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Formation of aerobic granular sludge under adverse conditions: Low DO and high ammonia

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Abstract

In this study, two adverse environments: low dissolved oxygen (DO) and high ammonia concentration, were employed to investigate the morphology, interspecies quorum sensing, extracellular polymers (EPS) characterization and microbial communities in the formation of aerobic granular sludge. Results showed that low DO could promote filamentous bacterial outgrowth. Under high ammonia concentration aerobic granular sludge (AGS) could still be cultivated, although it was looser and lighter than the control group. During the early stage of the AGS cultivation process, AI-2 activity reached a peak value in all three reactors, and ultrasonic pre-treatment was not beneficial to the release of AI-2. During AGS formation, the production of polysaccharide exhibited increases from 12.2 % to 40.3 %, 49.6 %, and 29.3 %. And PS in R2 was the highest as the result of sludge bulking. PS/PN was 1.5~8 in the three reactors. Three-dimensional EEM fuorescence spectroscopy variation indicated the change of protein in EPS, and the highest intensity of Peak T1 was obtained. The location shift of Peak T1 was not obvious, and Peaks A, C, and T2 shifted toward longer wavelengths (red shift) of 5~60 nm, or shorter wavelengths (blue shift) of 10~25 nm on the emission scale and / or excitation scale in all three reactors. This provided spectral information on the chemical structure changes. Bacteria in R3 had the highest species diversity, and all bacteria in β-Proteobacteria were identified as genus *Thauera*, which suggested that simultaneous nitrification and denitrification occurred in R3. The filamentous bacteria in seed sludge and R2 were species-richer. There was a low abundance of filamentous bacteria in R1 and R3, which contributed to the granule structure stability.

Key words

Aerobic granular sludge, Low dissolved oxygen, High ammonia concentration, Interspecies quorum sensing, Microbial communities

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Introduction

Recently, aerobic granular sludge (AGS) has become a new biological approach for wastewater treatment. AGS is an aggregation of microbes with a typically rounder and more regular appearance, which are generally much larger and denser than bioflocs (Mishima and Nakamura, 1991). AGS could allow reactors to operate at a higher biomass concentration, which could reduce the volumetric requirement for phase separation, and facilitate the simultaneous removal of COD, nitrogen, and phosphorus (Jiang *et al*., 2002; Yilmaz *et al*., 2008). Extensive research has been carried out on the formation mechanisms of AGS. Many reports show that shear force and cell hydrophobicity play important role in the cell-immobilization system. Cell surface hydrophobicity might contribute to the ability to aggregate, i.e., increased cell-to-cell adhesion (Liu *et al*., 2003; Tay *et al*., 2001a). In addition, extracellular polymers (EPS) were also key factors in the formation of AGS. This resulted from the property of the materials bound to the cell surfaces (McSwain *et al*., 2005).

Previous studies have shown that the characteristics and stability of AGS were closely related to its growth environment, including DO (Tay *et al*., 2001b), growth medium (Tay *et al*., 2002), water temperature (de Kreuk, 2005), organic loading (Moy *et al*., 2002), and ammonia concentration (Yang *et al*., 2004). At lower oxygen concentrations, aerobic organic

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substrate uptake was obviously compensated by anoxic acetate uptake. Nitrogen removal was favoured by decreased oxygen concentrations, but lower oxygen concentration saturation could lead to granule disintegration and biomass wash out (Corral *et al*., 2005). Free ammonia can result in a significant decrease of cell hydrophobicity and repress the production of cell polysaccharides, which is responsible for the failure of aerobic granulation in high free ammonia concentrations (Yang *et al*., 2004).

Some concepts of microbial ecology, such as quorum sensing (QS), were applied to in-depth research on the formation and abilities of microbial aggregates like biofilm and AGS. Many bacteria synthesize and secrete small molecules, i.e. autoinducers (AIs), into the surrounding environment. As the population density increases, accumulation of these molecules eventually reache a threshold concentration, which regulates gene expression. Three types of AIs have been identified: oligopeptides, N-acylhomoserine lactones (AHLs), and autoinducer-2 (AI-2). AI-2 is produced by a large number of bacterial species and is universally used for interspecies communication for both Gram-positive and Gram-negative bacteria. Many bacteria possess homologs of the luxS gene, which is responsible for the production of AI-2 (Bassler *et al*., 1993). Previous studies have reported that QS control could successfully reduce or prevent biofilm formation on surfaces such as medical devices (Baveja *et al*., 2004) and membrane bioreactors (Yeon *et al*., 2009). The formation of AGS also could be promoted by the QS system (Zhang *et al*., 2011). However, no information is available on the features and formation of AGS under certain adverse environments that occasionally happen in wastewater treatment plants.

In light of the above the present study aimed to investigate the formation process of aerobic granules under adverse environmental conditions, specifically low DO and high ammonia concentration. These investigations are likely to provide a clearer insight into the microbial granules, and may facilitate engineering and manipulation of the granulation, providing a potentially promising technology for biological wastewater treatment.

Materials and Methods

In this experimental study, research was conducted on the physical, biochemical characteristics, and microbial community structure of AGS formation in SBR to explore the influence of DO and ammonia concentration on aerobic granulation. First, low DO and high ammonia concentrations were applied to SBR. To evaluate the AGS formation process, a three-dimensional excitation–emission matrix (EEM) and microbial DNA extractions were conducted. This was followed by polymerase chain reactions (PCR) and clone library analysis. QS autoinducer activity and EPS sample features were analyzed, as was the bacterial community structure of AGS.

Cultivation of aerobic granules: In the study, three columns (120 cm high, 50 mm in diameter) with a working volume of 1.96 l were used as sequencing batch reactors (SBRs). The SBRs were seeded with fresh activated sludge obtained from a domestic wastewater treatment plant (loading rate, 3 kg m^{-3} d^{-1}) in Xiamen city, PR China. They were operated at a cycle time of 4 hr, composed: of 5 min settling, 5 min discharging, 7 min filling, and 223 min of aeration. The same volume exchange ratios of 40% were applied. The DO in reactor 1 (R1) and reactor 3 (R3) were each 6~7 mg l^{-1}, while the DO of reactor 2 (R2) was about 1 mg l^{-1}. R1 was used as the control, and R2 and R3 were the tested units. Synthetic wastewater with the following composition was used: Sodium acetate as the sole carbon source, 1.27 g l^{-1}; NH_4Cl, 96.8 mg l^{-1} (R1 and R2), 386.5 mg l^{-1}(R3); K_2HPO_4, 56 mg l^{-1}; $MgSO_4.7H_2O$, 14 mg l^{-1}; $FeSO_4.7H_2O$, 11 mg l^{-1}; $CaCl_2.H_2O$, 17 mg l^{-1}; and trace solution 1 ml l^{-1}. This gave a total COD of 5 kg m^{-3} d^{-1}. The composition of the trace solution was: $FeCl_3.6H_2O$, 1.5 g l^{-1}; $CuSO_4.5H_2O$, 30 mg l^{-1}; KI 30 mg l^{-1}, $MnCl_2.4H_2O$, 120 mg l^{-1}; $Na_2MoO_4.2H_2O$, 60 mg l^{-1}; $ZnSO_4.7H_2O$, 120 mg l^{-1}; and $CoCl_2.6H_2O$, 150 mg l^{-1}. Due to a holiday, three reactors were shut down on day 42, and the sludge was chilled at 4 °C in the freezer for 30 days. Following the holiday, the three reactors continued to run until the cultivation time of the AGS in reactors reached 63 days.

Assay of AI-2 : The AI-2 activity variation under two different conditions were investigated the whole cultivation process for 63 d in three reactors, with aeration for 6.5 hr continually occurring only for R1 before shut down. 2 ml mixture in the SBR was then collected and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through a 0.22 μm filter and then transferred to a clean tube as cell-free culture. The AI-2 activity in the aerobic granular sludge was measured using the *Vibrio harveyi* BB170 bioluminescence reporter assay, where *Vibrio harveyi* BB120 used as positive control. The AI-2 detection mechanism of *Vibrio harveyi* is described by basic protocol (Michiko, 2005). *Vibrio harveyi* strain BB170 and BB120 (AI-2+) from glycerol frozen stocks were inoculated into 5 ml AB medium for 14 hrs at 30 °C with aeration until the culture was turbid (OD_{600}=0.7 to 1.2). Examination in a dark room verified that the culture was bright. Cell-free culture was collected from *Vibrio harveyi* strain BB120 and filtered through 0.22 μm filter for use as a positive control. AB medium was used as a negative control. The BB170 over night culture was diluted at 1:5000 into fresh AB medium. Mixtures containing 20 μl cell-free culture fluids and 180 μl diluted BB170 culture were prepared in a 96-well microtiter plate, and incubated at 30 °C with aeration. AB medium used here was filtered through a 0.22 μm filter. The bioluminscence of *Vibrio harveyi* BB170 was measured using a multifunctional

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microplate reader (Spectra Max M5, USA) at a wavelength of 490 nm. Measurement was taken every 30 min for 5 to 7 hr, until the luminescence value ratio between the samples and AB medium control sample reached its highest value.

Extraction and assay of EPS from mixed liquor: About 15 ml of mixed suspended flocs or aerobic granular sludge liquid, which was collected once a week from the SBRs, was centrifuged at 9000 rpm for 10 min to remove bulk solution. After discarding the supernatant, the remaining pellet was washed and re-suspended in 20 ml EPS extraction buffer (8.5% NaCl and 2% EDTA, pH 8.0). After sonication at 100 W (8 s pulse and 2 s interval for 30 cycles), the tubes were centrifuged at 9000 rpm for 10 min. EPS solution was the centrifuged supernatant. Before storage at 4 °C, the supernatant was filtered through 0.22 μm filter and then transferred to a clean tube. The dry weight of pellet was obtained by washing once with 0.5% formalin solution and drying out at 60 °C for 48 hr. This weight was used to calculate the yield ratio of EPS from sludge.

The protein content was examined using a reagent kit (Thermo, USA) based on BCA methods (Smith *et al*., 1985). The carbohydrate was determined by the anthrone-sulfuric acid method (Koehler, 1952). The three-dimensional excitation-emission matrix (EEM) of the EPS samples were determined using protocol mentioned in previous literature (Liu *et al*., 2009).

Analysis of bacterial community structure: From the seed sludge and the cultivated sludge, DNA was extracted using a bacterial DNA extraction kit (Bioteke, China) following kit instructions. DNA quality was determined by electrophoresis and spectrophotometry. The polymerase chain reaction (PCR) of the full length 16S rDNA was performed as mentioned by Layton *et al*. (2000). The PCR was conducted under the following conditions: 95 °C for 10 min, then 25 cycles including 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, and finally 72 °C for 10 min for prolongation. Each sample was amplified in three independent reactions and then the triplicate PCR products were mixed. The products were purified by a DNA gel retraction kit (Solar bioscience and Technology Co., Ltd., Beijing, PR China). Four 16S rDNA libraries were constructed: that of the seed sludge and those of the cultivated sludge. The purified full length 16S rDNAs were ligated top MD-19T vectors (TaKaRa, Japan) and the vectors were transformed into competent *Escherichia coli* top 10 cells. After blue/white screening on ampicillin plates, the white colonies were picked out into fresh liquid LB medium supplemented with 100 μg ml^{-1} ampicillin. For each library, 30 colonies were picked out. After growth for 16 hr, the transformants were examined for the insertion of the 16S rDNA sequences through PCR using the primer pair from the vector, and electrophoresis. For each library, the DNA segments were sequenced using the ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). These sequences were analysed by comparison with 16S rDNA gene sequences in the GenBank using a BLAST search (National Centre for Biotechnology Information, US National Library of Medicine). They were also checked with the Ribosomal Database Project II (RDP) Chimera Detection Program (Maidak et al., 2001) for species identification.

Other analytical methods: Mixed liquid suspended solids (MLSS), sludge volume (SV %), and sludge volume index (SVI) were determined by standard methods (Eaton *et al*., 1995). The size of the granular sludge was measured by a laser particle size analysis system (Master-size 2000, Malvern, Britain). The morphology of the sludge was observed with an Olympus SZX9 microscope.

Results and Discussion

Granular sludge properties: Fig. 1 shows the granular sludge properties in the three reactors. In R1 and R3, the size of the granule increased as culture time increased (Fig. 1G). Before 42 days of cultivation, the average diameter of the granules in R3 was larger than the average granule diameter of granules in R1. But after the holiday, the situation was reversed until 63 days of cultivation. On day 28, AGS in R1 and R3 were tight and regular (Fig. 1A, C). On day 63, AGS in R1 was tighter and denser than AGS in R3 (Fig. 1D, E). The influence of ammonia nitrogen on the process of AGS cultivation has been scarcely investigated prior to this study, thus providing only a few references. Previous studies have demonstrated that an excessive ammonia concentration can inhibit microbial growth (Hansen *et al*., 1998). Yang *et al*. (2004) demonstrated that aerobic granules formed only when the free ammonia concentration was less than 23.5mg l^{-1}. In our research, AGS could still be cultivated successfully when the ammonia concentration was 96.8 mg l^{-1} in R1. It could be cultivated successfully only until the concentration reached 386.5 mg l^{-1}. AGS was looser and lighter in R3, which perhaps resulted from different synthetic wastewater composition and operation conditions. Because of the looser structure of AGS in R3, MLSS in R1 was higher than MLSS in R3. At the same time, SV% and SVI in R1 were lower than in R3 (Fig. 1H, I and J). Filamentous bacteria could be obviously recognized at the edge of AGS in R3 (Fig. 1F). Fig. 1G shows that the diameter of AGS in R3 is close to the diameter of AGS in R1. It was speculated that the loose structure of AGS in R3 was beneficial in transfer of nutrients and excretion of metabolites, which promoted the growth of AGS in R3.

In the SBR with a high height to diameter ratio, the circumstance of low DO could not supply enough shear force to form AGS.and could promote filamentous bacteria blooming so as to destroy sludge granulation (Tay *et al*.,

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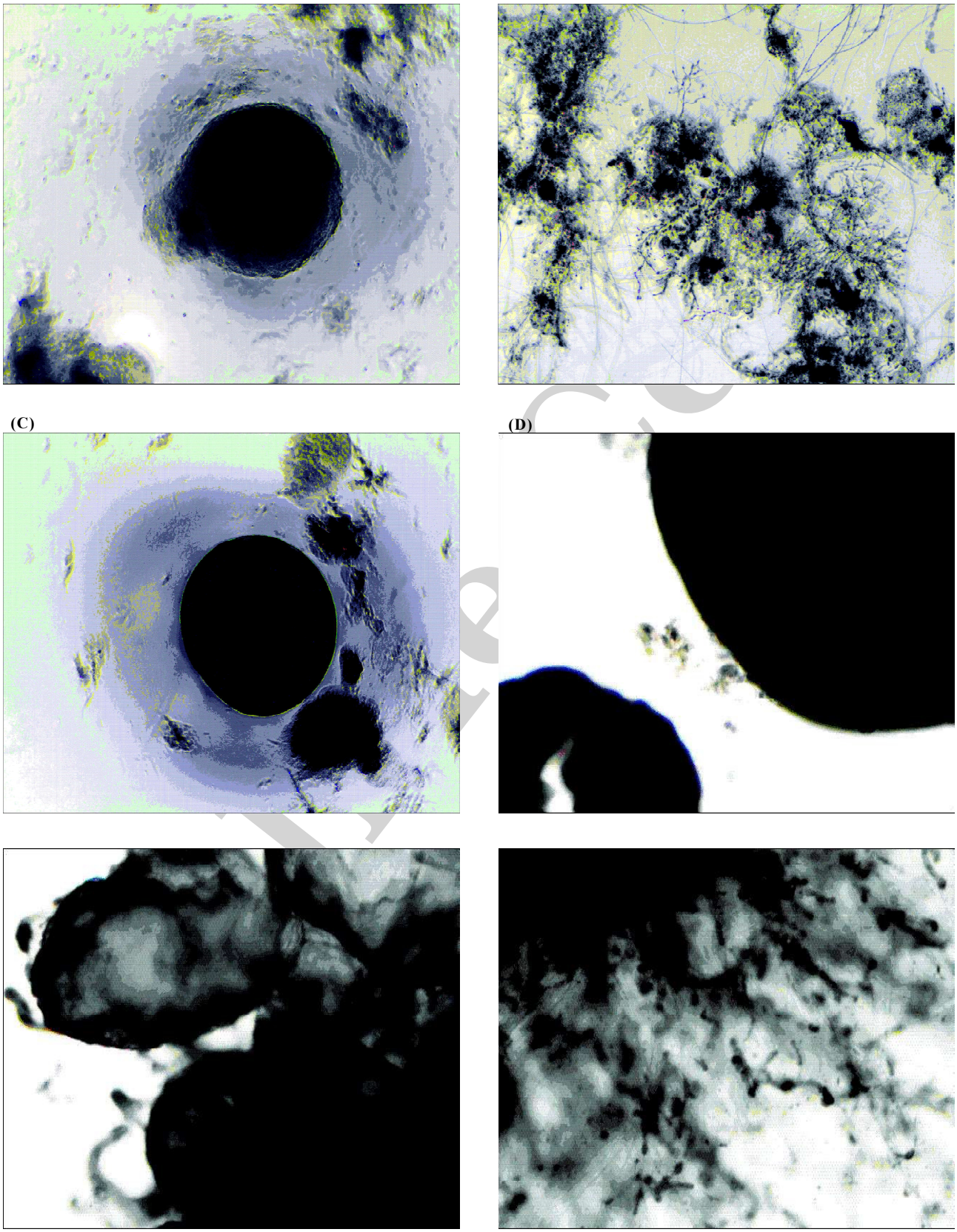

Fig. 1 (A - F) : The growth of aerobic granular sludge during 63 day cultivation in R1, R2, and R3. Morphologies of sludge sampled at day 28 (A: R1, B: R2, C: R3); 63 day (D: R1, E: R2, F: R3). Bar=500 μm in (A), (B), (C), (D), and (E); and bar=50 μm in (F)

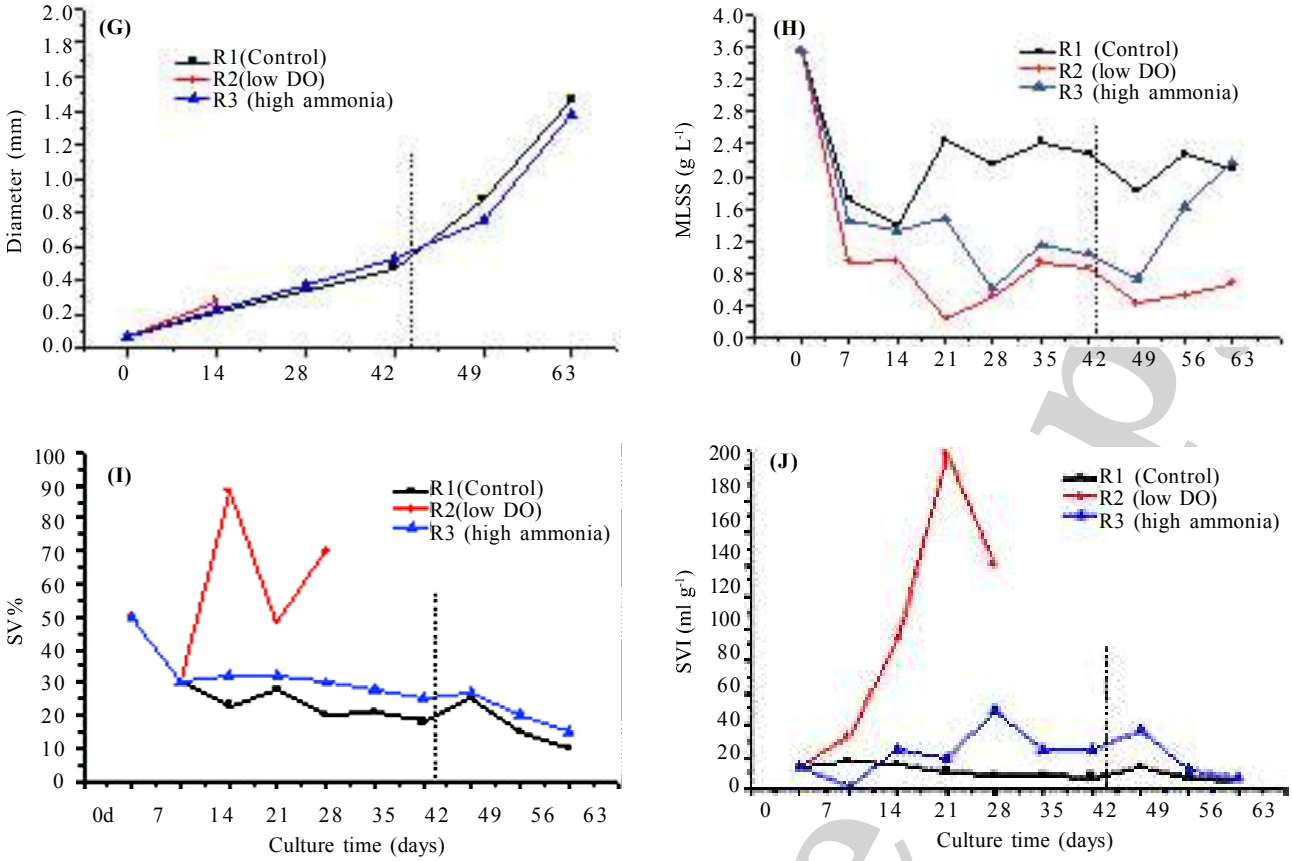

Fig. 1 (G - J) : Average granular diameter change is shown in (G). MLSS, SV%, and SVI vs. culture time are shown in H, I and J, respectively. The dash line in G, H, I and J represents the 30 day time in which the sludge was chilled in the refrigerator

2001b). Because of the anoxic environment in R2, filamentous bacteria increased quickly, and granulation of suspended sludge was destroyed gradually. Therefore the diameter of biomass recognized by the laser particle size analysis system was meaningless from day 14 onward (Fig. 1G). In addition, R2 was full of filamentous bacteria, and biomass bulking happened obviously on day 28 (Fig. 1B). For the filamentous bacterial blooms, considerable biomass was washed out of R2, and thus MLSS was lowest in R2. Furthermore, in R2, SV% and SVI were highest among three reactors (Fig. 1H, I and J). After day 28, the flocs in R2 could not deposit completely, and therefore SV % and SVI analysis was not conducted from then on.

It should be noted that lower temperature (less than 10 °C) can result in filamentous and non-filamentous sludge bulking. Filamentous sludge bulking was related to the excessive growth of filamentous bacteria, while non-filamentous sludge bulking was related to the growth of Zoogloea colonies (Peng *et al*., 2003; Novak *et al*., 1993). The SBR operation at 8°C produced granules with irregular shape, and caused excessive filamentous bacterial growth. This caused unstable operation via severe biomass washout (de Kreuk, 2005). During the holiday, slight sludge bulking occurred because of the lower temperature in the freezer. So following the holiday, AGS diameter increased more quickly than it did before refrigeration. This was followed by slow sedimentation. As MLSS slightly decreased, SV% and SVI slightly increased (Fig. 1G, H, I and J).

Characterizations of AI-2: Bassler *et al*. (1993) suggested that AI-2 can serve as a universal inter–species quorum sensing signaling molecule. Zhang *et al*. (2011) has demonstrated aerobic sludge granulation along with the release of AI-2 molecules from aerobic sludge cells. Fig. 2A shows the AI-2 activity in three reactors, which indicates that sludge cells under anoxic and high ammonia nitrogen concentration conditions could also release AI-2 molecules. Such circumstances obviously influenced the AI-2 activity of sludge cells. From day 0 to day 14, AI-2 activity was lowest in R2. From day 14 to day 63, though there was serious sludge bulking, sludge cells still released a good amount of AI-2 molecules. The lowest MLSS in R2 led to the highest AI-2 activity per gram sludge. It is worth mentioning that though AGS in R3 had already formed, AI-2 activity per gram sludge in R3 was still higher than it was in R1 due to the minor AGS density in R3. During the early stage of the AGS cultivation process, AI-2 activity reached

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a peak value in all three reactors (for example, R1 on Day 14), which was consistent with previous research (Zhang *et al*., 2011). The same situation also happened after refrigeration. As Fig. 2B shows, AI-2 activity in R1 during continual aeration for 6.5 hr also reached a peak value at 1.5 hr, like that in the three reactors (Fig. 2A). Su and Yu (2005) demonstrated that the formation of granules was a four-phase process: acclimating, shaping, developing and maturating. And a modified logistic model could well fit the granule growth by diameter. The initial shaping of AGS was an important stage that represented the beginning of granulation. In the present study, in both the 63 day cultivation and the cultivation with aeration for 6.5 hr, AI-2 activity reached peaks at the early stage of cultivation. This was consistent with previous research (Su and Yu, 2005). The peaks of AI-2 activity could be regarded as the beginning of the shaping stage. At the peak of AI-2 activity, interspecies communication strengthened, which meant that the speed and driving force of suspended sludge granulation reached the highest value.

Fig. 2B also shows that before aeration for 1.5 hr, i.e. before the AI-2 activity peak, the AI-2 activity of the ultrasonic (US) pre-treatment sample was higher than that of the no-US sample. After 1.5 hr, the situation reversed. Production of AI-2 is the only QS system shared by both Gram-negative and Gram-positive bacteria (Bassler *et al*., 1993). Thus it was easily speculated that ultrasonic may be beneficial to the release of AI-2 molecules from inside the cell or from the EPS composed of AGS. But in the present study, AI-2 activity of the US pre-treatment sample was lower than that of no-US sample after 1.5 hr. AI-2 is produced from *S*-adenosylmethionine to 4,5-dihy-droxy-2,3-pentanedione (DPD) and homocysteine in at least three enzymatic steps. As the precursor of AI-2, DPD probably forms sacyclicmolecule, and possibly undergoes further rearrangements to yield AI-2 (Chen *et al*., 2002). To date, ultrasonic has always been applied to degrade organic matter in wastewater (Naffrechoux *et al*., 2000; Destaillats *et al*., 2000). Here, we speculated that US could destroy the structure of organic matter on the AI-2 synthetic route (such as *S*-ribosylhomocysteine or DPD etc.), and therefore we deviated from the initial hypothesis. The relationship between AI-2 activity and ultrasonic requires further study. Considering that AI-2 is produced from *S*-adenosylmethionine in at least three enzymatic steps (Chen *et al*., 2002), whether the enzymes required in the AI-2 synthetic route were inactivated by ultrasonic still requires investigation.

Analysis of EPS: EPS are metabolic products accumulating on the surface of bacterial cells, which could alter the physico-chemical characteristics of the cellular surface like charge, hydrophobicity, and other properties. Their complicated nature is reflected by the wide range of proteins, polysaccharides, lipids and DNA (Liao *et al*., 2001; Sheng *et al*., 2008). With regard to the location of PS and PN, many studies shared a conclusion that polysaccharides were localized at the outer edge of granules, whereas the center was comprised mostly of proteins (McSwain *et al*., 2005; Adav *et al*., 2007). It is generally believed that cell polysaccharides can mediate both bacterial cohesion and adhesion in AGS formation. Thus it has a decisive role in building and maintaining the structural integrity of a microbial community.

The contents of cell polysaccharide (PS), cell protein (PN) and PS/PN in EPS of different culture times are shown in Fig. 3. During the 63 day period in which reactors were running, the production of polysaccharide exhibited

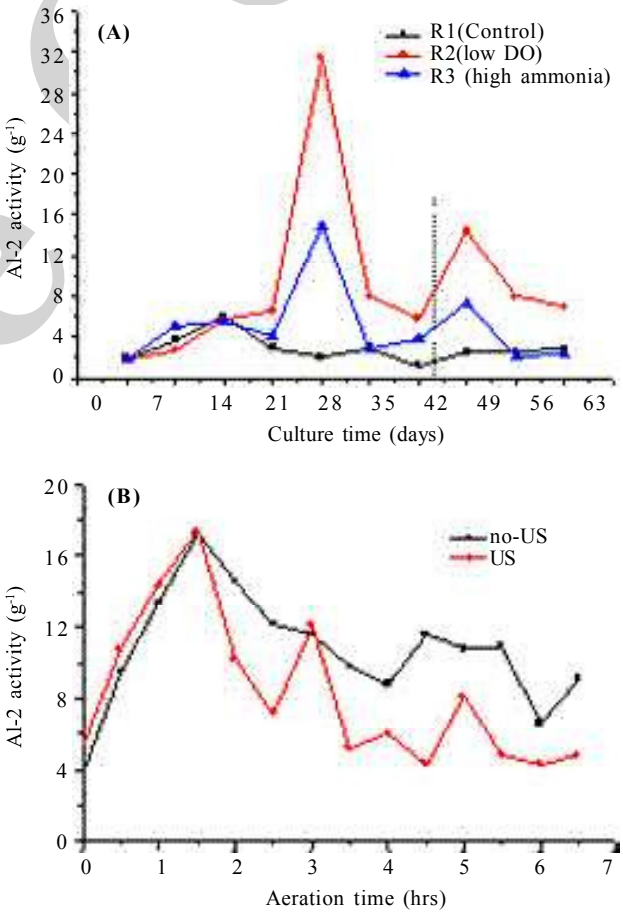

Fig. 2 : Variation of AI-2 activities in R1, R2 and R3 sampled at different culture times. (A) Variation of AI-2 activities sampled at one cycle for 6.5 hr (only in R1), and US meant that after sampling, 2 ml mixture in the SBR was sonicated (Branson Sonifier W-450) at 100 W (8 s pulse and 2 s interval for 30 cycles), and then centrifuged at 12,000 rpm for 10 min. (B) The AI-2 activity was represented by the highest luminescence value ratio between the samples and the AB medium control sample of per gram sludge (g^{-1}). The luminescence was measured using the *V.harveyi* BB170 AI-2 bioassay

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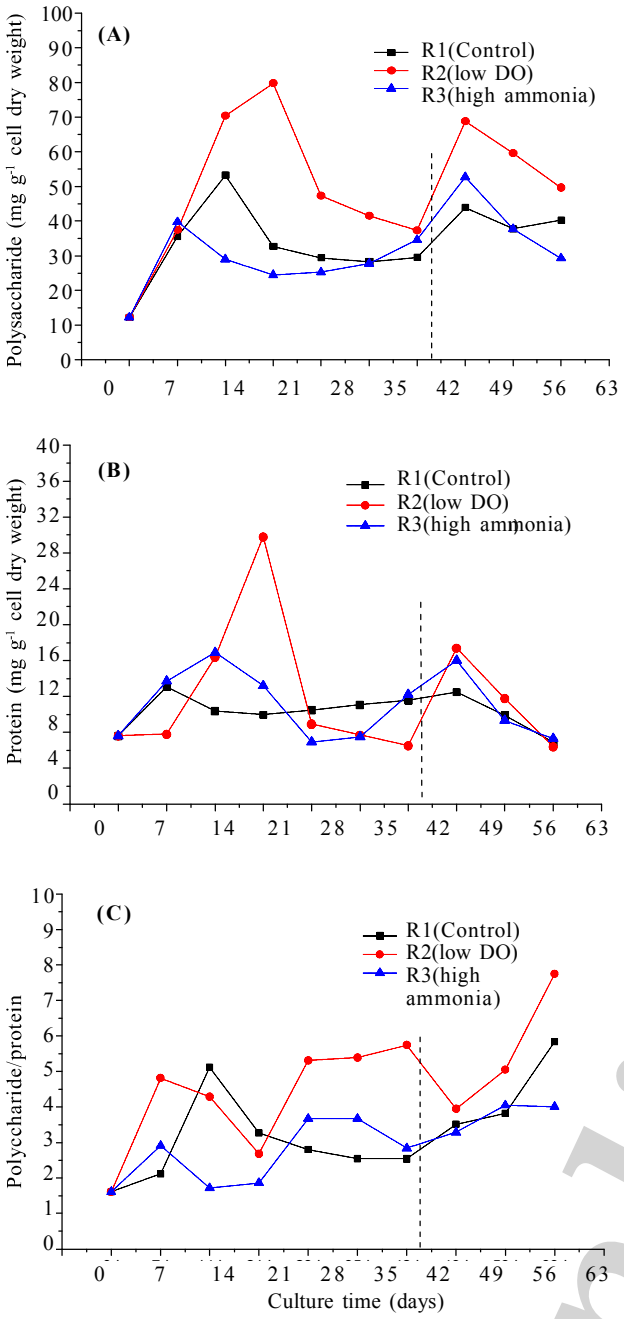

Fig. 3 : Change of EPS contents in R1, R2 and R3 sampled at different culture times. (A) Polysaccharide (PS); (B) protein (PN); (C) Polysaccharide/protein (PS/PN).

increase from 12.2 to 40.3 %, 49.6 and 29.3 %, but with an intermittent increase pattern. PS reached peaks at the early stage of cultivation, which was consistent with AI-2 activity (Fig. 2A). The peaks of PN could also be regarded as the beginning of the shaping stage, as AI-2 activity could be. It was noteworthy that PS in R2 was highest during the cultivation process. Higher SVI represented sludge bulking and higher PS content in activated sludge (Forster and Dallas, 1980). Regarding lower DO, sludge bulking occurred in R2, and PS content was proportional with SVI, which could explain the highest PS occurring in R2. Yang *et al.* (2004) confirmed that free ammonia resulted in a significant decrease of cell hydrophobicity, and also repressed the production of cell polysaccharides. But in our research, PS in R1 and R3 were equal, and ammonia did not distinctly inhibit PS. PN in the three reactors had no obvious difference, though PN in R1 was more constant than PN in R2 and R3. Many different conclusions about PS/PN in the AGS cultivation process have been drawn in previous research. Some research demonstrated that PS was greater than PN, and that PS/PN was 1~13. This was consistent with our result (PS/PN was 1.5~8) (Liu and Tay, 2007; Wang *et al.*, 2006; Tay *et al.*, 2001b; Zhang *et al.*, 2007). Other research concluded that PN was greater than or equal to PS, and that PS/PN was 0.8~1 (McSwain *et al.*, 2005; Adav and Lee 2008). Such different results perhaps resulted from different methods applied to extract EPS.

Three-dimensional EEM fluorescence spectroscopy has been successfully utilized to identify the chemical composition of EPS, because of its ability to distinguish certain classes of organic matter. Four key fluorescence peaks: fiuorophores A, C, T1, and T2, are commonly observed in the EPS of active sludge samples (Liu *et al.*, 2011). As shown in Table 1, peaks A and C are related to humic-like substance derived from the break down of plant material (Lee *et al.*, 2008). Protein-like fiuorophores, including tryptophan-like (Peak T_1) and aromatic protein-like (Peak T_2) materials, are usually detected at enhanced levels in domestic sewage-impacted water (Baker *et al.*, 2003). The fluorescence parameters, including peak locations and fluorescence intensity, were extracted from EEM fluorescence spectra and summarized in Table 1. These values could be employed for quantitative analysis.

Generally, an increase of the fluorescence peak intensity during the cultivation process indicates the production of fiuorescing material. Tryptophan is a hydrophobic amino acid, and previous studies have shown

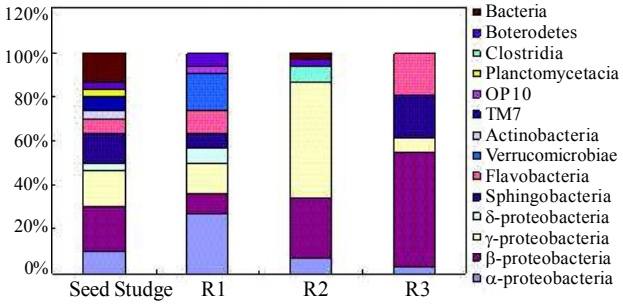

Fig. 4 : Bacteria community structure in SBRs at the class and phylum level

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that hydrophobicity is the main driving force for biogranulation (Liu *et al*., 2003). Table 1 shows that the fluorescence intensities of Peaks T1 and T2 were higher than the intensities of Peak A and Peak C. This indicated that the content of protein in EPS was higher than that of humic acid. Metzger *et al*. (2007) showed that protein played the most important role in MBR biofouling formation. As microbial aggregates, AGS has similar properties with MBR biofouling. Table 1 also showed an increase in the fluorescence intensities of Peak T_1 by about 70% in R1 and R3 during the 63 days. However, the fluorescence intensity of Peak T_1 decreased by about 35% in R2. To our knowledge, therefore, the regular growth of AGS in R1 and R3 may be attributed to the hydrophobicity from hydrophobic substances represented by tryptophan, the reduction of which perhaps resulted from sludge bulking in R2. The highest intensity of peak T_1 could also be obtained as AI-2 activity, PS, and PN during the early cultivation stages, however the times at which the highest intensities were reached varied.

Table 1 shows that the fluorescence intensity of Peak T2 increased by about 57% in R1 during the 63 days, while the fluorescence intensities of Peak T2 decreased by about 45% in R2 and 35% in R3. Peak T2 is related to an aromatic protein-like substance, which includes hydrophobic and hydrophilic amino acids. The fluorescence intensity variation of Peak T2 might suggest that hydrophilic amino acids were dominant in the EPS extracted from AGS in R3 under the duress of high amino. However the fluorescence intensity of Peak T2 may decrease with the increase of AGS diameter.

Location shifts of fiuorscence peaks provide spectral information on the chemical structure changes of samples (Table 1). The location shift of peak T_1 was not obvious, especially the excitation scale, which proved that the structure and chemical property of tryptophan-like matters in EPS were relatively stable and therefore hydrophobic. So they could be commonly used to evaluate the formation of AGS. The location of peak T_2 shifted toward the shorter wavelengths (blue shift) to 10~25 nm on the emission scale in the three reactors. But this was not significant on the excitation scale. The location of peak A shifted toward longer wavelengths (red shift) to 5~30nm on the emission scale and / or excitation scale in the three reactors, but this was not significant on the emission scale for R3. The location of peak C shifted toward longer wavelengths (red shift) to 30~60 nm on the emission scale and / or excitation scale in the three reactors, and the shift was regular and distinct. A red shift is related to an increase of carbonyl, hydroxyl, alkoxyl, amino, and carboxyl groups in the structures of fiuorophores (Chen *et al*., 2002; Uyguner and Bekbolet, 2005). A blue shift is ascribed to the elimination of particular functional groups such as carbonyl, hydroxyl and amine, a reduction in the degree of π-electron systems, and the decrease in the number of aromatic rings and conjugated bonds in a chain structure (Swietlik *et al*., 2004). Previous research demonstrated that ozonation resulted in a significant location shift of all peaks in EEM fluorescence spectra for MBR internal foulants (Liu *et al*., 2011). Such

Table 1 : Variation of the fluorescent intensity of the four typical peaks in the EEM profiles of extracted EPS from sludge in R1, R2 and R3 with different culture times

Reactor	Samples	Peak T_1		Peak T_2		Peak A		Peak C	
		E_X/E_m	Int	E_X/E_m	Int	E_X/E_m	Int	E_X/E_m	Int
R1	0d	275/345	822.9	230/345	269.4	250/435	201.3	310/420	179.4
	14d	275/340	513.2	225/335	276.8	250/450	77.1		
	28d	275/340	1750			270/465	256.4	375/455	80.7
	42d	275/335	1105	230/330	181.6	270/465	255.4	365/455	87.5
	49d	275/330	1764	235/335	210.3	270/460	346.5	370/460	126.6
	63d	275/330	1410	230/335	422.6	270/465	75.8		
R2	0d	275/345	822.9	230/345	269.4	250/435	201.3	310/420	179.4
	14d	275/330	1118	230/320	238.1				
	28d	275/335	1074			265/455	174.7	365/455	68.8
	42d	280/340	511.5			260/445	432.9	360/450	373.7
	49d	275/335	649.3	230/335	283.6	260/465	279.6	365/450	115.2
	63d	275/330	534.6	230/325	149.1	275/445	63.8		
R3	0d	275/345	822.9	230/345	269.4	250/435	201.3	310/420	179.4
	14d	275/350	399.6	225/350	357.6				
	28d	275/340	2193						
	42d	275/335	291.8	220/345	209.7	255/445	63.1	360/450	95.3
	49d	275/345	728.8	230/325	145.7	255/435	175.9	360/460	62.4
	63d	275/345	1343	230/320	176.7	270/430	335.3	345/435	124.3

*Blank means the fluorescent intensity was less than 50.0

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Table 2 : Filamentous bacteria populations in sludge

Clone No. *	Accession number of the closest sequence	Similarity%	Taxonomy**
S-2	DQ836253.1	99	*Comamonadaceae*
S-6	JN679085.1	100	*Haliscomenobacter*
S-12	EU177725.1	97	*Haliscomenobacter*
S-13	JN217075.1	98	*Terrimonas*
S-23	AB076847.1	98	*Comamonadaceae*
S-30	AB076847.1	99	*Comamonadaceae*
R1-7	EF523466.1	87	*Haliscomenobacter*
R1-23	BX842648.2	98	*Bdellovibrio*
R2-3	JN371671.1	100	*Clostridia*
R2-4	EU640512.1	100	*Comamonadaceae*
R2-7	JN544144.1	100	*Comamonadaceae*
R2-14	HM991518.1	96	*Comamonadaceae*
R2-15	JN679128.1	97	*Comamonadaceae*
R2-26	EU325622.1	99	*Brachymonas*
R3-21	EF523439.1	95	*Haliscomenobacter*

* S-signifies clone numbers in seed sludge, and R1-, R2-, R3- signify clone numbers in R1, R2, and R3 separately. ** The taxonomy of the corresponding sequences was obtained from the Ribosomal Database Project II (RDP) database (http://rdp.cme.msu.edu/classiûer/classiûer.jsp, Maidak et al., 2001) The confirmed taxonomy was intercepted with the confidence threshold at 80%

shifts resulted from the structural and chemical property variations of soluble organic materials in EPS after ozonation. The mechanism of the location shifts of Peaks T_2, A, and C accompanied with granulation requires further study.

Microbial communities for seed sludge and granules in three reactors: Fig. 4 shows the bacteria community structure of seed sludge and AGS or suspended sludge in the three reactors at the phylum or class level. More than half of the bacteria belonged to *Proteobacteria*, which included α-, β-, γ- and δ-subgroups. Besides that, two classes, including *Flavobacteria* and *Sphingobacteria* in phylum *Bacteroidetes*, were present in all four samples.

In the seed sludge bacteria, majority of the bacteria were grouped with members of *Proteobacteria*, with 10% in α-*proteobacteria*, 20% in β-*proteobacteria*, 16.7% in γ-*proteobacteria*, and 3.3% in δ-*proteobacteria*. The next two main groups clustered with *Flavobacteria* (6.7%) and *Sphingobacteria* (13.3%). Bacteria from the classes α-*proteobacteria* and β-*proteobacteria* have commonly been found in conventional activated sludge (Bond *et al*., 1999; Snaidr *et al*., 1997), which was consistent with our identifications. In the bacteria of R1, *Proteobacteria* had a higher proportion of 56.7% than the proportion in seed sludge. Bacterial species were fewer than in seed sludge, and many species that exhibited low proportions were not present: including *Planctomycetacia*, TM7, and *Actinobacteria*. This could be explained by the following: with the sludge granulation process, the species and quantity of microbial populations in activated sludge were prone to stablize gradually (Xavier *et al.*, 2007). In the bacteria in R2, *Proteobacteria* had the highest proportion at 76.2%, higher than that in seed sludge, R1 and R3. However, there was an unexpected phenomenon in the bacteria in R2: γ-*Proteobacteri*a made up an overwhelming proportion of 51.7 %. In the bacteria in R3, bacteria species diversity was the lowest; only five classes were identified. Previous research showed that the reactor with the highest substrate loading rate had the lowest species diversity, while the reactor with the lowest substrate loading rate had the highest species diversity (Li *et al*., 2008). In our research, the seed sludge was obtained from a wastewater treatment plant, and the organic loading rate was lower than that in the three reactors. In addition, the ammonia-nitrogen loading rate in R3 was the highest. Therefore, seed sludge had the highest species diversity, while R3 had the lowest species diversity. Most surprising was that β-*Proteobacteria* had an overwhelming proportion of 51.6 %, and all of the bacteria in β-*Proteobacteri*a were identified as genus *Thauera* (data not shown). The genus *Thauera* has been found to be a highly abundant species in AGS fed with acetates (Liu *et al*., 2010). *Thauera* is denitrifying bacterium, and is generally present in biological organic oxidation and nitrifying–denitrifying activated sludge (Li *et al*., 2008). Under the high ammonia stress, *Thauera* became the dominant species, and simultaneous nitrification and denitrification was realized in the AGS of R3 (Mosquera-Corral *et al*., 2005; Cassidy and Belia, 2005).

Filamentous growth has been commonly observed in aerobic granular sludge SBR (McSwain *et al.*, 2004; Schwarzenbeck *et al*., 2005). Once filamentous bacteria outgrowth occurs in the reactor, settleability of aerobic granules becomes poor, and subsequent biomass washout and eventual disappearance of aerobic granules occurs. Thus, filamentous growth leads to instability of aerobic granules. Previous researchers demonstrated that many factors cause filamentous growth. These factors include wastewater composition (Chudoba *et al*., 1973), low substrate availability (Knoop and Kunst, 1998), dissolved oxygen concentration (Rossetti *et al*., 2005), solids retention time (Tandoi, 1998) and nutrient deficiency (Chudoba *et al*., 1973). Although filamentous growth is a common phenomenon in aerobic granular sludge SBR, low-levels and moderate-levels of filamentous growth do not cause operational problems and may even stabilize the granule structure (Li *et al*., 2006). The populations of filamentous bacteria in seed sludge and the three reactors are listed in detail in Table 2. The most filamentous bacteria were present in R2, and its population was simplest. This showed that filamentous bacteria outbroke under low DO. The filamentous bacteria population in seed sludge was also species-richer than in R1 and R3, proving that activated

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sludge from the wastewater treatment plant was composed of filamentous bacteria and non-filamentous bacteria. In addition, the family *Comamonadaceae* (belongs to β-*Proteobacteria*) was abundant in seed sludge and R2, and especially in R2. However, the family *Comamonadaceae* was not present in R1 or R3. Another remarkable phenomenon was that there was a low abundance of filamentous bacteria in R1 and R3: *Haliscomenobacter* and *Bdellovibrio* in R1, and only *Haliscomenobacter* in R3. Although AGS in R3 was looser than that in R1, AGS was still formed successfully in R1 and R3, and of course, filamentous bacteria contributed to the stability of granule structure.

This study represents the first time that low DO and high ammonia concentration were employed to investigate the morphology, interspecies quorum sensing, EPS characterization, and microbial communities in the formation of aerobic granular sludge. Our result indicated that though it was looser and lighter, AGS still could be cultivated under high ammonia concentration. Meanwhile, filamentous bacteria outbroke under low DO. As for QS, AI-2 activity had a peak value in all three reactors, and AI-2 activity of the ultrasonic pre-treatment sample was consistently lower than that of no-US sample. PS in R2 was the highest, resulting from the filamentous bacteria. Three-dimensional EEM fluorescence spectroscopy has been successfully utilized to identify the variation of EPS chemical composition, and the location shift of the fiuorscence peaks elaborated the chemical structure changes of EPS. The filamentous bacteria in low DO conditions were species-richer, and in high ammonia concentration conditions, *Thauera* was the most abundant. Furthermore, this research attempts to shed light on the potential for developing technology that would be able to cultivate granules of adequate structural stability for practical applications.

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