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Operation of a sequencing batch reactor for cultivating autotrophic nitrifying granules

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Abstract

The granulation of nitrifying sludge in a sequencing batch reactor (SBR) fed with NH$_4^+$-N-laden inorganic wastewater was investigated. After 120-day operation spherical and elliptical granules with an average diameter of 0.32 mm were observed. The hydrophobicity surface, settling velocity and specific gravity of the matured granules increased with the processing of sludge granulation. Spatial distribution of bacterial species within the autotrophic granules was analyzed with fluorescence in situ hybridization. Both ammonia- and nitrite-oxidizing bacteria were observed in the granular sludge. The Michaelis-Menten equation was used to describe their NH$_4^+$-N utilization rate, and the kinetic coefficients were calculated to be $v_m = 18.0$ mg/g-VSS/h and $K_m = 36.7$ mg/l. Taking into account the NH$_4^+$-N utilization rate and removal efficiency together, an NH$_4^+$-N concentration range of 100-250 mg/l was found to be favourable for the operation of the SBR to cultivate nitrifying granules.

Keywords: Aerobic granule; Ammonia-oxidizing bacteria (AOB); Autotrophic; Kinetics; Nitrite oxidizing bacteria (NOB)

1. Introduction

Nitrogen compounds like ammonia and nitrate can be found in many wastewaters and need to be removed in order to prevent oxygen depletion and eutrophication of surface waters. Nitrification, the biological oxidation of ammonia, was described already a century ago. Extensive reviews on autotrophic nitrification and nitrogen
removal from wastewater are available (van Benthum et al., 1996). The rate-determining step in this process is nitrification, which is accomplished by autotrophic nitrifying bacteria under aerobic conduction (Ruiz et al., 2003; Ni et al., 2008). It is difficult to obtain and maintain sufficient nitrifying bacteria in wastewater treatment plants due to their very low growth rates.

In order to solve the problem, various techniques for retaining nitrifying bacteria with high density in a reactor have been recently proposed, e.g., entrapment in a hydrogel matrix of polyvinyl alcohol (Myoga et al., 1991) or polyethylene glycol (Sumino et al., 1992; Isaka et al., 2007). However, development of a simpler and more effective immobilization method for nitrifying bacteria is still demanded.

Aerobic granulation represents an innovative cell immobilization strategy in biological wastewater treatment and it is attracting increasing interests (Beun et al., 1999; Zheng et al., 2005; Su and Yu, 2005; Wang et al., 2007; Liu et al., 2009). Aerobic granules are self-immobilized microbial aggregates that are usually cultivated in sequencing batch reactors (SBR) without adding a carrier material. Many researchers reported aerobic granulation for efficient treatment of organic wastewater (Zheng et al., 2005; Su and Yu, 2005; Wang et al., 2007). However, the information on the nitrifying bacteria granulation with inorganic wastewater rich in ammonium is limited (Tsuneda et al., 2003; Liu et al., 2008; Ni and Yu, 2008). Tsuneda et al. proved that nitrifying bacteria could be self-immobilized in an aerobic upflow fluidized reactor. Spherical, pseudocubic and elliptical granules with a diameter of 0.35 mm were produced at the bottom of the reactor after 300 days of operation. The reactor
was operated continuously at a hydraulic retention (HRT) of 7.6 h and influent
NH$_4^+$-N of 500 mg/l. Liu et al. also cultivated nitrifying granules in an SBR (Tsuneda et al., 2003). Although autotrophic nitrifying granules for nitrification have been
developed, the formation of nitrifying granules and the distribution of
ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the
nitrifying granules are still not clear up to now yet.

Therefore, the main objective of this work was to cultivate nitrifying granules in
an SBR and elucidate the distribution of AOB and NOB in the granules. The physical
properties of granules, such as hydrophobicity of cell surface, settling velocity and
specific gravity, were also investigated. It is expected that the work would be useful to
better understand the mechanisms responsible for the granulation of nitrifying cultures
and apply them for the treatment of NH$_4^+$-N-laden inorganic wastewaters.

2. Materials and methods

2.1. Reactor set-up and operation

The SBR had a working volume of 3.4 l with an internal diameter 6.0 cm and a
height of 130.0 cm. The reactor was operated for 6 h each circle with a HRT of 12 h.
Effluent was drawn at 60.0 cm from the bottom, resulting in 1.7 l of mixed liquor left
in the reactor after effluent withdrawal. The filling and withdraw time were 2 min and
5 min respectively. The settling time was varied from 20 to 5 min and the remainder
was the reaction time. The seeding sludge, taken from an aeration tank in
Wangxiaoying Municipal Wastewater Treatment Plant, Hefei, China, was
pre-cultivated in a batch reactor supplied with ammonium and inorganic carbon for 6
weeks. The pre-cultivated sludge had a mixed liquor suspended solids (MLSS)
concentration of 12.0 g/l and a sludge volume index (SVI) of 42.8 ml/g. Sludge of 1.7
l was inoculated into the SBR, resulting in an initial MLSS concentration of 7.0 g/l in
the reactor. The experiment was performed in a temperature-controlled room at
25±1°C. Air was introduced through an air diffuser by an air pump at the bottom of
the reactor.

A synthetic wastewater was used based on the previous studies (Tsuneda et al.,
2003; Liu et al., 2008). This wastewater, with a similar characteristics to tannery
wastewater (Carrera et al., 2003), had the following compositions: NH₄Cl, 764 mg/l;
NaHCO₃, 2200 mg/l; MgSO₄, 25 mg/l; FeSO₄, 5 mg/l; CaCl₂, 5 mg/l and
microelement solution 1.0 ml/l. To satisfy the growth requirement of nitrifying
bacteria, the ratio (w:w) of bicarbonate to ammonium-nitrogen was kept over 8.0. The
microelement solution contained: H₃BO₃, 0.15 mg/l; ZnCl₂, 0.05 mg/l; CuCl₂, 0.02
mg/l; MnSO₄·H₂O, 0.05 mg/l; (NH₄)₆Mo₇O₂₄·4H₂O, 0.06 mg/l; CoCl₂·6H₂O, 0.15
mg/l; FeCl₃, 0.05 mg/l and NiCl₂, 0.04 mg/l.

2.2. Identification of nitrifying bacteria composition

The granule samples, taken from the reactors 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 (PBS). The granules were then exposed to 50% ethanol in PBS 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 temperature, hybridization was conducted following the established method (Sekiguchi et al., 1999).

A ROX-labeled NSO190 probe (5′CGATCCCCTGCTTTTCTCC3′) targeting AOB and a FITC-labeled NIT3 probe (5′CCTGTGCTCCATGCTCCG3′) targeting Nitrobacter were used. The hybridization image was captured using a fluorescence microscope (Leica, DM6000B). For quantitative analysis of FISH images, about 10 images were scanned and averaged by image processing software (IMT i-Solution, version 3.0).

2.3. Analysis

The MLVSS and SVI were measured according to the Standard Methods (APHA, 1998). Ammonium, nitrate and nitrite concentrations were determined colorimetrically following the Standard Methods (APHA, 1998). Changes in morphology of the granules, size, specific gravity, cell hydrophobicity and settling velocity were determined according to the methods reported by Su and Yu (2005).
3. Results and discussion

3.1. Formation of nitrifying granules

The seeding sludge with a mean floc size of 0.10 mm had a fluffy, irregular and loose-structured morphology. After 120-day operation, spherical and elliptical granules were formed. These granules increased in size and their average diameters reached 0.32 mm. The nitrifying granules had a compact and round-shaped structure with a clear outer shape. No filamentous bacteria were observed on the granule surfaces.

The aerobic granulation, i.e., from dispersed sludge to mature granules, is a gradual and slow process. Previous studies showed that the settling time had a significant influence on the aerobic granulation and that a short settling time was favourable for the granule formation (Lei et al., 2004). The settling time required for successful aerobic granulation would not be longer than 5 min. However, for a too short settling time a large volume of nitrifying population could not be maintained efficiently for the granulation because of their low growth. Thus, the settling time of the SBR was gradually decreased from 20 to 8 min. When the biomass concentration and SVI reached a pseudo-steady state, the settling time was fixed at 8 min. The changing patterns of MLVSS and SVI in the continuous operation of the SBR are
After seeding, the biomass concentration in term of MLVSS in the reactor decreased gradually because some microorganisms with poor setting properties were washed out of the reactor, and reached a relatively stable level of about 2.0 g/l on day 40 (Fig. 1). The initial SVI of seeding sludge was 42.8 ml/g. As shown in Fig. 1, the SVI first gradually increased and then decreased. At the end of experiment, the SVI decreased to only 36.4 ml/g, suggesting that the mature granular sludge had a more excellent settling capacity compared with the seeding sludge.

It took a long time period for the nitrifying bacteria with low growth rates to become granulation. Washout of flocs from an SBR is one of the essential strategies for aerobic granulation. Suitable aeration volume and hydrodynamic shear force are usually favorable to promote the nitrifying granulation (Tay et al., 2001). The present study demonstrates that at an HRT of 12 h, an air flow of 3.0 l/min, settling time of 8 min and a load of 0.235 kg NH₄⁺-N /l/d were appropriate to the formation of nitrifying granules.

3.2. Comparison between seed sludge and nitrifying granules

Table 1 summarizes the characteristics of the seeding sludge and the nitrifying granules at the end of experiment. The specific gravity of sludge increased from 1.005 g/cm³ at the beginning of the experiment to 1.018 g/cm³. Such a significant improvement of specific gravity indicates their highly compact structure. The
decrease in SVI and the increase in settling velocity clearly show that the sludge settling ability improved considerably as the granulation progressed.

Hydrophobicity of cell surface is considered to play an important role in the self-immobilization and attachment of cells to a surface. The seeding sludge had a mean contact angle value of 35.4° and increased to 72.8° on day 180. A significant difference in cell hydrophobicity between the seeding sludge and the nitrifying granules reveals that the formation of the nitrifying granules was coupled to an increase in the cell surface hydrophobicity. Similar results have been also found in other studies (Tay et al., 2001; Zheng et al., 2005).

3.3. FISH image analysis of nitrifying bacteria

Figure 2 illustrates the FISH images of the nitrifying granules collected from the SBR on day 180. The nitrifying granules were simultaneously hybridized with NSO190, AOB domain specific probe, labeled with ROX (Fig. 2b) and NIT3, Nitrobacter domain specific probe, labeled with FITC (Fig. 2c). The FISH images (Fig. 2a) illustrate that the AOB were found close to the granule surface, while that the NOB, i.e., Nitrobacter, were found in the deeper layer of granules, which might take advantage of the product (nitrite) formed by the AOB. Quantitative FISH image analyses of samples on day 180 show that the AOB occupied 62.7-63.6% in the total bacteria, while Nitrobacter occupied only 14.8-15.5%.

A typical changing pattern of NH₄⁺-N, NO₂⁻-N and NO₃⁻-N concentrations in one
operating cycle of the SBR is illustrated in Fig. 3. The NH$_4^+$-N concentration decreased rapidly and input NH$_4^+$-N was converted to nitrite and nitrate. Furthermore, a temporary nitrite accumulation with respect to nitrate formation was observed, indicating that activities of both AOB and NOB in granules were high. The results without nitrite accumulation suggest that, in addition to *Nitrobacter*, other NOB might also be present in the granules, converting nitrite to nitrate. Recently, *Nitrospira* has been found to play a role in nitrite oxidation in engineered systems (Kim and Kim, 2006). Coskuner and Curtis (2002) reported that *Nitrospira* and *Nitrobacter* could be coexisted in a full scale activated sludge plant. Thus, both *Nitrospira* and *Nitrobacter* might be present in the nitrifying granules.

The maximum number of the AOB and *Nitrobacter* occupied 79.1% of the total bacteria, suggesting that other AOB, NOB and heterotrophs might exist in nitrifying granules, despite of no organic matter in the feeding solution. Extracellular polymeric substances, produced by these heterotrophs, could be favorable to nitrifying granulation through stabilizing the scaffold of the granule and maintaining the three-dimensional structure (Tsuneda et al., 2003).

3.4. *Kinetics of substrate utilization*

Nitrifying bacteria are strictly aerobe, sensitive to substrate concentration and other operating conditions such as temperature and pH. In order to investigate the effects of substrate concentration on nitrification, NH$_4^+$-N concentration in the feeding...
solution was varied from 50, 100, 150, 200, and 250 to 300 mg/l. At each NH$_4^+$-N concentration, the SBR was operated for 2 weeks. For this SBR, its NH$_4^+$-N utilization rate is directly related to the nitrifying bacteria concentration in the granules and the NH$_4^+$-N concentration surrounding the granules. The NH$_4^+$-N concentrations of influent and effluent (after reaction for 2 h) were examined and recorded as $S_o$ and $S_e$ respectively. The substrate utilization rate is ration of the difference of $S_o$ and $S_e$ and the sludge concentration within 2 h. Fig. 4 shows the nitrifying rate increased rapidly as the NH$_4^+$-N concentration was increased from 50 to 200 mg/l and remained constant from 250 to 300 mg/l. Variations of the NH$_4^+$-N removal efficiency with the influent NH$_4^+$-N concentration are also illustrated in Fig. 4. On the contrary, an increase in NH$_4^+$-N concentration resulted in a decrease in NH$_4^+$-N removal efficiency.

Furthermore, the Michaelis-Menten equation was used to model the NH$_4^+$-N utilization as follows (Wu et al., 2002):

$$ v = \frac{v_m S}{K_m + S} $$

(1)

where $v$ is the NH$_4^+$-N utilization rate (mg/g-VSS/h), $v_m$ is the maximum NH$_4^+$-N utilization rate, $k_m$ is the dissociation constant (mg/l) and $S$ is the NH$_4^+$-N concentration. The double reciprocal form of Eq. (1) can be given:

$$ \frac{1}{v} = \frac{K_m}{v_m S} + \frac{1}{v_m} $$

(2)

Plotting $1/v$ against $1/S$, a straight line was obtained with an intercept of $1/v_m$ and a slope of $K_m/v_m$. From the slope and intercept of the best-fit line, $v_m$ and $K_m$ could be
estimated and thus the kinetics equation of substrate utilization was determined.

According to Eqs. (1) and (2), with a plot $1/v$ against $1/S$, the values of $v_m$ and $K_m$ were estimated as 18.0 mg/g-VSS/h and 36.7 mg/l, respectively. The regression line had a correlation coefficient of 0.987 implying the applicability of Eq. (1). Therefore, the kinetic equation of the NH$_4^+$ utilization was:

$$v = \frac{S}{2.0343 \cdot 0.0555}$$

(3)

The $K_m$ value represents the NH$_4^+$-N level required to reach 50% of the maximum NH$_4^+$-N utilization rate and could be used for adjusting the most appropriate NH$_4^+$-N level. The $v_m$ value of 18.00 mg/g-VSS/h is significantly higher than a value of 3.29 mg g-VSS/h in an airlift reactor as reported by Carvallo et al. (2002). This difference suggests that the nitrifying granules cultivated in the present work had a high nitrification rate. Thus, taking into account both NH$_4^+$-N utilization rate and removal efficiency together, an NH$_4^+$-N concentration range of 100-250 mg/l was appropriate for the effective operation of the SBR with nitrifying granules.

4. Conclusions

- After 120-day of operation, compact nitrifying granules were formed. Their surface hydrophobicity, settling velocity and specific gravity increased with the sludge granulation.
- The AOB were close to the granule surface, while the NOB were found in the
deeper layer of granules. The total number of *Nitrobacter* was much smaller than that of the AOB.

- The Michaelis-Menten equation could appropriately describe the NH$_4^+$-N utilization rate of the granules with a $v_m$ of 18.0 mg/g-VSS/h and $K_m$ of 36.7 mg/l.
- An NH$_4^+$-N of 100-250 mg/l was found to be appropriate for the operation of the nitrifying SBR.

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**References**


Table 1 Characteristics of the seeding sludge and the nitrifying granules

<table>
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<tr>
<th>Item</th>
<th>Seeding sludge</th>
<th>Nitrifying granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLVSS (g/l)</td>
<td>6.0±0.3</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>MLVSS/MLSS (%)</td>
<td>70.0±4.5</td>
<td>81.9±5.3</td>
</tr>
<tr>
<td>SVI (ml/g)</td>
<td>92.0±4.3</td>
<td>36.4±2.1</td>
</tr>
<tr>
<td>Average diameter (mm)</td>
<td>&lt;0.1</td>
<td>0.323±0.018</td>
</tr>
<tr>
<td>Settling velocity (m/h)</td>
<td>&lt;1.0</td>
<td>1.7-2.8±0.5</td>
</tr>
<tr>
<td>Specific gravity (g/cm³)</td>
<td>1.005±0.001</td>
<td>1.018±0.003</td>
</tr>
<tr>
<td>Contact angle</td>
<td>35.4°±3.5</td>
<td>70.2°±4.8</td>
</tr>
</tbody>
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Figure captions

Figure 1 Changing patterns of SVI and MLVSS in the continuous operation of the SBR.

Figure 2 FISH images of aerobic granules on 180th day. (a) ROX-labeled probe NSO190 (red) and FITC-labeled probe NIT3 (green); (b) ROX-labeled probe NSO190; and (c) FITC-labeled probe NIT3.

Figure 3 Nitrification profiles observed in a cycle phase

Figure 4 Variations of the substrate utilization rate and NH$_4^+$-N removal efficiency at different influent NH$_4^+$-N concentrations: ● substrate utilization rate, and ○ NH$_4^+$-N removal efficiency.
Fig. 1. Changing patterns of SVI and MLVSS in the continuous operation of the SBR
**Fig. 2.** FISH images of aerobic granules on 180th day. (a) ROX-labeled probe NSO190 (red) and FITC-labeled probe NIT3 (green); (b) ROX-labeled probe NSO190; and (c) FITC-labeled probe NIT3
Fig. 3. Nitrification profiles observed in a cycle phase
Fig. 4. Variations of NH₄⁺-N removal efficiency and the substrate utilization rate at different influent NH₄⁺-N concentrations: ○ the substrate utilization rate, and ● NH₄⁺-N removal efficiency