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The modified mechanism for denitrifying granular sludge formation in a UASB reactor

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ABSTRACT

Physico-chemical and microbial mechanism of sludge during granulation process in upflow sludge bed reactor were investigated in this study. Mature granules achieved high settling velocity of 186–217 m/h, low VSS/SS of 0.34 and a nitrate loading rate of 2.61 kgNO_3^--N/(m^3·d). Scanning electron microscopy photographs showed that granular sludge surface was dominated by rod-like bacteria wrapped by filamentous substances such as extracellular polymeric substances (EPS) and fungus. Ca and Mg content of sludge increased from 50.3 to 127.6 mg/g SS and from 4.6 to 8.1 mg/g SS, respectively. EPS content increased from 68 to 88 mg/g VSS, and the ratio of protein to polysaccharides increased by 3-fold. Isolated *Fusarium oxysporum* fungi-1 played a key role in granulation process. Base on the results, a modified five-step granulation mechanism to describe the denitrifying granular sludge was proposed based on mechanism of extracellular polymer hypothesis.

Keywords: Granulation mechanism; Denitrifying granular sludge; EPS; Ca accumulation; Fungi

1. Introduction

Bio-granulation is a promising biotechnology since it has an excellent settleability, high biological activity and high treatment efficiency. According to oxygen demand, granular sludge can be divided into anaerobic, aerobic and anoxic (denitrifying) granular sludge. Anaerobic sludge granulation has been successfully achieved in up-flow anaerobic sludge blankets (UASB) for decades to treat various organic wastewaters [1–3]. Anoxic denitrifying granular sludge technology in the UASB reactor has been investigated for the removal of nitrate from wastewater [4–6].

Compared with anaerobic granular sludge, denitrifying granular sludge (anoxic) can be developed within a few weeks and have the ability to remove nutrients efficiently. However, the denitrifying granule sludge is not stable [7]. Therefore, it is important to study the granulation mechanism and understand the critical factors that have an influence on the characteristics of denitrifying granules.

The granulation mechanism of anaerobic and aerobic granular sludge has been widely investigated, and several theories have been proposed, for e.g., the crystal nucleus hypothesis [2], the four-step extracellular polymer hypoth-

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esis [8], cell hydrophobicity related hypothesis [9], hydraulic selection pressure hypothesis [10], and the filamentous fungi promoted mechanism [11]. Among these hypotheses, the extracellular polymer hypothesis is an important mechanism [8]. According to the extracellular polymer hypothesis, the granulation processes are divided into four steps: (1) transport of cells to the substratum, (2) reversible adsorption to the substratum by physicochemical force, (3) irreversible adhesion of the cells to the substratum, and (4) cell multiplication and granules development [8].

Anaerobic granulation process can be accelerated and UASB start-up time can be shortened, if any of these four steps can be improved [8]. However, the granulation mechanism of denitrifying sludge is not fully understood. The application of hypotheses are limited to certain conditions, such as the reactor type, the inoculum, and wastewater characteristics. Therefore, it is important to understand the granulation mechanism for denitrifying granular sludge.

In this study, the granulation mechanism of denitrifying granules was investigated. Firstly, denitrifying granules were cultured in an UASB reactor, and then the changes of metal and extracellular polymeric substances (EPS) contents were investigated during sludge granulation process. Secondly, microorganisms were isolated to investigate the dominant strains of mature granular sludge. Ultimately, the process of granulation was discussed to understand the granulation mechanism of denitrifying granular sludge, which may facilitate the development of granular sludge formation mechanism.

2. Materials and methods

2.1. UASB set up

A lab-scale UASB reactor with a working volume of 3.5 L was used to investigate the granulation mechanism of denitrifying sludge (Fig. 1). The height and inner diameter of the UASB reactor were 800 mm and 75 mm, respectively. The reactor consisted of four sampling ports, and was placed in an incubator at (35±1) °C.

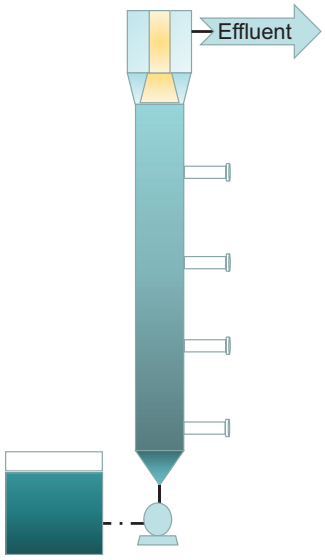

Fig. 1. Layout of the UASB reactor.

2.2. Seed sludge and synthetic influent composition

The seed sludge was collected from return sludge of the Xianyang Road Wastewater Treatment Plant (Tianjin, China) which treats municipal wastewater. The seed sludge was steeped by tap water for three days, and then steeped by the synthetic influent ($NaNO_3$-N 50 mg/L) for ten days, changing the water every day. The reactor was inoculated with 32.7 g volatile mixed-liquor suspended solids (MLVSS) seed sludge. The ratio of volatile mixed-liquor suspended solids to suspended solids ratio (VSS/SS) was 0.64, and sludge volume index (SVI) was 96 mL/g.

The influent was prepared with CH_3OH, $NaNO_3$ and KH_2PO_4 in tap water. The NO_3^--N varied from 50 to 250 mg/L, while the ratios of COD/N and the concentration of KH_2PO_4-P were maintained at 4 and 2 mg/L, respectively. Otherwise, according to the metal element concentration in the seed sludge, the concentration of Ca, Mg, Fe and Cu in influent were adjusted to 50.3±4.9 mg/L, 4.6±0.8 mg/L, 6.16±0.73 mg/L, and 0.35±0.09 mg/L with $CaCl_2$, $MgSO_4$, $FeCl_3$ and $CuSO_4$, respectively. The pH of feeding solution was approximately 7.1–7.3.

2.3. Experimental procedure

The lab-scale UASB reactor was operated for a period of 120 d including three experimental phases. In Phase I (days 1–35), the influent COD and NO_3^--N concentrations were 200 and 50 mg/L, respectively. The reactor was started-up by gradually increasing inflow, with the inflow being controlled at 0.5, 1.0, and 1.5 L/h during the 1st to 12th day, 13th to 27th day, and 28th to 35th day, respectively. The hydraulic retention times (HRT) were 7.0, 3.5 and 2.3 h with a nitrate loading rate (NLR) of 0.17, 0.34 and 0.52 kgNO_3^--N/(m^3·d). The upward-flow velocities were 0.11, 0.23 and 0.34 m/h, respectively.

In Phase II (days 36–70), the HRT was maintained at 2.3 h. The influent NO_3^--N concentration increased and maintained at 100 mg/L with the NLR of 1.04 kgNO_3^--N/(m^3·d) during the 36th to 57th day. Then, it increased to 150 mg/L with the NLR of 1.57 kgNO_3^--N/(m^3·d) during the 36th to 57th day.

In Phase III (days 71–120), the HRT was controlled at 2.3 h with the backflow ratio of 100%. The influent NO_3^--N concentration increased to 250 mg/L with NLR of 2.61 kgNO_3^--N/(m^3·d). The upward-flow velocity was 0.68 m/h.

2.4. Analytical methods

Nitrate, COD, mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) levels were determined according to the standard methods [12]. The granules size, settling velocity and physical strength were measured using the wet sieving method, precipitation method and integrity coefficient (%) method, respectively [13].

The morphology of the mature granular sludge was investigated using the scanning electron microscopy (SEM) (HITACHI, S-4800-I). The granules were separated into the surface part and the interior part, were dried as Jiang et al. [14], and then were observed by SEM.

Metals in liquid samples and biomass were determined using the atomic absorption spectroscopy (Shimazu,

Table 1
The experimental procedure of UASB reactor

Phases	Days	Influent COD mg/L	Influent NO_3^-N mg/L	NLR $kgNO_3^-N/(m^3 \cdot d)$	HRT hour	Upward flow m/h	Inflow L/h	Backflow ratio %
Phase I	1–12	200	50	0.17	7.0	0.11	0.5	–
	13–27	200	50	0.34	3.5	0.23	1.0	–
	28–35	200	50	0.52	2.3	0.34	1.5	–
Phase II	36–57	400	100	1.04	2.3	0.34	1.5	–
	58–70	600	150	1.57	2.3	0.34	1.5	–
Phase III	71–120	1000	250	2.61	2.3	0.68	1.5	100

AA-6800). Granule samples were dried, then re-dissolved in a hot concentrated HNO_3 (36.5%) solution, and filtered into 50 mL volumetric flasks. Samples were analyzed by the atomic absorption spectroscopy using hollow cathode lamps and acetylene/air flame.

The formaldehyde+NaOH method was applied to extract EPS [15], while TOC was used to characterize EPS content. The phenol-sulfuric acid method was used to quantify polysaccharides, with glucose as the standard. Proteins were measured by the Bradford method using bovine serum albumin (BSA) as the standard [13].

Spread-plating method was used for strain isolation and purification in the granules with a modified agar medium. Granules taken out of UASB reactor were triturated mechanically. The triturates were diluted with sterile distilled water, and then 0.1 mL aliquots of the 10^{-3} and 10^{-4} dilutions were spread on modified agar media, to which 100 mg/L NO_3^-N were added. The Luria-Bertani (LB) agar medium and the Rose Bengal agar medium were used for bacteria and fungi isolation, respectively. Methylene blue solution (3%, 2 mL/L) was added to agar medium to identify denitrification reaction. The plates were incubated at 30°C for 4 d. Pure isolates were obtained by re-culturing individual colonies several times on fresh agar medium to produce single colonies. The isolates were conducted for genomic DNA extraction and PCR amplification (Shanghai Sangon, Ltd). 16S rRNA (for bacteria) genes and 18S rRNA (for fungi) genes of isolates were PCR amplified with primer pairs 7F/1540R (27F: 5′-CAGAGTTTGATCCTGGCT-3; 1540R:5′-AGGAGGTGATCCAGCCGCA-3) and NS1/NS6 (NS1:5′-GTATCATATGCTTGTCTC-3′; NS6:5′- GCATCACAGACCTGTTATTGCCTC-3′), respectively. 16S rRNA and 18S rRNA gene sequences from isolates were analyzed using the Nucleotide-nucleotide BLAST (Blastn) database (http://www. ncbi.nlm.nih.gov/BLAST). Sequences and their closest matches retrieved from the database were aligned using the software Clustal X 2.1, and phylogenetic trees were constructed by the neighbor-joining method with the software MEGA 5.0. Evolutionary distances of nucleotide sequences were calculated with the p-distance model, and bootstrap values were obtained with 1000 re-samplings.

3. Results and discussion

The nitrate removal performance of UASB reactor after 120 d of operation as shown in Fig. 2. Nitrogen removal efficiency was above 70% at the beginning of start-up,

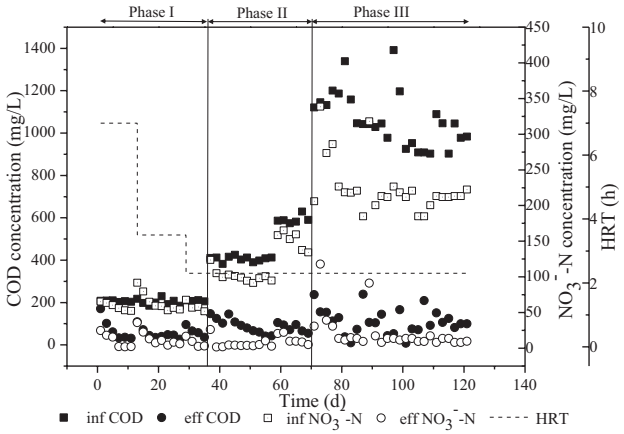

Fig. 2. Performance of UASB reactor during the granulation process.

and it again reached above 70% within 5–7 d, when NLR increased. During the Phase I-II, the effluent concentrations of COD and NO_3^-N were below 50 and 10 mg/L when the USAB reached to stable status. It took approximately 55 days for flocculent sludge transforming into granular sludge during phase II. In phase III, increasing NLR had no effect on nitrogen removal efficiency and the granular sludge stability. Nitrogen removal efficiency was over 90% (effluent concentration of less 10 mg/L) without any granule foaming or breaking up. However, the average effluent COD concentrations increased to 110.7 mg/L with the efficiency of 89.5%.

3.1. Character and morphology of denitrifying granular sludge

SEM was used to observe the morphology of the mature denitrifying granular sludge in UASB reactor (Fig. 3). Figs. 3A–3C were taken from different regions of granule surface, while Fig. 3D was the interior of granular sludge. Fig. 3A shows that granular sludge surfaces were dominated by rod-like bacteria, which were embedded in a large number of staggered filaments just as pupae interspersing among their honey comb. As shown in Fig. 3B, the microorganisms were surrounded by a number of filaments and were bounded the neighboring microorganisms together. However, the granular sludge surface exhibited another type of filaments, which were larger in morphol-

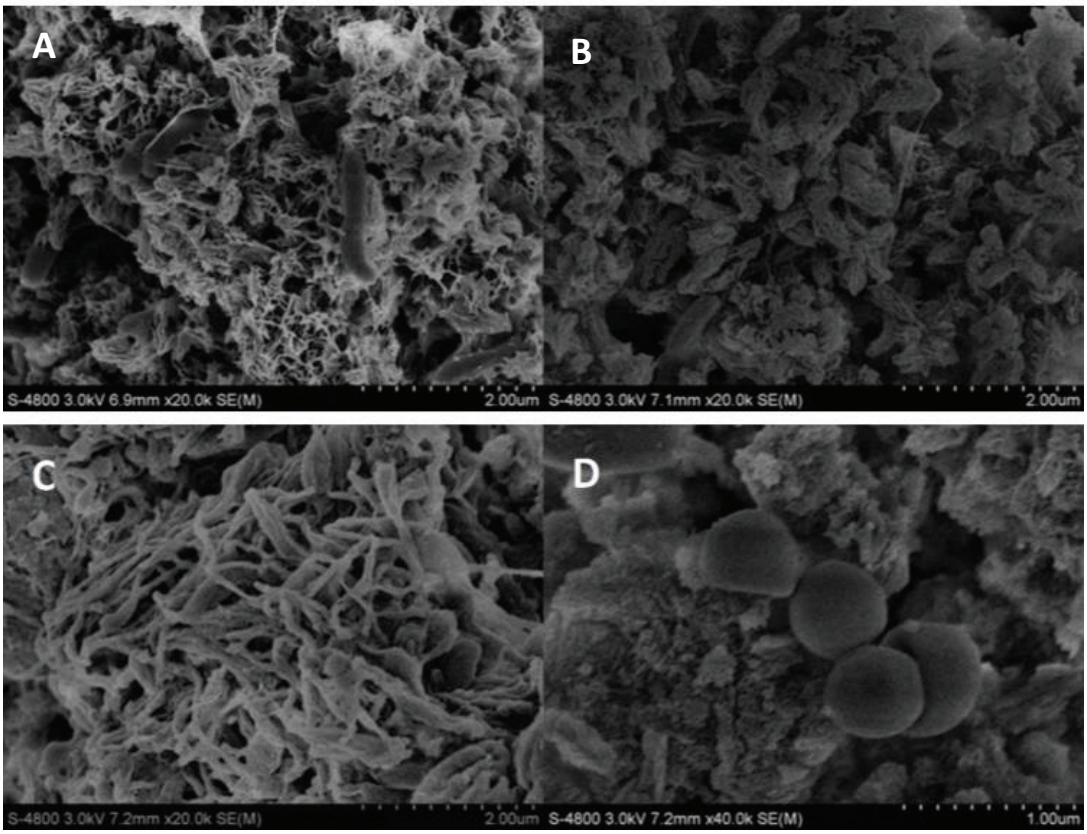

Fig. 3. SEM images of granular sludge in day 120 (A–C surface of the granule (20.0 k); D interior of the granule (40.0 k)).

ogy (Fig. 3C). On the other hand, Fig. 3D shows the interior of granular sludge, which was mostly filled with abiotic substances with a small number of spherical bacteria. The SEM photograph shows that the rod-shaped microorganisms were homogeneously distributed on the surface of denitrifying granular sludge, which was consistent with previous findings [16]. The filaments, which were reported to have influence on the binding of cell-cell [17], wrapped around bacteria were likely to be EPS as shown in Fig. 3A and Fig. 3B. Compared with filaments shown in Fig. 3A and Fig. 3B, filaments in Fig. 3C were significantly bigger and thicker, suggesting that they were likely to be fungal hyphae. These fungal hyphae-like filaments might play a role in accelerating granulation and enhancing granule structure.

The pictures showing the transition course of sludge granules are listed in Fig. S1 (Supporting information). The particle size > 0.8 mm accounted for more than 90% of granular sludge. The particle size distribution in 120th day is listed in Fig. S2 (Supporting information). The characteristic parameters of granular sludge cultured in 120th day are shown in Table S1 (Supporting information). The volatile matter of the sludge was 34% of its dry weight with calcium (Ca) and magnesium (Mg) contents of 127.6 and 8.1 mg/g SS, respectively, and that the proportion of volatile matter was generally lower than that of the traditional granular sludge (30%–90%) [18]. Furthermore, the granule settling velocity was over 180 m/h, which is significantly higher the velocity reported in the literature [8]. Generally, the granule settling velocity is considered to be in the range of 18–100 m/h, typically between 18–50 m/h. The high settling velocity can be because of high inorganic content in the sludge. Furthermore, the granule strength (integrity ratio %) was 97.3%. Liu et al. [19] also reported that Ca accumulation could enhance the density and strength of granular sludge, resulting in an excellent settling performance.

3.2. Variation in sludge and metal element concentration during granulation

The variations of MLSS, MLVSS and VSS/SS in the UASB reactor were determined during granulation process. As shown in Fig. 4A, the MLSS increased from 25.4 to 47.7 g/L after 120 d operation, while the VSS/SS decreased from 0.64 on 1th day to 0.34 on 55th day, indicating that the inorganic substances were continuously accumulated and that the materials with poor settling performance were taken out of the UASB reactor during the granulation process. The MLVSS increased from 11.9 g/L on 55th day to 16.4 g/L on 120th day because of the multiplication of dominant microorganism. The VSS/SS ratio reached the lowest point of 0.34 on 55th day, and was steady afterwards, suggesting the maturity of denitrifying granules. The relatively low VSS/SS ratio was caused by increasing inorganic substance accumulation [19].

Ca and Mg were reported to influence sludge characteristics and granulation time [13,14,19,20]. Therefore, Ca, Mg, Fe and Cu contents in sludge were investigated during

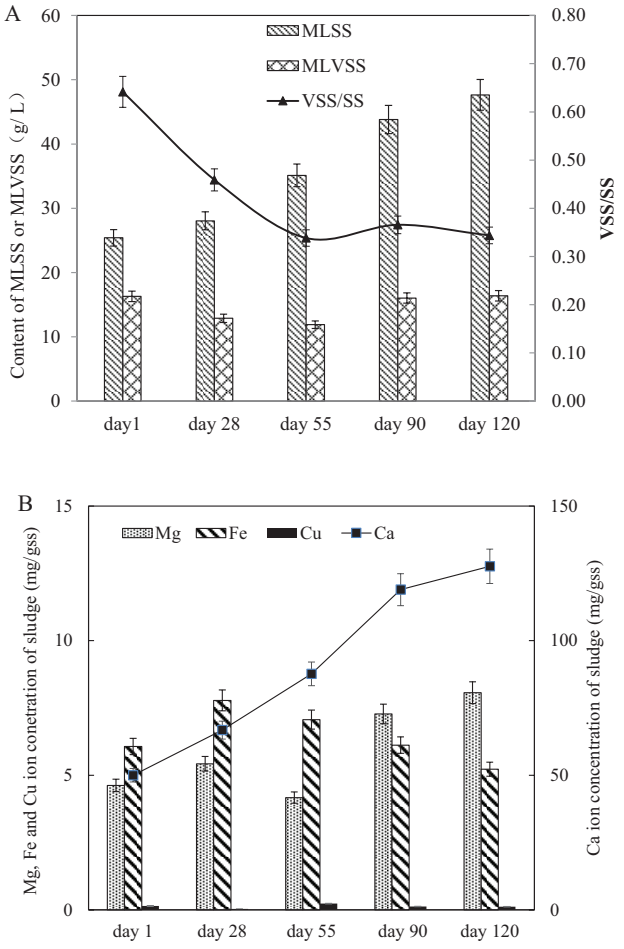

Fig. 4. Variation in (A) sludge and (B) metal element concentrations during granulation process.

the granulation process (Fig. 4B). The Ca content in sludge increased from 50.3 mg/g SS on 1st day to 127.6 mg/g SS on the 120th day, which is an increase of 1.5 times comparing to that in seed sludge. This result demonstrates that Ca accumulation occurred during the granulation process. Similar phenomenon was also observed in aerobic sludge granulation process [20]. The high settling velocity, high strength and the low VSS/SS ratio of denitrifying granular sludge cultured in UASB reactor might be caused by the accumulation of Ca precipitation [19]. Grotenhuis et al. [21] removed Ca with ethylene glycol-bis (b-aminoethyl ether)-N-N-tetraacetic acid (EGTA), and observed the gradual decline in granular sludge strength and integrity. This demonstrates the positive effect of Ca content on the stability and integrity of granular sludge. Research studies showed that Ca promoted the formation of different types of granular sludge and shorten the start-up of reactors [13,14]. The results indicate that Ca accumulation affects granular sludge formation and structural stability.

Fig. 4B shows the Mg accumulation during granulation process. Mg content in sludge increased from 4.6 mg/g SS on 1st day to 8.1 mg/g SS on 120th day. Mg acted as a biological enzyme activator benefitting microbial metabolism in granular sludge [13]. In contrast, Fe and Cu contents

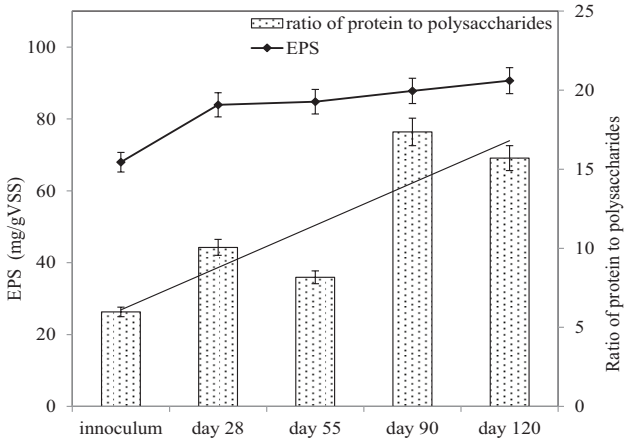

Fig. 5. Change in EPS during granulation process.

decreased during the granulation process. The color change of sludge, from black to brown and then to yellow white, was also related to the gradual decrease of Fe concentration in the sludge [8].

3.3. Change of EPS of sludge during granulation process

EPS, which are sticky polymer materials that are secreted by cells, are major components of the matrix material in both aerobic and anaerobic granules [9,13,14]. Therefore, the variations of EPS and its components in sludge were investigated to understand the mechanism of granulation process (Fig. 5). During the sludge granulation process, EPS content increased from 68 to 88 mg/gVSS, suggesting that there was a positive relationship between the formation of granular sludge and the components of sludge EPS. With increased EPS content, the binding force between neighboring microorganisms was enhanced, which might further strengthen the structural integrity of granular sludge. A large amount of EPS like filaments were found to form a cross-linked network between neighboring microorganism. Li et al. (2013) also found similar phenomenon in the study of denitrifying granular sludge. EPS played an important role in material adsorption and cell aggregation by providing electrostatic binding sites and extensive surface area, which were beneficial for microorganisms to attract the organic and inorganic materials [9,23].

The EPS content could change cell surface charge, which is considered to be a crucial factor affecting the microbial aggregates [9]. Generally, microorganisms carry negative charge on the surface, and a repulsive force generally prevents the cell from approaching another. High content of EPS could decrease the negative charge of cell surface [8]. This is because of the existence of electropositive functional groups such as amino in protein in EPS, and the neutralization of negative charge of cell surface by multivalent metal cation such as Ca^{2+} and Mg^{2+} adsorbed by EPS. Therefore, increased EPS content can enhance the aggregation potential of cells by decreasing the electrostatic repulsion between cells.

The ratio of extracellular protein to polysaccharides in mature denitrifying granular sludge was 17, which is nearly 3 times higher than that of seed sludge (Fig. 5). This

indicates that the ratio of extracellular protein to polysaccharides exhibited a rising trend during the granulation process of denitrifying sludge, consistent with previous report [17]. As two dominant components of EPS, the ratio of extracellular protein to polysaccharides and their contents can influence hydrophobic characteristics of cell surface. Extracellular protein and polysaccharides are reported to be hydrophobic and hydrophilic, respectively [24]. Hence, increasing the ratio of extracellular protein to polysaccharide in EPS could result in increasing hydrophobicity of sludge surface. Increased hydrophobicity would decrease surface free energy for cells gathering and enhance the binding affinity between cells, which could finally trigger the formation of cell aggregates and accelerate granular sludge formation [25].

In summary, the gradual increase of EPS and the ratio of extracellular protein to polysaccharide was advantageous to the aggregation cells and accelerated the formation of granular sludge.

3.4. Microorganism community present in denitrifying granular sludge

The microorganism community present in denitrifying granular sludge was investigated to understand the denitrifying granulation mechanism. A denitrifying fungus (fungi-1, GenBank accession number: KF483525) and two denitrifying bacteria (GX-1 and GX-2, GenBank accession numbers: KF410592 and KF410593) were isolated from the mature granular sludge. Phylogenetic analysis suggested that fungi-1 belonged to the *Fusarium oxysporum* (Fig. 6A), while two denitrifying bacteria were from *Bacillus* and *Ochrobactrum anthropi* (Fig. 6B), respectively.

In the context of metabolism, microorganisms played different roles in denitrification process, which were decided by their metabolic characteristics. Denitrifying bacteria were sensitive to dissolved oxygen, while denitrifying fungi could remove nitrate under a micro-aerobic or an initially aerobic condition [26,27]. Fungal systems usu-

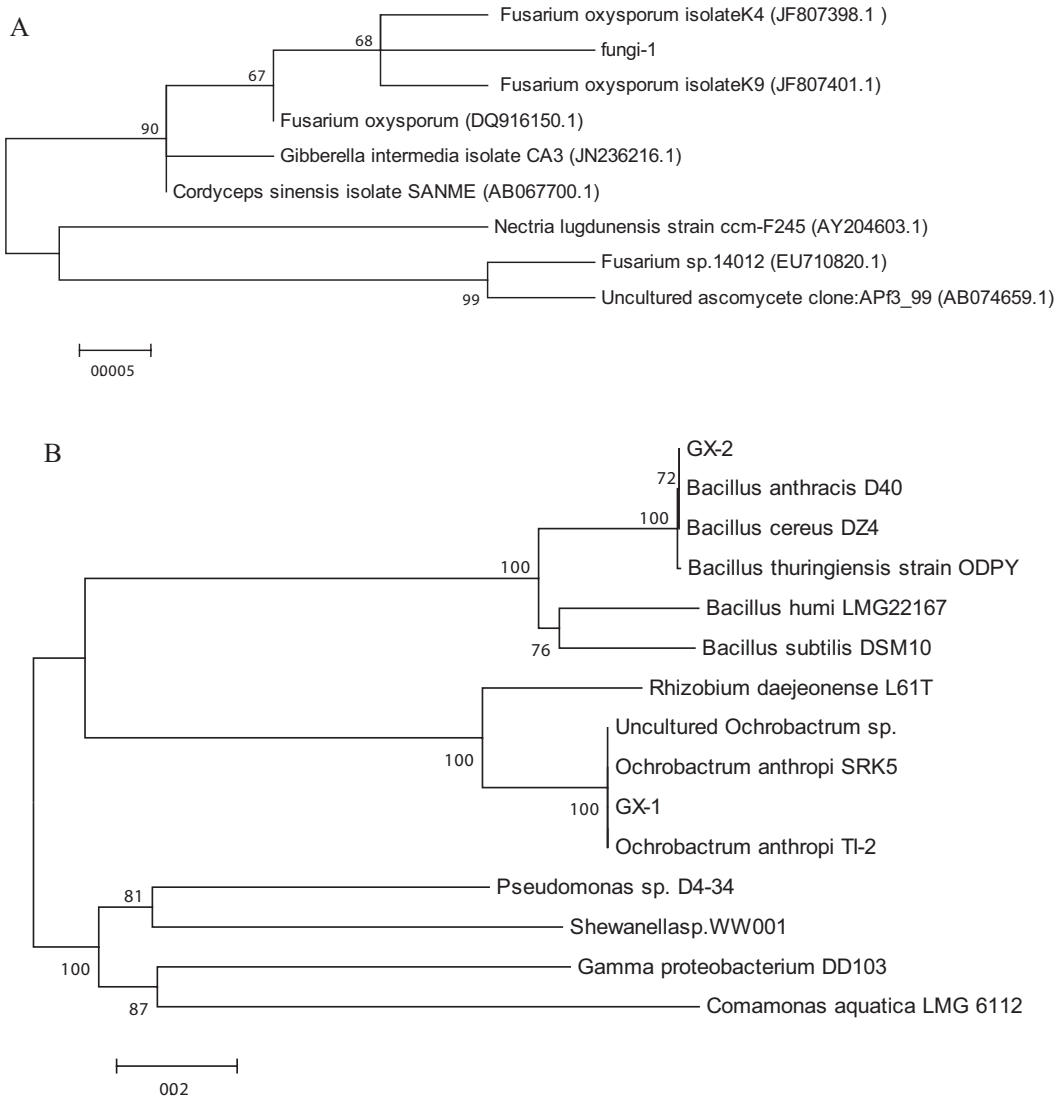

Fig. 6. Gene homologous evolutionary tree of (A) denitrifying fungus fungi-1, and (B) denitrifying bacteria GX-1 and GX-2.

ally lack N_2O reductase, leading to evolution of N_2O as the final denitrification product [28], while denitrifying bacteria could achieve complete denitrification. During denitrifying metabolism process in the UASB reactor, fungi-1 might eliminate the inhibition of oxygen for denitrifying bacteria, while denitrifying bacteria can complement the N_2O reductase deficiency of fungi-1 and achieve completely denitrification. Therefore, the co-existence of denitrifying bacteria and fungi resulted in excellent nitrate removal efficiency of over 90% in the UASB reactor.

In the context of physicochemical effects, fungi play an important role in granulation process due to their filamentous structure. As shown in Fig. 3C, denitrifying bacteria were wrapped by a large amount of fungal mycelium like filamentous substance. The fungi-1, which acted as a framework of granules, provided shelters for denitrifying bacteria from being washed out by the shear force in the UASB reactor. In addition, the big filamentous fungi adsorbed organic or inorganic substances on their surface, leading to increased adherent possibility and aggregation of bacteria and consequently resulting in increased formation of granules. Fungi alone could not form granular sludge with a good performance of stability [29]. Therefore, the results suggested that *Fusarium oxysporum* fungi-1 complemented the denitrifying bacteria to perform denitrification and granulation processes.

3.5. Granulation mechanism of denitrifying sludge

Sludge granulation is often considered as a process of microbial self-aggregation. Physicochemical characteristics and microorganism community of the sludge changed during the sludge granulation process, which can provide an insight about the granulation mechanism. During the denitrifying sludge granulation process, Ca and Mg contents in sludge increased significantly, while the components and concentrations of EPS varied. Furthermore, significant amount of *Fusarium oxysporum* fungi-1 were found in the denitrifying granular sludge. Therefore, it was hypothesized that there is a correlation between these changes and the granular sludge formation in the UASB reactor, and that the granular sludge formation is a combined result of these factors.

Based on the above discussion, a five-step granulation mechanism is proposed for denitrifying granular sludge formation in the UASB reactor, by modifying the four steps granulation process theory [8]. In the proposed mechanism, the filamentous fungi contributes to the formation of denitrifying granules due to their filamentous structure, which is novel and different from the four steps theory of granulation process. In the proposed mechanism, Ca, EPS and filamentous fungi are the main three driving forces for denitrifying sludge granulation. The granulation process is shown in Fig. 7 and described as follows:

Step I. Transport and Reversible Adsorption: Transportation of cells to the substratum surface and their adsorption on the substratum surface

At the beginning, dispersedly suspended cells are transported to substratum by external physico-chemical forces such as Brownian motion, convective transport caused by

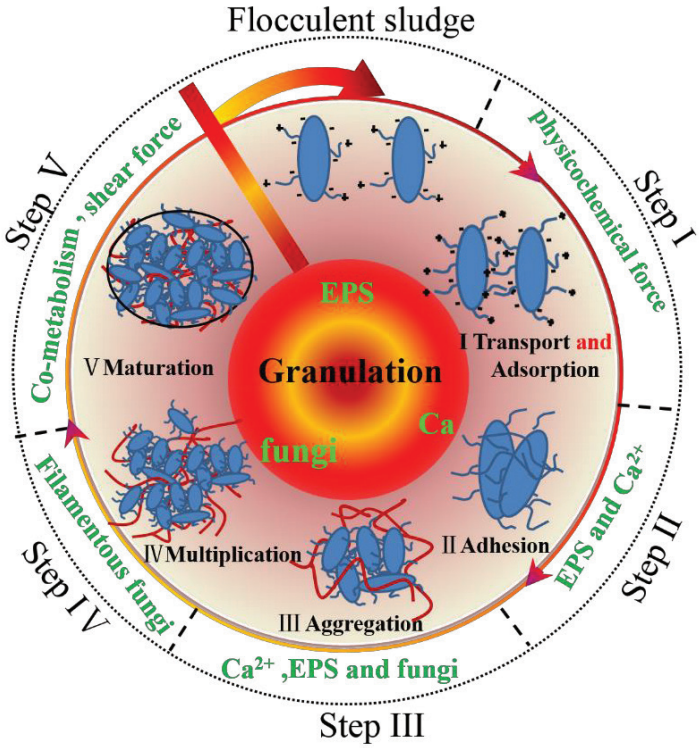

Fig. 7. Granulation mechanism diagram.

hydraulic force, gas floatation, and sedimentation [8]. Cells or inorganic substances such as straw in the sludge, and Ca precipitation such as $CaCO_3$ and $CaPO_4$ are generally considered to be the substratum in granular sludge [19]. The combined effect of up-flow fluid and gravity in the UASB reactor increases the chance of cell collision. When the cells and the substratum are close enough, adsorption can take place. The adsorption strength depends on different physico-chemical forces such as ionic, dipolar, hydrogen bonds and hydrophobic interactions [8]. In this study, the physico-chemical adsorption strength was enhanced due to the change of cell surface character caused by EPS and Ca^{2+}. Increased EPS content strengthens the attraction between cells and substratum by providing extensive binding area and attraction sites for cells. Increased protein to polysaccharide ratio improved the hydrophobicity, which enhanced the adsorption potential between cells and substratum. The electrostatic repulsion was also reduced with the increased protein to polysaccharide ratio and with the presence of positive ions, mainly Ca^{2+}. Thus, at this step, physico-chemical force promoted the cell transport and adsorption.

Step II. Irreversible adhesion: strong bonds between cells and substratum

According to the four-step extracellular polymer hypothesis reviewed by Schmidt and Ahring [8], bacterial fimbria, polymers and other holdfast structures establish strong bonds between substratum and cells. In the denitrifying sludge granulation process, EPS and Ca play key roles at Step II. As discussed in Section 3.3, the existence of Ca^{2+} benefited irreversible adhesion by enhancing the linkage function of EPS. Increasing EPS content in the granular sludge was conducive to the cross-linking adhesion of cell. Ca^{2+} could facilitate the linkage between cell-polysaccharide and polysaccharide-polysaccharide by combining various electronegative groups such as OH^- (the secondary function groups in polysaccharides), carboxyl and phosphate groups on bacterial surfaces [30]. Ca^{2+} was also beneficial to the folding of polymers by cross-bridging the polysaccharides of EPS [31], and consequently to strengthen tertiary structure of EPS, which further increased the bond between the polymer network and the cells in sludge. Therefore, Ca^{2+} and EPS enhance the irreversible adhesion step during the denitrifying sludge granulation process.

Step III. Aggregation: further adhesion among cells and adsorption to filamentous fungi

Through the irreversible adhesion process, the cell resistance to external unfavorable factors was greatly increased, which would benefit further cell adhesion [8]. With the increasing ratio of protein to polysaccharide in EPS, the hydrophobicity of cells increases, and then the adsorption process proceeds continuously as discussed in Section 3.5. In addition, the adhesion process also continues because of the increasing EPS and Ca accumulation. The adsorption and adhesion processes result in the growth of cell aggregation. However, it is important to note that filamentous fungi with large surface areas could supply appropriate platform for cells to adhere. Compared with single bacterium, the aggregation process is an advantage for cells to adhere on the fungi surfaces, and the aggregated cells with larger contact area can easily be wrapped by fungal hyphae. As shown in Fig. 3C, fungi acted as the framework in the granular sludge, winding and wrapping aggregated cells intensely, which can greatly improve the strength of granular sludge. Therefore, except for Ca and EPS, fungi also play a key role in the aggregation process.

Step IV. Multiplication: cell aggregation multiplication and the development of granular sludge

During the cell aggregation process, increased number of cells adhere, wrap and fill in the frame made up by filamentous fungi. As discussed in Section 3.4, fungi compliment bacteria to increase the metabolism rate by enhancing the substance transformation and energy utilization efficiency. The mutually-beneficial symbiosis between fungi and bacteria improves the growth and metabolic of microbial cells in the cell aggregates. Thereafter, the enhanced proliferation of microbial cells leads to multiplication of cell aggregation which increases size of cell aggregation and forms the initial denitrifying granular sludge.

Step V. Maturation: stabilizing the structure of granular sludge and establishing the balance of metabolism

The multiplication of microorganisms and the accumulation of inorganic substance is finite and controlled by hydraulic shear force, substrate limitation, and the metabolism balance of fungi and bacteria. The hydraulic shear force drives the granular sludge to become smooth, round/streamline shape, and keeps the EPS content and the size of granular sludge in a reasonable range [9,32]. The EPS is adhered to the surface of granular sludge, which is beneficial to strengthen the structure of granular sludge and increase the efficiency of nutrient transport. However, the over-produced EPS was considered to help to block the gas tunnels, prevent gas escaping out of the granules and adhere small gas bubbles, which made the sludge density decreased and finally caused the sludge floatation [33]. The accumulation of Ca precipitation results in the excellent settleability and compaction to the interior structure of granular sludge, which is consistent with the prevised study [34]. Fungi and bacteria complement each other to establish the metabolism balance and accomplish denitrification process. In the end, the mature denitrifying granular sludge with excellent denitrification performance and stability structure is formed.

4. Conclusions

The granulation mechanism of denitrifying granules was investigated in this study. The main conclusions in this study are summarized as follows (1) the rod-shaped microorganisms and the filamentous material were distributed on the surface of denitrifying granular sludge, which might play a role in accelerating granulation and

enhancing granule structure. (2) the concentration of Ca accumulated from 50.3 to 127.6 mg/g SS, which may affects granular sludge formation and structural stability. (3) EPS content increased to 88 mg/g VSS, and the ratio of protein to polysaccharides increased by 3-fold, which was advantageous to the aggregation cells and accelerated the formation of granular sludge. (4) *Fusarium oxysporum* fungi-1 played a key role in accelerating granulation process and enhancing granule structure. (5) A novel modified five-step granulation mechanism, including Step I Transport and Reversible Adsorption, Step II Irreversible Adhesion, Step III Aggregation, Step IV Multiplication and Step V Maturation, was proposed for denitrifying granular sludge. In the mechanism, Ca, EPS and fungi were the main driving forces. This granulation mechanism is beneficial to understand the denitrification granulation process.

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Supporting information

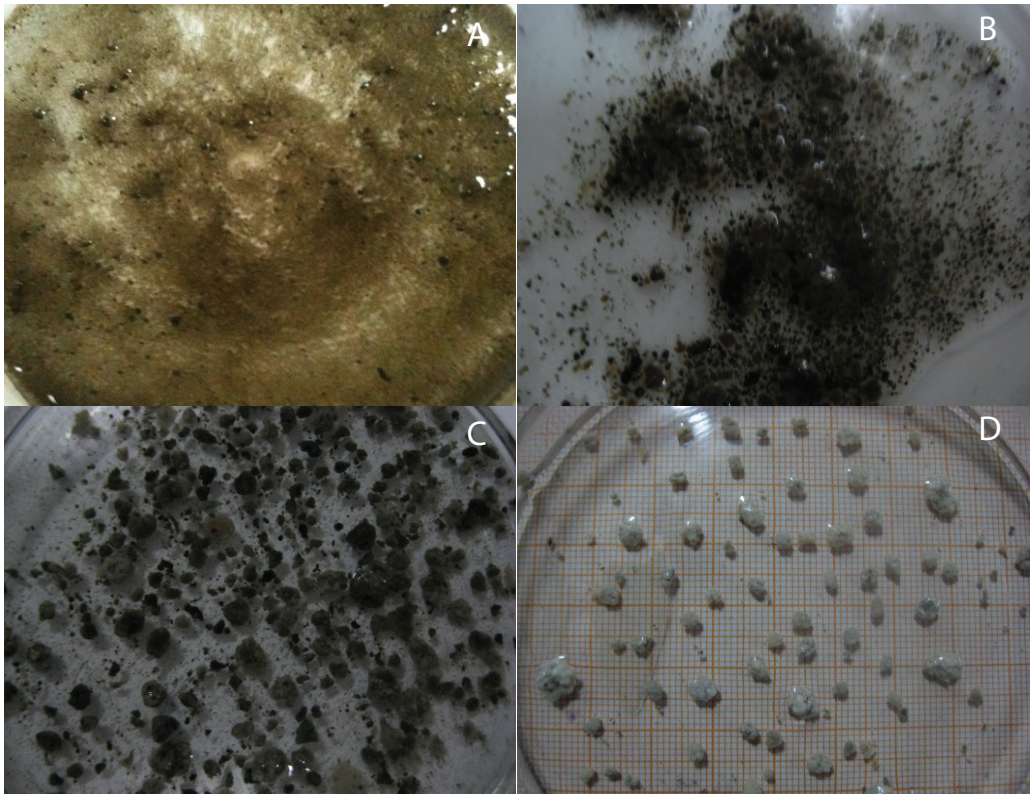

Fig. S1. Morphological changes of sludge during granulation process. (A: seed sludge, B: sandy granular sludge, C: initial granular sludge, D: mature granular sludge).

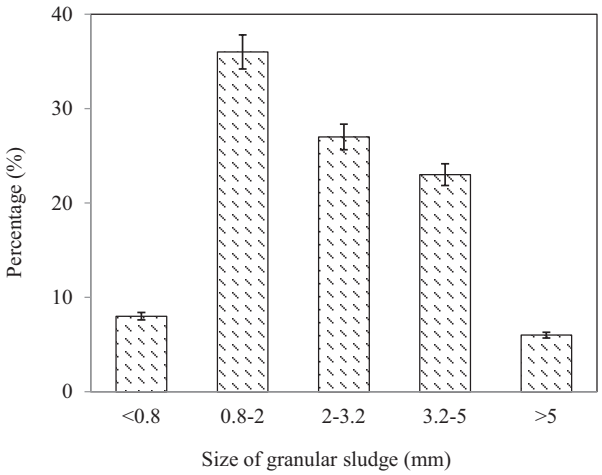

Fig. S2. Size distribution of denitrifying granular sludge.

Table S1
Characteristic parameters of denitrifying granular sludge

Parameters	Results
Wet density (g/cm^3)	1.097
Settling velocity (m/h)	186–217
Granule strength(integrity ratio %)	97.3
VSS/SS	0.34
Ca (mg/g SS)	127.6
Mg (mg/g SS)	8.1