Enhanced aerobic nitrifying granulation by static magnetic field

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A B S T R A C T

One of the main challenging issues for aerobic nitrifying granules in treating high strength ammonia wastewater is the long granulation time required for activated sludge to transform into aerobic granules. The present study provides a novel strategy for enhancing aerobic nitrifying granulation by applying an intensity of 48.0 mT static magnetic field. The element analysis showed that the applied magnetic field could promote the accumulation of iron compounds in the sludge. And then the aggregation of iron decreased the full granulation time from 41 to 25 days by enhancing the setting properties of granules and stimulating the secretion of extracellular polymeric substances (EPS). Long-term, cycle experiments and fluorescence in-situ hybridization (FISH) analysis proved that an intensity of 48.0 mT magnetic field could enhance the activities and growth of nitrite-oxidizing bacteria (NOB). These findings suggest that magnetic field is helpful and reliable for accelerating the aerobic nitrifying granulation.

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1. Introduction

Biological nitrogen removal from wastewater by nitrification and denitrification is the most common and well-known treatment process. Nitrifying bacteria have a low growth rate and tend to be washed out from reactors, making the nitrification the rate-limiting step of the entire process. Therefore, it is necessary to keep a large amount of nitrifying biomass in reactors to ensure an efficient nitrification, especially in treatment with high strength ammonia wastewater. Immobilization is an efficient technique to retain large numbers of slow-growing organisms in reactors. Researchers have done many studies on granulation of nitrifying bacteria and they obtained good achievements (Belmonte et al., 2009; Shi et al., 2010; Tsuneda et al., 2003). However, the nitrifying granulation rate is slow due to the extremely low growth rate of nitrifying bacteria. In an aerobic upflow fluidized bed (AUFB), nitrifying granules with a diameter of 0.2 mm were observed after 100 days (Tsuneda et al., 2003). In a pulsing sequencing batch reactor (SBR), after 400 days of operation, only 1.07 g VSS/L of nitrifying granules with a mean diameter of 0.9 mm was accumulated (Belmonte et al., 2009). When the wastewater contains organic compounds, granulation rate can be accelerated. But the growth rate of aerobic nitrifying granules decreased sharply when the substrate N/COD ratio increased (Liu et al., 2004). When ammonium--nitrogen concentration was 150 mg/L, the specific growth rate of granules was merely about 0.04 per day and the mean diameter of mature granules was below 0.5 mm. Such slow granulation rate hinders the application of aerobic nitrifying granules in the treatment of high strength ammonia wastewater. Thus, it is necessary to look for some strategies to accelerate the aerobic nitrifying granulation process.

Previous researches have demonstrated that magnetic field could affect the growth and biodegradation ability of microbe, thus the biological effects of magnetic field have attracted attentions in the field of wastewater treatment (Ji et al., 2010; Lebkowska et al., 2011; Tomska and Wolny, 2008). Among them, most studies focused on the biodegradation of organic substrates and demonstrated that the use of magnetic could enhance microbial activity and accelerate degradation of organics (Ji et al., 2010; Lebkowska et al., 2011). In addition, the exposure of magnetic field to activated sludge was found to accelerate nitrification rate (Tomska and Janosz-Rajczyk, 2004; Tomska and Wolny, 2008). Consequently, it is believed that proper intensity of magnetic field could promote microbial metabolism and further enhance the granulation of nitrifying bacteria.

Therefore, the aim of this work was to explore the feasibility of enhancing aerobic nitrifying granulation by using a static magnetic field. The influences of magnetic field on extracellular polymeric substances (EPS) and nitrification performance of aerobic nitrifying granules were also monitored. For the purpose of investigating the influences of magnetic field on the distribution and abundance of ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), the fluorescence in-situ hybridization (FISH) assay was further conducted, and the results were analyzed with a program.
Image Pro Plus (version 6.0). It is expected that the information provided here will be useful for the cultivation and application of aerobic granules in the future.

2. Methods

2.1. Reactor set-up and operation

Experiments were performed in two identical 3.5 L internal-circulating sequencing batch reactors (SBRs, 100 cm in height and 8 cm in diameter for external down-comer, 70 cm in height and 5.4 cm in diameter for internal riser) with volumetric exchange ratio of 50% at room temperature 22 ± 3 °C. The two reactors were operated sequentially in 6 h cycles, with 12 min of influent filling, 336 min of aeration, 9 min of settling and 3 min of effluent withdrawal. The settling time was decreased from 9 to 3 min on day 16 for a short settling time would be beneficial for aerobic granulation. Air bubbles for aeration were introduced through a diffuser located at the reactor bottom with an air flow rate of 0.1 m³/h. Permanent magnets (cuboid, 25 × 100 × 150 cm) were only attached to the exterior reactor body of R1 with an intensity of 48.0 mT magnetic field. A distance of 2 m was kept between the two reactors to avoid effects of magnets to the control reactor (R2).

2.2. Inoculated sludge and synthetic wastewater

The two reactors were inoculated respectively with 1.0 L of activated sludge taken from the secondary settling tank of the Jinan Municipal Wastewater Treatment Plant of China, with an initial mixed liquor suspended solids (MLSS) concentration of 3.68 g/L; MLVSS/MLSS of 0.67 and sludge volume index (SVI) of 77.32 mL/g. Synthetic wastewater mainly consisted of 0.26 g/L glucose, 0.53 g/L sodium acetate, ammonium chloride, sodium bicarbonate (used to adjust influent pH at 7.8 ± 0.1) and other necessary mineral-salts medium including FeSO₄, 40 mg/L; KH₂PO₄, 22 mg/L; (used to adjust influent pH at 7.8 ± 0.1) and other necessary mineral-salts medium including FeSO₄, 40 mg/L; KH₂PO₄, 22 mg/L; MgSO₄·7H₂O, 2.2 mg/L; EDTA, 20 mg/L. The influent COD was fixed at 500 mg/L (50% contribution each of glucose and sodium acetate) and NH₄⁺−N was stepwisely increased from 100 to 400 mg/L.

2.3. Analysis

The measurements of mixed liquor suspended solids (MLSS), volatile suspended solids (MLVSS), COD, Y and N concentrations were conducted according to standard methods (APHA, 1998). The granule size was measured using an image analysis system (Image-Pro Express 6.0, Media Cybernetics) with an Olympus CX41 microscope and a digital camera (Olympus, Japan). Digital images were analyzed with a program Image Pro Plus (version 6.0). For each condition, three samples were measured for average and at least 10 different fields were examined for each sample.

2.4. Three-dimensional excitation and emission matrix (EEM) fluorescence spectroscopy

To investigate influences of magnetic field on the compositions of EPS, three-dimensional EEM spectroscopy was applied in this experiment. All EEM spectra were measured with an F-4600 fluorescence spectrophotometer (Techcomp Ltd., Japan). EEM spectra were collected with subsequent scanning emission spectra from 200 to 600 nm at 5.0 nm increments by varying the excitation wavelength from 200 to 600 nm at 5.0 nm increments. The spectra were recorded at a scan rate of 30,000 nm/min, using excitation and emission slit bandwidths of 5 nm. The voltage of the photomultiplier tube (PMT) was set to 400 V for high level light detection. The blank scans were performed at an interval of 10 using deionized water.

2.5. FISH analysis

To characterize AOB and NOB in the granules, FISH assay was further conducted. Granules were fixed in 4% freshly prepared paraformaldehyde solution for 4–6 h at 4 °C, and then washed twice with phosphate-buffered saline (PBS). The granules were then embedded in OCT (Optimal Cutting Temperature) compound (Tissue-Tek) at −20 °C for at least half an hour. Sections of 20 μm thick were cut with a rotary microtome (Microm HM 550) and mounted on gelatin-coated glass slides. The fixed granules were dehydrated by successive passages through 50%, 80%, and 100% ethanol (three times). After air drying at room temperature, hybridization was conducted following an established method (Sekiguchi et al., 1999).

DAPI (4,6-diamidino-2-phenylindole) was used to stain all the DNA containing organisms as background for total biomass determination. The 16S rRNA-targeted oligonucleotide probes were as follows: a FITC-labeled Nso190 for AOB (Mobarry et al., 1996), Cy3-labeled Ntspa662 and Cy3-labeled Nit3 for NOB (Daims et al., 2001; Wagner et al., 1996). Hybridized samples were viewed with a confocal laser scanning microscope (LEICA TCS SP2, Germany). Digital images were analyzed with a program Image Pro Plus (version 6.0). For each condition, three samples were measured for average and at least 10 different fields were examined for each sample.

3. Results and discussion

3.1. Formation and stability of aerobic nitrifying granules

The seeding sludge had a fluffy and irregular structure when initially inoculated into the two reactors. Previous studies have demonstrated that the settling time plays an important role in the formation of granules and a short settling time is beneficial for aerobic granulation (Adav et al., 2009; Qin et al., 2004). Therefore, settling time was decreased from 9 min to 3 min on 16th day, and then sludge with poor setting properties in R2 was washed out. It could observe clearly that there was a sharp decrease of MLSS in R2 as it decreased from 5.58 to 3.32 g/L (Fig. 1). With regard to R1, little sludge was washed out profiting from good setting property. After 18 days operation, some small granules were firstly observed in R1, and full granulation achieved on day 25 when round-shaped granules had become the dominant form of biomass (Fig. S1A). In comparison, the time for achieving full granulation in R2 was obviously longer, which required 41 days (Fig. S1B). The microstructures of the aerobic granules was further examined by SEM. SEM observations on 60th day revealed that bacteria of granules in R1 hold dense and compact bacterial structures, with cocci bacteria spreading all over the granules (Fig. S2a and b). Meanwhile granules in R2 were dominant by bacillus bacteria, and structures of granules were loose (Fig. S2c and d). This indicated that magnetic field could influence the dominant bacteria of granules.

After then, change trends of physical characteristics of sludge in two reactors were similar, with a gradual increase of mean
diameter and MLSS (Fig. 1a). The mean diameter of the granules in R1 and R2 stabilized at 2.1 and 1.3 mm finally, respectively. However, as shown in Fig. 1b, settleability of sludges in R1 was improved significantly by magnetic field as SVI was decreased from 77.32 mL/g to only 20.93 mL/g at the end of the experiment. This was consistent with the effect of magnetic field on the settleability of activated sludge reported by previous studies (Hattori et al., 2001a,b; Xu and Sun, 2008). And that may be the reason that why settling time was decreased from 9 to 3 min on 16th day, amount of sludge was washed out from R2 while the phenomenon did not occur in R1. In R2, there was not enough sludge for effective granulation as a result of the sludge loss. Thus, it can be reasonably inferred that 48.0 mT MF enhanced setting properties of sludge and further accelerated the formation process of granules.

As the magnets could attract metals such as iron and nickel, the compositions and contents of elements in granules were analyzed by SEM/EDX. It was clearly showed in Fig. 2 that iron content of granules cultivated in magnetic field represented 5.2% while it was only 1.2% in the control. There were no obvious differences of other elements between granules in two reactors. Because that there were 40 mg/L FeSO₄ and 1.5 mg/L FeCl₃·6H₂O in the synthetic wastewater, some iron compounds would attach to activated sludge in reactors. Iron compounds in activated sludge could be magnetized with exposure of magnetic field, and then the floc size was enlarged by assembling the iron compounds together by the magnetic forces between them (Hattori et al., 2001a,b). This aggregation of iron compounds resulted in a faster granulation process. Furthermore, granules with more iron content in R1 would hold greater densities and suffer the attraction of the magnet fitted in the bottom of R1, which can explain why granules in R1 hold better setting properties in the whole process.

Previous research have indicated that the divalent metal ions such as Ca²⁺ and Mg²⁺ can accelerate granulation process (Li et al., 2009; Liu et al., 2010). It was likely that Fe²⁺ could also accelerate the granulation process as granulation times in both the two reactors in present study were obviously shorter than previous studies (Belmonte et al., 2009; Tsuneda et al., 2003). As mentioned above, more iron content was discovered in granules cultivated in magnetic field, and then the increased Fe²⁺ and Fe³⁺ compounds in sludge would also accelerate the granulation process. Compared with the previous methods used for enhancing sludge granulation, exposure to magnetic field will be a potential alternative as the magnetic field can accelerate the process of granulation.

### 3.2. EPS of aerobic nitrifying granules

As sticky metabolic products secreted by bacteria, EPS are mainly composed of proteins, polysaccharides, humic acids and lipids. According to previous studies, EPS are beneficial to aerobic granules, such as providing nutrition to microorganism and
protecting microorganism toward toxic shocks (Sheng et al., 2010). In view of this, the effects of magnetic field on EPS contents of granules were measured in this study. EPS contents determined at different stages were presented in Fig. 3. It could be observed that the contents of proteins and polysaccharides of granules increased during the operation in both of the two reactors. But it was worthwhile to note that EPS content of granules in R1 was enhanced by a large margin under the influence of magnetic field. At the end of the experiment, the contents of proteins and polysaccharides in R1 were 60.33 and 18.21 mg/gVSS, which was 25.4% and 33.3% more than that in R2, respectively.

To characterize the EPS extracted from granules in R1 and R2, three-dimensional EEM spectroscopy was also applied in this experiment. EEM spectroscopy is collection of a series of emission spectra over a range of excitation wavelengths, and it can be used to identify organic material such as proteins and humic substances in EPS (Meng et al., 2011). The EEM fluorescence spectra of EPS extracted from granules in R1 and R2 were presented in Fig. 4. The peaks located at the excitation/emission wavelengths (Ex/Em) of 235/290–295 nm (peak A), 275/295–300 nm (peak B) and 260–265/365–370 nm (peak C), and was considered as aromatic protein-like substances, tyrosine protein-like substances and humic-like acids, respectively (Chen et al., 2003; Sheng and Yu, 2006). It could be observed that the peak locations of R1 were the same as that of R2 and had no changes in the whole process, indicating that magnetic field had no significant influences on the components of EPS. The peak locations were independent of the EPS concentration, but the peak intensities had a close relation with the EPS concentration. Clearly, there was a trend that the fluorescence intensities in R1 were higher than that in R2 in the whole process. This result was consistent with Fig. 3, which confirmed the conclusion that magnetic field had increased the EPS contents in granules once again.

It has been reported that when the microbial communities were subjected to certain selection pressures, EPS production would be substantially enhanced, and the stimulated EPS in turn accelerated the formation of biogranules (Adav et al., 2008; Sheng et al., 2010). To date, a number of selection pressures including hydraulic retention time, hydrodynamic shear force, substrate composition and settling time have been demonstrated to stimulate bacteria to secrete more EPS (Ni and Yu, 2010; Wang et al., 2006). In the present study, it seemed that magnetic field could also enhance the EPS production.

The stimulated EPS production in anaerobic granules has been found to be related to iron accumulation within granules (Liu et al., 2011). Then iron compounds in granules in R1 were likely to accumulate in the form of iron precipitates in granules with the gradual granular formation and maturation. Juang et al. (2010) has detected large amounts of iron precipitates in aerobic granule interiors in continuous-flow reactors. As there were more iron contents in the sludge, more EPS would be bounded and immobilized in the sludge. This provides a possible explanation for the stimulated EPS production in aerobic nitrifying granules under magnetic field exposure. And the stimulated EPS contents would further contributed to the faster granulation of nitrifying bacteria as mentioned above.

3.3. Nitrification performance of aerobic nitrifying granules

In order to investigate the effect of magnetic field on nitrification performance of aerobic nitrifying granules, long-term monitoring experiments were carried out. Similar to previous report (Wang et al., 2007), aerobic nitrifying granules exhibited excellent performances in removing ammonia, with removal ratios of both reactors were above 99% from day 30 to the end of the experiments (Fig. 5). But when influent ammonium concentration increased to 400 mg/L on day 80, there was an increase of nitrite concentration in the effluent of R2. The result revealed that nitrite could not be translated into nitrate successfully as before.

To better understand the transformation of nitrogen and the differences between the two reactors, cycle experiments were carried out on 55th day and 110th day. Fig. S3 exhibits similar nitrogen transformation processes in the two reactors. But after quantification of the oxidation rates of ammonia and nitrite (Table...
S1), it can be found that the nitrite oxidation rate in R1 was 4.7 mg/g SS h and 5.8 mg/g SS h on 55th day and 110th day respectively, much higher than 3.7 mg/g SS h and 4.3 mg/g SS h in R2. Meanwhile there were no obvious differences between the ammonia oxidation rates in the two reactors.

Results above suggested that magnetic field could enhance activities NOB. Similar results were also reported by other researchers, Tomska and Janosz-Rajczyk (2004) and Tomska and Wolny (2008) found that the nitrification rate of activated sludge exposed to magnetic field was higher than the control, and the rate of oxygen uptake by NOB was intensified more significantly. Though there are many reports about the influence of magnetic field on bacteria, the mechanism of low magnetic field affects biological systems is still not clear now. Previous studies demonstrated that magnetized water had higher pH, electric conductivity and osmotic pressure than general water, and thus had stronger permeability through cell membrane (Gonet, 1985; Lednev, 1991). These changes of water made by magnetic field may further influence the metabolic activities of bacteria such as enzyme activities, respiration and other metabolic processes. This may be the reason that NOB in R1 took possession of higher activities.

### 3.4. Identification of nitrifying bacteria composition – FISH analysis

To investigate the influences of magnetic field on the distributions and quantities of AOB and NOB, FISH assay was further conducted. It could be easily found in FISH images (not shown) that the distributions of bacteria in nitrifying granules picked from R1 and R2 were approximately the same: AOB was mainly located in the surface area of the granules while NOB were mainly located in the inner area of the granules, was just adjacent to the AOB. Similar distributions of bacteria in nitrifying granules were also reported by Shi et al., 2010. Although bacteria distributions of granules in two reactors were similar, there were still obvious differences in the distributions and qualities of bacteria. FISH images show that some void spaces were in inner area of granules in R1 and this indicated that there was no presence of active bacteria in the cores of granules in R1. As discussed above, the accumulation of iron compounds in the inner part of granules in R1 was probably responsible for the absence of active bacteria.

Quantitative FISH image analyses showed that aerobic nitrifying granules in R1 and R2 possessed similar relative abundance of ammonia-oxidizing bacteria, but NOB represented 28 ± 2.5% in R1 while it took up only 16 ± 1.8% in R2. The phenomenon revealed that 48.0 mT magnetic field might be beneficial to the growth of NOB. Then it could explain why there were sharp increases of nitrite concentration and decreases of nitrate concentration in the effluent of R2 while R1 run steadily as showed in Fig. 5.

### 4. Conclusions

The present study provides a novel strategy for speeding up the granulation process by applying a 48.0 mT static magnetic field. It was observed that magnetic field improved settleability of granules and stimulated EPS production by accumulating iron compounds in the sludge. The long-term, cycle experiments and quantitative analysis of FISH images demonstrated that 48.0 mT MF was beneficial to the growth and activity of NOB. This strategy which was simple and practical will be helpful for the cultivation and application of aerobic granules in the future.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.01.108.

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