EPS. The EPS content of sludge would decrease under anaerobic conditions (Nielsen et al., 1996). It is reported that activated sludge flocs tend to disintegrate under oxygen limitation or depletion conditions. Such a disintegration might be caused by the suppression of EPS production or the hydrolysis of EPS as well. Shin et al. (2001) compared the EPS production of activated sludge in three bioreactors and found that at a high dissolved oxygen level the production of carbohydrates in EPS increased with time, whereas the protein content remained unchanged. At a low dissolved oxygen level, the concentrations of both carbohydrates and proteins are kept the same.

6. Relationship between EPS and functions of microbial aggregates

6.1. Mass transfer in microbial aggregates

In wastewater treatment reactors, EPS cover the surface or fill in the interior of cells of microbial aggregates. Li and Ganczarczyk (1990) observed the presence of plenty of EPS in the interior of activated sludge flocs with amorphous-phase surrounding cells. This suggests that substrate must pass through the EPS layer for transferring to cells. The pores in granular sludge can also be clogged by the EPS, which decreases the mass transfer efficiency of the substrates (Mu et al., 2006; Zheng and Yu, 2007). Generally, the component diffusion coefficients of EPS are lower than those of water, which means that EPS may influence the import of nutrients and the export of metabolic products. Characklis et al. (1990) claimed that as impermeable substances, EPS may prevent the permeation of dye to cells. EPS significantly influence the effective diffusion coefficients of substrates. A high level of EPS is not beneficial for substrate mass transfer. The permeability of anaerobic granules was found to be lower at a higher level of EPS (Mu and Yu, 2006). However, as EPS can adsorb organic substances and increase their concentration in the region of the cell surface, the role of EPS in mass transfer must be carefully considered (Hinson and Kocher, 1996).

6.2. Surface charge of microbial aggregates

As there are many charged functional groups in EPS, their content and composition influence the surface charge of microbial aggregates. However, the physicochemical characteristics of the different components in EPS are not identical, and thus they have different effects on the surface charge of aggregates. Usually, the EPS content had a positive effect on the net negative surface charge of sludge (Jia et al., 1996; Mikkelsen and Keiding, 2002a). Wilen et al. (2003) found that the total EPS content and the individual components both had a positive effect on the negative charge of sludge, and that the effects of proteins and humic substances were the most significant. Liao et al. (2001) reported that the carbohydrate content of EPS had a positive relationship with the net surface charge. On the contrary, Wang et al. (2005) investigated changes in EPS and the surface characteristics of sludge in the aerobic sludge granulation process, and found that the total EPS content and the protein and carbohydrate contents had a negative effect on the net surface charge of sludge. On the contrary, the DNA content had no significant effect on either the surface charge or the hydrophobicity of the sludge.
The ratios among EPS components have a more significant effect on surface charge of microbial aggregates than the content of individual components (Morgan et al., 1990). The proteins/carbohydrates ratio was found to have a negative effect on net surface charges of sludge, while total EPS content was found to have no influence (Liao et al., 2001). This attributed to the unique charge properties of proteins. The amino groups in proteins are positive and can neutralize the negative charges from carboxyl and phosphate groups, and thus decrease the net negative surface charges of sludge.

6.3. Flocculation ability of microbial aggregates

The flocculation ability of microbial aggregates is a key to the achievement of a low turbidity and a high quality of effluent. The interactions between EPS and cells have a significant effect on microbial flocculation ability (Morgan et al., 1990). In a study of the deflocculation of activated sludge under anaerobic conditions, bacteria and EPS were found to make up the main part of the deflocculated matter (Wilen et al., 2000), which indicated that EPS play an important role in flocculation.

There are two flocculation mechanisms induced by cations: double layer compression and ion bridging through EPS. A high ionic concentration would promote the bacterial flocculation and that such a phenomenon was attributed to the compressing double electric layer (Liu et al., 2007a). The ion bridging interactions between multivalent cations and EPS also play a key role in microbial flocculation (Sobek and Higgins, 2002; Nguyen et al., 2007). Multivalent cations (e.g., Ca\(^{2+}\) and Mg\(^{2+}\)) tend to bridge with EPS and thus improve the flocculation of microbial aggregates (Higgins and Novak, 1997; Liu et al., 2007a). An increase in the monovalent cations in activated sludge would deteriorate the sludge characteristics and floc structure (Kara et al., 2008). The dispersed cells in activated sludge tended to reflocculate with the addition of Ca\(^{2+}\) (Zita and Hermansson, 1994). To improve the flocculation ability of sludge, direct addition of multivalent cations could be one useful approach (Higgins et al., 2004).

The role of EPS composition in relation to flocculation has also been studied. Microbial aggregates tend to deflocculate after the removal of their surface proteins. Addition of a small amount of protein-hydrolyzing enzyme to a reactor would lead to the sludge deflocculation, while dose of a carbohydrate-degrading enzyme caused much less deflocculation (Higgins and Novak, 1997). Nucleic acids may also play an important role in flocculation. The bacterial flocculation ability worsened after the degradation of nucleic acids in the EPS of *Rhodovulum* sp., which was treated by nucleic acid hydrolase (Watanabe et al., 1998). Wilen et al. (2003) found that the flocculation ability of sludge increased with an increase in the protein content or a decrease in the humic substance content, and decreased with an increase in the total EPS content. These results imply that the influence of individual EPS components on the flocculation of microbial aggregates is complex. The ratios of the main EPS components may be more influential on the microbial flocculation (Liao et al., 2001). The authors also emphasized that readily extracted EPS are more beneficial for flocculation. Li and Yang (2007) indicated that the LB-EPS had a negative effect on sludge flocculation and excessive EPS in the form of LB-EPS could weaken cell attachment and result in poor flocculation.

The microbial cell flocculation can be described using the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) or extended DLVO theories (Bos et al., 1999; Liu et al., 2007a). In the DLVO theory the total energy of adhesion is the result of the van der Waals attractive forces and the generally repulsive interactions due to the interpenetration of the electrical double layers (Rijnaarts, et al., 1999), while the van der Waals forces, polar interactions, electrical double layer interaction and Brownian movement forces are taken into consideration by the extended DLVO theory. If the cell kinetics could overcome the total energy barrier in the DLVO curves, the cell could aggregate and flocculation would occur. The DLVO theory provides an effective way to evaluate the contribution of EPS to the sludge flocculation (Fig. 2) (Liu et al., 2010).

6.4. Settleability of microbial aggregates

Many studies have demonstrated EPS to have a negative effect on the settleability of microbial aggregates (jin et al., 2003). As EPS are negatively charged, a high concentration of EPS increases the surface charge of microbes, which results in an increase in the repulsive forces between cells and a decrease in the settleability of microbial aggregates (Morgan et al., 1990). The LB-EPS are also found to have a negative effect on the sludge settleability, due to the fact that an increase in LB-EPS content may bring more bound water into the aggregates, and therefore produce highly porous flocs with a low density (Yang and Li, 2009). The sludge volume index (SVI) is often used to characterize the settleability of sludge, with a low SVI value denoting good settleability.

Generally, the SVI of microbial aggregates increases as the EPS content increases. However, up to now, the effects of the main components of EPS on the settleability of microbial aggregates have not been well elucidated. Proteins and DNA contents in EPS would have more significant effects on the settleability of microbial aggregates. The DNA and protein contents in EPS were found to have a positive relationship with the SVI (Bura et al., 1998; Martinez et al., 2000; Liao et al., 2001), while the effect of the carbohydrate content in EPS on SVI was not significant (Liao et al., 2001; Hoa et al., 2003).

6.5. Dewatering ability of microbial aggregates

EPS can be regarded as key factors in the thickening and dewatering processes of sludge (Houghton et al., 2001; Mikkelsen and Keiding, 2002a). Two types of binding mechanisms between water molecules and EPS are involved: electrostatic interactions and hydrogen bonds. The former are active between the permanent dipole of the water of the functional groups of the EPS, and the latter are active between EPS hydroxyl groups and water molecules (Neyens et al., 2004).

Generally, the pressure-filtration is the main means for sludge dewatering. The specific resistance to or capillary suction time is commonly used to characterize the dewatering ability of sludge through press. An increase in EPS generally leads to a poorer sludge dewatering ability, possibly because the steric force that is generated by EPS prevents contact between cells. In addition, the
macromolecules in EPS cause the retention of much water in sludge flocs and increase the amount of interstitial water in such flocs. EPS can also form a stable gel that prevents water seepage from the pores of flocs, which deteriorates the dewatering ability of sludge. After EPS were removed, the sludge dewatering ability would be improved (Chen et al., 2001).

However, some studies have shown that sludge dewatering ability improves as the EPS content increases (Mikkelsen and Keiding, 2002a; Jin et al., 2004). With a higher EPS content, activated sludge had a lower shear sensitivity and lower degree of dispersion, leading to a good dewatering ability (Mikkelsen and Keiding, 2002a). Houghton et al. (2001) proposed that the effect of EPS on the dewatering ability of sludge depended on the content of EPS in sludge. Using sludge samples from eight wastewater treatments, they found that the dewatering ability of activated sludge initially increased with the EPS content, but then decreased once the EPS content exceeded a certain threshold. This suggests that a lower EPS content is more beneficial for flocculation, as this causes the cells to bind more tightly. As the EPS content further increased and exceeded a certain threshold, the water that was retained by the EPS significantly increased, which resulted in a lower sludge dewatering ability (Houghton and Stephenson, 2002).

The various components in EPS have different effects on the dewatering ability of microbial aggregates. The proteins had a high water-holding capacity. Thus, a reduced protein fraction in sludge improved the sludge dewatering ability (Sponza, 2002; Cetin and Erdincler, 2004). Increasing carbohydrate fraction in EPS would worsen the sludge dewatering ability (Cetin and Erdincler, 2004; Jin et al., 2004). The effect of humic substances on dewatering ability of sludge was insignificant.

6.6. Stability of microbial aggregates

The stability of microbial aggregates is essential to the solid/liquid separation process in biological wastewater treatment systems (Seka and Verstraete, 2003). Stability is defined as the ability of microbial aggregates to resist hydrodynamic and mechanical shear (Liao et al., 2002; Mikkelsen and Keiding, 2002b; Sheng and Yu, 2006c). Particles, bacteria, and EPS are eroded from the surface of aggregates as a result of hydrodynamic shear force (Mikkelsen and Keiding, 2002b). The structure of microbial aggregates is an important factor that governs the stability of flocs and the solid/liquid separation process (Chu and Lee, 2004; Sheng et al., 2006a). The EPS are involved in the structure of microbial aggregates and the interactions between cells. In addition, the main intermolecular interactions between cells, including polymer entanglement (Mikkelsen and Nielsen, 2001), bridging through EPS, and electrostatic interaction (Klausen et al., 2004; Sheng et al., 2006a), as well as van der Waals force and hydrogen bonds (Mayer et al., 1999), contribute to the stability of microbial aggregates. EPS are thus expected to govern to a certain extent the stability of microbial aggregates (Stoodley et al., 2002; Adav et al., 2008). A higher EPS content in sludge would result in greater sludge stability (Mikkelsen and Nielsen, 2001). Sheng et al. (2006a) proposed that the sludge had a multiple-layer structure with two distinct regions, as illustrated in Fig. 3. The outer region was a dispersible part, in which the dispersible sludge cells were glued by the readily extractable EPS. The inner region was a stable one, where the residual sludge cells were glued by other non-readily extractable EPS. After exposed to shear, the outer region would disperse. Thus, the readily extracted EPS also have a close relationship with sludge stability.

6.7. Adhesion ability of microbial aggregates

The microbial surface properties are important to the interfacial interactions between the cells and the solid surface, which is of crucial importance for biofilm formation in the aquatic environment. The adsorption of EPS to a material surface would alter the substrata physicochemical characteristics and hence influence the initial bacterial adhesion process (Gomez-Suarez et al., 2002; Omoike and Chorover, 2006). The presence of EPS on cell surfaces could enhance cell deposition in solid surface (Long et al., 2009; Tong et al., 2010). The carboxylate, phosphate and amine functional groups were found to contribute to the adhesion of bacteria to solid surface (Leone et al., 2006; Parikh and Chorover, 2006). After EPS were removed from the sludge surface, the number of attached microbial cells decreased (Park et al., 2000). With a similar surface charge density the EPS-rich strains had a much greater bacterial adhesion potential in porous media over the EPS-deficient strains (Liu et al., 2007b). The adhesion of microbial cells to a surface resulted from EPS deposition would also lead to biofilm formation or biofouling (Sand and Gehrke, 2006).

Both electrostatic and chemical bonding interactions are associated with the bacterial adhesion (Poortinga et al., 2002; Tsukada et al., 2003) found that if the EPS amount was relatively small, cell adhesion onto solid surfaces was inhibited by the electrostatic interaction, and that if it was relatively large, cell adhesion was enhanced by the polymeric interactions. Furthermore, the suppression of electro-repulsive forces promotes the adhesion (Tsukada et al., 2004). These interface force could be explored using classical DLVO or extended DLVO theories (Bos et al., 1999). The adhesion of EPS to solid surface was controlled by the non-DLVO forces, in addition to the DLVO interactions (Zhu et al., 2009). However, in both theories, the polymeric interactions and ion bridging are not taken into account, which are also the dominant interactions in microbial adhesion (Ong et al., 1999; Rijnants et al., 1999). Thus, more studies should be carried out to evaluate the contribution of EPS onto the bacterial adhesion.

6.8. Formation of microbial aggregates

Sludge granulation refers to the self-immobilization of microbes in biological wastewater treatment reactors, which results in a compact structure of aerobic and anaerobic granules. Microscopic pictures show that there are plenty of EPS in the interior of these aerobic and anaerobic granules (de Beer et al., 1996; Tan et al., 2001; McSwain et al., 2005). The interactions between EPS and microbial cells can influence the formation of granular sludge. Quarby and Forster (1995) found that the carbohydrate content in EPS and the granular strength both decreased simultaneously, which suggested that EPS played crucial roles in sludge granulation and the maintenance of the structure of granular sludge. Zhou et al. (2006) found that a slight overloading in UASB reactors would stimulate the EPS production and shorten the period of granulation. Addition of exogenous EPS to anaerobic reactors with deteriorated granules would lead to a significant recuperation of operational performance of the reactors
In the aerobic sludge granulation process, the EPS content increased with cultivation time at the initial stage, but remained constant after the granules had matured (Wang et al., 2005). All of these indicate the essential roles of EPS in microbial granulation. The composition and distribution of EPS influence the formation of microbial granules. EPS can bind cells closely through ion bridging interactions, hydrophobic interactions, and polymer entanglement, which serves to enhance and promote the formation of microbial granules, as shown in Fig. 4.

Bacterial biofilms are structured communities of cells enclosed in self-produced hydrated EPS matrix adherent to an inert surface. Large amounts of EPS are associated with the cells and contribute to the biofilm heterogeneity (Sutherland, 2001b). EPS are viewed as important mediators in the adhesion of bacteria to surfaces. They are involved in the adhesion process during biofilm formation, maintaining the structural integrity of biofilms and therefore the overall stability of biofilm communities (Wolfaardt et al., 1999). Recently, extracellular DNA was found to be a key component for bacterial biofilm formation and was required for the initial establishment of Pseudomonas aeruginosa biofilms (Whitchurch et al., 2002). The extracellular DNA served as a cell-to-cell interconnecting matrix component in biofilms (Allesen-Holm et al., 2006). It also has a spatial filamentous network structure, allowing access to suspended particulate organic matters and cooperative behavior and interactions in a community (Bockelmann et al., 2006).

7. Future work needed

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:

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.

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.

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.

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.

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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