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Characteristics of aerobic granules rich in autotrophic ammonium-oxidizing bacteria in a sequencing batch reactor

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ABSTRACT

Aerobic granules with high carbon- and ammonia-oxidizing activities were cultivated in a sequencing batch reactor. The granule size slowly increased with the operating time and the mean diameter reached 0.85 mm after 120 days of operation. Most of the ammonia was converted to nitrite and less nitrate was generated. The numbers of ammonia-oxidizing bacteria increased significantly in the formation of matured granules, and the fluorescence in situ hybridization image analysis demonstrates that the ammonia-oxidizing bacteria became the dominant nitrifying bacteria in the granules. Filamentous bacteria were mainly located on the outer surface of granules and cocci were dominant in their inner. The porosity and pore size distribution analysis with a size exclusion chromatography show that the granules had an excellent permeability for substrates with a molecular weight <1000 Da. The ammonia-oxidizing bacteria were enriched as the dominant nitrifying bacteria in the granules for simultaneous carbon and nitrogen removal in the reactor.

1. Introduction

In biological wastewater treatment processes for nutrient removal, nitrification is usually the rate-limiting step, which is achieved by two types of bacteria, i.e., ammonia-oxidizing bacteria (AOB) responsible for nitrite formation, and nitrite-oxidizing bacteria (NOB) for conversion of nitrite to nitrate [1,2]. Modern nitrogen removal processes, such as single high ammonia removal over nitrite (SHARON) and anaerobic ammonium oxidation (ANAMMOX), have several advantages in saving costs associated with aeration and organic carbon sources for denitrification [3]. It was reported that denitrification rate with nitrite was 1.5–2 times greater than that with nitrate [4].

Both SHARON and ANAMMOX processes need partial nitrification of ammonia to nitrite, which can be achieved through disequilibrating the activities or numbers of AOB and NOB [5]. Since wastewaters often contain both organics and nitrogen, aerobic granules that are capable of simultaneously removing organic carbon and accumulating nitrite are highly desired. Compared with the conventional activated sludge flocs, the aerobic granules have better settling ability, higher biomass retention in bioreactors and greater ability to withstand shock-loading rates [6,7]. However, little information is available about the formation of aerobic granules rich in the AOB.

Heterotrophic and nitrifying bacteria could coexist in microbial granules [2,8]. An increased substrate N/COD (chemical oxygen demand) ratio results in a signification shift among the three populations within granules [2]. Furthermore, an appropriate controlling substrate N/COD ratio is important to improve the aerobic granule stability. At a high N/COD ratio, aerobic granules had a compact mushroom-like structure, but had no a non-clustered structure at a low N/COD ratio [9].

The main objective of this work was to cultivate aerobic granules with capacity of simultaneous organic carbon removal and partial nitrification, and to explore their microbial and physicochemical characteristics. The distribution of the AOB and NOB in the granules was also determined by the most probable number (MPN) and fluorescence in situ hybridization (FISH).

2. Materials and methods

2.1. Reactor set-up

A sequencing batch reactor (SBR) used in this study had a working volume of 2.4 L with an internal diameter 6.0 cm and a height of 110.0 cm. The reactor was operated for 4 h each circle.

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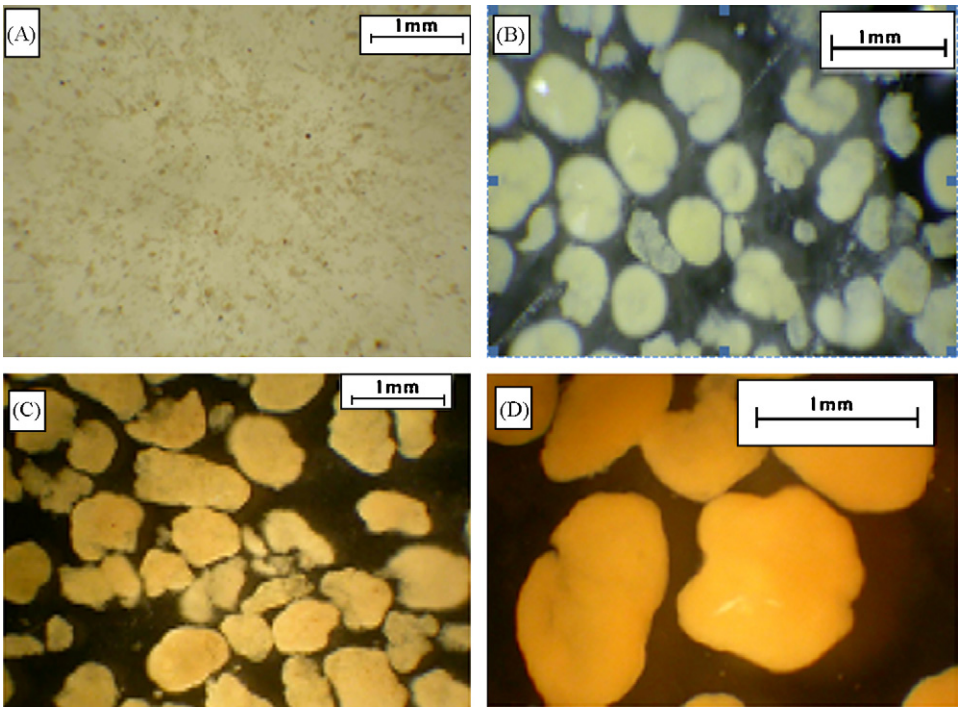

Fig. 1. Images of sludge in the granulation process: (A) seed sludge; (B) sludge on day 30th; (C) sludge on day 90th; (D) sludge on day 180th.

The filling and withdraw times were 2 and 5 min, respectively. The settling time varied from 6 to 2 min and the remaining time was for aeration. Effluent was drawn from the reactor middle and the exchange volume was 50%, resulting in a hydraulic retention time (HRT) of 8 h. The seeding sludge was collected from another SBR reported previously [10], which had a mixed liquor suspended solids (MLSS) concentration of 4.0 g L^{-1} and a sludge volume index (SVI) of 65.0 mL g^{-1}. Sludge of 0.8 L was seeded to the SBR used in the present work, resulting in an initial MLSS concentration of 2.3 g L^{-1} in the reactor. The experiment was performed at 25 °C. Air was introduced through an air diffuser at the reactor bottom by an air pump.

2.2. Wastewater

The composition of the synthetic wastewater was as follows: sucrose, 893 mg L^{-1}; NH_4Cl, 764 mg L^{-1}; $NaHCO_3$, 2000 mg L^{-1}; $MgSO_4$, 25 mg L^{-1}; $FeSO_4$, 5 mg L^{-1}; $CaCl_2$, 5 mg L^{-1} and microelement solution 1.0 mL L^{-1}. Sucrose was used as the carbon source in this work, because it could be readily utilized by activated sludge and also it is a main component of some food processing wastewaters. The N (NH_4^+-N)/COD ratio in weight of the wastewater was 1:5. To ensure the growth requirements of nitrifying bacteria, the ratio of bicarbonate to ammonium-nitrogen was kept above 8.0 mg mg^{-1}. The microelement solution contained H_3BO_3, 0.15 g L^{-1}; $ZnCl_2$, 0.05 g L^{-1}; $CuCl_2$, 0.02 g L^{-1}; $MnSO_4{\cdot}H_2O$, 0.05 g L^{-1}; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.06 g L^{-1}; $CoCl_2{\cdot}6H_2O$, 0.15 g L^{-1}; $FeCl_3$, 0.05 g L^{-1} and $NiCl_2$, 0.04 g L^{-1}.

2.3. Analytical methods

2.3.1. Chemical and microscopy analysis

The COD, MLSS, mixed liquor volatile suspended solid (MLVSS), SVI, ammonium, nitrite and nitrate concentrations were measured according to the Standard Methods [11]. The oxygen uptake rate (SOUR) was determined as described by Liu et al. [9]. Sucrose of 400 mg L^{-1}, NH_4Cl of 20 mg L^{-1} and $NaNO_2$ of 20 mg L^{-1} were, respectively, used as a substrate for determination of $(SOUR)_H$ (heterotrophs), (SOUR)-NH_4 (AOB) and (SOUR)-NO_2 (NOB). The DO concentration was recorded against time and the SOUR was calculated from the DO-time curve and the aerobic granule concentrations. Specific gravity and settling velocity of granules were determined using the method described by Zheng et al. [12].

The granule morphology was observed by using an optical microscope (Olympus CX41). The microbial compositions of granules were observed using a scanning electron microscopy (SEM, Akashi-SX-40). The granule size was measured with the approach reported by Su and Yu [10].

2.3.2. MPN

The numbers of AOB in seed sludge and granules were determined with an MPN method. Samples of 20 mL taken from the SBR were centrifuged at 9000 × *g* for 10 min and washed several times with sterile 0.9% sodium chloride solution until no NO_2^--N was detected in supernatant with Griess reagent. The MPN analyses were performed in serum tubes by preparing triplicate dilutions (10^{-3} to 10^{-7}) of stable consortia in a medium appropriate for the particular MPN. Each liter of the medium consisted of the following nutrients (mg): $(NH_4)_2SO_4$ 700; NaCl 300; Na_2CO_3 2000; $FeSO_4$ 50; Na_2HPO_4 100; $MgSO_4$ 50; $CaCl_2$ 50. NO_2^- test was conducted with the Griess–Illosvay method and the MPN was calculated using a MacGrady table [13].

2.3.3. FISH

FISH technique was used to investigate the microbial community of AOB and NOB in granules. The granule samples, withdrawn from the reactor on the 180th day, were fixed in 4% freshly prepared paraformaldehyde solution for 6 h at 4 °C and then washed twice with phosphate-buffered saline. They were then exposed

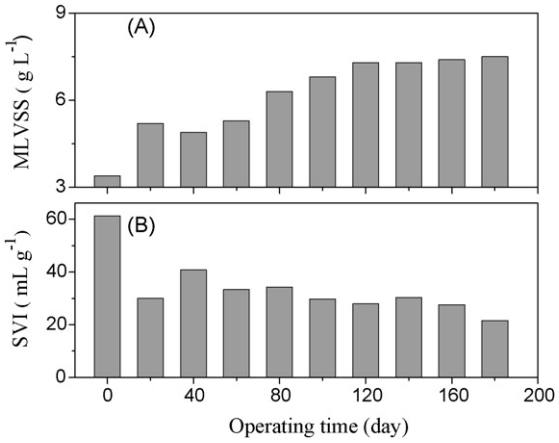

Fig. 2. Performance for the SBR: (A) MLVSS and (B) SVI.

to 50% ethanol in the saline for 12 h at −20 °C. The fixed granules were dehydrated by successive passages through 50, 80, and 100% ethanol (three times), 50:50 (vol/vol) ethanol-*tert*-butyl alcohol, and 100% *tert*-butyl alcohol (three times) and embedded in melted paraffin wax. The sections of 20 μm thick were cut with a rotary microtome and mounted on gelatin-coated glass slides. The sections were dewaxed through 100% xylene (two times) and 100% ethanol (two times). After air drying at room temperatures, hybridization was conducted following the established method [14].

A ROX-labeled NSO190 probe (5′-CGATCCCCTGCTT TTCTCC-3′) targeting ammonia-oxidizing bacteria and a FITC-labeled NIT3 probe (5′-CCTGTGCTCCATGCTCCG-3′) targeting *Nitrobacter* were used. The hybridization image was captured using a fluorescence microscope (Leica, DM6000B). For quantitative analysis of FISH images, approximately 10 images were scanned and averaged using image processing software (IMT i-Solution, Version 3.0).

2.3.4. Size-exclusion chromatography (SEC) measurement

Porosity is closely related to substrate and product transport in granules. It could be quantitatively determined with an SEC measurement [15]. The granules with mean diameters of 0.85 mm, taken from the SBR on the 180th day, were used for SEC measurement following the procedures reported by Zheng and Yu [15].

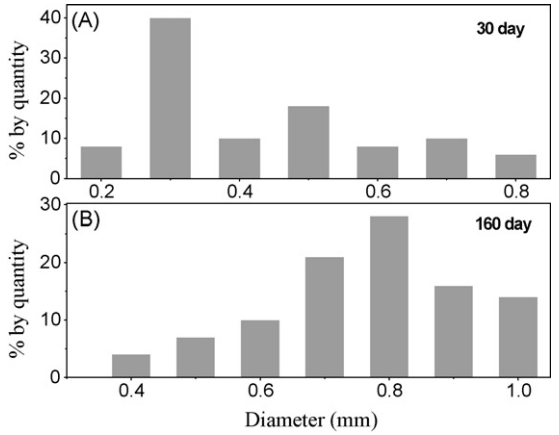

Fig. 3. Size distributions of the granules on: (A) day 30th and (B) day 160th.

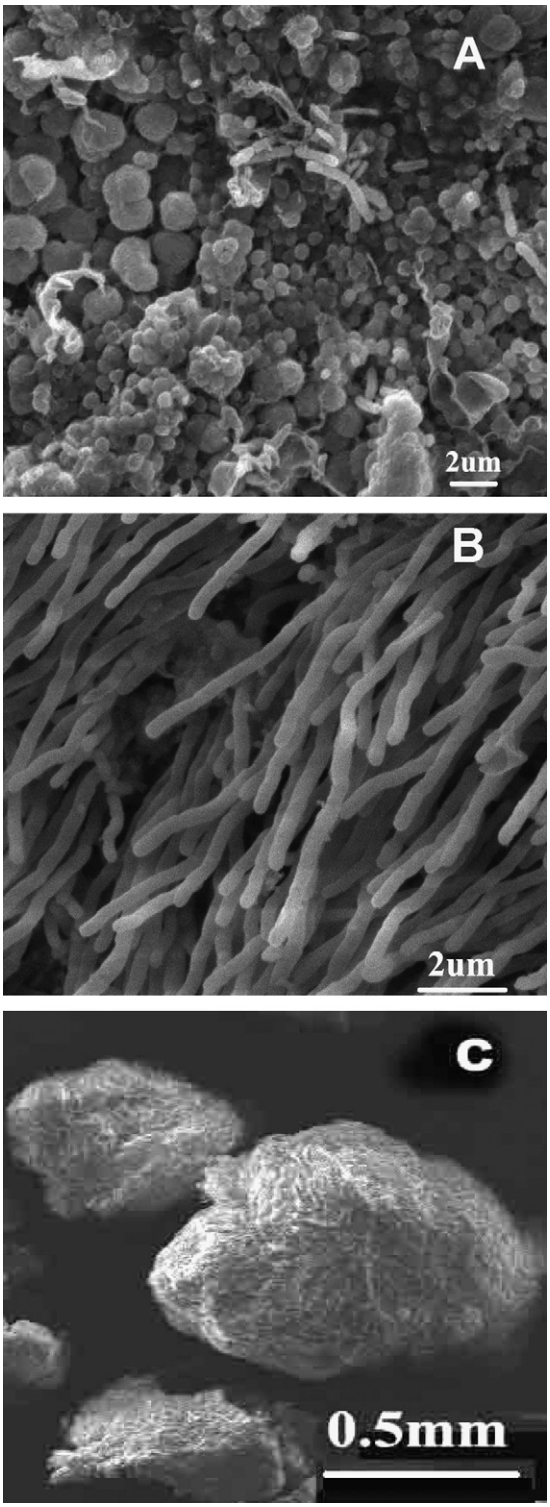

Fig. 4. SEM of the granules: (A) inner structure; (B) outer structure; (C) morphology.

3. Results

3.1. Granulation in the SBR

The seed sludge with a mean particle size of 0.10 mm showed a fluffy, irregular and loose-structured morphology (Fig. 1A). After 30 days of operation, aerobic granules with a diameter of 0.2–0.3 mm were formed. Thereafter, the numbers and average diameter of the

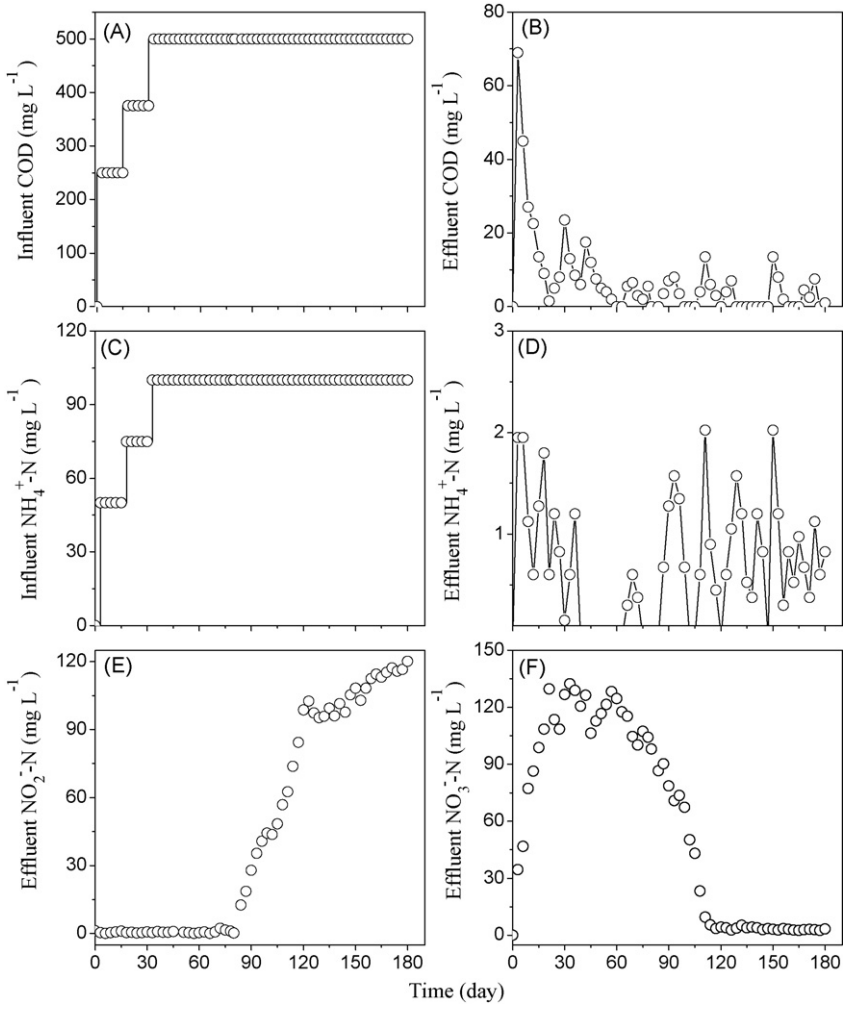

Fig. 5. Profiles of: (A) influent COD concentration; (B) effluent COD concentration; (C) influent NH_4^+-N concentration; (D) effluent NH_4^+-N concentration; (E) effluent NO_2^--N concentration; (F) effluent NO_3^--N concentration.

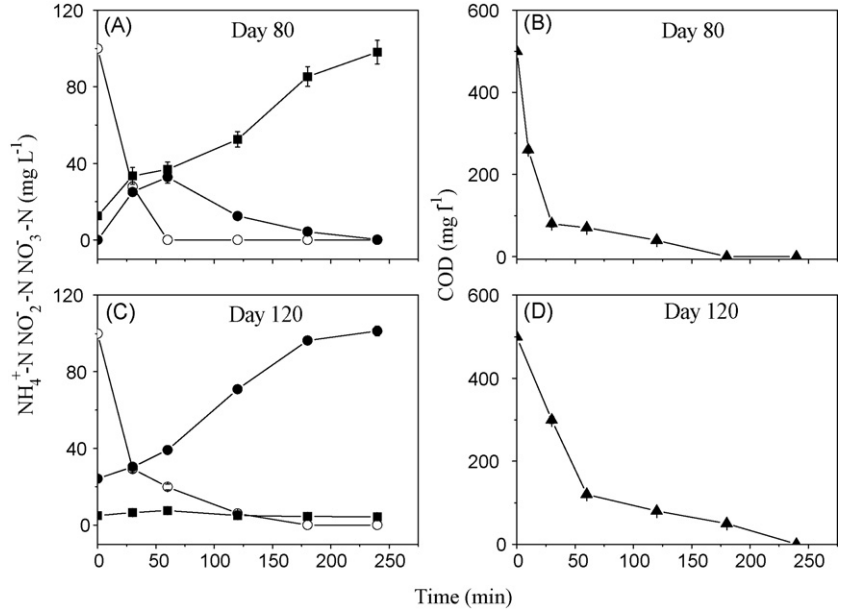

Fig. 6. Profiles of COD and nitrification in a cycle on days 80th and 120th (○) NH_4^+-N; (●) NO_2^--N; (■) NO_3^--N; (▲) COD.

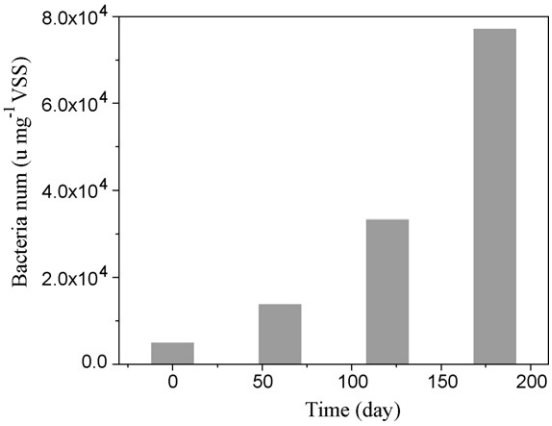

Fig. 7. AOB number profiles in the SBR operation.

bioparticles kept increasing (Fig. 1B). Afterwards, granules kept growing at a lower speed to the end of the operation. On day 180th, the granules had an average diameter of 1.1 mm (Fig. 1D).

The MLVSS and SVI profiles in the operation of SBR are shown in Fig. 2. After seeding, the MLVSS concentration in the reactor increased gradually and reached a relatively stable level of 6.5 g L^{-1} on day 80th. The initial SVI of seed sludge was 61.2 mL g^{-1}. After 20-day operation, the SVI decreased to 30.0 mL g^{-1}, and then decreased to only 21.6 mL g^{-1} at the end of experiment, implying that the granules had an excellent settling capacity.

In the granulation process, sludge size is an important parameter. The granule size distribution by number on days 30th and 160th was measured with 100 granule samples using an image analysis, and the results are shown in Fig. 3. Most of the granules were smaller than 0.5 mm in diameter on day 30th (Fig. 3A). The size distribution curve on day 160th became a normal distribution curve with an average diameter of 0.85 mm. The granule size increased slowly with the operating time.

The aerobic granules had a compact and round-shaped structure with a clear outer shape (Fig. 1B–D). An SEM image of a granule, its surface and interior at a high magnification in Fig. 4 shows that it had regular shape with some filamentous microorganisms (Fig. 4B and C). As a result, the granules had rough surface. As shown in Fig. 4A and B, cocci were predominant in the granule interior.

3.2. Partial nitrification

The profiles of the influent COD and NH_4^+-N, and effluent COD, NH_4^+-N, NO_2^--N and NO_3^--N) are shown in Fig. 5. The reactor performance was continuously improving in terms of COD and NH_4^+-N removal efficiencies in the operation (Fig. 5A and C). After 10 days of operation, both COD and NH_4^+-N removal efficiencies reached 97%. In the initial 80 days after inoculation, the influent NH_4^+-N concentration decreased rapidly and was converted to nitrite and nitrate (Fig. 5E and F). Fig. 6A and B illustrate a typical changing pattern of NH_4^+-N, NO_2^--N and COD concentrations in one cycle on days 80th and 120th. The NH_4^+-N concentration decreased rapidly and it was converted to nitrite and nitrate (Fig. 6A). Furthermore, a temporary nitrite accumulation with respect to nitrate formation was observed, indicating that both activities of AOB and NOB in the granules were high. The COD concentration decreased sharply after 30 min in a new cycle. However, after the 80th day NO_2^--N began to accumulate and NO_3^--N decreased gradually in the effluent, implying that the activity of the NOB was reduced (Fig. 5E). Such an accumulation of NO_2^--N was in accord with

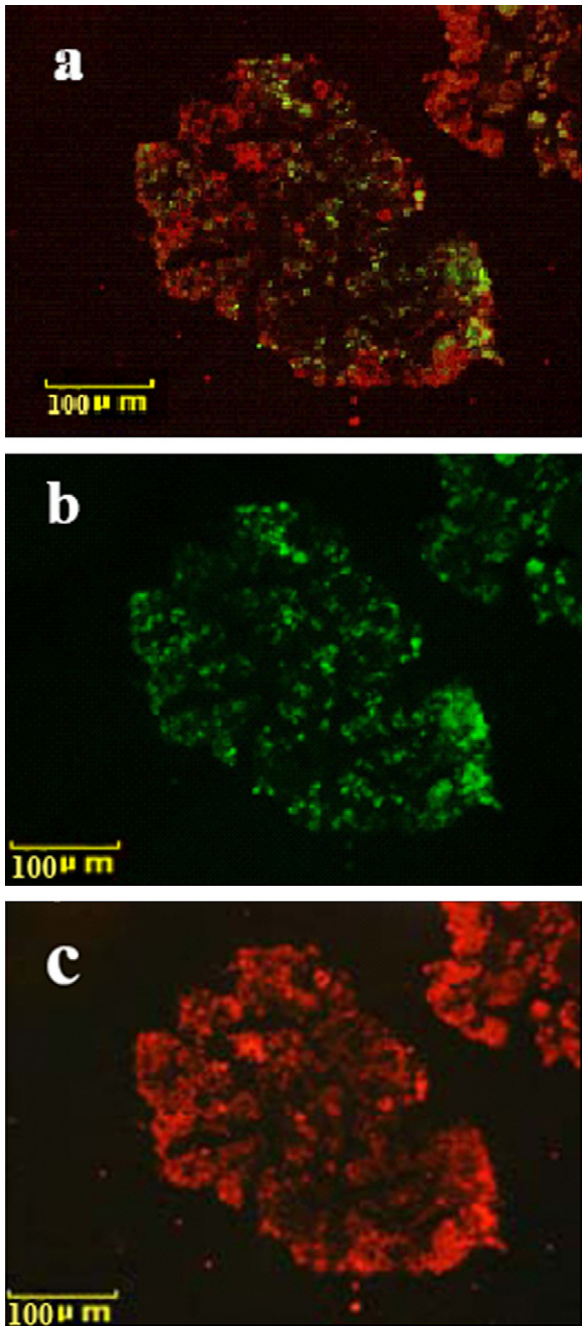

Fig. 8. FISH images of the granules on day 180th: (a) ROX-labeled probe NSO190 (red) and FITC-labeled probe NIT3 (green); (b) FITC-labeled probe NIT3; (c) ROX-labeled probe NSO190. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the increase in the AOB number. As shown in Fig. 7, the number of the AOB increased from 5000 μ mg^{-1} VSS in the seed sludge to 77210 μ mg^{-1} VSS in the granules at the end of experiment.

The results above indicate that the nitrifying populations in the sludge significantly increased. The ratio of the specific oxygen utilization rate $(SOUR)_H$ of the heterotrophs to the sum of specific oxygen utilization rates by the AOB $(SOUR)_{NH_4}$ and the NOB $(SOUR)_{NO_2}$, i.e., $(SOUR)_N$, $(SOUR)_H/(SOUR)_N$, represents the overall activity of the nitrifying populations. This index could be used to describe the fraction of the nitrifying bacteria in aerobic granules. On day 180th, the value of $(SOUR)_H/(SOUR)_N$ reached 0.8,

Table 1
Characteristics of the seed sludge and aerobic granules

	Seed sludge	Aerobic granules	
		90th day	180th day
MLVSS ($g\,L^{-1}$)	1.9 ± 0.2	7.5 ± 0.2	8.0 ± 0.3
MLVSS/MLSS (%)	70.0 ± 2.1	95.0 ± 2.5	96.2 ± 1.7
SVI ($mL\,g^{-1}$)	60.0 ± 3.5	27.5 ± 1.9	21.6 ± 2.4
Average diameter (mm)	<0.1	0.50 ± 0.08	0.85 ± 0.07
Settling velocity ($m\,h^{-1}$)	<1.0	2.3–4.0 ± 0.8	18.4–28.1 ± 2.1
Specific gravity ($g\,cm^{-3}$)	1.005 ± 0.001	1.028 ± 0.003	1.036 ± 0.005

suggesting that the nitrifying bacteria were enriched in the aerobic granules.

3.3. Community analyses

The spatial distribution of the AOB and NOB in granules was analyzed by the FISH. The granule was simultaneously hybridized with NSO190, the AOB domain specific probe, labeled with ROX (Fig. 8c) and NIT3, *Nitrobacter* domain specific probe, labeled with FITC (Fig. 8b). The FISH images (Fig. 8a) illustrate that the AOB were mainly located on the granule surface, while the NOB, i.e., *Nitrobacter*, were found to be located in the deeper layer of the granule. The total numbers of *Nitrobacter* were much smaller, compared with those of the AOB. A quantitative FISH image analysis of the nitrite-accumulating granule samples on the 180th day show that the AOB and *Nitrobacter* accounted for 69.4–70.8% and 16.2–18.3% of the total nitrifying bacteria in the granule, respectively. The results suggest that the AOB became the most dominant nitrifying bacteria in the matured granules.

3.4. Characteristics of aerobic granules

Table 1 summaries the characteristics of seed sludge and the granules sampled at two different stages. The specific gravity of sludge increased after granulation. It was 1.005 $g\,cm^{-3}$ at the beginning of experiment, and increased to 1.028 and 1.036 $g\,cm^{-3}$ after 90 and 180 days of operation, respectively. The significant improvement of the specific gravity for the sludge indicates that a highly compact structure was formed.

A total of 43 aerobic granules with a diameter of 0.6–1.3 mm were analyzed to determine their settling velocities in water. The settling velocities of the granules increased with their diameter and were much greater than those of the sludge flocs. The mature granules were well settled after 2–3 min of settling, leaving a clear supernatant in the reactor.

3.5. Porosity and pore size distribution

Fig. 9 illustrates the SEC test results. For acetate, its partition coefficient (K_{av}) is 98%. As shown in Fig. 9, the elution volume of blue dextrans, i.e., void column volume, was 64 mL, while the total column volume was 100 mL. The exclusion limit and total avail volume of the granules are given in Table 2. The molecules lower than

Table 2
Porosity characteristics of the granules using tap water as eluant

Solute	Molecular weight (Da)	Available porous volume (K_{av}) (%)
Acetate	82	98.2
PEG-400	400	94.4
PEG-1000	1000	88.9
PEG-4000	4000	66.7
PEG-10000	10,000	33.3
PEG-20000	20,000	11.1

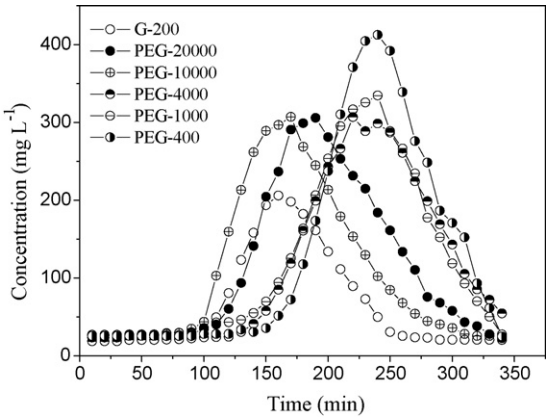

Fig. 9. Elution profiles of solutes with different molecular weights on the column packed with granules.

1000 Da could readily penetrate the granule pores. The granules had a higher exclusion limit for molecules larger than 20,000 Da. In general, if gel was used as the packing materials of column, plotting K_{av} against logarithm of the molecular weight (log M), a straight line was obtained. However, for the granules, the relationship between K_{av} and log M, as an indication of the pore size distribution, is not linear. Thus, the experimental data were subjected to a stepwise regression analysis (Fig. 10), and the following regression equation was obtained:

$$Y = -0.936 + 1.462X - 0.284X^2 \quad (1)$$

According to Eq. (1), a maximum log M value of approximately 4.4 was calculated when K_{av} was 0. This suggests that the exclusion limit was 25,118 Da for the aerobic granules.

4. Discussion

Both SHARON and ANAMMOX processes via nitrite pathway rely on the competition, elimination or inhibition of the NOB, so that the oxidation of nitrite to nitrate is reduced or blocked. Free ammonia (FA) and oxygen limitation are known to inhibit nitrification, especially nitrite oxidation [16]. The FA concentration in the SBR is estimated using the following equation [17]:

$$\mathrm{FA}(mg\,L^{-1}) = \frac{[NH_4\text{-N}] \times 10^{pH}}{\exp[6334/273 + T] + 10^{pH}} \quad (2)$$

where $[NH_4^+\text{-N}]$ is the ammonium-nitrogen concentration and T is temperature.

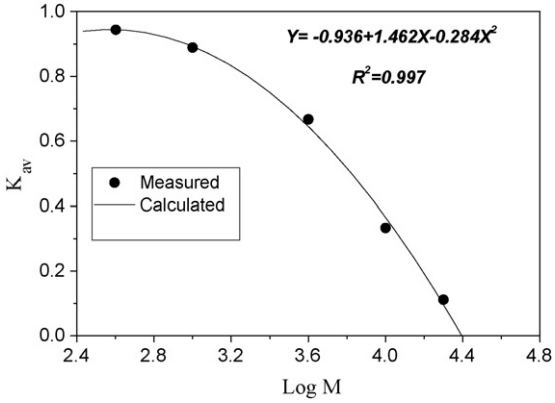

Fig. 10. Relationship between log of molecular weight (M) and partition coefficient (K_{av}).

After 80 days of operation, the FA concentration reached 0.51 mg L^{-1}. Previous work showed that the FA inhibition threshold was 0.1–4.0 mg L^{-1} for the NOB [16]. Thus, the change of nitrifying activity of the granules, which had a high activity for the AOB and low activity for the NOB (Fig. 5), can be conjectured in two ways. First, with the granulation process, AOB can outgrow NOB because of the high FA level in the SBR and accordingly to accumulate nitrite and achieve partial nitrification. Secondly, few NOB might be present in the granules, attributed to the wash-out of the NOB as explained above. On the other hand, the numbers of the AOB increased. This result was supported by both MPN and FISH analyses. In this work, oxygen limitation can be eliminated by maintaining a sufficiently high level of dissolved oxygen at 5.6–6.0 mg L^{-1}. However, previous studies have demonstrated that oxygen penetration depth is limited inside aerobic granules even when oxygen concentration in the bulk liquid is high [7]. Therefore, oxygen might also play a crucial role in affecting activity and distribution of the AOB and NOB in the granules. A further work is warranted to find out whether oxygen limitation can be eliminated or not.

Variations of the AOB counted with the MPN (Fig. 7) confirm that the AOB were enriched in the granules. Such a change was favourable for SHARON and ANAMMOX processes, compared with conventional nitrification–denitrification. Nitrite accumulation and the FISH analysis confirm that the NOB, e.g., *Nitrobacter*, were gradually washed out from the SBR, probably attributed to the selective inhibition of FA. In addition to *Nitrobacter*, *Nitrospira* is reported to play an important role in nitrite oxidation in activated sludge systems [5]. In this work, *Nitrospira* was not detected due to the limitation of these probes (NSO190 and NIT3).

However, despite of the great number of the AOB, ammonia remained unconverted in the reactor. This might be attributed to the inhibitory effect of the product accumulation on the conversion of ammonia to nitrite. Thus, both ammonia and nitrite would be accumulated with the increasing influent ammonia concentration. The conversion of NH_4^+-N to NO_2^--N relies heavily on the bicarbonate/ammonium ratio. The molar stoichiometric relationship for the complete ammonium oxidation should be 2 mol HCO_3^-/mol NH_4^+-N [18]. In our work, the bicarbonate/ammonium ration of 2.36 is beneficial to NO_2^--N accumulation. Through properly controlling pH, the influent NH_4^+-N concentration and the biomass retention in the SBR, the effluent composed of NH_4^+-N and NO_2^--N in a ratio of 1:1 could be achieved. In this case, partial nitrification of ammonia to nitrite could be a possible way to meet the requirement for the ANAMMOX process [18].

Aerobic granules contained channels and pores that penetrated to a depth of 900 μm below the granule surface. It was reported that the nitrifying population was mainly located at a depth of 70–100 μm from the granule surface [19]. These channels and pores would facilitate the transport of oxygen and nutrients into and metabolites out of the granules [2]. From an application point of view, the diffusion of dissolved oxygen and substrates are the major concerns of engineers. In this work, the matured granules with a relatively small diameter had favourable nitrification activity. Smaller granules might be more effective for nitrification in wastewater treatment, as these granules have more live cells within a given volume of granules [19].

The porosity and pore size distribution have a significant effect on the distribution and growth of the microorganisms in granules. The SEC tests show that available porosity (K_{av}) of the granules exceeded 0.88, indicating that they were highly porous. This observation was partially supported by the SEM images in Fig. 4, which show that there were some filamentous bacteria or fungi on the granule outer surface and that lots of cavities were present. These cavities could enhance substrate transfer from the bulk to granules and intermediate or by-product transfer from inside granules to the bulk. The extent to which substrate can penetrate a granule might be one of the main factors influencing the granules characteristics. Since the AOB were mainly located on the granule surface (Fig. 8), they were more advantageous for obtaining oxygen and nutrients than the NOB. This could be another possible reason why the AOB became dominant in the granules. The results of the present study also reveal that the heterotrophs and autotrophs could co-exist in the aerobic granules.

5. Conclusions

Aerobic granules performing partial nitrification were successfully cultivated in an SBR. The MPN and FISH analyses reveal that AOB could become the dominant nitrifying bacteria in the granules because of the long term inhibition of the NOB. With the granulation of activated sludge, its specific gravity increased, whereas the granular size increased slowly and the mean diameter was 0.85 mm on day 180th. The reactor could be kept in stable operation over 6 months. The SEC analytical results show that the granules had an excellent permeability for substrate with a molecule weight <1000 Da. The NOB could be selectively removed from the SBR to enrich the AOB in the granules. This study demonstrates the possibility for the effective use of alternative nitrogen removal technologies via nitrite from wastewaters.

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