Rapid cultivation of aerobic granular sludge by bone glue augmentation and contaminant removal characteristics
Shuo Wang, Wenxin Shi, Shuili Yu and Xuesong Yi

ABSTRACT
To achieve a quick start-up and stable operation, aerobic granular sludge (AGS) was cultivated in a sequencing batch airlift reactor (SBAR) with the addition of bone glue augmentation. Adding an amount of bone glue (40 mg L\(^{-1}\)) can accelerate granulation, which advanced by 10 d on average. Aerobic granules of size 0.5–3.0 mm were dominant in the SBAR and the settling velocity acquired a better correlation with the size of the AGS. In addition, the content of total polysaccharides was 19.54 mg gMLSS\(^{-1}\) (grams of mixed liquor suspended solids) (an increase of 34.0%), the content of total protein was 60.59 mg gMLSS\(^{-1}\) (an increase of a factor of 33) and the total proteins/total polysaccharides ratio was 3.3. The relatively high protein content was an essential feature for cultivation of AGS, which may indicate that extracellular polymeric substance was the mechanism for granulation due to the adhesion of microorganisms by bone glue. AGS possessed better chemical oxygen demand, NH\(_4^+\)-N and PO\(_4^{3-}\)-P removal efficiency (of 86.7, 90.6 and 93.8%, respectively) and no nitrite accumulation was observed in the whole process.

INTRODUCTION
Aerobic granular sludge (AGS) is a special biofilm structure which was developed nearly 20 years ago. Compared with activated sludge, the clear surface and compact structure of AGS confer many advantages such as better settling properties, lower consumption and higher biomass retention and treatment efficiency (Beun et al. 2002). The AGS technique has therefore been widely used in organic wastewater treatment (Moy et al. 2002) and the treatment of wastewater with heavy metal or toxic substances (Xu et al. 2005; Sun et al. 2011). To date, many pilot-scale AGS reactors have been established all around the world (Lin et al. 2010; Jungles et al. 2011).

Liu & Tay (2002) proposed four steps corresponding to the formation process of AGS at ambient temperature: (1) microbe-to-microbe contact; (2) aggregates formed by physical and chemical forces; (3) microbial forces to form aggregates by the secretion of extracellular polymeric substance (EPS); and (4) hydrodynamic shear force. EPSs are sticky materials secreted by microorganisms, comprising polysaccharides, proteins, humic acids and lipids (Adav et al. 2008). Accumulation of EPS is correlated with the occurrence of biological adhesion and microbial aggregation, which promotes aerobic granulation. Many studies considered that polysaccharides were the key component in aerobic granulation since they facilitate cell-to-cell adhesion (Tay et al. 2001; Liu & Tay 2002); many others found that proteins were the significant component as they were enriched in the sheared granules (McSwain et al. 2005; Chen et al. 2007). No conclusions can therefore be drawn about which components in EPS are more important in the formation process of AGS.

In addition, the complex cultivation of AGS and the quick start-up and stable operation of the AGS reactor have restricted its development and application. Consequently, it is of great importance to focus on the formation mechanism of AGS and the rapid cultivation of AGS by adding divalent metals, trivalent metals or coagulant aids.

Li et al. (2009) and Wang et al. (2012) reported that divalent metals and trivalent metals could be helpful for aerobic granulation because of their important roles in the self-immobilization of microorganisms, which can decrease the formation time of AGS and enhance biomass retention and
efficiencies of contaminants removal. No studies have, however, focused on the functions of coagulant aids in aerobic granulation. Bone glue is a type of low-cost coagulant and is completely safe and environmentally benign. In this study, the formation of AGS enhanced by bone glue and the characteristics of AGS and its EPS are studied. Furthermore, the contaminants removal by AGS and the size distribution of AGS have been investigated. It is expected that this study could provide some fundamental information on the feasibility and practical application of AGS by bone glue augmentation.

**METHODS**

**Experimental set-up**

Compressed air was supplied via a diffuser at the bottom of a sequencing batch airlift reactor (SBAR, working volume 5.0 L) at a flux of 0.20 m³ h⁻¹; dissolved oxygen (DO) (close to saturation) was not controlled in the reactor. Influent was introduced at the bottom of the reactor by means of a peristaltic pump. Effluent was discharged from the middle port of the reactor with a volumetric exchange ratio of 50%. The operation cycle time was 6 h, including 30 min for idle, 30 min for feeding, 5 min for settling, 5 min for effluent discharge and the rest of the time for aeration. The operation temperature of the SBAR was controlled at 25 ± 1 °C by a water bath. Accordingly, influent pH and solids retention time were adjusted to 7.0–7.2 and 30 d, respectively.

The seed activated sludge was taken from the aerobic tank of Wenchang (Harbin, China) wastewater treatment plant with an anoxic/oxic process. The components and their concentrations in the synthetic wastewater were listed as (mg L⁻¹): NaAc 850.0, CaCl₂ 60.0, MgSO₄ 42.0, NH₄Cl 240.0, EDTA 42.0, NaHCO₃ 250.0, K₂HPO₄ 58.0 and KH₂PO₄ 24.0. Element solution of 1 mL gave a total chemical oxygen demand (COD) concentration of 1,200 mg L⁻¹; NH₄⁻N concentration 100 mg L⁻¹ and PO₄³⁻-P with a concentration of 16 mg L⁻¹. The trace element solution (Wang et al. 2012) contained the following components (g L⁻¹): FeCl₃·6H₂O 1.5, H₂BO₃ 0.15, CuSO₄·5H₂O 0.03, KI 0.03, MnCl₂·4H₂O 0.12, Na₂MoO₄·2H₂O 0.06, ZnSO₄·7H₂O 0.12 and CoCl₂·6H₂O 0.15.

**Experimental procedure**

After 2 months’ domestication, activated sludge under different bone glue concentrations (0, 10, 20, 30, 40, 50 and 60 mg L⁻¹) was inoculated in seven SBARs (working volume of 5.0 L) referred to as R0–R6. The operation cycle time, the components and their concentrations of the wastewater and trace element solution were all the same as the synthetic wastewater. The reactors were operated for more than 6 months to investigate the long-term performance of AGS under different bone glue concentrations.

The property of AGS was evaluated by two major parameters: mean diameter growth rate and specific oxygen uptake rate (SOUR). The following equations define the average diameter \(d_{pj}\) and mean diameter growth rate \(\mu\) of AGS:

\[
d_{pj} = \frac{\sum_{j=1}^{n} \Delta p_j d_j}{\sum_{j=1}^{n} \Delta p_j}
\]

\[
\mu = \frac{d_{(t+10)} - d_{t}}{t}
\]

where \(d_{pj}\) (mm) and \(\mu\) (μm d⁻¹) are the predicted size and growth rate of AGS; \(d_j\) (mm) is the mean diameter of AGS in group \(j\); \(\Delta p_j\) (%) is the total suspended solids (TSS) for group \(j\); \(d_i\) and \(d_{(t+10)}\) are the mean diameter of AGS at time \(t\) (d) and 10 d after time \(t\) respectively.

**Analytical methods**

COD, NO₃⁻-N, NO₂⁻-N, NH₄⁺-N, PO₄³⁻-P and mixed liquor suspended solids (MLSS) were measured according to Standard Methods (APHA 1998). Granule size and their distribution were determined with a standard sample sieve according to Laguna et al. (1999). The microstructure and morphology of the aerobic granules were observed by scanning electron microscope (SEM; S-4800N, Japan). SOUR by heterotrophs was measured using the method described by Zeng et al. (2007). Sludge volume index (SVI) was determined according to the settled bed volume after 30 min settling and the dry biomass weight (Bao et al. 2009). The extraction of EPS from AGS and the total polysaccharides (PS) and total protein (PN) contents of the EPS are described by Adav et al. (2008). The characteristics of AGS and its EPS were both measured by the excitation-emission matrix (EEM).

The simultaneous nitrification and denitrification (SND) capacity of AGS, \(E\) and \(R\), respectively, are defined:

\[
E_{(SND)} = \frac{\text{NH}_4^+\text{(oxidized)} - \text{NO}_x^-(\text{produced})}{\text{NH}_4^+(\text{oxidized})} \times 100\%
\]
\[ R_{(\text{SND})} = \frac{\text{NH}_4^{+}(\text{oxidized}) - \text{NO}_x^{-(\text{produced})}}{t} \]  

(4)

where \( E \) and \( R \) are measured in units of \% and mmol L\(^{-1}\) h\(^{-1}\), respectively; \( \text{NH}_4^{+}(\text{oxidized}) \) and \( \text{NO}_x^{-(\text{produced})} \) are the values of \( \text{NH}_4 \) oxidized during the aerobic phase and produced at the end of aeration (mg L\(^{-1}\)); and \( t \) (h) is the aeration time.

RESULTS AND DISCUSSION

Optimization of bone glue concentration

Since bone glue is widely used in drinking water and wastewater treatment processes as a type of coagulant, it was utilized to investigate the augmentation of aerobic granulation. As can be seen in Figure 1, the mean diameter growth rate (MD growth rate) of AGS increased to 27.62 \( \mu \)md \( h \)–1 when bone glue concentration was 50 mg L\(^{-1}\), but decreased to 13.96 \( \mu \)md \( h \)–1 when bone glue concentration was 60 mg L\(^{-1}\). SOUR of AGS reached the peak value of 43.4 mgO\(_2\) gMLSS \( h \)–1 when bone glue concentration was 40 mg L\(^{-1}\) and fell to 39.2 and 33.1 mgO\(_2\) gMLSS \( h \)–1 when bone glue concentration was 50 and 60 mg L\(^{-1}\), respectively.

As shown in Table 1, the mean diameter of AGS enhanced by bone glue was higher (2.2–2.8 mm) than for the granules without the addition of bone glue; moreover, sludge granulation time was reduced (from 40 to 30 d) when bone glue was the adjunction. Consequently, 40 mg L\(^{-1}\) bone glue was optimal for the functioning of AGS at ambient temperature. This result implied that the presence of bone glue could enhance the formation process of AGS by allowing aggregates to form earlier and to gain a larger size; microorganisms can adhere to bone glue, which results in a rapid granulation and also a quick SBAR start-up.

However, the mechanism of rapid granulation by bone glue is quite different from those of divalent or trivalent metals (Yu et al. 2003; Jiang et al. 2003). Augmentation by such metals was through the physico-chemical functions of adhering the negative charge groups present on the surface of the microorganism and inside its polysaccharides (Wang et al. 2012). The advantage of using bone glue is that its concentration can be easily controlled (the effluent would be turbid for concentrations >40 mg L\(^{-1}\)). The addition of bone glue was terminated when AGS became mature and the granules could maintain good morphology and smooth appearance; bone glue is only added during the granulation process.

SVI and MLSS

A concentration of 40 mg L\(^{-1}\) bone glue was added with the influent, the reactor was inoculated with 2.5 L of activated sludge and the initial MLSS was 2.0 g L\(^{-1}\). Small and yellow granular sludge could be seen at day 9 after start-up.

### Table 1 | Granule characteristics under different bone glue concentrations

<table>
<thead>
<tr>
<th>Bone glue concentration (mg L(^{-1}))</th>
<th>R0</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean diameter (mm)</td>
<td>2.2</td>
<td>2.2</td>
<td>2.4</td>
<td>2.7</td>
<td>2.8</td>
<td>2.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Sludge granulation time (d)</td>
<td>40</td>
<td>39</td>
<td>36</td>
<td>30</td>
<td>30</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>COD removal(^a) (%)</td>
<td>84.4</td>
<td>83.8</td>
<td>86.2</td>
<td>85.8</td>
<td>86.7</td>
<td>85.9</td>
<td>87.3</td>
</tr>
<tr>
<td>NH(_4)-N removal(^a) (%)</td>
<td>85.0</td>
<td>89.0</td>
<td>88.1</td>
<td>89.6</td>
<td>90.6</td>
<td>91.4</td>
<td>90.5</td>
</tr>
<tr>
<td>PO(_4)-P removal(^a) (%)</td>
<td>89.2</td>
<td>91.8</td>
<td>92.5</td>
<td>92.1</td>
<td>93.8</td>
<td>94.0</td>
<td>93.6</td>
</tr>
</tbody>
</table>

\( ^a\)Contaminant removal efficiencies measured when the operation of SBAR was stable.
With the gradual wash-out of the activated sludge, AGS with a compact structure and smooth surface dominated and became mature in the SBAR at day 30. The result implied that bone glue augmentation can accelerate granulation at ambient temperature. During this period, SVI decreased from 97.8 to 44.9 mL g\(^{-1}\) and MLSS increased from 2.0 to 6.3 g L\(^{-1}\) (Figure 2(a)). After day 35, SVI was stable around 42.9–45.4 mL g\(^{-1}\) and MLSS still increased to nearly 8.0 g L\(^{-1}\), indicating that the mature aerobic granules had better settling properties and higher biomass retention at ambient temperature.

In the formation process of AGS, different microenvironments caused by mass transfer and oxygen transfer are better for different kinds of microorganisms. It is clear from Figure 2(b) that the layered structure existed from the outer sphere to the inner space of AGS, and there were more pores on the surface and hollow structure inside AGS which are both better for mass transfer.

### EPS

Bacteria can secrete sticky materials called EPS, comprising proteins, polysaccharides, humic acids and lipids, which assist cell adhesion and are therefore useful in initiating the aerobic granulation process (Wang et al. 2012). As can be seen from Figure 3, the PN content in EPS of AGS increased from 1.77 to 60.59 mg gMLSS\(^{-1}\) (due to the bone glue addition) at day 30 and then advanced to 63.22 mg gMLSS\(^{-1}\). However, there was no obvious variation in PS content: the concentration varied over the range 14.58–19.54 mg gMLSS\(^{-1}\) during the whole process. Moreover, the PN/PS ratio was 3.3 in AGS. This result illustrated that the high total protein content could be the mechanism by which AGS formed. The relatively high protein content was essential for cultivation of AGS, consistent with the results of Adav et al. (2008) and McSwain et al. (2005).

Figure 4 shows that the EEM characterization of AGS and its EPS demonstrated two major peaks in the spectrum with excitation–emission (Ex/Em) wavelengths at 220–230/340–350 nm (peak A) and 270–280/340–350 nm (peak B). Based on the classification scheme developed by Chen & Strevett (2003), peaks A and B were in regions II (aromatic proteins) and IV (soluble microbial by-product), respectively, proving that protein was the key component for aerobic granulation. Note that protein content in AGS (Figure 4(a)) is remarkably higher than that in EPS (Figure 4(b)) under the same dilution ratio (total organic carbon or TOC < 1 mg L\(^{-1}\)). This indicates that the bone glue, which is basically composed of protein, could enhance neighbouring microbial cells to form a cross-linked network by attraction of organic and inorganic materials, and could help granulation and stability (Liu & Tay 2004).

However, the contents of total protein and total polysaccharides were remarkably lower than those of others (Adav et al. 2008; Li et al. 2009), showing that the augmentation of bone glue greatly affected the amount of EPS; the...
concentrations of polysaccharides and protein secreted by microorganisms were therefore less.

**Granule size**

Figure 5(a) highlights that granular sludge size in the range 0.5–3.0 mm was dominant in SBAR, which was 81.4% in all AGS, and the mean diameter was 2.8 mm. The result indicated that AGS enhanced by bone glue possessed a larger size than that of others since bone glue can absorb microorganisms and then form granulation by hydrodynamic shear force (Adav et al. 2008). However, the specific surface area of AGS would decrease when granule size increased; moreover, mass transfer in AGS could reduce the contaminant removal efficiency (Wang et al. 2012). In addition, wet density and settling velocity both increased with the addition of bone glue, which could improve separation efficiency of wastewater and AGS.

Figure 5(b) demonstrates that settling velocity acquired a better correlation with the size of AGS, which could be described by the equation:

\[ y = 8.844x + 10.016 \]

where \( y \) is the predicted settling velocity (m h\(^{-1}\)) of AGS at ambient temperature and \( x \) is the coded value of AGS size (mm).

Substrates and DO are limited in AGS, which leads to the poor microbial activity of AGS. Li & Liu (2008) reported that the substrate utilization rate of AGS (size 0.5 mm) was twice as large as the AGS (size 1.0 mm). AGS of size 5.0–7.0 mm (Yang et al. 2008) would be disintegrated by the

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**Figure 4** | Fluorescence regions of (a) aromatic proteins and (b) soluble microbial by-product in AGS and EPS of AGS (TOC < 1 mg L\(^{-1}\)).

**Figure 5** | (a) Size distribution of AGS augmented by bone glue and (b) relation between AGS size and settling velocity.
serious inhibition of mass and DO transfer. Granules enhanced by bone glue would not only possess a better settling property but also maintain higher bioactivity for long-term operational stability.

Contaminant removal process

Initially the NH$_4^+$-N and PO$_4^{3-}$-P removal efficiencies were low (Figure 6(a)). DO consumption was slow at a level of 2.5 mg L$^{-1}$ throughout the entire feeding phase, which could be attributed to the lower bioactivity of activated sludge and lower biomass retention. Subsequently, with the progress of the SBAR operation, COD, NH$_4^+$-N and PO$_4^{3-}$-P removal efficiencies kept increasing. This indicated that the substrates can be gradually stored in the form of poly-$\beta$-hydroxybutyrate consumed in the famine period, giving an advantage over filamentous bacteria (Beun et al. 1999). A feast–famine regime therefore operated under these conditions (de Kreuk & van Loosdrecht 2004; McSwain et al. 2005), which was considered better for granulation and important for controlling the overgrowth of filamentous bacteria. Note that there were no filamentous bacteria grown during the formation process of AGS by adding bone glue, which is quite different from the previous studies (Liu et al. 2003). Furthermore, SOUR, NH$_4^+$-N and PO$_4^{3-}$-P removal gradually increased with the operation of the bioreactor, demonstrating that the microbial activity and stability of AGS were good. The addition of bone glue in the formation process of AGS was more suitable for bio-reactor long-term steady operation.

Figure 6(b) indicates that effluent COD, NH$_4^+$-N and PO$_4^{3-}$-P were 68.1, 4.2 and 1.0 mg L$^{-1}$, respectively, during the stable process of SBAR operation, and the respective COD, NH$_4^+$-N and PO$_4^{3-}$-P removal efficiencies were 86.7, 90.6 and 93.8%. As listed in Table 1, COD, NH$_4^+$-N and PO$_4^{3-}$-P removal efficiencies of AGS were enhanced by 2.7, 6.6 and 5.2% after the addition of 40 mg L$^{-1}$ bone glue. Furthermore, the nitrification and denitrification co-efficiency was 85.4% and the SND rate was 0.49 mmol L$^{-1}$ h$^{-1}$. These results are almost the same as those reported by de Kreuk & van Loosdrecht (2004) and Cassidy & Belia (2005), highlighting that the augmentation of bone glue did not result in any differences in COD, NH$_4^+$-N and PO$_4^{3-}$-P removal. This result also showed that AGS demonstrated higher nitrogen and phosphorus removal due to the mass transfer and oxygen transfer as well as pH and temperature gradient in AGS (Kreuk et al. 2005). Thus, the formation of AGS at ambient temperature was not caused by a single factor but by the synergism of different prerequisites, indicating that the augmentation of bone glue could only enhance granulation in the formation process of AGS.

CONCLUSIONS

AGS enhanced by 40 mg L$^{-1}$ bone glue with influent was successfully cultivated in 30 d, a significant reduction of 10 d. The addition of bone glue in the formation process of AGS was more suitable for bioreactor long-term steady operation. The granules can maintain good morphology and smooth appearance, so the bone glue can only be added during the granulation process. The relatively high protein content was an essential feature for AGS formation. AGS possessed better COD, NH$_4^+$-N and PO$_4^{3-}$-P removal efficiencies; however, the augmentation of bone glue did not result in any differences in contaminant removal. The morphology of various microorganisms and complex microenvironments may enforce different physiological and biochemical responses to achieve the contaminant removal.
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