Comparison of extracellular polymeric substances (EPS) extraction from two different activated sludges
Leiyan Zhang, Hongqiang Ren and Lili Ding

ABSTRACT

The characteristics of extracellular polymeric substances (EPS) extracted with five different extraction protocols from two different activated sludges were studied. The results showed that the major EPS constituent extracted by centrifugation was protein for the sludge in sequencing batch reactor treating chemical wastewater, and nucleic acid for the sludge in moving bed biofilm reactor treating synthetic urban wastewater. The order of EPS extraction amounting from the two sludges was formaldehyde + NaOH > formaldehyde + heating > EDTA > heating > centrifugation. The different extraction methods, the wastewater type, and activated sludge source greatly affected the amount and composition of EPS. The chemical extracted methods were more effective than the physical methods in extracting EPS for the two sludges. Moreover, formaldehyde combined NaOH was most effective in extracting EPS for the two sludges. However, chemical extraction could contaminate the EPS solution, which was pointed out by infra-red analysis and was also proved by cell lyses during EPS extraction and carrying over of the chemical extractant. Therefore, this study highlights that the choice of EPS extraction method should consider both the extraction yield and content and the contamination of extracting reagents to the EPS solution. The extraction procedures should be optimized and most effective.

Key words | extracellular polymeric substances, extraction method, sludge

INTRODUCTION

Extracellular polymeric substances (EPS) are metabolic products, resulting from active secretion from mainly sessile bacterial cells (Ying et al. 2010). EPS has various actions in the bacterial living system. EPS could play a protective role in harsh environments, and could also provide nutrition for microorganisms (Kazy et al. 2002). EPS connects organic compounds to each other by bridging inorganic ions such as Ca or Mg (Flemming & Wingender 2001). EPS was also found to be crucial to the flocculation (Frølund et al. 1996; Liu et al. 2001), sedimentation and dewatering of the activated sludge (Neyens et al. 2004; Li & Yang 2007), and membrane fouling (Drews et al. 2006a).

The EPS were composed of a variety of organic compounds (Frølund et al. 1996). Carbohydrate and proteins were major constituents of EPS (Comte et al. 2006a), and nucleic acids were also EPS constituents (Zhang et al. 1999). Liu & Fang (2002) indicated that the EPS normally contained small quantities of deoxyribonucleic acids (DNA), which were released from the dead cells after lysis. However, large quantities of DNA in EPS may be an alarming indication that cells were lysed during the harsh extraction process. Therefore, the DNA content in EPS could indicate the quality of the EPS extraction protocol. However, there is a lack of systematic research regarding their concentration in EPS.

Some research currently describes the advantages and disadvantages of the EPS extracted methods. For instance, the use of heat to extract EPS could induce hydrolysis of a part of EPS and decrease the amount of EPS (Comte et al. 2007). D’Abzac et al. (2006b) and Jahn & Nielsen (1995) explain that Na resin exchanges Na⁺ for divalent ions (mainly Ca²⁺ and Mg²⁺), which bind to EPS or the bacterial cell and could increase Na content in EPS solutions, and Na⁺ causes a rise in osmotic pressure of the liquid sodium and may induce cell lysis. Liu & Fang (2002) demonstrated that formaldehyde + NaOH was most effective in extracting EPS, where NaOH could increase the pH resulting in EPS solubility in water. However, Pan et al. (2010) indicated that ethylenediaminetetraacetic acid (EDTA) or formaldehyde + NaOH was used to extract low quantities of protein and polysaccharide. To date, a standardized
The extraction procedure has not been established. The amount and composition of EPS also strictly depends on the wastewater type, activated sludge source, sludge loading rate, and sludge age (Drews et al. 2006b).

Summarizing the previous literature, the main EPS extraction methods are used in this study, including centrifugation, heating, formaldehyde + heating, formaldehyde + NaOH, and EDTA, adjusting the amount of the EPS extracted solutions and the extractant in order to optimize the EPS extraction. This study chooses two different activated sludge sources, one is from treating synthetic urban wastewater and the other is from treating chemical wastewater. To date, the study of EPS extracted from activated sludges treating chemical wastewater is rare.

This study was conducted to compare the effectiveness of five extraction procedures for EPS and the difference in the amount and composition of EPS between the two different activated sludges. The contents of carbohydrate, protein, nucleic acid, and DNA in the extracted EPS samples were analyzed for comparison. An infrared (IR) investigation was also performed to highlight a potential modification of the EPS solution by the extraction procedure.

MATERIALS AND METHODS

Source of sludges

Two activated sludges were sampled from a moving bed biofilm reactor (MBBR) treating synthetic urban wastewater, and from a sequencing batch reactor (SBR) treating chemical wastewater. MBBR had 25 days of sludge age and 6 h of hydraulic retention, the process removed 95–97% chemical oxygen demand (COD) and 89–93% nitrogen from wastewater. SBR had 11 days of sludge age and 48 h of hydraulic retention, the process removed 21–90% COD from wastewater.

Extraction protocol of EPS

The EPS procedure was carried out according to methods developed by Liu & Fang (2002), D’Abzac et al. (2010b) and Pan et al. (2010). This included two physical extraction and three chemical extraction procedures. Two physical extraction procedures were performed: centrifugation by itself as the control method, and heating. Three chemical extraction procedures were performed: formaldehyde and then heating; formaldehyde and then NaOH, and EDTA. For the control, EPS was extracted from 20 mL sludge simply by high-speed centrifugation (19,900 g) for 20 min (CT15RT, Shanghai Tianmei Analytical Instruments Co., China), followed by the removal of supernatants and microbial cells through a 0.22 μm cellulose nitrate membrane and a dialysis membrane of 3,500 Da (TX01052, USA) before being stored at −20°C. For the other methods, the sludges were first extracted by heating (80°C, 10 min), formaldehyde + heating (0.6 mL, 37%, 1 h, 4°C), formaldehyde + NaOH (10 mL, 1 mol/L, 3 h, 4°C), or EDTA (20 mL, 2%, 3 h, 4°C). The extracted solutions were treated following the same procedures as the control.

Chemical analysis of extracted EPS

The carbohydrate content of EPS was measured by the anthrone method using glucose as the standard (Gaudy 1962). The content of protein in EPS was measured by the modified Lowry method (Frolund et al. 1995) using ovalbumin as the standard. The nucleic acid content in EPS was achieved by subtracting the inorganic phosphate from the total phosphate (Chen et al. 2005). The DNA content in EPS was measured by the diphenylamine colorimetric method using fish sperm DNA sodium salt as the standard (Chen et al. 2005).

IR spectrometry

EPS extracts were freeze-dried. Approximately 1 mg of EPS was mixed with about 180 mg of KBr to compress and form a pellet and was analyzed using a spectrometer (NEXUS870 American-Nicolet Co.).

Extraction yield calculating

The percentage (%) of dry weight (DW) of EPS to the dry weight of sludge was the extraction yield (DW EPS/DW sludge).

RESULTS

EPS quantities extracted from different sludges

Table 1 shows the amounts of EPS extracted from the two different activated sludge samples by five methods. The results indicated that the amount of EPS in the sludge was strongly dependent on the extraction method. The EPS content in each gram of volatile solid of activated sludge varied from 24.71 mg to 199.36 mg. The order of EPS extraction...
amount from the two sludges was formaldehyde + NaOH > formaldehyde + heating > EDTA > heating > centrifugation. Apart from formaldehyde + NaOH, the EPS quantities in the sludges of MBBR treating synthetic urban wastewater were much higher than that of SBR treating chemical wastewater.

For formaldehyde + NaOH, 191.99 mg and 199.36 mg EPS were extracted from each gram of volatile solids in the MBBR and SBR reactor, respectively. For comparison, the control method only extracted 54.04 mg and 24.71 mg EPS, respectively. The content extracted by the formaldehyde + NaOH method was three to eight times more than the control, one to three times more than formaldehyde + heating, one to four times more than EDTA and 41–80% more than heating.

**Constituents of EPS extracted from different sludges**

Table 2 compares the EPS constituents in the two activated sludges extracted by five methods. The results primarily analyzed the main composition of carbohydrate, protein, and nucleic acid, plus small quantities of DNA. Results showed that formaldehyde + NaOH was most effective in extracting protein and nucleic acid for the activated sludges of MBBR, and was most effective in extracting protein, carbohydrate and nucleic acid for the activated sludges of SBR. In extracting DNA, formaldehyde + heating was most effective for the two activated sludges.

Comparing the extraction methods of formaldehyde + NaOH and formaldehyde + heating, NaOH enhanced the extraction of protein and nucleic acid of EPS by formaldehyde in the two sludges, and for the sludge in SBR it also enhanced the extraction of carbohydrate. However, formaldehyde combined with heating extracted DNA of EPS 2–24 times as high as formaldehyde combined with NaOH for the two activated sludges.

In the two sludges, carbohydrate and DNA of EPS extracted using formaldehyde + heating were higher than that by heating alone. The contents of protein and nucleic acid of EPS in the sludge of MBBR and carbohydrate, protein, nucleic acid, and DNA of EPS in the sludge of SBR with EDTA were higher compared with the control method.

Concerning the sludge in MBBR, more than 60% organic matter of EPS was extracted by the extraction method, apart from EDTA. However, for the sludge in SBR, the percentage of the extracted organic matter of EPS was less than 54% with the five extraction methods. The maximal content of extracted organic matters of EPS from the two activated sludges all appeared in the chemical extraction methods.

---

**Table 1** | EPS extracted from the activated sludges in MBBR and SBR (mean ± SD)

<table>
<thead>
<tr>
<th>Extraction</th>
<th>MBBR EPS (mg per g VS)</th>
<th>SBR EPS (mg per g VS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>54.04 ± 3.31</td>
<td>24.71 ± 0.35</td>
</tr>
<tr>
<td>Heating</td>
<td>112.35 ± 6.88</td>
<td>39.54 ± 0.55</td>
</tr>
<tr>
<td>Formaldehyde + heating</td>
<td>185.45 ± 11.23</td>
<td>54.37 ± 0.76</td>
</tr>
<tr>
<td>Formaldehyde + NaOH</td>
<td>191.99 ± 11.75</td>
<td>199.36 ± 2.79</td>
</tr>
<tr>
<td>EDTA</td>
<td>167.81 ± 10.27</td>
<td>47.78 ± 0.67</td>
</tr>
</tbody>
</table>

**Table 2** | Constituents of EPS in each gram of the activated sludges in MBBR and SBR (mg/g) (mean ± SD)

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Protein</th>
<th>Carbohydrate</th>
<th>Nucleic acid</th>
<th>DNA</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MBBR sludge</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation</td>
<td>7.07 ± 0.11</td>
<td>3.75 ± 0.06</td>
<td>21.34 ± 0.48</td>
<td>0.95 ± 0.52</td>
<td>20.93 ± 4.47</td>
</tr>
<tr>
<td>Heating</td>
<td>53.98 ± 1.61</td>
<td>7.14 ± 0.02</td>
<td>9.41 ± 5.47</td>
<td>1.02 ± 0.41</td>
<td>40.79 ± 2.62</td>
</tr>
<tr>
<td>Formaldehyde + heating</td>
<td>32.43 ± 0.80</td>
<td>19.61 ± 4.93</td>
<td>26.90 ± 2.48</td>
<td>75.46 ± 0.41</td>
<td>29.04 ± 13.29</td>
</tr>
<tr>
<td>Formaldehyde + NaOH</td>
<td>76.74 ± 0.46</td>
<td>14.50 ± 0.30</td>
<td>50.40 ± 0.37</td>
<td>31.59 ± 0.41</td>
<td>18.76 ± 10.94</td>
</tr>
<tr>
<td>EDTA</td>
<td>10.49 ± 0.11</td>
<td>1.82 ± 0.60</td>
<td>31.06 ± 1.21</td>
<td>0.15 ± 0.00</td>
<td>124.30 ± 10.99</td>
</tr>
<tr>
<td><strong>SBR sludge</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation</td>
<td>2.17 ± 0.29</td>
<td>0.02 ± 0.01</td>
<td>0.74 ± 0.45</td>
<td>0.76 ± 0.22</td>
<td>21.03 ± 0.71</td>
</tr>
<tr>
<td>Heating</td>
<td>5.39 ± 0.43</td>
<td>0.51 ± 0.30</td>
<td>2.71 ± 0.88</td>
<td>0.95 ± 0.56</td>
<td>29.98 ± 0.14</td>
</tr>
<tr>
<td>Formaldehyde + heating</td>
<td>3.70 ± 0.05</td>
<td>0.80 ± 0.04</td>
<td>0.76 ± 0.61</td>
<td>24.57 ± 1.34</td>
<td>24.54 ± 1.41</td>
</tr>
<tr>
<td>Formaldehyde + NaOH</td>
<td>31.18 ± 1.49</td>
<td>2.65 ± 0.09</td>
<td>10.54 ± 0.02</td>
<td>1.34 ± 0.78</td>
<td>153.65 ± 1.97</td>
</tr>
<tr>
<td>EDTA</td>
<td>5.33 ± 0.14</td>
<td>0.39 ± 0.16</td>
<td>9.89 ± 9.23</td>
<td>0.95 ± 0.04</td>
<td>31.23 ± 8.53</td>
</tr>
</tbody>
</table>
IR analysis of EPS extracts

The EPS extracted by the centrifugation method from the two activated sludges investigated (Figure 1) showed similar IR spectra from a qualitative point of view. Several characteristic bands can be noticed: peaks at 3,200–3,420, 2,930–2,940, 1,630–1,660, and 1,130–1,160 cm⁻¹.

Figure 2 presents IR spectra of EPS samples extracted by the four protocols from the two activated sludges. EPS extracted by heating showed no notable difference from the control. IR spectra of the three chemical methods extracted EPS from the sludges in MBBR and SBR displayed different adsorption bands and was different from that of the control. The three IR spectra of EPS extracted by chemical methods showed particular bands at 1,550–1,600, near 1,350, and 1,040–1,080 cm⁻¹.

EPS extraction yield in the different sludges

Table 3 summarizes the extraction yields calculated from the dry weight of the two activated sludges and the dry weight of EPS obtained from the two activated sludges. These EPS extraction yields differed according to the sludge in the different reactor and there was a larger difference between the values in the two sludges. Furthermore, the EPS extraction yields differed according to the extraction protocol used, with the same trends for the two sludges: formaldehyde + NaOH > formaldehyde + heating > EDTA > heating > centrifugation. Chemical extraction yields were generally higher than the physical extraction ones. The formaldehyde combined with NaOH gave the highest extraction yields and allowed an effective extraction.

DISCUSSION

Organic constituents of EPS in the different activated sludges

The activated sludges sampled from MBBR treating synthetic urban wastewater and from SBR treating chemical wastewater were both in flocculent form. The results showed that the major EPS constituent extracted by centrifugation was protein for the sludge in SBR, and nucleic acid for the sludge in MBBR. However, in recent research, carbohydrate and protein were considered the main constituents of EPS in the activated sludge (Comte et al. 2006b).

<table>
<thead>
<tr>
<th>Extraction</th>
<th>MBBR (±SD)</th>
<th>SBR (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugation</td>
<td>4.19 ± 0.2</td>
<td>0.69 ± 0.0</td>
</tr>
<tr>
<td>Heating</td>
<td>8.70 ± 0.3</td>
<td>1.10 ± 0.0</td>
</tr>
<tr>
<td>Formaldehyde + heating</td>
<td>14.2 ± 0.5</td>
<td>1.52 ± 0.0</td>
</tr>
<tr>
<td>Formaldehyde + NaOH</td>
<td>14.9 ± 0.5</td>
<td>5.56 ± 0.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>13.0 ± 0.5</td>
<td>1.33 ± 0.0</td>
</tr>
</tbody>
</table>
These differences mainly depended on the nature and composition of the wastewater of these activated sludges treatment and the operating conditions of the reactors (Sponza 2003).

The ratio of protein and carbohydrate for the sludge in MBBR ranged from 1.71 to 7.56, and that in SBR was between 4.65 and 141.55. This compared with the reported ratios of 0.20–14.1 (D’Abzac et al. 2010a), 0.5–21.2 (Comte et al. 2006b) in previous studies. The discrepancy could be due to the failure to take account of humic substance in this study, leading to the overestimation of protein content (Liu & Fang 2002).

Comparing the sludge in MBBR and SBR, more than 60% of organic matter of EPS was extracted from the sludge in MBBR by the extraction methods apart from EDTA, but the sludge in SBR was less than 54% with the five extraction methods as the different wastewater compositions could affect the contents of EPS extracted. For example, a high level of protein (70 mg/g volatile suspended solids (VSS)) and a low DNA content (6 mg/g VSS) were measured in the EPS of the municipal activated sludge, while lower levels of protein and higher levels of DNA content were measured in the chemical floc EPSs (Sponza 2003).

IR spectra

Similar IR spectra were represented in the EPS extracted by the control method from the two sludges (Figure 1). The figures show five main characteristic bands. The large band between 3,200 and 3,420 cm\(^{-1}\) was assigned to the stretching vibration of OH into polymeric compounds, and another large band between 1,150 and 1,160 cm\(^{-1}\) was assigned to the stretching vibration of C-O-C into polysaccharide structures (Comte et al. 2006b). The visible weak peak between 2,930 and 2,940 cm\(^{-1}\) was assigned to asymmetric stretching vibration of CH\(_2\) related to aliphatic chains of proteins, carbohydrates, lipids, and humic acids. Both bands at 1,630–1,660 cm\(^{-1}\) assigned to stretching vibration of C=O and C=N (Amide I) and 1,540–1,550 cm\(^{-1}\) assigned to stretching vibration of C-N and deformation vibration of N-H (Amide II) were identified as protein (D’Abzac et al. 2010a).

Figure 2 shows the IR spectra of one physical method and three chemical methods. The IR spectra of the heating and control method were similar. However, IR spectra of the three chemical extracted EPS methods displayed different adsorption bands compared with spectra of the control method. In addition to the five main bands of the control method, they also showed another three main bands. For instance, the bands at 1,600–1,550 cm\(^{-1}\) related to carboxyl functions, a peak near 1,350 cm\(^{-1}\) related to the vibration of C=N, and the bands at 1,040–1,080 cm\(^{-1}\) related to stretching vibration of OH assigned to polysaccharide structures (D’Abzac et al. 2010a). There were several bands visible below 1,000 cm\(^{-1}\) in the IR spectra of the five extracted EPS methods which were assigned to phosphate, carbonate or sulfur functional groups.

The IR spectra obtained for EPS extracted by the formaldehyde + NaOH and formaldehyde + heating methods showed some bands such as 2,832, 2,493, 1,444, 1,384, and 1,123 cm\(^{-1}\), and some bands below 1,000 cm\(^{-1}\). These bands did not appear in the control spectra which may be interpreted as specific bands for formaldehyde or the result of a formaldehyde and EPS reaction (Comte et al. 2006b).

For the IR spectrum obtained for EPS extracted by the EDTA method, a band around 1,300 cm\(^{-1}\) was present and was characteristic of C-N stretching. Moreover, many fine bands below 1,000 cm\(^{-1}\) were attributed to inorganic functional groups. This demonstrated contamination of EPS by the chemical reagent (EDTA), due to the formation of the chelating reagent between EDTA and the EPS. The EDTA–EPS complexes were not removed by membrane separations, and thus the EDTA remained in the EPS after lyophilization (Liu & Fang 2002).

Effectiveness of EPS extraction

The efficiency of the extractions was mostly related to the extraction yield. The highest extracted yields were obtained with the chemical extraction procedures, in the form of formaldehyde combined with NaOH, as formaldehyde could react with the amino, hydroxyl, carboxyl, and sulfhydryl groups of protein and nuclei acids of the cell membrane and prevent cell lysis (Sutherland et al. 2008). Also, the presence of NaOH increased the pH and may allow the extraction of protein such as exoenzymes which surrounds the cells and could increase the EPS solubility (Wingender et al. 1999). Therefore, a great deal of EPS was extracted by this method. However, the chemical methods used for EPS extraction could contaminate EPS samples due to extracting reagents, which was pointed out by infra-red analysis (Figure 2).

The second most efficient extraction was formaldehyde combined with heating which was higher than the heating alone, but the residual formaldehyde could remain in the EPS solution altering their properties. At the same time, this method also extracted the highest amount of DNA.
This may be because the heating process disrupted the sludges facilitating their release and this method could extract bound EPS (Comte et al. 2006b). This indicated the presence of an abnormal cell lyses during the EPS extraction procedure.

Chemical extraction protocols were the most efficient to extract EPS in terms of quantity and the extraction yield, but carry-over of chemicals from extraction solution toward the EPS solution had to be taken into account.

**CONCLUSIONS**

The results showed that the major EPS constituent was protein for the sludge in SBR treating chemical wastewater, and nucleic acid for the sludge in MBBR treating synthetic urban wastewater. Apart from that extracted by formaldehyde + NaOH, the EPS quantities in the sludges of MBBR were much higher than that of SBR. The amount and composition of EPS strictly depended on the different extraction methods, the wastewater type, and activated sludge source.

IR spectra indicated that there was a similar trend in the EPS extracted by the control method from the two sludges. It was similar in the IR spectra between the two physical methods, but IR spectra of the three chemical extracted EPS methods displayed different adsorption bands. Moreover, there were several bands of inorganic functional groups in the three chemical extracted EPS methods.

The highest extracted yields were obtained with the chemical extraction procedures, which was formaldehyde combined NaOH. The second most efficient extraction was formaldehyde combined with heating. The chemical extraction protocols were the most efficient to extract EPS in terms of quantity and the extraction yield. However, according to IR spectra, cell lyses during EPS extraction and carrying over of chemicals extractant, the chemical extraction methods of EPS should be considered carefully.

**ACKNOWLEDGEMENTS**

We thank Xu Ke, Hu Jin, and Xiao Chun for field and laboratory support. This study was supported by the Jiangsu Planned Projects for Postdoctoral Research Funds of China (1101001B), the Jiangsu Technology Support Program of China (BE2010042), and the Jiangsu Natural Science Foundation (BK2011016).

**REFERENCES**


First received 17 November 2011; accepted in revised form 20 April 2012