Fractionation and characterization of sludge bacterial extracellular polymers by FT-IR, $^{13}$C-NMR, $^1$H-NMR


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Abstract Sludge bacterial extracellular (BEP) polymers exhibit a strong affinity with metals in addition to their role in the bioflocculation of activated sludge. It is of utmost importance in elucidating the chemical characterization of sludge BEP prior to a better understanding of its environmental behavior. Sludge BEP extracted from activated sludge was characterized by FT-IR, $^1$H-NMR, and $^{13}$C-NMR and fractionated into 6 fractions (hydrophilic acid, base, and neutral; and hydrophobic acid, base, and neutral). The results indicated that sludge BEP contained more polysaccharides but fewer amino acids or proteinaceous materials compared to dissolved organic matter (DOM) derived from sludge. No aromatic substance was found in sludge BEP as indicated by NMR. Sludge BEP was dominated by hydrophilic fractions, accounting for 65% of total DOC of sludge BEP and hydrophilic neutral constituted about 58% of the hydrophilic fraction. The chemical fraction and characterization indicated that sludge BEP might exhibit a higher capability in enhancing the transport of the associated contaminants through porous media than that of sludge DOM.

Keywords Bacterial extracellular polymers; hydrophilic; hydrophobic; sewage sludge; spectroscopy

Introduction

There has been a growing interest in adopting phytoremediation as a clean-up technology for heavy metal contaminated soils in the last decade (Baker, 1997; Blaylock et al., 1997). One key area of research in phytoremediation is to enhance the mobility and availability of heavy metals in soil. Addition of chelating agents such as EDTA has been demonstrated to be an attractive means of increasing the extractability of plant available Pb in contaminated soil (Salt et al., 1997). Sludge bacterial extracellular polymers (BEP), which are often composed of polysaccharides, protein, RNA and DNA, also exhibit a high affinity towards certain metal ions causing an enhanced transportation of metals in the environment (Geesey et al., 1988, 1989; Brown and Lester, 1982; Rudd et al., 1984; Mittleman and Geesey, 1985; Lion et al., 1988). Chen et al. (1995) studied the mobility of extracellular polymer in an aquifer sand and showed that BEP were relatively mobile and could reduce metal adsorption by over 90% at a BEP concentration of 10.6 mg L$^{-1}$. Lion et al. (1988) reported that surface sorption between BEP and mineral surface occur more frequently at low pH condition by the electrostatic attraction, ligand exchange with hydroxyl groups on the surface or by ternary surface co-adsorption of metal ions and BEP. Similar phenomenon is also observed is soils receiving dissolved organic matter (DOM) from sludge, compost, animal wastes, and crop residues (Baham and Sposito, 1983; Fischer et al., 1998; Lamy et al., 1993; Temminghoff et al., 1997; Zhou and Wong, 2001). The ability of DOM in mobilizing metals depends on the nature of their sources (Ohno and Cranell, 1996; Fischer et al., 1998; Zhou et al., 2001). Therefore, it is important to investigate the chemical nature of sludge BEP by spectroscopic techniques and chemical fractionation procedure in order to elucidate or predict the metal binding capability of sludge BEP.
Materials and methods
The preparation of sludge BEP and DOM

Anaerobically digested activated sewage sludge was collected from Suojincun sewage treatment plant in Nanjing, PR China and stored at 4°C for subsequent experiments. Bacterial extracellular polymers were extracted from the sludge using methods as described by Pavoni et al. (1972) and Czajka et al. (1997). Sludge was rinsed twice with double-distilled water and crude sludge BEP was harvested by centrifuging the rinsed activated sludge for 20 min at 32,000 × g and 4°C to effect shearing of polymeric materials from the microbial surface. Bacterial extracellular polymers commonly have molecular weight of 10^5 to 10^6 dalton (Sutherland and Ellwood, 1979; Hsieh et al., 1990), therefore, dialysis tubing (Spectra/Por 7, Spectrum Industries, California, US) having a nominal molecular weight cut-off (MWCO) of 8000 dalton was used to remove the low molecular weight impurities. The supernatant was placed in 8000 MWCO dialysis tubing and dialyzed against 18.5 MΩ/cm DDI water with constant mixing for 3 days at 4°C. Eight water exchanges were made to remove low-molecular-weight impurities from the interior solution of dialysis tubing. After dialysis, the polymer solution was lyophilized and stored at 4°C in a sealed glass tube before use. The dissolved organic matter derived from the same sludge was also collected through extracting sludge with DDI water at 10:1 (v/w) for 16 h followed by centrifuging at 12,500 × g for 20 min and filtered through 0.45 µm membrane. A subsample of the filtrate was dialyzed and lyophilized using the method mentioned above to obtain the DOM fraction. A subsample of the filtrate was directly lyophilized for spectroscopic analysis and fractionation of DOM. The dissolved organic carbon concentration of sludge BEP and DOM were determined by using a TOC analyzer (TOC-5000A, Shimaduz).

Fractionation of BEP and DOM

The freeze-dried sludge BEP and DOM were dissolved in DDI water prior to fractionation by “polarity” method into 6 different fractions i.e., hydrophilic acid (HiA), neutral (HiN), base (HiB), and hydrophobic acid (HoA), neutral (HoN), and base (HoB) using a macroreticular exchange resin as described by Leenheer (1981) with modifications as described in Zhou et al. (2000). The molecular size distribution of sludge DOM was determined by dialysis method using dialysis bags (Spectra/Por 7, Spectrum Industries, California, US) having a nominal molecular weight cut-off of 1,000, 3,500, 8,000, 15,000 and 25,000 dalton. To each dialysis bag, 20 mL of DOM were added and dialyzed against 1L of 0.01M K₂SO₄ for 12 h at 4°C with the external solution being replaced twice after 4 h and 8 h. Then the external dialysis solution 0.01M K₂SO₄ was replaced with DDW and the DOM solution in the dialysis bag was further dialysed for another 28 h with frequent replacement of external DDW. After dialysis, portion of interior solution was withdrawn from each bag for DOC analysis. Fractionation was performed in triplicate for each DOM.

Characterization of sludge BEP and DOM

Fourier transform infra-red spectroscopy (FT-IR) was used to determine the functional groups of BEP and DOM. Samples were prepared by mixing 2 mg of the freeze-dried BEP or DOM with about 100 mg of drier KBr, which was then pelletized under a pressure of 10 tonnes for 1 min. The spectrum of each freeze-dried DOM or BEP was recorded using a Nicolet, Magna-IR 550.

The ¹H-NMR spectra were recorded on a Bruker AC250 spectrophotometer operating at 67.8 MHz. The samples were prepared by dissolving 100 mg BEP or DOM in 1mL of 0.1M NaOD/D₂O. The ¹H-NMR spectra were recorded with the preset sequence (Sanders and Hunter, 1987) in order to eliminate the signal caused by D₂O. All chemical shifts were
measured on the basis of the tetramethylsilane external standard. Liquid-state $^{13}$C-NMR spectra of the BEP and sludge DOM were recorded on the same spectrometer at a resonance frequency of 67.8 MHz. At 1.8-s pulse delay and inverse gated decoupling, about 32,768 scans were recorded for the sample.

**Elemental analysis and the measurement of the amount of carboxyl-containing compound**

The relative proportion of C, H, and N atoms in sludge BEP and DOM were measured by using a CHNO/S analyzer (Perkin Elmer, USA). Total carboxyl-containing organic compound including protonated carboxyl, carboxylate and amino acids in DOM was also determined by a modified colorimetric method (Liu and Mo, 1985).

**Results and discussion**

**Chemical composition, fractionation and spectroscopic characterization**

Results of chemical analysis of sludge BEP and DOM are given in Table 1. The atomic ratio of C/N, H/C and H/N of sludge BEP were 1.84, 1.58 and 2.91 times that of those in DOM, respectively, indicating that there is relatively more H than N in sludge BEP. Sludge BEP contained higher amount of carboxyl-containing compound than sludge DOM, implying that there would likely be relatively more carbohydrates, especially polysaccharides, but less proteins or N-containing compounds than sludge DOM.

The FT-IR of sludge BEP and DOM demonstrated similar band contours but difference in the relative intensity of absorption bands (Figure 1). The main absorption bands of these spectra are: a broad band at 3,000–3,500 cm$^{-1}$ (stretching of H-bonded OH), a sharp peak at 2,100–2,400 cm$^{-1}$ (N-H stretching of amide groups, possibly originating from proteinaceous materials), a sharp peak at 1,600–1,700 cm$^{-1}$ (stretching C = O and N-H bending), a peak at 1,450–1,560 cm$^{-1}$ (–NH$_3^+$ or –NH$_2^+$-bending), a peak at 1,400 (C-H deformation of aliphatic group and –C=C– stretch of aromatic ring), a strong absorption at 1,000 to 1,150 cm$^{-1}$ (symmetric and asymmetric C-O stretch in polysaccharides and carboxyl), and a band and moderate strong peak at 570 to 670 cm$^{-1}$. (N-H out of plane bending, C-H

**Table 1** Atomic ratio among C, H, and N, and the amount of carboxyl-containing compound in BEP and DOM

<table>
<thead>
<tr>
<th></th>
<th>C/N</th>
<th>H/C</th>
<th>H/N</th>
<th>–COO$^-$ compound meq/g DOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEP</td>
<td>2.15* (0.22)#</td>
<td>13.40 (1.46)</td>
<td>28.81 (4.25)</td>
<td>25.9 (3.56)</td>
</tr>
<tr>
<td>DOM</td>
<td>1.17 (0.05)</td>
<td>8.47 (0.63)</td>
<td>9.91 (0.33)</td>
<td>17.1 (2.18)</td>
</tr>
</tbody>
</table>

* Values are means of triplicates
# Values in parentheses are standard deviation

**Figure 1** FT-IR spectra of sludge BEP and DOM
bending in aromatic ring or C-H deformation of carbohydrates). The interpretations of the FT-IR spectra were based on Morrison and Boyd (1983). Specific regions of interest are summarized in Table 2.

The absorption at 2,000–2,300 cm⁻¹, together with the relative strong bands at 600–670 cm⁻¹, suggested that –NH containing proteinaceous materials existed preferentially in BEP and DOM, while it showed that aromatic nuclei were not major contributors to the overall structure. Differences observed in the relative intensity of absorption bands for BEP and DOM can be ascribed to structural and chemical alteration due to different origins. The stronger peak at 1,640 cm⁻¹ and a stronger and broad peak at about 1,100 cm⁻¹ (–C=O of polysaccharides) suggested that sludge BEP had a considerably higher amount of aliphatic carboxylate than that of sludge DOM. On the other hand, more –NH substance such as ammonia and proteinaceous materials were found in sludge DOM as compared to sludge BEP because of the stronger and broader band at 1,400 cm⁻¹ for sludge DOM. Peak-height ratios can be used as crude measures for relative changes in functional group contents of humic substances and aqueous extracts of forest litter (Inbar et al., 1989; Gressel et al., 1995). Two reference peaks for FT-IR peak-height ratio calculations: –NH₃⁺ or –NH₂⁺ peaked at 1,406 cm⁻¹ and C-O peak of polysaccharide peaked at 1,100 cm⁻¹ were chosen. It was found that the 1,100/1,406 peak ratio (2.72) for BEP was greater than that in sludge DOM (1.47), indicating that BEP have more polysaccharides and fewer amino acids or proteinaceous material than sludge DOM. The results of the IR spectra were further supported by the results of chemical analysis that sludge BEP exhibited higher C/N and H/C atomic ratio, and higher –COO⁻ concentration. Other researchers also found that bacterial polymers are comprised mainly of polysaccharides, and to a less extent protein, DNA and RNA. The relatively higher –NH-containing compounds in sludge DOM can perhaps be contributed to proteinaceous material and formation of biodegradation products during sludge digestion. Kuo and Parkin (1996) noted that some typical chelating functional groups found in BEP from anaerobic treatment were carboxylates, hydroxyles, amines, sulhydral, and phenols.

It is however noted that aromatic rings as well as polysaccharides, carboxyl and amino sugar might all be present in IR regions such as 570 –670, 1,600–1,700 and 3,200–3,400 cm⁻¹. Therefore, it is important to validate the IR data with ¹H and ¹³C–NMR data. Many researchers indicated that ¹H and ¹³C–NMR spectroscopy provides more specific chemical structural information than any other techniques (Wershaw, 1994, 1996; Li and Shuman, 1996). The spectra of ¹H-NMR of BEP was given in Figure 2 and its assignments are based on related literatures (Zech and Alt, 1986; Kögel-Knabner, 1987; Candler et al., 1988). The strong signals in the 2- to 5-ppm region indicated carbohydrate structures instead of aromatic ring structure (Figure 2). This was consistent with the infrared spectra results and suggested that carbohydrates such as polysaccharides were generally one of the most important contributors to the chemical structure of BEP.

### Table 2 Possible FT-IR band assignments

<table>
<thead>
<tr>
<th>Band (cm⁻¹)</th>
<th>Proposed assignment</th>
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</thead>
<tbody>
<tr>
<td>3,235; 3,395</td>
<td>–OH and H-bonded O-H groups in amino sugar, polysaccharides, and phenols etc.</td>
</tr>
<tr>
<td>2,273; 2,372</td>
<td>N-H stretching, possibly originating from proteinaceous materials</td>
</tr>
<tr>
<td>1,640; 1,670</td>
<td>C=O stretching of carboxylate, –C=C– stretching of aromatic ring</td>
</tr>
<tr>
<td>1,560</td>
<td>–NH₃⁺ or –NH₂⁺-bending</td>
</tr>
<tr>
<td>1,406; 1,436, 1,408</td>
<td>–NH₃⁺ or –NH₂⁺-bending, symmetric and asymmetric C-H bending of aliphatic carbo-hydrates</td>
</tr>
<tr>
<td>1,018–1,123</td>
<td>symmetric and asymmetric C-O stretch in polysaccharides or carboxyl</td>
</tr>
<tr>
<td>570–643</td>
<td>N-H out of plane bending, C-H bending in aromatic ring or C-H deformation of carbo-hydrates</td>
</tr>
</tbody>
</table>
Figure 3 illustrates the characteristic $^{13}$C-NMR absorption of BEP. According to Kaiser et al. (1997), Wilson (1987), and Orem & Hatcher (1987), the $^{13}$C-NMR spectra of natural organic matter consisted of four regions: a) the alkyl region (0–50 ppm), mainly representing C atoms bonded to other C atoms (methyl, methylene, and methine groups); b) the O-alkyl region (50–110 ppm), mainly representing C bonded to O (carbohydrates, alcohol, and ethers); c) the aromatic region (110–160 ppm), representing C in aromatic systems and olefins; and d) the carbonyl region (160–210 ppm), including carboxyl C (160–190 ppm). The weak signals of the $^{13}$C-NMR spectra given in Figure 3 were due to limited solubility of freeze-dried BEP in D$_2$O. Almost all signals of the $^{13}$C-NMR spectra of BEP were in the range of 50–110 ppm, suggesting that carbohydrates such as polysaccharides should be a main contributor to BEP structure. The higher –C=O groups in sludge BEP as compared to sludge DOM were mainly come from carboxyl groups as confirmed from the obvious signal in the range of 160–200 ppm, which agreed with the above FT-IR spectra and chemical analysis. The combination of FT-IR and NMR spectra demonstrated that sludge BEP consisted mainly of polysaccharides and to a less extent substances containing a carbonyl group such as carboxyl, while aromatic substances were almost absent.
Sludge BEP and DOM were fractionated into 6 fractions including hydrophilic acid (HiA), base (HiB), neutral (HiN), and hydrophobic acid (HoA), base (HoB), and neutral (HoN). Sludge BEP was dominated by hydrophilic fractions instead of hydrophobic fractions. The former accounted for 65% of total DOC in sludge BEP (Table 3). Among the hydrophilic fractions, HiN displayed the highest percentage (37%) in sludge BEP, which accounted for almost 58% of hydrophilic fraction, while HiA and HiB had an almost equal share of the remaining hydrophilic fraction. However, for hydrophobic fractions of sludge BEP, HoB and HoN represented the two major components, which, respectively, constituted about 50% and 40% of the hydrophobic fraction of sludge BEP. It was concluded that the neutral fraction especially HiN was the major fraction of sludge BEP, while the acid fraction i.e., HiA + HoA, constituted less than 20% of sludge BEP. In contrast, almost the same amount of hydrophilic and hydrophobic fraction was found in sludge DOM. Hydrophilic and hydrophobic fractions were, respectively, predominated by HiA i.e., 72% of hydrophilic fractions and HoA i.e., 84% of hydrophobic fractions. Hence, the total acid fractions (HiA + HoA) were the major components of sludge DOM followed by base fractions.

According to Keefer et al. (1984), HiA is mainly comprised of uronic acids, simple organic acids, polyfunctional acids, or polyhydroxy phenols; HiN of carbohydrates, polysaccharides, polyfunctional alcohols or phosphate salts; HiB of mainly amino acids, amino sugar, low molecular weight amines, pyridine; while HoA is aromatic phenols, certain organic acids, anionic detergents, aromatic acid; HoN is hydrocarbons, fat, waxes, oils, amides, chlorinated hydrocarbons; and HoB is complex aromatic amines, nucleic acids. Therefore, sludge BEP was expected to contain much more carbohydrates and polysaccharides than sludge DOM because of the higher HiN fraction, which was in agreement with the results from FT-IR and NMR spectra. Similar results were also reported by other researchers, that sludge BEP contained high amounts of polysaccharide or proteinaceous materials (Pavoni et al., 1972; Norberg and Enfors, 1982; Brown and Lester, 1979; Czajka et al. 1997; Hejzlar and Chudoba, 1986). It is well documented that hydrophilic fractions of DOM had a greater ability to bind metals and their mobility were less retarded by soil as compared to hydrophobic fractions (Liang et al., 1996; Gu et al., 1995; Kaiser and Zech, 1997). It was implied that sludge BEP might enhance the transport of the associated contaminants through porous media more easily than sludge DOM because it might exist in the mobile form. Jardine et al. (1989) and McCarthy (1993) reported that if the DOM was immobilized by soil matrix during the transportation process, it would provide an adsorption site for pollutants. As a result, the mobility of the associated pollutants would be impeded.

The molecular size distribution of sludge DOM is listed in Table 4. In this study, sludge DOM existed predominantly in the molecular size fraction of <1,000 Da or >25,000 Da, whereas the intermediate molecular size fractions comprised less than 3.2% of total DOM. This distribution pattern was similar to that obtained for composts, leachate of waste disposal sites, soils and biosolids-amended soils in other studies (Han and Thompson, 1999;...
Homann and Grigal, 1992; Raber and Kögel-Knabner, 1997). Baham and Sposito (1983) also noted that approximately one-half of the organic compounds in the DOM from anaerobically digested sewage sludge had relative molecular masses of <1,500 Da. The higher amount of DOM fraction with a molecular size >25,000 Da for peat and sewage sludge can be explained by the degradation of low molecular weight compounds during the formation of peat and the anaerobic digestion of sewage sludge. Although no molecular distribution was done for sludge BEP, other studies showed that sludge BEP had a high molecular weight of $10^5$ to $10^6$ dalton (Sutherland and Ellwood, 1979; Hsieh et al., 1990).

The present study showed that sludge BEP contained more polysaccharides, and higher hydrophilic fraction through chemical characterization and fractionation study. It is expected that BEP would exhibit a higher affinity with metals and higher mobility in soil environment as compared to sludge DOM. This will require further experiments to demonstrate the chemical behaviour of BEP in soil and its influence on the mobility of heavy metals in soil.

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References


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Table 4: Distribution of molecular size (dalton) fractions of sludge DOM (%)

<table>
<thead>
<tr>
<th>Molecular Size Fraction</th>
<th>&lt;1,000</th>
<th>1,000–3,500</th>
<th>3,500–8,000</th>
<th>8,000–15,000</th>
<th>15,000–25,000</th>
<th>&gt;25,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sewage sludge</td>
<td>42.1</td>
<td>3.15</td>
<td>3.06</td>
<td>2.31</td>
<td>1.07</td>
<td>48.3</td>
</tr>
</tbody>
</table>


