Microbial population dynamics of granular aerobic sequencing batch reactors during start-up and steady state periods

Y. Q. Liu, Y. H. Kong, R. Zhang, X. Zhang, F. S. Wong, J. H. Tay, J. R. Zhu, W. J. Jiang and W. T. Liu

ABSTRACT

This study investigates microbial population dynamics in granular sequencing batch reactors (GSBR). The experimental results of DGGE fingerprint of sludge demonstrated that the microbial community structure of sludge shifted significantly during granulation period and nutrient removal improvement period. After reactor performance and physical characteristics of sludge reached steady state, microbial population of sludge became relatively stable. The high similarity of microbial community structure between co-existed flocculated sludge and granular sludge in GSBR at different operation phases indicated that similar microbial consortium could exist in compact aggregated form or in amorphous flocculated form. Therefore, strong selection pressure was still required to wash out flocs to maintain the stability of reactor operation. In addition, it was found that substrate type had considerable impact on microbial species selection and enrichment in granular sludge. The clone library of granular sludge showed that microbial species in divisions of α-*Proteobacteria*, β-*Proteobacteria*, γ-*Proteobacteria* and *Bacteroidetes* existed within acetate-fed granule communities and *Thauera* spp. from β-*Proteobacteria* accounted for 49% of the total clones in the whole clone library. It is thus speculated that *Thauera* spp. are important for the formation of acetate-fed granules under the conditions used in this study, maintaining the integrity of granules or substrate degradation.

Key words | flocculated sludge, granular sequencing batch reactor (GSBR), granular sludge, microbial population dynamics

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INTRODUCTION

Aerobic granules are a type of microbial aggregates with well-defined appearance and compact structure, which grow under aerobic conditions. The distinct difference between aerobic granules and biofilm is that the formation of aerobic granules does not need any external carrier or nucleus for microbes to attach for aggregate formation. Due

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to their higher density, bigger size and excellent settling ability, aerobic granules can be retained easily in the reactor so that a higher biomass concentration can be achieved even though reactor is operated with a short settling time (Liu & Tay 2004; Adav *et al.* 2008*a*). Furthermore, the compact structure of granules, high biomass concentration and presence of starvation period enable granular sequencing batch reactor (GSBR) to adapt to various fluctuating conditions. In addition, the presence of aerobic and anoxic zones inside the granules enables different biological processes to occur simultaneously in the same system (de Kreuk *et al.* 2005; Wang *et al.* 2009). Due to these advantages as compared to activated sludge system, aerobic granular sludge technology is getting more attention and several pilot-scale GSBRs have been built. However, the mechanism of aerobic granulation is still not fully understood (Liu & Tay 2004) and the effective guidance based on the mechanism understanding has still not been established. Therefore, the operation and optimization of GSBR still mainly depends on experience.

In the last decade, some information on EPS distribution in granules (McSwain *et al.* 2005; Adav *et al.* 2008*b*), microstructure of granule (Tay *et al.* 2001; Liu *et al.* 2006), the spatial distribution of some bacteria with specific functions such as ammonia oxidizing bacteria, nitrite oxidizing bacteria, and phosphorus removal bacteria (Lemaire *et al.* 2008), and even some isolates with specific functions such as phenol degradation (Adav & Lee 2008) have been disclosed for better understanding of granulation. However, it is still unclear how microbial community evolves during the transformation of activated sludge to granular sludge with real wastewater. In addition, the information on the microbial population of flocs in GSBR and bacteria species of stable granules is absent. This study aims to address these questions from the perspective of microbial community structure during start-up period and steady state with different substrate types to deepen the understanding of granulation.

MATERIALS AND METHODS

Experimental setup

Three bubble columns (R1, R2 and R3) were used to cultivate aerobic granules. For R1 and R2, the reactor diameter was 20 cm with a 36 L volume capacity. For R3, the reactor diameter was 5 cm with a working volume of 2 L. Both R1 and R2 were fed with wild-fluctuated real wastewater from houses, factories, commercial and industrial premises with COD from 300 to 1,800 mg l^{-1} and ammonia nitrogen from 40 to 110 mg l^{-1} while R3 was fed with synthetic wastewater, which consisted of COD (sodium acetate) 1,000 mg l^{-1}, NH_4-N 26 mg l^{-1}, PO_4-P 4 mg l^{-1}, $CaCl_2{\cdot}2H_2O$ 15 mg l^{-1}, $MgSO_4{\cdot}7H_2O$ 12.5 mg l^{-1}, $FeSO_4{\cdot}7H_2O$ 10 mg l^{-1} and trace elements. The trace elements contained (mg l^{-1}): H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$, 0.03; $MnSO_4{\cdot}H_2O(NH_4)_6$, 0.05; $Mo_7O_2{\cdot}4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2{\cdot}6H_2O$, 0.05 and NiCl, 0.05.

Three reactors were operated in a sequential batch mode with a cycle time of 4 h, which included feeding, aeration, settling and discharging. Fine air bubbles for aeration were supplied through an air sparger at the reactor bottom with an upflow air velocity of 1.2 cm s^{-1} to all reactors. The effluent was discharged from the middle port of the reactors with a volumetric exchange ratio of 50%. The organic loading rate (OLR) was controlled precisely at 3 g COD l^{-1} d^{-1} in R3 while OLR in R1 and R2 fluctuated around 3 g COD l^{-1} d^{-1}. Reactor R2 and R3 were started up by step-wise decreasing settling time until to 2 min while reactor R1 was started up with a fixed short settling time of 2 min, which led to the similar minimum settling velocity such as 25–28 cm/min in three reactors at the steady state. Three reactors were inoculated with the same sludge taken from the local aeration tank for treating real wastewater with a SVI of 150 mg l^{-1} and an average size of 120 μm. All operating conditions were same when the three reactors reached steady state phase.

Analytical procedures

Chemical oxygen demand (COD), sludge volume index (SVI) and biomass dry weight (MLSS) were analyzed according to standard methods (APHA 1998). Average particle size and floc volume percentage with size below 200 μm were measured by a laser particle size analysis system (Malvern MasterSizer Series 2,600, Malvern instruments, Malvern) or an image analysis (IA) system (Image-Pro Plus, V4.0, Media Cybernetics). Morphology of sludge

was observed, and sludge photos were taken by the IA system with the Olympus SZX9 microscope (Olympus SZX9).

DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE)

Approximate 1 g of sludge sampled from reactors was washed with distilled water and TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) subsequently in a 2 ml EP tube. After centrifugation at 2, 300 × g for 5 min, the sludge was preserved in 588 μl DNA extraction buffer (0.1 M Tris-HCl, 0.1 M Na_2-EDTA, 0.1 M sodium phosphate, 1.5 M NaCl, pH 7.5) and stored at −20°C until extraction. Bead-beater was applied to rupture bacterial cells and DNA of samples was extracted with lysozyme, proteinase K, SDS and sodium dodecyl sulphate treatment, followed by phenol/chloroform/isoamyl alcohol (25: 24: 1) extraction and isopropanol precipitation.

20 ng DNA were amplified by polymerase chain reaction (PCR) using PCR primers P2: 5′-ATT ACC GCG GCT GCT GG-3′; and P3: 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′ as described by Muyzer *et al.* [18]. 50 μL PCR mixture proceeded with TAKARA PCR kit (TAKARA BIO INC, Japan), including 0.5 μl of each primer (10 mM), 5 μl of PCR buffer, 4 μl of dNTP (2.5 mM), 0.5 μl of Ex Taq polymerase (TaKaRa, 2.5 U/μl) and 37.5 μl of double-distilled water. PCR process was performed by activation at 95°C for 3 min, 30 cycles with each including denaturation at 95°C for 0.5 min, annealing at 55°C for 0.75 min, elongation at 72°C for1 min, and a final step of 72°C for 10 min. Successful PCR was confirmed by 1.5% agarose gel electrophoresis.

40 μl of PCR product was loaded with 8 μl loading dye to a 8% acrylamide, 30–70% urea-formamide denaturing gel and run in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM Na_2-EDTA, pH 8.0) for 900 min at 85 V by DGGE system (Bio-Rad Decode, USA) at 61°C. After electrophoresis, the DGGE gel was stained with EB (ethidium bromide) for 30 min and photographed with an EDAS 290 gel imaging system (Eastman Kodak, USA). Every DGGE gel was performed at least twice to make sure the reliability of result. Gelcompare II (Applied Math, Belgium) was used for analysis of DGGE band pattern.

Construction of clone library

From the extracted DNA, 16S rRNA genes were PCR-amplified by a primer set specific for bacteria: 8f (5′-AGA GTT TCC TGG CTC AG-3′) and 1492r (5′-TAC GGT TAC CTT GTT ACG ACT-3′). The PCR products purified by Quick PCR purification kit (Qiagen, Chatsworth, CA) were cloned into plasmid vector with the TOPO TA cloning system (Invitrogen, Carlsbad, CA) according to the protocol described by the manufacturer. A total of 191 clones were randomly selected and sequenced. The results were then compared with available database sequence using the program BLASTn of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the RDP Classifier of Ribosomal Database Project II (http://rdp.cme.msu.edu/) for initial phylogenetic assignment in Dec 2008.

RESULTS AND DISCUSSION

Shift of microbial community structure in reactor R1

The microbial population of sludge during the whole process of GSBR operation was analyzed by DGGE and shown in Figure 1. In the DGGE gel, the number, precise position, and intensity of the bands in each lane gave an estimate of the number and relative abundance of numerically dominant species in the sample (Muyzer *et al.* 1993). It was found that microbial population shifted greatly in the first month, within which granule fraction increased from 0 to above 80%. It is clear that the transformation of

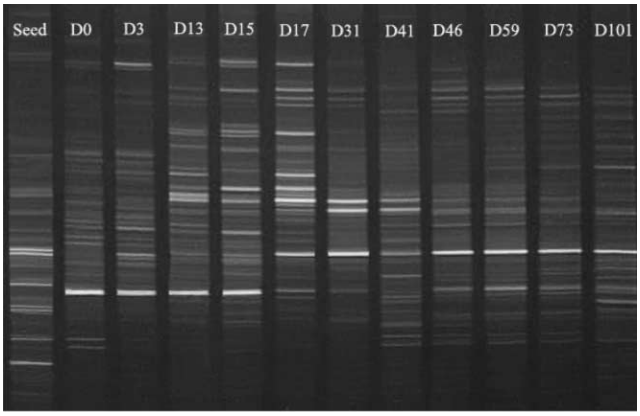

Figure 1 | DGGE profiles of ribosomal DNA fragments obtained after enzymatic amplification of DNA extracted from sludge in R1 with operation time.

activated sludge to granules was accompanied by the change of microbial population of sludge in the reactor. Li *et al.* (2008) also observed the microbial population change of sludge during the granulation period in GMBR fed with synthetic wastewater with glucose as carbon source.

Although the shift of microbial population during granulation and the stable microbial community of aerobic granules during steady state have been reported (Williams & de los Reyes 2006; Zhang *et al.* 2008*a*,*b*), it is still unclear when the transformation of microbial community structure occurs from significant change stage to a relatively stable stage. In this study, COD removal efficiency was above 90% after one-month operation, but ammonia removal efficiency was still as low as 40% as shown in Figure 2. With the biomass accumulation over time, ammonia removal efficiency increased to 99% after a 50-day operation. All process parameters including COD removal, ammonia removal, biomass concentration, granular sludge proportion and physical properties of granular sludge such as SVI and granule size were stabilized after 50-day operation, indicating that the system reached steady state after the 50-day operation.

The DGGE fingerprints were analyzed for percent similarity by using a software program (GelCompare II) capable of similarity/dissimilarity calculation and cluster analysis features. It was found that there was more than $71 \pm 6\%$ similarity among most samples during day 46 to 101, indicating a highly stable microbial community. Evidently, the stable microbial community structure of granular sludge was in good agreement with the stability of

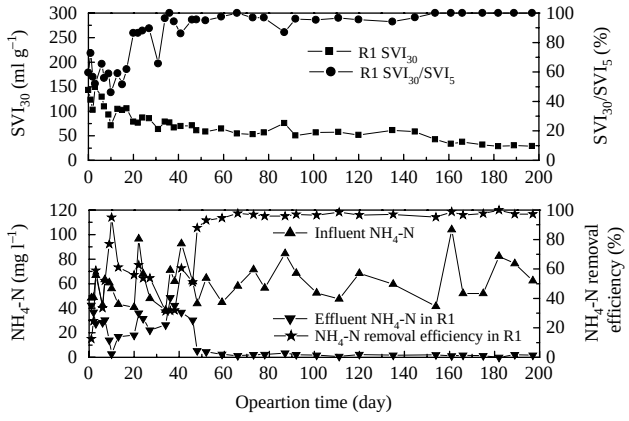

Figure 2 | Evolution of SVI_{30} and SVI_{30}/SVI_5 of sludge in R1 and nitrification in R1 with operation time.

sludge properties and process performance in reactor R1. Although real wastewater with wild fluctuated influent quality was used in R1 in this study, the change of microbial community structure of sludge in the reactor is consistent with that reported with a granular reactor started up with synthetic wastewater, which has stable influent quality (Li *et al.* 2008). Therefore, it could be speculated that the change of microbial community structure of sludge in the reactor is mainly related to the change of sludge morphology or specific substrate degradation in GSBR.

Aerobic granulation occurs only under specific operational conditions with high selection pressure such as short settling time, high shear stress and above a certain organic loading rate (Liu *et al.* 2005). A kind of natural selection with survival of the fittest species is thus involved in granulation in response to the specific environment in GSBR such as high minimum settling velocity, in which easily aggregated species were selected to remain in the reactor by forming granules with better settleability while suspended species were washed out due to poor settleability. In fact, it was observed during the granulation as shown in Figure 1 that some dominant bands in seed sludge disappeared or their intensity weakened over time while some bands appeared or became stronger in intensity. It was also interesting to note that some bands were abundant in both seed sludge and steady-state granules. Therefore, granulation is actually a process to build a new ecosystem by microbial selection and enrichment under selected operational pressure. Here, it should be pointed out that not all bacteria in aerobic granules are necessarily easily aggregated bacteria. Bacteria with poor aggregation ability contributing to substrate degradation or metabolism pathways of easily aggregated bacteria still can co-exist in the ecosystem of aerobic granules. Jiang *et al.* (2004) isolated pure bacteria from granules for treating phenol containing wastewater and found that bacteria with poor aggregation ability but high phenol degradation ability coexisted with easily aggregated bacteria with poor phenol degradation ability. Therefore, it is highly possible that the evident microbial population shift after granulation up to 50 days (ammonia removal efficiency improved from 40 to 99% during this period) mainly build a microbial population balance between ammonia oxidizing bacteria and bacteria for maintaining the structure of granules and COD degradation.

Microbial community structure of flocculated sludge and granular sludge in reactors R1 and R2

During the long-term operation of reactors R1 and R2, it was noted that flocculated sludge always co-existed with granules. The proportion of flocculated sludge only accounted for 2–10% of the total biomass at most times at the steady state and occasionally reached over 20%. The evident interface was observed between granular sludge and flocculated sludge after settling in the reactors. To analyze the difference of microbial population of sludge with different flocculated states in GSBR, flocculated sludge, granular sludge, the mixed sludge, sludge collected in the discharged effluent from the reactor R2 at the steady state were sampled and analysed. Their DGGE fingerprint profiles were shown in Figure 3.

Although the microbial community structure of sludge changed slightly from day 640 to 773 as shown in Figure 3, band number and the intensity of dominant bands of flocculated sludge were almost the same to those in granular sludge on different operation days. In addition, the microbial population of effluent biomass, which was mainly flocculated sludge and easy to be washed out, was similar to that of sludge retained in the R2. Furthermore, the DGGE fingerprints of flocculated sludge and granular sludge coexisted in R1 at steady state were almost the same as well (Figure 4). It was obvious that microbial community structure of co-existed flocculated sludge and granular sludge had no significant differences at different operation periods. These observations are noteworthy as it is thought that microbial selection exists for granulation and microbial population of granules is of great difference with that in amorphous flocculated sludge (Morval *et al.* 1992; Liu *et al.* 2004). Currently, it is still unclear why similar microbial consortia at different states can co-exist in GSBR. One possible explanation is that bacteria tend to be in amorphous form to obtain food easily rather than in aggregation form due to less substrate transfer resistance. For example, the specific growth rate of granular sludge in the suspended and granule sludge co-existed system is $0.022\,h^{-1}$ while that of suspended sludge can reach up to $0.190\,h^{-1}$ under the same conditions (Liu *et al.* 2004). So it is likely that the superiority of flocculated sludge on microbial growth kinetic leads to the presence of flocs in GSBR. However, relatively poor settleability of flocculated sludge results in washout of majority of them thus leading to the presence of flocs in sludge effluent. Generally, floc fraction in the mixed biomass in GSBR is less than 10%. It was observed that the floc ratio increased rapidly once floc fraction exceeded 20%. Granular sludge is thus easy to be replaced by floc because of the inferiority of

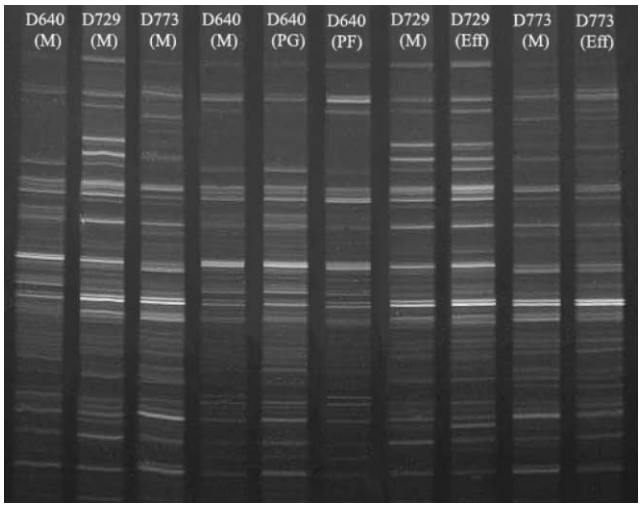

Figure 3 | DGGE fingerprint profiles of sludge in R2; M, PG, PF, and Eff represent mixed sludge in R2, pure granules and pure flocs separated from the mixed sludge in R2, and effluent biomass collected from discharging stream of R2, respectively; D represents operation day.

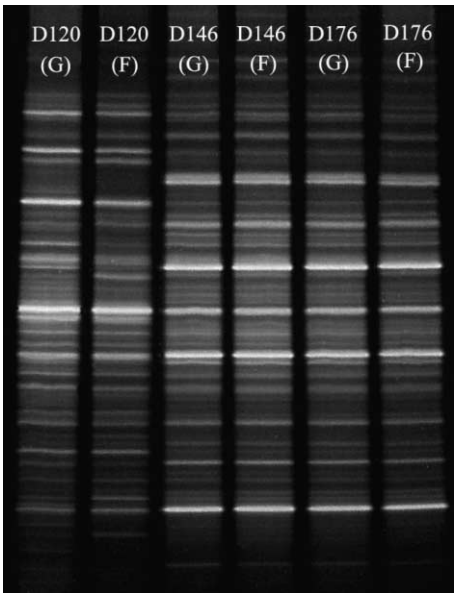

Figure 4 | DGGE profiles of sludge in R1; G and F represent pure granules and pure flocs separated from the mixed sludge in R1, respectively. D represents operation day.

granules on substrate utilization and microbial growth, which finally leads to the collapse of reactor operation. Therefore, reactor operation still needs to be controlled to wash out flocs even after granules form. It is no doubt that selection pressure is very important for washing out flocs continually to maintain the long-term stability of granules.

The clone library of acetate-fed granules at steady state

So far, the reported species in aerobic granules distribute in divisions or subdivisions of *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes* under different operation conditions (Jiang *et al.* 2004; Adav & Lee 2008; L *et al.* 2008; Zhang *et al.* 2008*a*; Zhang *et al.* 2008*b*; Song *et al.* 2009). Eukaryal species were also reported in aerobic granules (Williams & de los Reyes 2006). All these information was obtained by sequencing excised DGGE band or isolating some specific bacteria. To fully investigate the microbial population in aerobic granules, steady-state granules in R3 fed by synthetic wastewater with acetate as model substrate were sampled and used for the construction of 16S rRNA gene clone library. It was found that *Thauera* spp. from *β-Proteobacteria* accounted for 49% of the 191 clones in the whole clone library. *α-Proteobacteria*, *γ-Proteobacteria* and *Bacteroidetes* accounted for 8.9%, 5.8% and 11%, respectively. It was thus speculated that *Thauera*-related spp. are important for granule formation, maintaining the integrity of the acetate-fed granules or substrate degradation. DGGE profile of granular sludge from R3 as shown in Figure 5 also suggested that *Thauera*-related spp. were the most abundant bacteria during the 6-month steady-state period, which was in good agreement with clone library result. However, no corresponding band to *Thauera* spp. was found in DGGE fingerprint of sludge from R1 and R2. In addition, microbial communities of acetate-fed granules at steady state were distinctly different to those of granules fed with real wastewater even though all three reactors were run under the same operational conditions after granules formed. This indicated that dominant bacteria in granular sludge are possibly influenced by the type of wastewater under the same operational conditions. Inoculum also determines the microbial community structure of stable granules. Besides substrate and inoculum, operational conditions such as temperature and organic loading rates have considerable impact on species selection and microbial community structure (Chen *et al.* 2008; Li *et al.* 2008; Song *et al.* 2009).

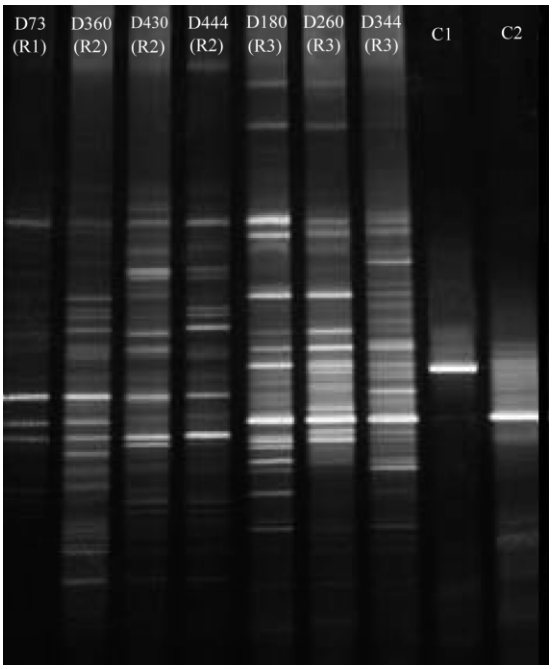

Figure 5 | DGGE profiles of steady-state granular sludge in three reactors at different operation days; D, R, C1 and C2 represents operation day, reactor, Clone *Aquimonas voraii* and clone *Thauera* spp. from the sludge on day 180 in R3, respectively.

CONCLUSIONS

This paper aimed to invesitigate the microbial population dynamics of granular aerobic sequencing batch reactors during start-up and steady state periods. Aerobic granules were successfully developed in all GSBRs with synthetic/real wastewater under different adjustment strategies of settling time. It could be concluded that:

- The microbial community structure of sludge in GSBR reached relatively steady state parallel to reaching steady state in reactor performance and physical characteristics of granules.
- The co-existed flocculated sludge and granular sludge had highly similar microbial community structure in different operation stages at steady state. The same microbial consortium thus could be present in compact aggregated form or in amorphous flocculated form.

- Substrate type could have an impact on microbial community structure of granule at steady state.
- Microbial species of *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria* and *Bacteroidetes* were abundant in acetate-fed granules, especially *Thauera* spp. from *β-Proteobacteria* accounted for 49% of the total clones in the whole clone library, which indicated that *Thauera* spp may play an important role in granules, either for granulation or for substrate removal.

Processing the complicated information on microbial ecology in granules at different stages under different conditions is not easy for disclosure of granulation. Combination of the traditional method such as isolation of pure culture with modern molecular techniques could be useful for the physiological and functional investigation of bacteria in granular sludge to deepen understanding of granulation.

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