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Effects of seed sludge properties and selective biomass discharge on aerobic sludge granulation

Guo-ping Sheng[a,b], An-jie Li[a], Xiao-yan Li[a,*], Han-qing Yu[b]

[a] Environmental Engineering Research Centre, Department of Civil Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong, China
[b] School of Chemistry, University of Science and Technology of China, Hefei 230026, China

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ABSTRACT

This study was carried out to investigate the effects of seed sludge properties and the selective sludge discharge method on aerobic granulation in biological wastewater treatment. Small-loose flocs and larger-denser flocs were separated from raw activated sludge by sedimentation in a settling column. The two types of sludge were used as seed biomass in two laboratory batch reactors for the granulation experiment. A fixed daily sludge discharge ratio of around 10% of slow-settling sludge was applied to the two reactors. The results showed that aerobic granules could be formed in the reactors from both seed sludge of different structural and settling properties. The initial washout of small-loose sludge flocs during the start-up of the bioreactors did not appear to be a crucial factor for granulation. The key operating parameter was the daily discharge of relatively slow-settling biomass from the reactors. PCR-DGGE analysis showed insignificant differences between the bacterial communities of the mature granular sludge in the two reactors. It implied that continuous discharge of small and slow-settling flocs removed these competitors for substrate uptake from the system and hence made the substrate more available for large and compact flocs and granules. This selective sludge discharge facilitated the growth and accumulation of denser sludge in the reactor, leading to complete granulation.

1. Introduction

Aerobic granular sludge formation is a novel, compact and high-rate biological wastewater treatment technology that has attracted increased research attention in recent years [1–5]. Compared to conventional activated sludge flocs, aerobic granules have a dense structure and better settleability. An aerobic granular bioreactor allows a high level of biomass enrichment and can cope with high-concentration organics and shock loadings [6]. From an engineering economics point of view, aerobic sludge granulation is a promising process that has the potential to become one of the next-generation biological wastewater treatment technologies for both organic degradation and nutrient removal [5].

Despite the advantages and potential of aerobic granulation, the mechanisms of granule formation are not well understood. Aerobic granules are cultivated in sequencing batch reactors (SBRs) treating organic wastewater. Many factors, such as the type of organic substrate, loading rate, aeration intensity, fluid shear rate, hydraulic retention time, feast-famine regime and length of the sludge-settling phase, are believed to influence the granulation process [3,7–12]. Operating parameters are often chosen carefully in SBR to provide a combination of conditions favourable for granule formation and accumulation. However, the crucial determining factor and underlying mechanisms for granulation are still unclear.

In using SBRs for sludge granulation, a short settling period is commonly adopted to force the discharge of small-loose sludge flocs from the reactors [3,9,11]. A short settling time often results in significant sludge washout in the initial phase of SBR operation [1,13]. This early washout of small and slow-settling sludge from the raw seed sludge is thought to be essential to the start-up of sludge granulation [6,9]. However, it is not known whether granulation can be achieved using these small and slow-settling flocs as the seed sludge. In other words, the effects of seed sludge with different structural and settling features on the granulation process remain to be evaluated.

In the present laboratory study, small-loose sludge flocs and larger-denser flocs were separated from raw activated sludge by sedimentation. These two types of sludge were used as seed inoculums in two small batch reactors for the granulation experiment. A fixed daily sludge discharge rate of around 10% of slow-settling biomass was applied to the two reactors. The aims of the experimental study were to examine the effects of seed sludge with different structural and settling properties on the process of granule formation and to investigate the role of selective sludge discharge in granulation and the related mechanisms.

* Corresponding author. Tel.: +852 2859 2659; fax: +852 2559 5337.
E-mail address: xlia@hkucc.hku.hk (X.-y. Li).
URL: http://web.hku.hk/ xlia/ (X.-y. Li).

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Table 1
Characteristics of the three sludge samples: raw activated sludge, small-loose sludge flocs and larger-denser sludge flocs.

	Raw activated sludge	Small-loose flocs	Larger-denser flocs
Settling velocity (m/h)	0.5–10	<1.0	>6.0
MLVSS/MLSS	0.73	0.79	0.70
Mean size (μm)	434.7	250.6	465.0
SVI (mL/g MLSS)	149.0	166.7	119.9
Percentage (% in MLSS) in raw sludge	100	3.2	12.4

2. Materials and methods

2.1. Separation of small-loose and larger-denser sludge flocs

Small-loose sludge flocs with a slow-settling velocity and larger-denser sludge flocs with a fast-settling velocity were obtained from activated sludge by separation using a settling column based on their settling velocities. Raw activated sludge was collected from a domestic wastewater treatment plant, Stanley Sewage Treatment Works, in Hong Kong. Prior to sludge separation, the sludge suspension with a mixed liquor suspended solids (MLSS) concentration of 4 g/L was filtered through a 0.45-mm screen to remove large debris. For each run of the sludge separation, 50 mL of the sludge suspension was transferred gently to the surface of the water column in a 1-L graduate cylinder. After 2 min of settling, the top 100 mL of suspension, which contained small and slow-settling sludge flocs, was collected using a 100-mL syringe. Subsequently, the next 800 mL suspension from the top was withdrawn and discarded from the settling column, and the remaining 100 mL of sludge suspension left at the bottom containing the denser and fast-settling flocs, was collected. More raw sludge was processed following the settling column procedure to obtain a sufficient amount of seed sludge for inoculation of the bioreactors. The main properties of the fast- and slow-settling flocs are shown in Table 1. The fast-settling flocs were found to be larger in size, more compact in structure and faster in settling velocity than the slow-settling flocs.

2.2. Aerobic sludge granulation experiment

Two laboratory batch reactors, R1 and R2, were used for growing aerobic sludge granules. Each reactor was a graduated cylinder small column (H 30 cm × i.d. 3.6 cm) with a working volume of 200 mL. The two portions of sludge, the small and slow-settling flocs and the larger and fast-settling flocs, were seeded into reactors R1 and R2, respectively, at the same initial MLSS concentration of 2 g/L. Aeration was supplied from the bottom of each column through an air diffuser by an air pump at a flow rate of 12 L/min. The reactors were fed once every 12 h. Synthetic acetate-based wastewater was used as the feed influent, and consisted of (in mg/L): CH_3COONa 1282, NH_4Cl 191, K_2HPO_4 27.2, $NaH_2PO_4{\cdot}2H_2O$ 18.7, $CaCl_2$ 29.6, $MgCl_2{\cdot}6H_2O$ 54.2 and 1.0 mL of a trace element solution. The feeding solution had an organic concentration in chemical oxygen demand (COD) of 1000 mg/L, and the corresponding volumetric organic loading of the reactors was about 2 g COD/L/d.

Discharge of small and slow-settling sludge flocs was conducted at the end of each 12-h cycle from the column reactors. During the phase of sludge discharge, the sludge was allowed to settle in the column without aeration. After 2 min or less of settling, around 30 mL of the sludge suspension was withdrawn with a 50-mL syringe from the surface downward in the column. The slow-settling sludge in the suspension was therefore removed from the reactors. The amount of the daily sludge discharge and the sludge concentration in each reactor were measured. It was intended for each reactor that the biomass sludge be discharged everyday at a ratio of 10% which consisted mainly of slow-settling flocs. The objective of this operation was to selectively remove the slow-settling sludge from the mixture while controlling the apparent solid retention time (SRT) in the reactors at about 10 days. After the selective sludge discharge, the remaining sludge suspension was allowed to settle in the column for another 30 min. The supernatant was then withdrawn from the reactors, and the feed solution was added into each reactor to restore the original volume of 200 mL.

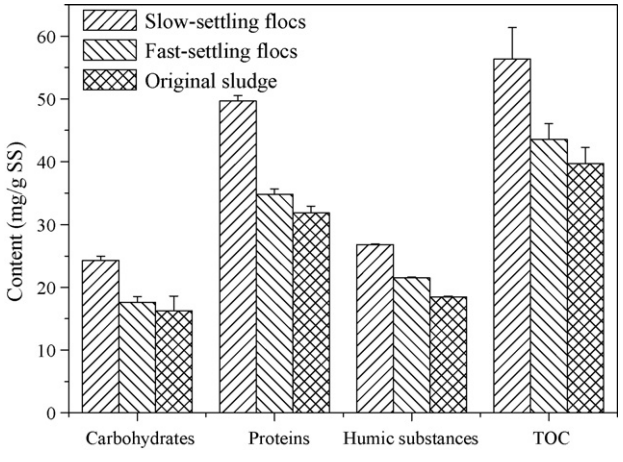

Fig. 1. Comparison of the microbial EPS content of the raw activated sludge, small-loose sludge flocs and larger-denser sludge flocs.

2.3. Denaturing gradient gel electrophoresis (DGGE) and bacterial species identification

The microbial population was analysed for the sludge samples from the two reactors on experimental days 1, 30 and 50. The genomic DNA of the biomass in the sludge and granule samples was extracted using a beadbeater (Mini-beadbeater™, Biospec, Bartlesville, OK, USA) and micro-centrifuge (MiniSpin plus®, Eppendorf, Hamburg, Germany) [14]. The bacterial 16S rDNA gene sequence (V3 region, corresponding to positions 341–534 of *Escherichia coli* sequence) was amplified by polymerase chain reaction (PCR) (PTC-200, MJ Research, Waltham, MA, USA) following the procedure detailed previously [15]. The forward primer sequence was 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′, and the reverse primer was 5′-ATTACCGCGGCTGCTGG-3′. The PCR amplified DNA products were then separated by DGGE through 8% polyacrylamide gels with a linear gradient of 30–60% denaturant using the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The gels were run for 6 h at 130 V in 1× TAE buffer at 60 °C, and then stained with ethidium bromide for 10 min and visualised by an UV illuminator. The DGGE images were acquired using the ChemiDoc (Bio-Rad) gel documentation system.

Selected DNA bands from the DGGE gels were re-amplified using the same PCR procedure described previously [15]. The PCR products were sequenced using BigDye Terminator (ABI PRISM® BigDye™ Terminator V3.1 Kit, Applied Biosystems, Foster City, CA, USA). The sequences were then analysed using the ABI PRISM®

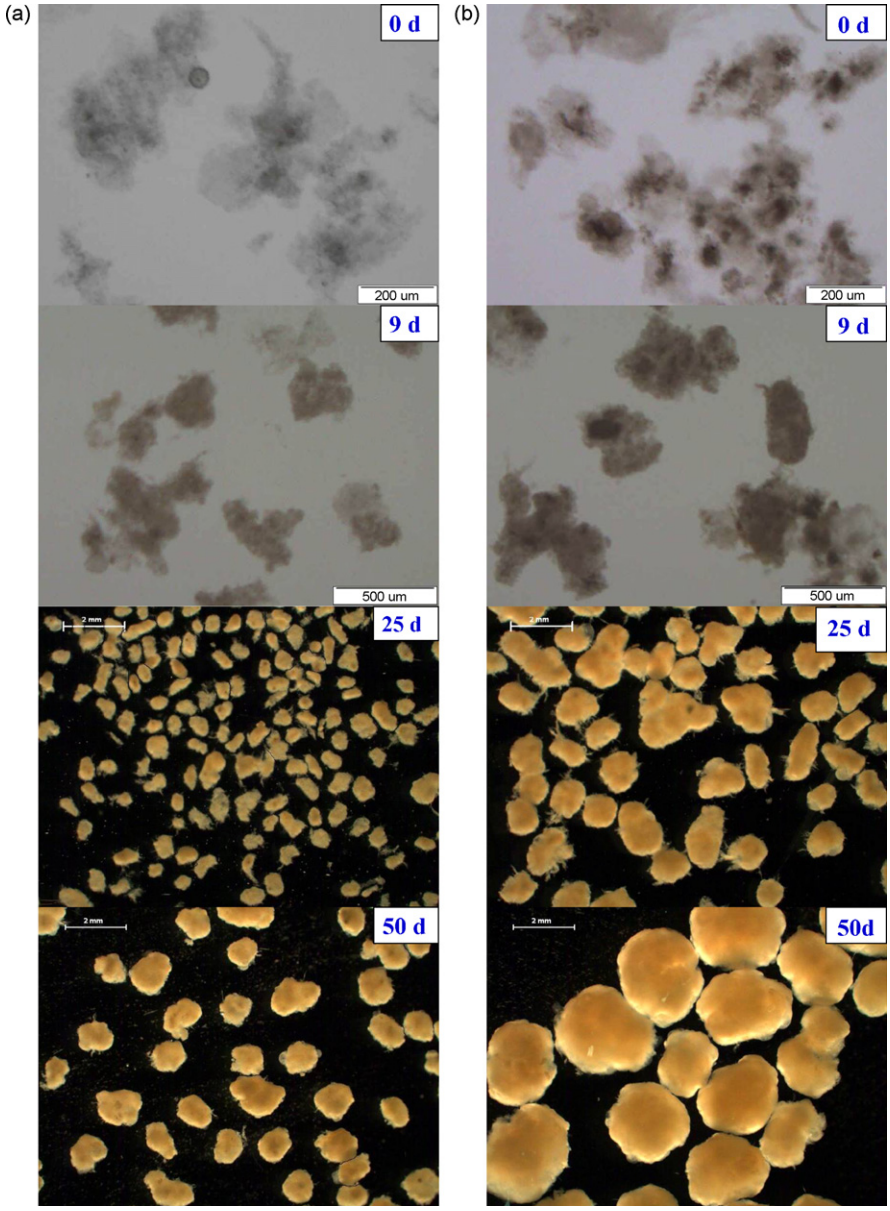

Fig. 2. Formation and evolution of aerobic granules in (a) R1 and (b) R2.

3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) for comparison with the 16S rDNA sequences in the GenBank with database search (National Center for Biotechnology Information, US National Library of Medicine) for identification [15].

2.4. Analytical analyses

The morphology of the sludge flocs in the reactors was analysed under an optical microscope (BX60, Olympus, Tokyo, Japan) equipped with a digital camera (DP10, Olympus). The size distribution of the sludge flocs was measured by a laser diffraction particle size analyser (Delsa 440SX, Bechman Coulter, Fullerton, CA, USA). Following sludge granulation in the reactors, the morphology of the granules was examined under a stereomicroscope (S8 APO, Leica, Wetzlar, Germany) equipped with a digital camera (EC3, Leica). The size and shape factor of the sludge flocs and aerobic granules were determined by a PC-based image analysis system (AnalySIS 3.1, Olympus Soft Imaging Solutions, Germany).

COD, MLSS, mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI) measurements were performed according to APHA-AWWA-WEF Standard Methods [16]. The settling velocity was determined by recording the time taken for the flocs or granules to settle through a distance of 20 cm in a column filled with water. The total organic carbon (TOC) of the water samples was measured using a TOC Analyzer (IL550, Lachat, Milwaukee, WI, USA).

The extracellular polymeric substances (EPS) of the sludge flocs and granules were extracted following a heat extraction procedure as described previous [17]. The sludge flocs was harvested by centrifugation at 4000 rpm for 5 min and washed twice with a 0.1% NaCl solution prior to extraction. For the granular sludge, the sample was homogenised by grinding to break down the structure of the granules. The sludge suspension was then heated to 60 °C in a water bath for 30 min. After centrifugation at 4000 rpm for 15 min, the supernatant was collected, which was regarded as the EPS extract. The TOC concentration of the EPS solution was measured. In addition, the carbohydrate content of the extract was determined by

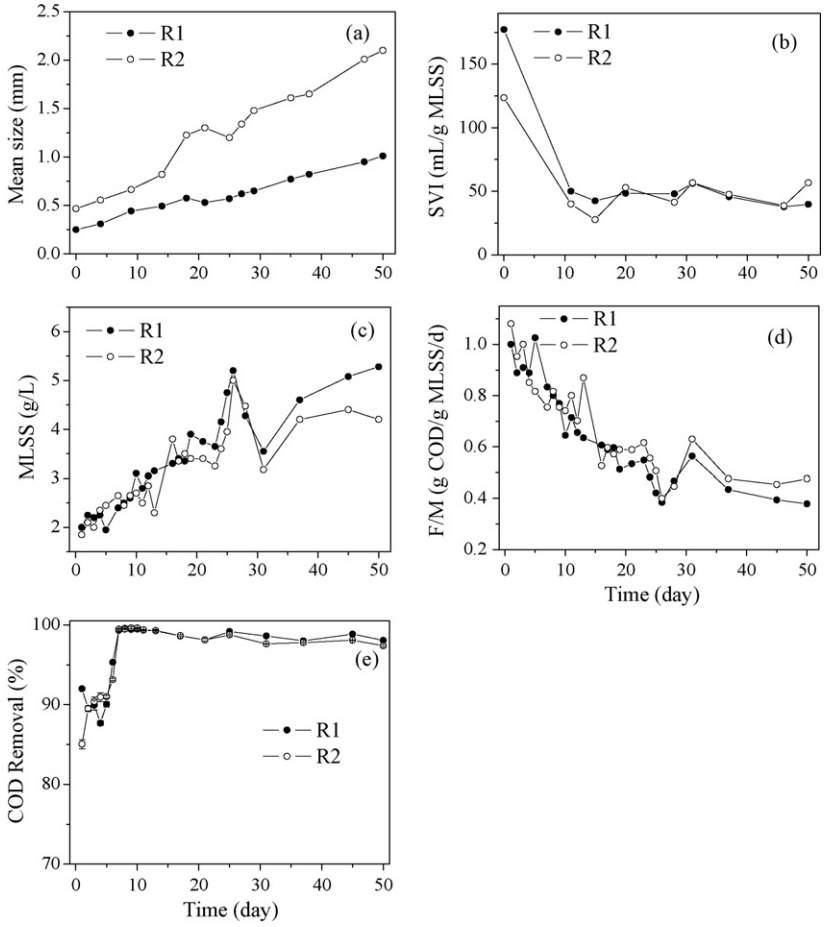

Fig. 3. Performance of reactors R1 (seeded with small-loose sludge flocs) and R2 (seeded with larger-denser sludge flocs) during the granulation process: (a) mean size, (b) SVI, (c) MLSS concentration, (d) F/M ratio and (e) organic removal efficiency.

the phenol–sulfuric method and expressed as the glucose equivalent, and its protein and humic substance content were measured according to the modified Lowry method [17].

As the amount of daily sludge sample obtained from each reactor was limited due to the small volume of the reactors, the sample analyses were arranged as follows. MLSS and MLVSS concentrations were measured on a daily basis. The size distribution (and morphology), SVI and the EPS content of the biomass were analysed once about every 5 days. The DGGE analysis was conducted on the biomass samples for days 1, 30 and 50. Thus, despite the small volume of the batch reactors, the amount of biomass sample obtained from the daily (10%) sludge discharge from a reactor was sufficient for the pre-determined chemical and biological analyses.

3. Results and discussion

3.1. Comparison of the seed sludge: small-loose flocs vs. larger-denser flocs

Of the sludge separated from the same raw activated sludge using the settling column, 12.4% was obtained as larger-denser flocs with a fast-settling velocity and only 3.2% as small-loose flocs with a slow-settling velocity. The denser flocs had a settling velocity faster than 6 m/h, whereas the small flocs had a settling velocity of less than 1 m/h. The two portions of sludge flocs were also largely different from each other in size, SVI and morphology (Table 1). The slow-settling flocs were smaller, with a rather loose structure and higher MLVSS/MLSS ratio, whereas the fast-settling flocs were larger, with a much more compact structure and lower MLVSS/MLSS ratio. Accordingly, the larger-denser flocs had a SVI considerably lower than that of the small-loose flocs. In previous studies of aerobic granulation, the small and slow-settling flocs in seed sludge have been washed out from the reactors in the initial phase of SBR start-up for sludge granulation [9,13].

The small-loose sludge had EPS content higher than that of the larger-denser sludge (Fig. 1). The results of the comparison between the portions of sludge were in agreement with the finding that EPS generally have a negative effect on the settleability of sludge [18,19]. Proteins were the main EPS component, followed by humic substances and carbohydrates. The carbohydrate, protein and humic substance ratios of the EPS of the raw activated sludge, small-loose sludge flocs and larger-denser sludge flocs were 1:1.96:1.14, 1:2.05:1.11 and 1:1.98:1.22, respectively, which shows that there were no differences in EPS compositions for the three types of sludge.

3.2. Aerobic granulation in the two bioreactors

As described previously, a fixed ratio at around 10% of daily discharge of slow-settling sludge was applied to the two batch reactors. As a result of the selective sludge discharge, aerobic granulation was achieved well in both reactors – R1, seeded with small-loose sludge flocs, and R2, seeded with larger and more compact flocs. Microscopic observation revealed the abundant of filamentous bacteria in both seed sludge samples, which disappeared gradually during the granulation process. The mean size of the sludge flocs in the two reactors increased continuously during the granule formation. After 9 days of cultivation, the mean

Table 2
Characteristics of the mature aerobic granules cultivated in R1 and R2.

	R1	R2
MLVSS/MLSS	91.7 ± 5.0%	93.5 ± 2.3%
Mean size (mm)	1.0 ± 0.2	2.2 ± 0.4
SVI (mL/g MLSS)	46.0 ± 6.7	48.1 ± 7.0
Settling velocity (m/h)	15.3 ± 4.1	33.4 ± 4.6
Shape factor	0.66	0.70

sizes of the flocs in R1 and R2 increased to 440 and 660 μm, respectively. After 25 days, regular-shaped granules with a smooth surface became dominant in the reactors. After 50 days, the mature granules increased further in size, with a rather compact microstructure (Fig. 2).

Granules were formed from both types of seed sludge. However, granulation in R2 occurred at a faster rate than in R1, and the granules in R2 were consistently larger than those in R1 (Fig. 3a). After 50 days, the mean sizes of the sludge had increased From 0.25 to 1.01 mm in R1 and from 0.46 to 2.16 mm in R2 (Table 2). Sludge granulation brought about improvement in sludge settleability. Due to the size difference, the R2 granules settled faster than did the R1 granules (Table 2). After 10 days, the SVI of the sludge in both reactors had decreased to a level of around 50 mL/g MLSS (Fig. 3b). Meanwhile, the biomass concentration in the two reactors increased gradually from about 2 to 5 g/L by day 26 (Fig. 3c). Hence, sludge granulation is shown as an effective means of biomass enrichment in biological wastewater treatment.

During granule cultivation, the MLVSS/MLSS ratios of the sludge in both reactors increased to about 0.90, suggesting an increase in the biomass fraction in the granular sludge. With biomass enrichment by sludge granulation, the food-to-micro-organism (F/M) ratios in the two reactors decreased gradually from 1.0 to 0.4 g COD/g MLSS-d (Fig. 3d). Accordingly, the organic removal efficiency of both R1 and R2 improved from an initial 80–90% to a high level of 96–98% (Fig. 3e).

Organic removal could be achieved rather rapidly in both granular reactors after sludge granulation. Within 2 h or less, the organic in both reactors was removed almost completely (Fig. 4). On average, the organic removal rates of the granular sludge in R1 and R2 were 976 and 639 mg COD/L h, respectively. The higher effectiveness of R1 granules in organic uptake and removal than that of R2 granules might be attributable to the different substrate transfer rates into granules of different sizes. It has been indicated that the substrate transfer rate is size dependent, i.e., the rate increases as the size of granules decreases [20,21]. The mean size of the aerobic granules in R2 was larger than that in R1, which likely led to a slower substrate transfer rate and thus a lower organic removal rate in R2 compared to those observed in R1.

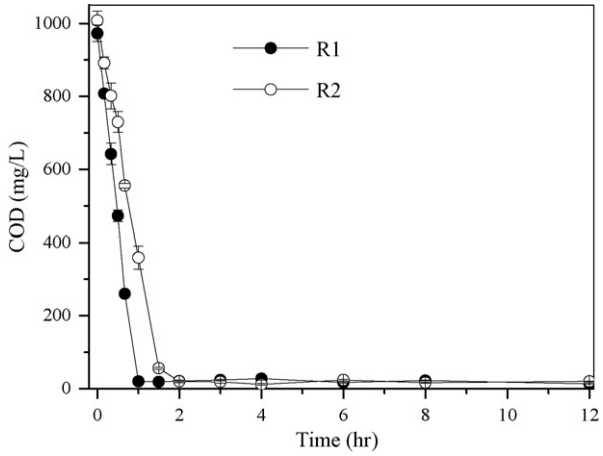

Fig. 4. Organic removal by the granular sludge during one feeding cycle in the two reactors after 50 days of sludge cultivation.

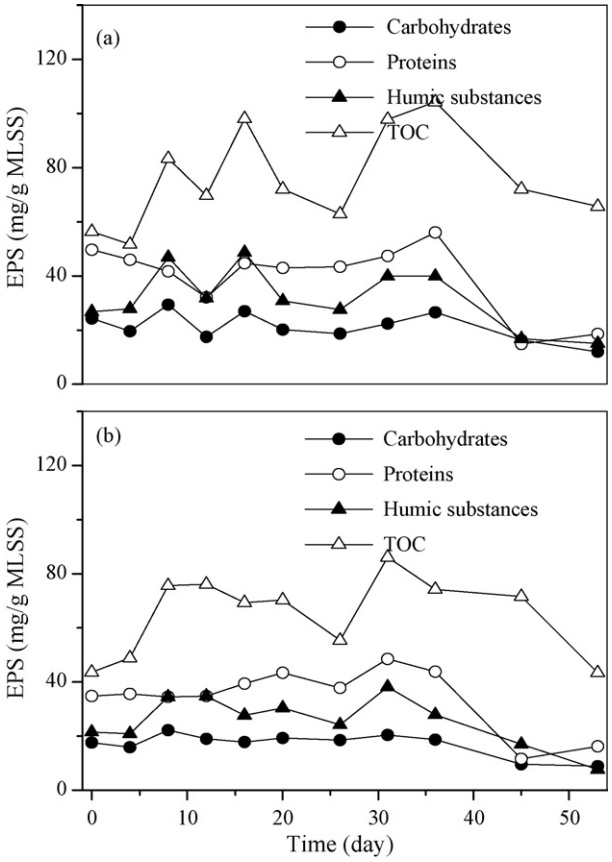

Fig. 5. Changes in the compositions of the microbial EPS during granular sludge cultivation in (a) R1 and (b) R2.

The microbial EPS of the sludge in R1 and R2 showed a similar trend of change during granulation (Fig. 5). The EPS content of the sludge in both reactors increased for the first 10 days and then

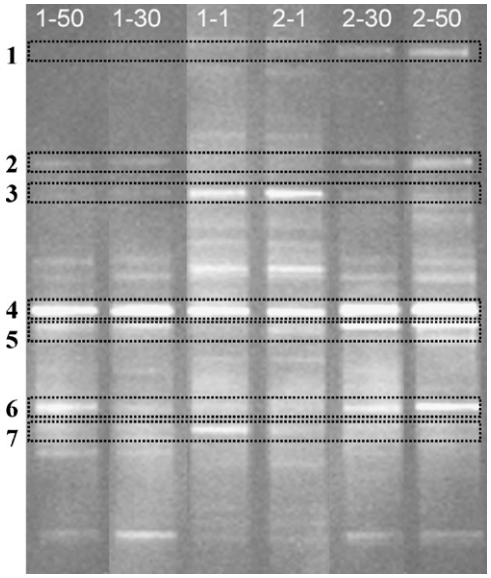

Fig. 6. DGGE images of the microbial sludge from the two reactors during aerobic granulation. Note: *m-n* is for the sludge from reactor m (R1 or R2) after *n* days of cultivation, e.g., 1-30: sludge from R1 after 30 days.

Table 3
Phylogenetic analysis of the dominant bacterial DGGE bands (Fig. 6).

Band no.	Closest relatives (accession no.)	Identity (%)	Phylogenetic division
1	Unknown		
2	*Rhodobacter sp.* TUT3732 (AB251408)	96	α-Proteobacteria
3	*Acidovorax sp.* BSB421 (Y18617)	99	β-Proteobacteria
4	*Brevundimonas sp.* (AJ227800)	94	α-Proteobacteria
5	Unknown		
6	*Zoogloea ramigera* (D14257)	99	β-Proteobacteria
7	Unknown		

fluctuated till 35 days of cultivation. With the completion of granulation, the EPS content of the sludge decreased. After 50 days, the protein, carbohydrate and humic substance contents of the EPS of the granular sludge were similar to those in the seed sludge flocs. It is generally considered that microbial EPS play a crucial role in aerobic granulation [22]. They can bind cells closely to enhance the formation and structural stability of granules [23]. However, the results of this study suggest that the role of EPS in sludge granulation is complex. During the early phase of granule formation, the EPS content increased with time. For mature granules, however, the EPS abundance remained the same or decreased. The results were agreed with previous studies, which also reported that the EPS content increased with cultivation time in the initial stage of aerobic sludge granulation, but demonstrated little change after the granules had matured [24]. A decrease in the F/M ratio during granule cultivation (Fig. 4d) could also be a reason for the decrease in the EPS content of the granular sludge.

3.3. Microbial population dynamics during sludge granulation

Well-resolved DGGE bands were obtained for the biomass in R1 and R2 during the granulation experiment (Fig. 6). In a mixture of extracted DNA, less abundant sequences may not be amplified sufficiently to form visible DGGE bands [15,25]. In the present DNA-based analysis, archae and eurarya were not targeted. Recent laboratory results show that healthy aerobic granules are dominated by bacterial cells with little eukaryal presence [26]. In addition, archae may be of abundance in some natural systems; however, they are not expected to be important in aerobic wastewater treatment bioreactors. The DGGE profiles of the sludge samples showed the evolution of bacterial species during granule formation. The microbial communities in the two types of seed sludge in R1 and R2 were not very different, despite the apparent difference in the physical characteristics of the sludge. Nonetheless, band 7 (an unidentified species) in the DGGE image of the R1 seed sludge was not a major band in that of the R2 seed sludge. Generally speaking, the DGGE comparison did not suggest a difference in the microbial diversity and abundance between R1 and R2 granules after 50 days of cultivation.

A few of the dominant bacterial bands were selected from the DGGE profiles (Fig. 6) for phylogenetic analysis (Table 3). The position of some major bands for the sludge in R1 and R2 shifted during sludge granulation, indicating the change in microbial population. For instance, band 3 (*Acidovorax sp*. BSB421, *β-Proteobacteria*) in the DGGE of both types of seed sludge disappeared after 30 days of cultivation. However, band 2 (*Rhodobacter sp*. TUT3732, *α-Proteobacteria*) and band 6 (*Zoogloea ramigera*, *β-Proteobacteria*), which were not significant for the seed sludge, appeared in the granular sludge in both reactors after 30 days. Band 1 (an unidentified species) occurred in the DGGE for the granular sludge in R2 but not in that of R1. However, a bright band, band 4 (*Brevundimonas sp*., *α-Proteobacteria*), occurred in all DGGE profiles throughout the sludge granulation. Band 5 (an unidentified species), a very weak band in both types of seed sludge, became brighter in the DGGE after sludge granulation in both reactors.

3.4. Importance of the sludge property and the role of selective biomass discharge in aerobic granulation

It is generally believed that SBRs are the most suitable type of bioreactors for aerobic granule formation [5]. Initial washout of slow-settling sludge is essential to the start-up of the SBR for aerobic granulation [6,13]. In this study, granulation was achieved readily for the seed sludge of denser and fast-settling sludge flocs. However, granules were also produced from the seed sludge of small-loose flocs with a slow-settling velocity, which were expected to be washed out in the initial phase of the start-up of SBR granulation. Based on the PCR-DGGE analysis, the differences between the bacterial communities of the granular sludge in the two batch reactors with different seed sludge are small. After cultivation, the two reactors seeded with different seed sludge types showed little differences in process performance (e.g., F/M, SVI and microbial community). The main difference was that the granulation process in R2 was faster than that in R1 (Fig. 2). However, R1 might be seen as R2 with a delay of 25–30 days with respect to the granule size and properties. Thus, the type of the seed sludge could affect only the rate of aerobic granulation to a certain extent. The structural and settling properties of seed sludge, as well as the initial washout of small and slow-settling sludge flocs, may not be as crucial to sludge granulation as previously thought.

In addition, for both types of the seed sludge, granules were produced in simple batch reactors that were not typical SBRs for granule cultivation. Few studies have been reported about the complete granulation in such small reactors. Under the well controlled experimental conditions, the results show that the key operation for granule formation was the daily discharge of 10% of the relatively slow-settling sludge from each reactor. Such operation would also affect the microbial community of the sludge. Band 3 in the DGGE profiles of both reactors disappeared after 30 days (Fig. 6), indicating that the bacterial species was largely eliminated from the biomass. On the contrary, bands 2, 4, 5 and 6 became brighter after granulation, suggesting that the corresponding species of microorganisms were enriched through the course of selective sludge discharge.

Previous studies showed that a good hydraulic condition would accelerate the process of both aerobic and anaerobic sludge granulation [2,3,27]. In SBRs for aerobic granulation, a high aeration rate and a short settling time are commonly adopted to discharge small-loose sludge flocs, which are thought to be essential to the start-up of sludge granulation [6,9]. However, the present study showed that the small and slow-settling sludge also could be granulated using the selective sludge discharge strategy. Small and loose sludge flocs in a sludge mixture were found to have an advantage over larger and dense granules in substrate uptake, and they can easily outcompete dense flocs and granules for substrates [28]. However, if the selective sludge discharge method is adopted, these competitors would be removed from the system. This would make the substrates more available for uptake and utilisation by the biomass in the attached-growth mode, leading to continuous granulation formation and growth. Thus, the selective discharge of small and

loose sludge flocs should be a key operating strategy for the rapid start-up of aerobic granulation in bioreactors.

4. Conclusions

- Aerobic granules were well formed from two different types of seed sludge, small-loose flocs and larger-denser flocs, which were separated from actual activated sludge by sedimentation. The initial washout of small and slow-settling sludge during SBR start-up did not appear to be a crucial factor for aerobic granulation.
- Sludge granulation was achieved in simple and small batch reactors. The key operation was the daily discharge at a ratio of 10% of relatively slow-settling sludge flocs from the reactors. PCR-DGGE analysis revealed that there were no differences between the bacterial communities of the granular sludge in the two reactors with different types of seeding sludge.
- During the granulation process, discharge of small and slow-settling flocs removed these competitors for substrate uptake from the sludge mixture and hence the substrate was made more available for large and compact flocs and granules. This selective sludge discharge facilitates the growth and accumulation of denser sludge particles in attached-growth mode, resulting in granulation.

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