Chemical Engineering Journal 309 (2017) 108–117

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Cultivating granular sludge directly in a continuous-flow membrane bioreactor with internal circulation

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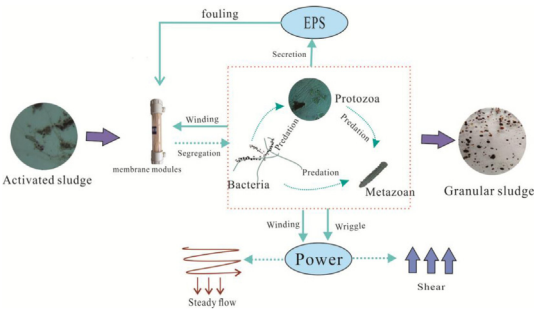
HIGHLIGHTS

- An MBR with internal circulation was used to cultivate granular sludge.
- Granular sludge was cultivated directly in this MBR in a continuous-flow mode.
- The resulting granular sludge was characterized by multi methods.
- Granular sludge remained stable for a long-term operation.
- Essential factors to influence the granulation process were illustrated.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 August 2016
Received in revised form 30 September 2016
Accepted 8 October 2016
Available online 11 October 2016

Keywords:
Granular sludge
Membrane bioreactor
Internal circulation
Microbial community
Continuous flow reactor

ABSTRACT

This research was conducted to cultivate granular sludge directly in a continuous-flow membrane bioreactor and explore the main factors influencing the granulation process. By establishing a suitable internal hydrodynamic circulation in a membrane bioreactor, granular sludge (GS) was successfully cultivated into mature granules with a compact structure and clear shape, in which extracellular polymeric substances played an important role in maintaining its integrity. The results showed that the main factors to determine the cultivation of GS included the total retention of sludge by the membrane module, the internal hydrodynamic circulation, and the entanglement of filamentous bacteria to sludge particles. Filamentous bacteria initiated a granulation process under the action of internal circulation, and maintained the stability of GS for a long period by wrapping biomass aggregates together. Even though filamentous bacteria were the major dominant microbial species in the bioreactor, the microbial community was richly biodiverse, and was responsible for the removal of organic pollutants and nutrients. Overall, the results demonstrated an alternative option for cultivating stable GS directly in a continuous-flow membrane bioreactor.

1. Introduction

Granular sludge (GS) is a collective of numerous self-immobilized cells in which a variety of functional microorganisms gather together to form microbial aggregates that have a tightly compact structure and diversified microbial communities. These aggregates are the main body that degrade various organic pollutants and convert nutrients from wastewater in a bioreactor [1]. A granular sludge bioreactor (GSBR), mainly composed of GS, possesses many dominant merits [2,3], including abundant microbial biodiversity, high retainable biomass concentration, large relative density, low sludge yield, excellent sludge settling ability, and robust ability to withstand high organic loading rate, which make

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http://dx.doi.org/10.1016/j.cej.2016.10.034
1385-8947/

it a promising means for treating both industrial and domestic wastewater [4,5], and attract many academic interests [2,6–8]. The successful implementation of a GSBR totally depends on the cultivation and proliferation of sufficient GS in the bioreactor. However, many factors have different impacts on the formation and the stability of GS, which lead to a very complex mechanism in forming stable and efficient GS [9].

In the past two decades, many studies have focused on exploring the effects of various operational parameters on the granulation process in sequencing batch reactors (SBR) or other reactors; these factors include hydrodynamic shear force (aeration intensity or stirring intensity), hydraulic retention time (HRT), reactor design, settling time, substrate composition, organic loading rate, and seed sludge [1,10,11]. Thus far, anaerobic and aerobic granular sludges have been successfully cultivated in up-flow anaerobic sludge bed reactor (UASB) [7] and SBR [1], and the latter has been regarded as the most successful way [4,8,9,12] to cultivate GS even in a full-scale municipal wastewater treatment plant (WWTP) [5]. From the prospective of an engineering application, the direct cultivation of GS in a continuous-flow reactor is more favorable than in a batch process due to its lower costs and more convenient operation. However, because continuous-flow reactors lack many conditions that are crucial for sludge granulation (such as alternating feast and famine conditions, and hydraulic selection pressure, among others), it is still very difficult to cultivate GS directly and maintain its long-term stability in a continuous-flow operating mode [8].

Combining granular sludge with a membrane bioreactor (MBR) creates a granular membrane bioreactor (GMBR). Such a new type of MBR can be operated in a continuous-flow mode [13] and also is a promising solution to mitigate membrane fouling [14] – a long-standing obstacle that still limits the application of MBRs [15,16]. Recently, Corsino et al. [17] used an MBR to investigate the stability of GS seeded from a column-type SBR, and showed undeniable difficulties for both the granule formation and the maintenance of GS in a continuous-flow mode. For maintaining the long-term stability of GS in a continuous-flow reactor, adding calcium and iron salts to form a strong granular core was verified to be effective [18], but the occurrence of inorganic salts in an MBR might also increase the possibility of permanent membrane fouling. To the best of our knowledge, almost all the reported GMBR must be inoculated with seed granular sludge from an SBR to start up the operation [19]. The direct cultivation of GS in a continuous-flow MBR is highly desirable, but is still quite difficult due to the complex granulation mechanism.

In regards to the formation mechanism and the stability of granular sludge, there still exist considerable disputes about the role of filamentous bacteria. Studying a continuous stirred tank reactor, Morales et al. [20] found that granular-type biomass could form under certain suitable conditions, but the presence of filamentous bacteria caused a failure in the formation of granular sludge. For inhibiting the negative effect of overgrowth of filamentous bacteria on the structural stability of granular sludge during long-term reactor operation, keeping the suspension alkaline was verified to be effective [21]. So far, several reports have demonstrated that the overgrowth of filamentous bacteria can cause poor settleability of granular sludge, resulting in sludge being readily washed out from the bioreactor and eventual failure of a GSBR [22,23]. However, other research has drawn a contradictory conclusion about the effect of filamentous bacteria on the formation of granular sludge. Li et al. [24] successfully cultivated compact granular sludge in an SBR even with the presence of filamentous bacteria, but adding 5% sodium chloride solution in the bioreactor was a prerequisite condition. Wang et al. found that numerous filamentous bacteria wrapped other bacteria together to form granules [25] with inorganic crystal-like material at the center to act as a nucleus. Based on this finding, they proposed a string-bag hypothesis to explain the formation mechanism of granular sludge. Figueroa et al. [26] compared the performance of granular sludge in three SBRs fed with different wastewater as influent, and revealed that filamentous bacteria acted as a structural backbone of granular sludge. Filamentous bacteria were also observed to play a key role in the formation of granular sludge in a conventional, continuous-flow completely mixed activated sludge system [27]. These investigations indicated that, under different scenarios, filamentous bacteria might either initiate the sludge granulation process or cause sludge bulking, with completely opposite effects on the formation of granular sludge and the successful operation of a GSBR.

This research aimed to cultivate GS directly in a continuous-flow MBR and explore the main factors influencing the granulation process. Through an experiment lasting 110 d, two closely related aspects of GS formation were mainly investigated: (1) cultivating GS directly in a continuous-flow membrane bioreactor; (2) characterizing the resulting GS through multiple methods and further illustrating the granulation process. These methods included a conventional optical microscope, scanning electron microscope (SEM), confocal laser scanning microscope (CLSM), and high-throughput sequencing (HTS). We hope the presented results would provide a useful reference for developing a convenient and cost-effective approach to cultivate GS directly and operate this bioreactor steadily in a long-term running.

2. Materials and methods

2.1. Bioreactor configuration and experimental conditions

The MBR used in the experiment was a rectangular bioreactor with an effective working volume of 36 L, which was divided into two chambers. One chamber, with about one-third of the total working volume, was an aeration zone. A membrane module (hydrophilic PVDF hollow membrane with a pore size of 0.22 μm and surface area of 0.5 m^2, MOF-Ib, Tianjin Motimo Membrane Technology Co., LTD, Tianjin, China) was mounted in the middle of this chamber with an aerator fixed below to release air bubbles and push the fluid upward. The other chamber was a mixing zone with a rotator mounted in the middle to provide mixing and drive the mixed liquor downward. With the simultaneous upward flow in the aeration chamber and downward flow in the mixing chamber, an internal hydrodynamic circulation was formed to circulate the fluid within the bioreactor. This configuration constituted a new kind of bioreactor – an internal circulation membrane bioreactor (IC-MBR). The configuration and controlling devices of the IC-MBR are shown in Fig. 1, and the operational conditions are listed in Table 1.

In the experiment, the used synthetic wastewater was prepared as substrate for the bioreactor by dissolving chemically pure glucose (103.42–372.31 mg/L), and other nutrients, including NH_4Cl (19.20–69.10 mg/L), KH_2PO_4 (4.41–15.90 mg/L), $NaHCO_3$ (160 mg/L), $MgSO_4$ (40 mg/L), $MnSO_4$ (12 mg/L), $CaCl_2$ (8 mg/L), and $FeSO_4$ (0.6 mg/L), in tap water. The synthetic wastewater had a carbon (as chemical oxygen demand, COD), nitrogen and phosphorus ratio (COD:N:P) of 100:5:1. Inoculated activated sludge was taken from the secondary sedimentation tank of a local WWTP (Lijiao municipal wastewater treatment plant, located in Haizhu District, Guangzhou, China). The initial concentration of the inoculated activated sludge was controlled at approximately 2500 mg/L mixed liquor suspended solids (MLSS), and the bioreactor was started up by feeding the prepared synthetic wastewater. The whole experimental period lasted for 110 d, which was divided into four phases (Phase 1, days 1–6; Phase 2, days 7–15; Phase 3, days 16–

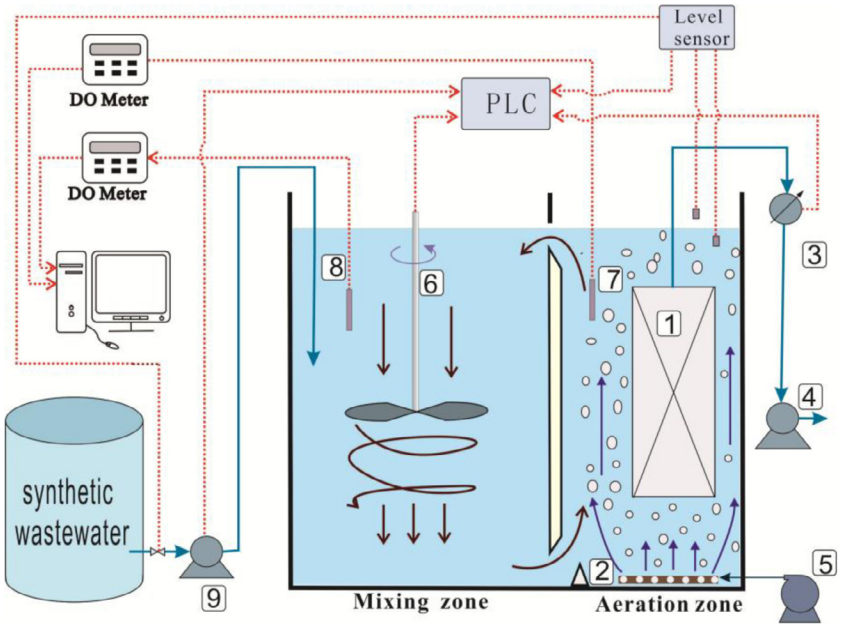

Fig. 1. Schematic diagram of the used MBR: (1). Membrane module; (2). Microporous aerator; (3). Vacuum meter; (4). Peristaltic pump of the effluent; (5). Air compressor; (6). Rotator; (7). DO probe in the aeration zone; (8). DO probe in the mixing zone; (9). Peristaltic pump of the influent.

Table 1
Operational condition.

Parameter	Value	Parameter	Value
Operation time (d)	110	HRT (h)	5
Temperature (°C)	25–31	SRT (d)	110
pH	7.0–8.0	Flux (L/(m^2·h))	12
Aeration rate (m^3/h)	1.0	Rotate speed (rmp)	50

28; Phase 4, days 29–110) according to the growth of biomass. During the whole period, no excess sludge was discharged from the bioreactor except that removed during sampling (100 mL each time) for analysis. The influent water quality at each operational phase is listed in Table 2.

2.2. Performance evaluation of the bioreactor and cleaning of the membrane module

The performance of the IC-MBR was evaluated in terms of the removal of organic pollutants and nitrogen-containing substances, including chemical oxygen demand (COD_{Cr}), and total nitrogen (TN). Ammonium nitrogen (NH_3-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N) were chosen as reference parameters to observe their variation in the effluent. The content of biomass was evaluated by measuring the concentration of MLSS and mixed liquor volatile suspended solids (MLVSS), and the settling ability of sludge was assessed by a commonly used parameter – 30-min sludge volume index (SVI). All indexes were measured according to the standard methods [28]. For the membrane fouling during the operation period, the operational pressure (trans-membrane pressure, TMP) increased gradually, which impeded the further operation, thus, the membrane module was taken out until the TMP reached at around 30 kPa and cleaned periodically according to the following protocol: (1) The module was rinsed for 10 min with clean water to remove the accumulated sludge; (2) The rinsed module was then placed in an ultrasound bath of clean water (KQ2200DE, Kun Shan Ultrasonic Instruments Co., Ltd, China) and cleaned at 100 W and 40 kHz for 10 min; (3) Next, the ultrasonically cleaned module was soaked in 0.1 M NaOH solution and sucked at a flow rate of 5 mL/min (lasting for 10 min), after which the module was sucked for 10 min in clear water again at the same flow rate.

2.3. Morphology observation of granular sludge

The morphology of sludge was observed using a multi-functional biological microscope imaging system (BK-FL, Chongqing OPTEC Instrument CO., LTD, Chongqing, China). A scanning electron microscope (SEM) (S-3400N (II), Hitachi Limited, Japan) was used to explore the surface structure of the formed sludge granules. Before each SEM observation, the sludge granule samples were fixed with a solution of glutaraldehyde 2.5% (v/v), and washed three times with phosphate buffer (PBS, pH = 6.8); then, the samples were dehydrated using ethanol solutions with increasing ethanol concentrations (50%, 70%, 80%, 90%, and 100% v/v). Thereafter, the ethanol in the dehydrated samples was displaced with ethanol in isoamyl acetate solutions (50% v/v) for 15 min, and then with pure isoamyl acetate (100% v/v) for 15 min. Finally, the samples were dried eight hours in a desiccator.

2.4. Analysis of granule size and granule-size distribution

After the sludge granules were cultivated and matured, samples of the granules were collected from both the mixing and aeration zones to measure their size (diameter) and analyze their size distribution using a laser particle size analyzer (MS2000, Malvern Instruments Ltd., UK). For comparison, the inoculated sludge was also sampled for size measurements using the laser analyzer under the same conditions as for the granular sludge.

2.5. Extraction and analysis of extracellular polymeric substances in GS

Extracellular polymeric substances (EPS) in GS were extracted according to a procedure described as follows: (1) First, a sludge sample was centrifuged at 5200×*g* for 5 min, and the EPS contained in the supernatant was termed as "loosely bound EPS" (LB-EPS); (2) Next, the sludge pellet left in the centrifuge tube was washed three times with deionized water, and then it was

Table 2
Condition of water quality in the influent at each operational phase.

Operational phase	COD (mg/L)	Organic loading rate (kg COD/m^3·d)	TN (mg/L)	TP (mg/L)
I (days 1–6)	100	0.26 ± 0.08	5.0	1.0
II (day 7–15)	150	0.32 ± 0.07	7.5	1.5
III (day 16–28)	200	0.27 ± 0.07	10.0	2.0
IV (day 29–110)	360	0.20 ± 0.13	18.0	3.6

Table 3
Scheme of staining EPS.

Dye	Excitation (nm)	Emission (nm)	Targets	Agency
Calcofluor white	400	410–480	β-1, 4 and β-1, 3 polysaccharides	Sigma (St. Louis, USA)
FITC	488	500–550	Protein, amino-sugars	Aladdin (China)
Nile red	514	625–700	Lipids, hydrophobic sites	Aladdin (China)
ConA conjugates	543	550–600	α-Mannopyranosyl, α-Glucopyranosyl sugars	Sigma (St. Louis, USA)

re-suspended in 0.05% NaCl solution to its original volume; (3) The re-suspended sludge sample was put in a water bath (60 °C) for 30 min, and then centrifuged at 5200×*g* for 30 min, and the EPS contained in the supernatant was termed as "tightly bound EPS" (TB-EPS).

After extraction, both the LB-EPS and TB-EPS were analyzed for protein (PN) and polysaccharide (PS) content. The content of PS in EPS was measured using the phenol-sulfuric acid method with glucose as the standard [29]. The content of PN in EPS was measured using the Bradford assay with bovine serum albumin as the standard [30].

For giving an *in-situ* description of the distribution of EPS in GS, the GS samples were collected from the bioreactor on day 76. After collection, the samples first were stained according to the procedure reported in the literature [31], after which, their 20-μm sections were cut on a cryomicrotome (HM560, Microm, Germany), and were observed with a CLSM (TCS SP5, Leica, Germany). The staining scheme is listed in Table 3.

2.6. DNA extraction and analysis of microbial community

To analyze the structure and evaluate the abundance of the microbial community in the bioreactor, the samples were taken from both the mixing and aeration zones of the MBR on day 76, then, the samples were separated into floc sludge (D < 0.15 mm) and granular sludge (D ⩾ 0.15 mm) by a 100-mesh sifter, and labeled as FS and GS, respectively.

The DNA in the sludge samples was extracted for analysis using an E.Z.N.A.™ Soil DNA Kit (Omega, Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Once the DNA was extracted, a Qubit 2.0 DNA Kit was used to exactly quantify the amount of DNA to confirm its addition in the PCR process. The primer of PCR coalesced the V3–V4 region of 16S rDNA gene in the Miseq HTS platform by using the primer sets 341F (CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG) and 805R (GACTGGAGTTCCTTGGCACCCGAG AATTCCAGACTACHVGGG TATCTAATCC). The first PCR amplification was performed containing 5 μL of 10 × PCR buffer, 0.5 μL of each dNTP (10 mM), 10 ng of Genomic DNA, 0.5 μL of Bar-PCR primer F (50 μM), 0.5 μL of Primer R (50 μM), and 0.5 μL of Plantium Taq (5 U/μL) and then add H_2O to 50 μL. The PCR amplification was carried out under the following conditions: initial denaturation at 94 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 30 s, primer annealing at 45 °C for 20 s, and primer extension at 65 °C for 30 s. Thereafter, PCR amplification was continually carried out by 20 cycles of denaturation at 94 °C for 20 s, primer annealing at 55 °C for 20 s, and primer extension at 72 °C for 30 s. A final extension step consisted of cooling at 4 °C.

In the second PCR amplification, the compatible primer of Illumina bridge type of PCR was drawn in. The addition of a second PCR amplification was conducted similarly to the first one, but the DNA was 20 ng. The second amplification was carried out by initial denaturation at 95 °C for 30 s, was followed by 5 cycles of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 15 s, and primer extension at 72 °C for 30 s. A final initial denaturation at 94 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 30 s, primer annealing at 45 °C for 20 s, and primer extension at 65 °C for 30 s. After that, it was continually carried out by 20 cycles of denaturation at 94 °C for 20 s, primer annealing at 55 °C for 20 s, and primer extension at 72 °C for 30 s. A final extension step was carried out at 72 °C for 5 min prior to cooling at 10 °C. The PCR products were sequenced in 1:1 (v/v) after electrophoresing. Following extraction, the extracted DNA was subjected to sequencing analysis by Miseq HTS of theV3−V4 region of 16S rDNA gene (Sangon Biotech Co., Ltd., Shanghai, China).

3. Results and discussion

3.1. Performance of the bioreactor

The bioreactor was operated continuously for 110 d without discharging any excess sludge except the small quantities removed during sampling for various measurements. For maintaining a stable food-to-microorganism ratio (F/M) within the bioreactor, the influent COD concentration was adjusted to match the growth of biomass, and thus, the whole operational period was divided into four phases according to each adjustment (Table 2). The performance results of the IC-MBR are shown in Fig. 2.

The membrane fouling is an important factor to affect the performance of the IC-MBR, thus, the periodic cleaning of membrane module is necessary. During the whole operational period, the time point to clean the membrane module was totally depended on the measured TMP, namely, the membrane module was not taken out until the TMP reached 30 kPa. The cleaning strategy was described in Section 2.2. The characteristics of membrane fouling during the operation period will be reported in our other investigation.

Fig. 2(a) shows a nearly linear growth trend of biomass in the bioreactor over the whole operational period. The successful start-up and stable operation of the IC-MBR indicated that the inoculated sludge totally adapted to the environmental conditions within the IC-MBR. At the initial phase of the operation (days 1–7), the value of SVI increased very quickly (to 328.65 mL/g), which indicated the overgrowth of filamentous bacteria and sludge bulking. However, during days 8–33, SVI obviously decreased to 105.62 mL/g, and then (from day 34), remained at a relatively

stable (at approximately 100 ± 20 mL/g) even when the concentration of MLSS reached a high value (greater than 7000 mg/L).

The content of organic substances contained in MLSS represents the amount of biomass; thus, the ratio of organic components (MLVSS) to the whole MLSS can be regarded as an approximation of the ratio of biomass contained in the sludge. Fig. 2(b) shows an increasing and a stable trend in the value of MLVSS/MLSS, which indicated that the conditions in the IC-MBR were quite suitable for the growth of microbes. Although the values of F/M fluctuated during the initial three stages of operation, the F/M remained stable at approximately 0.2 kg COD/(kg·MLSS·d) from day 40 until the end of the experiment. Even though the concentration of COD in the influent was adjusted for three times during the experiment (increasing it from 100 mg/L to 360 mg/L), the removal of COD remained at a high and stable efficiency except the initial two stages (Fig. 2(c)). After day 10, the removal of COD almost exceeded 80%, which indicated a good performance of the IC-MBR.

Totally converting nitrogenous substances to nitrogen gas requires an alternating oxic and anoxic environment to create a suitable nitrification-denitrification condition. Fig. 2(d) shows that the concentration of NO_3^--N was high in the first three phases, but gradually decreased to a low value thereafter, while the concentra-

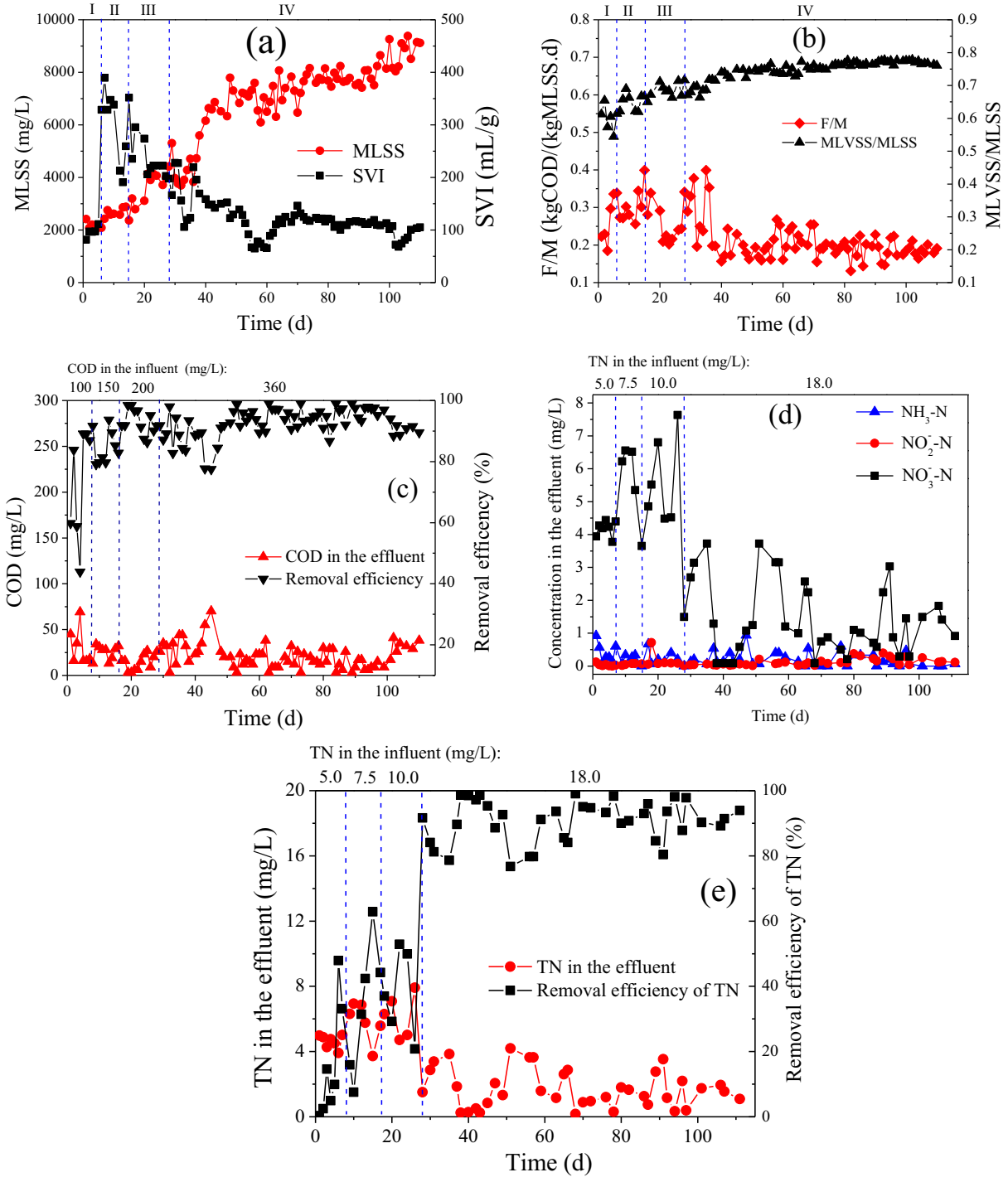

Fig. 2. Performance of the IC-MBR: (a). the growth of biomass and its settling ability; (b). the ratio of MLVSS/MLSS; (c). the removal of organic pollutants; (d). the concentration of NH_3^--N, NO_2^--N and NO_3^--N in the effluent; (e). the removal of TN.

tions of NH_3^--N, NO_2^--N always remained at low levels; these results implied a good nitrification condition formed in the bioreactor during the whole operating period. After 35 d, the removal of TN remained at above 80% (Fig. 2(e)), which demonstrated that a suitable condition for denitrification formed after the initial three phases.

3.2. Formation of GS in the bioreactor

With the total retention of sludge by the membrane module and in the presence of sufficient nutrients in the IC-MBR, the biomass grew very quickly. However, a rapid increase in the SVI value during the first phase (Fig. 2(a)) implied sludge bulking might have occurred. To reveal the granular sludge formation process, the sludge samples were collected from the IC-MBR at different time intervals to observe their morphology over the whole operation period. The results are shown in Fig. 3.

After 8 d, the value of SVI was 328.65 mL/g, which showed the actual occurrence of sludge bulking in the bioreactor. The image of sludge shown as Fig. 3(a) was taken on day 8, and shows the overgrowth of filamentous bacteria and the presence of only flocs (i.e., no sludge granules). This image gives a visual explanation for the phenomenon of sludge bulking. In the continuous internal hydrodynamic circulation of the IC-MBR, the sludge flocs and filamentous bacteria were subjected to continuous rotational shear, and their shapes might be totally altered by the shearing force. The circular shape of sludge flocs and filamentous bacteria shown in Fig. 3(b) confirmed the influence of rotational shearing. Under such operational conditions, filamentous bacteria may act as a skeleton to bring individual bacteria and tiny sludge particles together, and provide suitable habitats for the attached microorganisms. The flocculated bacteria and tiny sludge particles intertwined with the filamentous bacteria, and further secreted EPS to form a larger granule. After 37 d operation, sludge granules could be obviously observed (Fig. 3(c)), which confirmed the successful formation of GS, and also explained the reason why the SVI values decreased to approximately 100 ± 20 mL/g at phase IV. Fig. 3(d)–(f) illustrate the sludge granule development process, revealing that the granules were growing larger and their shape was gradually becoming more regular with a clear edge as the reactor operated longer. During the sludge granule development process, the filamentous bacteria still existed in the latter operational period.

The above results showed that the retention of the membrane module, the occurrence of filamentous bacteria and the internal circulation pattern in the IC-MBR played important roles during the formation and development of sludge granules. In phase I, the environmental conditions within the bioreactor were quite suitable for the growth of filamentous bacteria, they proliferated very quickly and caused sludge bulking. Because of the total retention by membrane module, the bulking sludge could not be washed out of the reactor by the flowing water, even under continuous-flow operation. The internal circulation caused the filamentous bacteria entangling tiny sludge particles and dispersed bacteria to form biomass aggregates, which led to the formation of larger sludge granules. As the reactor operation continued, the environmental conditions became more suitable for the development of sludge granules, and the granular sludge gradually became predominant within the bioreactor. This phenomenon reflected the succession of microbial communities from the original flocs sludge into that of the GS.

3.3. Granule size and size distribution

To further verify the formation of GS within the IC-MBR, after the bioreactor was operated for 76 days (Fig. 3(e)), sludge from both the mixing and aeration zones was sampled to measure the granule size and analyze its size distribution. These measurements were compared to similar measurements on the original sludge and the results are shown in Fig. 4.

As discussed in Section 3.2 and observed in Fig. 3, sludge granules have formed after 37 days of operation. The size measurements (Fig. 4) indicated that the sizes of sludge granules in both the mixing and aeration chambers were very similar, and that granules in the size range 0.20–0.40 mm comprised the largest

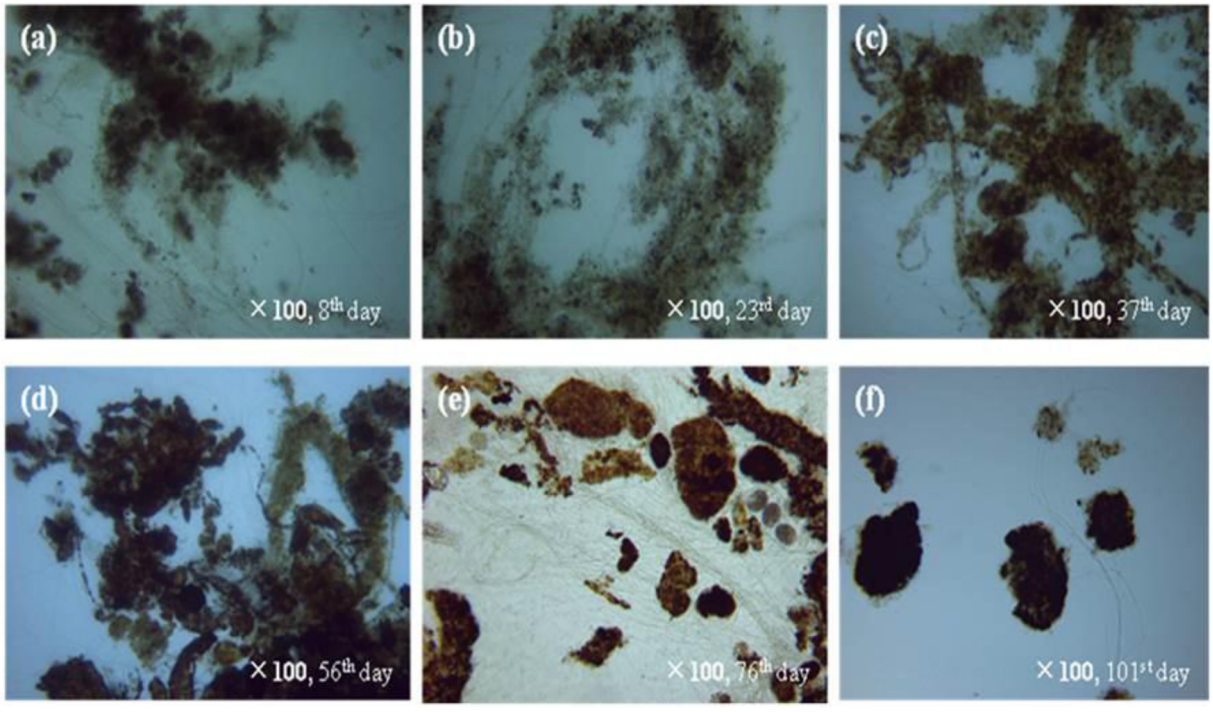

Fig. 3. Formation of granular sludge in the IC-MBR: (a)–(f) images of sludge granules on the 8th, 23rd, 37th, 56th, 76th and 101st day.

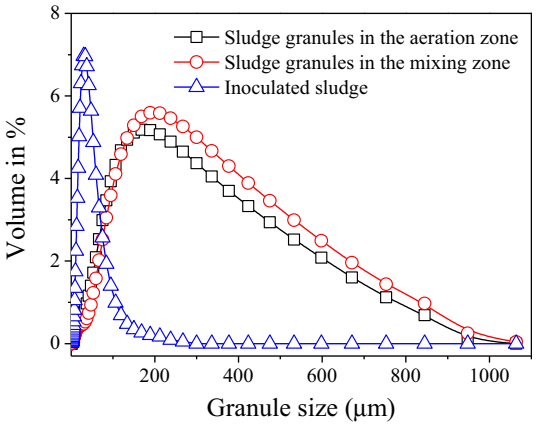

Fig. 4. Granule size and its distribution on the 76th day.

proportion of granules. The average size of granules in these two zones was 0.228 ± 0.014 mm, and the maximum diameter of the granules was 1.125 mm. The size of inoculated sludge particles was smaller than that of the sludge granules, and mostly in the range of 0.06–0.08 mm within a very narrow size distribution.

3.4. SEM observation

The surface of sludge granules was observed with an SEM under different magnifications to reveal its surface characteristics. The samples were collected on day 76, which was the same sampling date for size measurements (Section 3.3). The results are shown in Fig. 5.

Fig. 5(A) shows that the collected granule samples were large enough to be observed even with naked eyes, and they had a regular shape with a clear edge. At 200× magnification, the SEM image (Fig. 5(B)) shows that a sludge granule was an ellipsoid with a rough surface. Fig. 5 (C) and (D) reveal further the details about the surface of the sludge granules, showing that the sludge granules were entwined with filamentous bacteria, and contained many rod-like bacteria. Fig. 5 also verifies that filamentous bacteria played an essential role in forming and developing sludge granules [32].

3.5. EPS composition in granular sludge

The main components in EPS include protein (PN) and polysaccharides (PS), and generally EPS can be classified as "bound EPS" and "soluble EPS". These substances adhere to different microbes, including bacillus, zoogloea, protozoa, metazoa and other insoluble particles, and provide a habitat for the attached microorganisms, which play an important role in building the microbial community in GS. The bound EPS can also be further classified as TB-EPS and LB-EPS. The TB-EPS mainly attaches to the cell surface peripheral capsules, and is mainly responsible for keeping cells together in clusters. The LB-EPS sheds into the surrounding environment as an amorphous slime, whose main role is bonding different clusters to form stable micro-colonies. Variations of LB-EPS, TB-EPS, and the ratio of PN to PS (PN/PS) over time are shown in Fig. 6.

At a pre-set time interval, the content of LB-EPS, TB-EPS, and the ratio PN/PS were measured with the same granular sample. Fig. 6 reveals the TB-EPS content tended to increase until the middle of phase IV (approximately day 63), and then decreased to the end of the experiment. In contrast, the LB-EPS content remained low during the whole experimental period (and always lower than the TB-EPS content), which implied that TB-EPS might play a main role in the formation and development of GS. The PN/PS ratio was always less than 1, which indicated PS was a dominant component over PN in GS.

3.6. EPS profiles in sludge granule

In GS, EPS may act as a skeleton to hold various microorganisms together and constitute a microbial community. However, for the

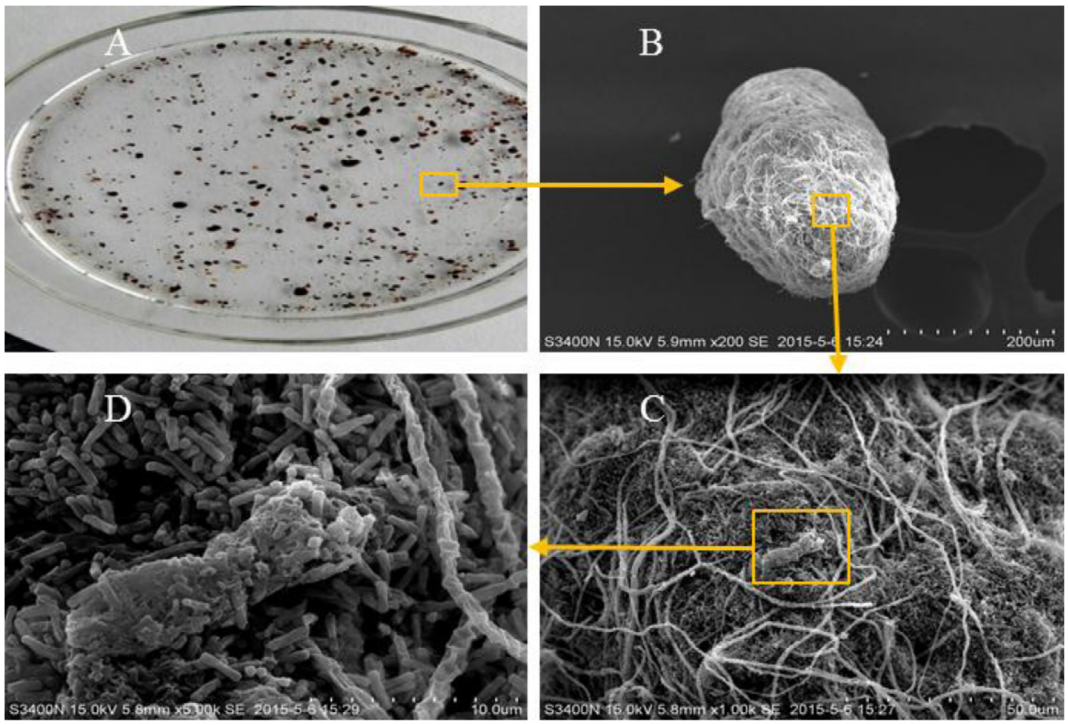

Fig. 5. Microscopic structure of the surface on granule sludge: (A). the photograph of GS; (B). the SEM image of GS (×200); (C). the SEM image of GS (×5000); (D). the SEM image of GS (×1000).

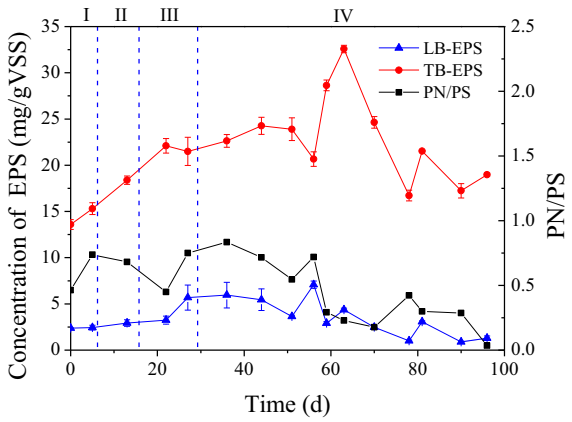

Fig. 6. EPS composition of granular sludge over time.

different selection pressures [33], the compositions of EPS are complex and distributed unevenly in the interior of sludge granules [31]. To reveal the distribution of various components of EPS in a sludge granule, a staining scheme (Table 3) was adapted. For the convenience of comparative analysis, the samples used for staining observation were collected on day 76 (as for other measurements). The results are shown in Fig. 7.

As shown in Fig. 7, proteins, α- and β-polysaccharides and lipids were all observed in the GS. According to the fluorescent intensity data presented in Fig. 7, proteins and β-polysaccharides were the main components in GS, and this observation was consistent with the data presented in Fig. 6. Fig. 7(a) and (b) shows that proteins and β-polysaccharides formed the core of a sludge granule and were distributed erratically over the GS. Fig. 7(c) reveals that α- polysaccharides mainly were located on a very small area of the granule and were less abundant than proteins and β-polysaccharides. Lipids were found nearly at the edge of GS (Fig. 7(d)) and functioned to maintain the stability of GS due to their adhesive force. Fig. 7(e) illustrates that the cultivated GS had a compact structure.

3.7. Microbial community

After being removed from the IC-MBR, sludge samples were separated into floc ($D < 0.15$ mm) and granular sludge ($D \geqslant 0.15$ mm) using a 100-mesh sifter, and labeled as "FS" and "GS", respectively. During IC-MBR operation, GS and FS coexisted and composed the microbial community within the bioreactor. The microbial community and its variation over time were revealed by using microscopic observation and an HTS method. Microscopic observation was carried out at a pre-set time interval during the whole experimental period, and analysis of the microbial community was based on the samples collected on day 76. The procedure of extracting DNA and analyzing microbial community is described in Section 2.6. After completion of HTS, the biodiversity of all samples was analyzed by using the software packages, including Prinseq 0.20.4, FLASH 1.2.3, Mothur 1.30.1, Uclust 1.1.579, Cytoscape 3.2, Qiime, R-project 3.2, Muscle 3.8.31, MEGAN 5.7.1, RDP classifier, Fasttree 2.1.3, RDP classifier database (16 s, fungal 28 s), Silva database (16 s, 18 s) and Unite database. The results are shown in Fig. 8.

Fig. 8(a) illustrates the variation of the main microorganisms within the IC-MBR over time. Obviously, filamentous bacteria were the dominant microorganism in the IC-MBR except during days 56–62. Some protozoa, including *Vorticella*, *Arcella* and *Epistylis*, were present during the whole experimental period. *Aeolosomatidae* appeared on day 16, and proliferated on day 56. *Water bears* appeared on day 69 when the biomass had accumulated to a relatively high concentration, and led to an oxygen mass transfer difficulty. GS and FS represented two different habitats within the IC-MBR. Results presented in Fig. 8(b) showed that, in terms of species level, *uncultured bacteria* occupied an absolute large ratio

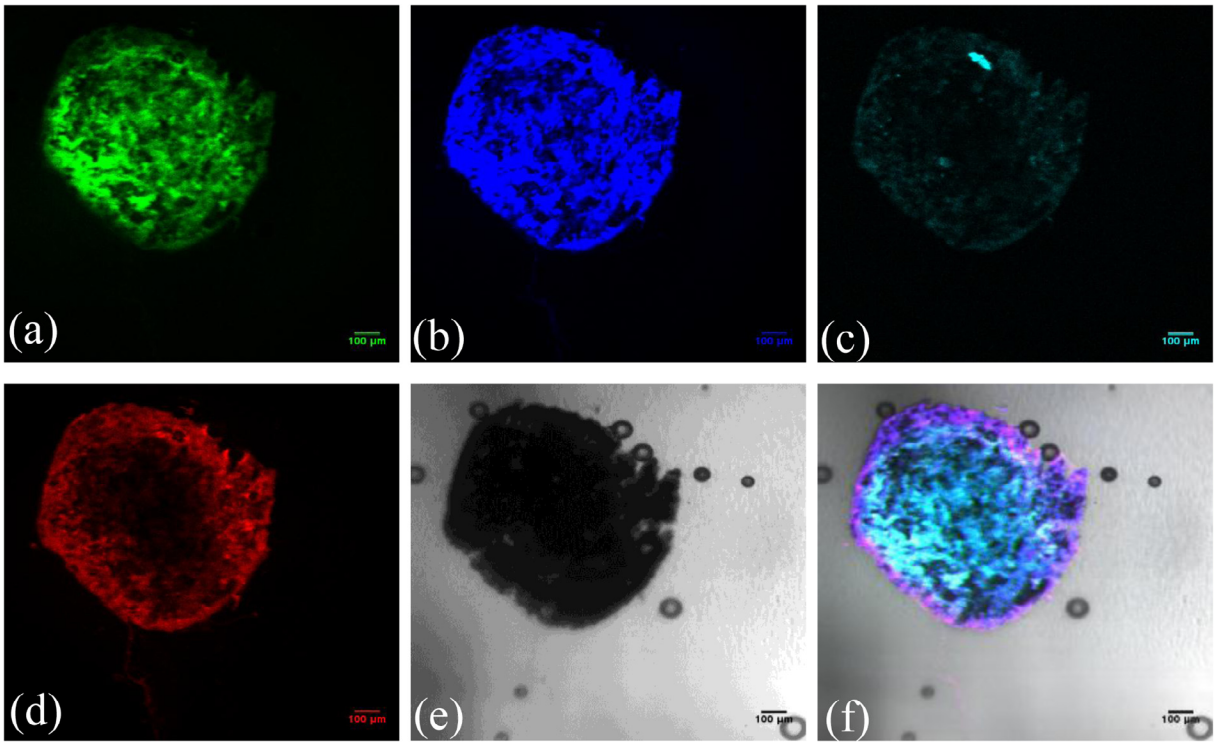

Fig. 7. EPS profile in a sludge granule: (a). CLSM image of proteins (FITC); (b). CLSM image of β-polysaccharides (Calcofluor white); (c). CLSM image of α-polysaccharides (Con A); (d). CLSM image of lipids (Nile red); (e). Phase contrast photograph; (f). Combined image of individual images in (a)–(e).

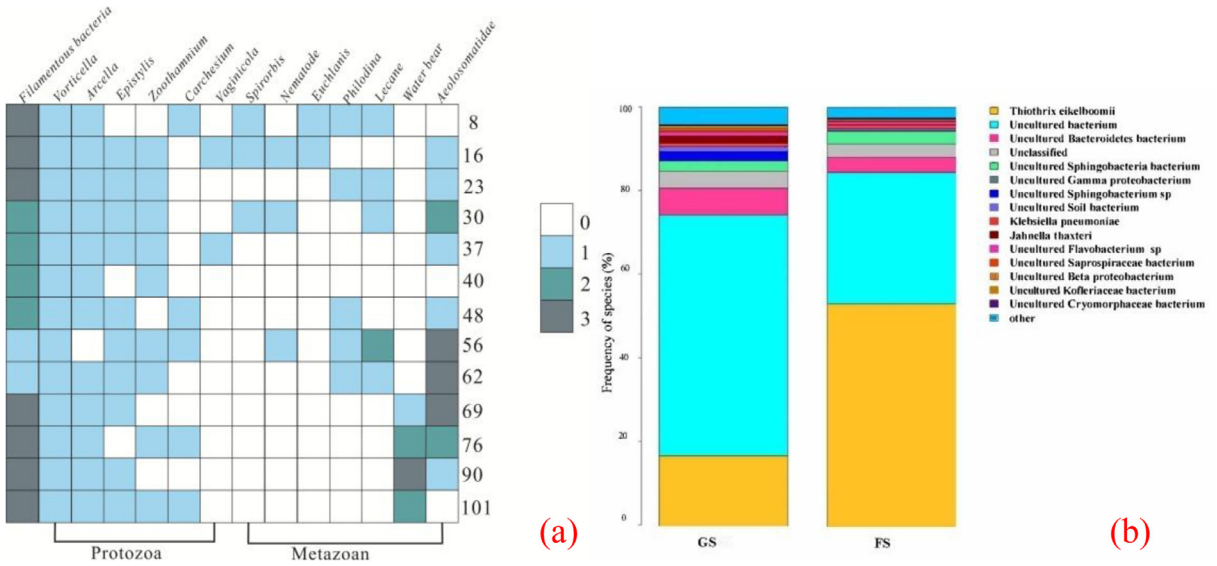

Fig. 8. Microbial community within the bioreactor: (a). abundance of biota over time (the color from "0" to"3" represents the variety of abundance: "0" means invisibility; "1"means visibility; "2"means high and"3"means higher); (b). microorganism distribution at species level.

(exceeding 50%) in GS, and *Thiothrix eikelboomii* was the second largest group. In FS, *Thiothrix eikelboomii* was the dominant microbial species.

The above results indicated that the occurrence of filamentous bacteria and their interrelationship with protozoa and metazoa were important for the sludge granulation process. Filamentous bacteria came from the inoculated sludge, and their winding and adhesive properties contributed to the formation of GS, which was crucial for sludge granulation. *Ciliates* survived more persistently than other protozoa under the steady flow and shear force in the reactor; because *Ciliates* could secrete EPS, they were regarded as the backbone of GS. In the IC-MBR four kinds of *Ciliates* were observed, including *Carchesium*, *Zoothamnium*, *Epistylis* and *Vorticella*; *Epistylis* were especially noticeable as appeared at start-up and remained until the end of the experiment. *Rotifer*, including *Lecane*, *Philodina* and *Euchlanis*, were always observed in the bioreactor, and they and *Vorticella* not only were regarded as an indicator of good effluent water quality [34], but also preyed on most of the dispersed bacteria [35]. *Arcella* also preyed on these dispersed bacteria and small floc sludge. Furthermore, most of the bacteria could not escape from the IC-MBR because of the membrane module. Instead, bacteria had to aggregate together to avoid the predation of protozoa, which might be another important stimulus for sludge granulation.

The results of HTS indicated that *Thiothrix eikelboomii*, a kind of filamentous bacteria, was dominant in the bioreactor; these bacteria utilize glucose and nitrate as their carbon and nitrogen sources [32]. Denitrifying bacteria (*Flavobacterium* and *Saprospiraceae*) were in greater abundance in GS than in FS, which was beneficial to the removal of TN. Overall, the main bacteria within the bioreactor could be classified as *Proteobacteria* and *Bacteroidetes*, but *Proteobacteria* were dominant. *Proteobacteria* included *Thiothrix Eikelboomii*, *Ganma proteobacterium*, *Sphingo bacterium*, *Sphingo bacteria bacterium*, *Klebsiella pneumonia* and *Beta proteobacterium*. Very few *Bacteroidetes* were detected in the bioreactor (only *Flavobacterium* sp and *Cryomorphaceae*).

4. Conclusions

GS was directly cultivated within an IC-MBR operated in a continuous-flow mode. On the 76th day, the average size of the cultivated GS was 0.228 ± 0.014 mm, and the maximum diameter was 1.125 mm. The total retention of sludge particles by membrane modules, the occurrence of filamentous bacteria and internal circulation were essential factors in forming GS. The formed GS had a compact structure, in which EPS played an important role in maintaining its integrity. The granular process could be divided into six steps, during which filamentous bacteria initiated a granulation process under the action of internal circulation, and maintained the stability of GS for a long period by wrapping biomass aggregates together. The microbial communities in the IC-MBR, including GS and FS, had rich biodiversity, which was responsible for the removal of organic pollutants and nutrients.

Acknowledgement

This work was supported by the National Natural Science Foundation of China [Grant number 21476050].

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