Effects of extracellular polymeric substances on granulation of anoxic sludge in sequencing batch reactor
Binbin Wang, Shunlian Liu, Hongmei Zhao, Xinyan Zhang and Dangcong Peng

ABSTRACT
Variations of extracellular polymeric substances (EPS) and its components with sludge granulation were examined in a lab-scale sequencing batch reactor (SBR) which was fed with sodium nitrate and sodium acetate. Ultrasonication plus cation exchange resin (CER) were used as the EPS extraction method. Results showed that after approximately 90 d cultivation, the sludge in the reactor was almost granulated. The content of extracellular polysaccharides increased from 10.36 mg/g-VSS (volatile suspended solids) at start-up with flocculent sludge to 23.18 mg/g-VSS at 91 d with matured granular sludge, while the content of extracellular proteins were almost unchanged. Polysaccharides were the major components of EPS in anoxic granular sludge, accounting for about 70.6–79.0%, while proteins and DNA accounted for about 16.5–18.9% and 4.6–9.9%, respectively. It is proposed that EPS play a positive role in anoxic sludge granulation and polysaccharides might be strongly involved in aggregation of flocs into granules.

Key words | anoxic granular sludge, cation exchange resin (CER), EPS, SBR

INTRODUCTION
Extracellular polymeric substances (EPS) have been investigated extensively over the past two decades and have been known as a major component of the organic fraction of activated sludge, in which various microorganisms (mainly bacteria) are embedded (Frølund et al. 1996). The EPS aggregate bacterial cells into flocs, granules and/or biofilms. They also form a protective layer for the cells against the harsh environment, and serve as carbon and energy sources during starvation (Laspidou & Rittmann 2002; Liu & Fang 2002). Furthermore, EPS also play a crucial role in flocculation and dewatering of activated sludge (Frolund et al. 1996).

EPS from bioaggregates is a heterogeneous material. In compositional analyses of activated sludge flocs, granules or biofilms, many components of EPS, such as proteins, carbohydrates, deoxyribonucleic acids (DNA), lipids, uronic acids and humic substances, have been reported (Nielsen & Jahn 1999). Polysaccharides are identified as the predominant constituent in the EPS for many pure cultures (Sutherland & Kennedy 1996; Cescutti et al. 1999). However, Frolund et al. (1996) state that proteins, instead of polysaccharides, are the largest EPS fraction in most activated sludges. The composition of EPS may be affected by the conditions under which the bioaggregates are grown. However, there is no doubt that EPS mainly consist of proteins and polysaccharides.

EPS have been detected in significant amounts in both aerobic granular sludge (Tay et al. 2001; McSwain et al. 2005; Chen et al. 2007; Adav et al. 2008a,b) and anaerobic granular sludge (Schmidt & Ahring 1996; Liu et al. 2002; Simon et al. 2009). Intensive research has shown that EPS are a major component for the biogranule matrix material in both aerobic and anaerobic granules (Liu et al. 2004). Schmidt & Ahring (1996) reported that the formation of granules is correlated with the production of EPS. Adav et al. (2008b) stated that the granule structure was stabilized by a network principally composed of β-polysaccharides as the backbone for embedded proteins, lipids, α-polysaccharides, and cells. On the contrary, McSwain et al. (2005) and Chen et al. (2007) demonstrated that granule formation and stability are dependent on a noncellular, protein core. It means that not all the components of EPS can assist granulation...
and enhance granule stability. The variations of EPS and its components during the whole granulation process may reveal which components play a major role. However, this information is limited, especially in anoxic conditions.

Nitrate contamination is a pervasive problem for water systems due to natural and human activities. High nitrate levels in water bodies can cause eutrophication and pose human health risks. Nitrate contamination of surface water and ground water has become widespread due to the extensive use of fertilizers in agriculture and improper discharge of sewage and industrial effluents. Removal of nitrate in high nitrate contaminated wastewater (such as fertilizer, explosives, pharmaceutical, metal finishing and nuclear industries wastewater) can limit the further contamination of water systems. Physical and chemical treatment processes such as ion exchange, reverse osmosis, membrane filtration and electrodialysis are effective for removing nitrate, but are expensive and produce waste streams with high levels of nitrate and other salts (Nancharaiah & Venugopalan 2011). Biological denitrification is the most acceptable method to remove the nitrate from water and wastewater. In biological treatment process, nitrate is converted to nitrogen gas and not just transferred to a secondary waste stream that requires subsequent treatment and disposal as physical and chemical treatment process. Due to the high concentration of nitrate in wastewater which is generated from industries, a biological denitrification system which has a high conversion rate of nitrate is desired.

In biological treatment systems, biomass in the form of granular sludge makes it possible to maintain high cell concentrations in the reactor and thereby achieve high conversion rates desired for the treatment of concentrated effluents. In addition, due to their compact structure, granules can tolerate high concentrations of pollutants. Therefore, anoxic granular sludge is gaining increasing interest in treating high nitrate contaminated wastewater. Wan & Sperandio (2009) evaluated and quantified the influence of denitrification of nitrate on the aerobic granular sludge development. It is reported that the faster growth of denitrifying bacteria would induce densification of the particles and enhance granule formation.

In this study, the variations of EPS and its components during the granulation of anoxic activated sludge in sequencing batch reactor (SBR) were examined. The objective is focusing on which components of EPS plays the major role in the granulation of sludge.

### MATERIAL AND METHODS

#### Granular sludge cultivation

Anoxic sludge was cultivated in SBR which had a working volume of 4 L with 30 cm in height and 18 cm in diameter. Liquor mixing was achieved using a vertical mixer running at 100 rpm. Peristaltic pumps were employed for influent feed and effluent withdrawal. The exchange ratio was controlled in 1/2 which corresponded to a 6 h of hydraulic retention time (HRT). The operating conditions, such as feed rate, mixing time, settling time and withdraw rate, were controlled automatically by programmable logic controller (PLC). The oxygen reduction potential (ORP) temperature and pH were monitored online by the probes (Mettler-Toledo, Switzerland) which connected to a data acquisition program. This reactor had worked with anoxic flocculent sludge for about 265 d for other research projects before this experiment. Sodium acetate and sodium nitrate were used as the carbon source and electron acceptor throughout this experiment. Sodium acetate and sodium nitrate were used as the carbon source and electron acceptor throughout the whole operation period. Small part of the sludge was found to granulate in previous study.

The compositions of synthetic wastewater and trace element used in this study are shown in Tables 1 and 2, respectively. The influent pH value was adjusted to 6.0–6.5 through dosing 6 M HCl. The reactor was operated with 6 min of influent filling, 163 min of stirring, 4 min of settling, 4 min of effluent withdrawing, and 3 min of idling. Setting a very short sedimentation time is aimed at washing out slow settling biomass and selecting fast settling granules in the reactor. The whole cultivation process was kept at 25±1°C.

#### EPS EXTRACTION

##### Selection of extraction methods

Literature screening showed that there are many methods for EPS extraction from sludge, such as centrifugation, reverse osmosis, membrane filtration, electrodialysis and chemical treatment processes such as ion exchange, reverse osmosis, membrane filtration and electrodialysis are effective for removing nitrate, but are expensive and produce waste streams with high levels of nitrate and other salts (Nancharaiah & Venugopalan 2011). Biological denitrification is the most acceptable method to remove the nitrate from water and wastewater. Wan & Sperandio (2009) evaluated and quantified the influence of denitrification of nitrate on the aerobic granular sludge development. It is reported that the faster growth of denitrifying bacteria would induce densification of the particles and enhance granule formation.

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#### Table 1 | Synthetic wastewater components

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD (NaAc)</td>
<td>200</td>
</tr>
<tr>
<td>NO3-N (NaNO3)</td>
<td>40</td>
</tr>
<tr>
<td>P (KH2PO4)</td>
<td>5</td>
</tr>
<tr>
<td>NH4-N (NH4Cl)</td>
<td>6</td>
</tr>
<tr>
<td>Trace element</td>
<td>0.05 (mL/L)</td>
</tr>
</tbody>
</table>
ultrasonication, alkaline treatment, ethylenediamine tetra-acetic acid (EDTA) treatment and cation exchange resin (CER) treatment. The methods for EPS extraction impact results greatly in some cases. Their effectiveness and limitations have been evaluated by many researchers. The CER method is considered as the most widely accepted EPS extraction method because of its high efficiency and low cell lysis (Frølund et al. 1996; Nielsen & Jahn 1999; Sheng et al. 2010). Compared with the flocculent sludge, granular sludge had more compact structure, so ultrasonication was used to disintegrate the granules before CER extraction (Dignac et al. 1998; D’Abzac et al. 2010).

Another point that needs to be addressed is that the extracted EPS in this study is mainly bounded EPS according to the extraction procedure. Total EPS can be classified into different categories, such as free EPS (first wash), half-bounded EPS (buffer wash) and bounded EPS. EPS is believed to be essential to biomass aggregates (such as floc, granule or biofilm) formation, but excessive EPS in the form of loosely bounded EPS (free or half bounded) may deteriorate cell attachment and weaken the aggregates structure (Li & Yang 2007). Bounded EPS should be the main part of the total EPS and play the major role in granulation of anoxic sludge. Therefore, bounded EPS was specifically determined in this study.

### Sludge handling and EPS extraction

The sample taken from the reactor was first washed with distilled water to remove any soluble components in the sludge and then centrifuged at 5,000 rpm for 15 min at 4 °C. After that, the sludge pellets were resuspended to 30 ml using a buffer consisting of 2 mM Na₃PO₄, 4 mM NaH₂PO₄, 9 mM NaCl and 1 mM KCl at pH 7 (Frolund et al. 1996) and then centrifuged at 5,000 rpm for 10 min. This process was repeated twice to obtain clean sludge.

The EPS extraction was performed as follows: the handled 50 ml sludge was sonicated at 20 W for 2 min. It was then transferred to extraction conical flask and CER (Dowex, 50 W×8, Fluka 44514) was added (60 g-CER/g-VSS). The flask was put into a shaker at a speed of 200 rpm for 2 h. After that, the mixture of CER and sludge was centrifuged for twice to remove the solids (5,000 rpm for 10 min at 4 °C; 12,000 rpm for 15 min at 4 °C). Finally, the supernatant was filtered by a membrane (0.45 μm) to remove any remaining insoluble substances.

### Chemical composition analysis

The Anthrone method (Raunkjær et al. 1994; Frolund et al. 1996) was applied for carbohydrate determination and glucose was used as the standard. The Lowry method (Frolund et al. 1996) was applied for protein determination and bovine serum albumin (BSA) was used as the standard. The DNA content in EPS was measured by the Diphenylamine colorimetric method (Liu & Fang, 2002) using calf thymus (CT) DNA as the standard.

### Other components

The particle size distribution of granular sludge was determined according to the method proposed by Laguna et al. (1999). All other components, such as chemical oxygen demand (COD), nitrate, nitrite, etc. were analyzed according to Standard Methods for the Examination of Water and Wastewater (APHA 1998).

### RESULTS AND DISCUSSION

### COD and nitrogen removal

The experiment lasted for about 100 d. The average removal rate of COD and nitrate were 91.49 and 98.02%, respectively. The profiles of NO₃⁻-N and COD variations during reaction period in a typical cycle (81 d) are shown in Figure 1. Nitrate in the mixed liquor was utilized quickly by the denitrifying bacteria in the first 20 min. Meanwhile,
there was an accumulation of nitrite (about 7.85 mg/L), which was rapidly consumed in the following 40 min. Change of COD corresponded well with nitrate consumption profile. A minimum COD was reached after approximately 30 min.

Change of ORP during a typical cycle (79 d) was also in well accordance with the NOx-N profile (Figure 2). As there might be a bit of dissolved oxygen (DO) in the influent synthetic wastewater, the ORP was 51 mV at the beginning of the reaction. However, the DO was consumed rapidly and ORP was maintained between −100−0 mV until the NOx-N was depleted. After that, due to the fermentation process, ORP dropped gradually to −212 mV in the following 90 min.

**Granulation of sludge and bounded EPS**

In order to evaluate sludge granulation, the particle size distribution of sludge in the reactor was determined for different time intervals (Figure 3). Flocculent sludge was the major component (accounting for about 81% of total suspended solid) in the reactor at the beginning of the experiment. After approximately 90 d cultivation, the sludge in the reactor was almost granulated. The granules which had a diameter between 1 and 2 mm accounted for about 78% of the total suspended solid on 91 d.

It is generally believed that the EPS normally contain small quantities of DNA, which are released from the dead cells after lysis. Large quantities of DNA in the EPS can be an alarming indication that the cells are lysed during the harsh extraction process (Liu & Fang 2002). Literature screening showed that the content of DNA accounts for about 3.8–16.5% of the total amount of EPS (Nielsen & Jahn 1999). In this study, the content of DNA in EPS is small, accounting for about 4.6–9.9% of total amount of EPS.

The variations of bounded EPS as well as its components with anoxic sludge granulation are shown in Figure 4. There was a significant increase of bounded EPS in the process of sludge granulation. The total amount of bounded EPS increased from 14.69 mg/g-VSS at start-up with flocculent activated sludge (5 d) to 29.37 mg/g-VSS at 91 d with granular sludge. Bounded EPS play a positive role in the process of anoxic granular sludge formation.

![Figure 1](image1.png) | Profiles of NOx-N and COD during reaction period in a typical cycle (81 d).

![Figure 2](image2.png) | The profile of ORP variations during reaction period in a typical cycle (79 d).

![Figure 3](image3.png) | Evolution of granular sludge particle size distribution in the lab-scale SBR.

![Figure 4](image4.png) | Bounded EPS, proteins, polysaccharides and DNA in different stages of granular sludge.
This result is consistent with previous studies on aerobic and anaerobic sludge granulation in which the accumulation of EPS has been correlated with biological adhesion and aggregation processes (Tay et al. 2001; Liu et al. 2002).

Results in Figure 4 also show that polysaccharides increased substantially (from 10.4 to 23.2 mg/g-VSS) with the granulation of anoxic sludge, while the content of proteins was almost unchanged. The increase of bounded EPS is mainly contributed by the increase of polysaccharides. This observation suggests that polysaccharides, rather than proteins, play a major role in the sludge granulation process. In fact, one of the most important functions of extracellular polysaccharides is supposed to be their role as fundamental structural elements of the EPS matrix determining the mechanical stability of biological aggregates. Therefore, polysaccharides might be strongly involved in aggregation of flocs into granules.

Another point that needs to be addressed is that protein was made of small fraction of bounded EPS (account for about 16.5–18.9%) in this study. It is contrary to the views that it is the predominant component of EPS (Frølund et al. 1996) and consistent with the views that EPS are mainly composed of carbohydrate (Sutherland & Kennedy 1996; Cescutti et al. 1999). This situation may be attributed to the fact that the substrate used in this experiment was acetate which has a relative molecular weight of less than 100 Da and could be taken up directly by bacteria. Little complex exoenzyme (protein) which is an integrated part of the EPS matrix (Frølund et al. 1995) is required.

CONCLUSIONS

Anoxic sludge was cultivated in a lab-scale SBR which was fed with sodium nitrate and sodium acetate. After about approximately 90 d cultivation, the sludge in the reactor was almost granulated. The content of bounded EPS in the sludge increases with the sludge granulation. Polysaccharides, rather than proteins, are the major components of bounded EPS in anoxic granular sludge and might be strongly involved in aggregation of flocs into granules.

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