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Salt-tolerance aerobic granular sludge: Formation and microbial community characteristics

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ABSTRACT

The salt-tolerance aerobic granular sludge (SAGS) dominated by moderately halophilic bacteria was successfully cultured in a 9% (w/v) salty, lab-scale sequence batch reactor (SBR) system. Influence of high salinity (0–9% w/v NaCl) on the formation, performance and microbial succession of the SAGS were explored. Crystal nucleus hypothesis, selection pressure hypothesis and compressed double electric layers hypothesis were used to discuss the formation mechanism of SAGS. Notably, salinity could be seen as a kind of selection pressure contributed to the formation of SAGS, while salinity also declined the performance of SAGS system. High throughput 16S rRNA gene analysis showed that the salinity had great influence on the species succession and community structure of SAGS. Moreover, *Salinicola and Halomonas* were dominant at 9% salt concentration, therefore moderate halophiles were identified as functional groups for the tolerance of hypersaline stress.

1. Introduction

Nowadays salinity is considered as a common stress factor in wastewater treatment plants (WWTPs), especially in industrial sectors, such as pickling, cheese manufacturing, seafood processing, tanning and pharmaceuticals (Moussa et al., 2006). Physical-chemical methods were conventionally used to treat the saline wastewater, while the cost was particularly high. Thus, choosing an alternative technology treating saline wastewater is urgent.

The traditional-activated sludge process has been proved to be a feasible, cost-effective treatment for degradation of organic pollutants in saline wastewater, which represents as much as 5% of worldwide effluent treatment requirements (Lefebvre et al., 2007). Besides, aerobic granular sludge, a promising biological treatment technology, has recently received more attention due to its remarkable settling characteristics, which can result in high sludge concentration and low reactor space (Abbas et al., 2015; Adav et al., 2008).

In recent years, much effort has been paid to study the effect of salinity on the performance and microbial community of aerobic granular sludge in SBR system (Lefebvre et al., 2006; Wang et al., 2015; Zhao et al., 2016). Besides, with the development of sequencing technique, high-throughput sequencing technology has been used to explore microbial communities under various environmental conditions. For example, high-throughput sequencing was used to study the performance and microbial community profiles in a SBR from 0 wt% to 3.0 wt% salinity, and found that *Actinobacteria* was dominant at salinity higher than 2.5 wt% (Zhao et al., 2016). Wang et al. (2016) reported that salinity had selective effects on the microbial community structure, and salt-resistant microbes contributed to the rising of richness and diversity at 2% and 4% NaCl stress. Salinity is known to influence the degradation rate through the inhibition of microbial activity or enzyme activity. Besides, with the increase of salinity, the water density correspondingly increased, causing the adverse effect on the settling characteristics. However, Moussa et al. (2006) reported that gradually increase salinity will stimulate the selection of dense flocs with minimum washout, and higher salt contents evolved a better settling sludge. Therefore, the hypersaline stress may not only shift the dominated microbial species, especially for some halotolerant bacteria, but also influence the formation process of granular sludge. In addition, the forming characteristics of salt-tolerant granular sludge and microbial community succession with elevated salinity were still unclear.

In this study, a lab-scale SBR was introduced to explore the performance, formation and microbial succession of salt-tolerance granular sludge respond to hypersaline stress (0–9% w/v NaCl). The 16S rRNA gene high-throughput sequencing was applied to decipher the species succession and community structure of salt-tolerance granular sludge and to identify the role of some functional salt-tolerant bacteria in salt-tolerance aerobic granular sludge system. The study provided detailed

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information of species succession for salt-tolerant granular system from 0% to 9% (w/v) salinity and a better understanding for the application of granular sludge in treating saline wastewater.

2. Materials and methods

2.1. Reactor set-up and operation optimization

A lab-scale SBR was operated for about 160 days, for culturing salt-tolerate granular sludge. The system was controlled by a programmable logic controller (PLC) and was maintained at room temperature (22 ± 2 °C). The reactor was 100 cm height with 8 cm internal diameter, its working height was 80 cm, so that the height/diameter ratio (H/D) was 10. The aeration was introduced by an air pump, and two peristaltic pumps were used to drive the influent and effluent water, the volumetric exchange ratio (VER) was 50% for each cycle. Every operated cycle of the reactor took 6 h. Generally each cycle included 5 min of feeding, 0–30 min of anaerobic phase, 150–180 min of aerobic phase, 3–30 min of settling time and 5 min of effluent discharge phase. To optimize the system performance, the reactor operation parameters were adjusted throughout the whole operation process, such as settling time, influent chemical oxygen demand (COD) and aerobic/anaerobic time (Long et al., 2014), and all operation parameters were summarized in Table 1.

2.2. Seed sludge and wastewater composition

The activated sludge was collected from the recycled sludge of the secondary clarifier in the Changqiao municipal wastewater treatment plant in Shanghai, China. Then, the inoculated sludge was consisted the 100 mL anaerobic granular sludge with the aerobic activated sludge. The initial mixed liquor suspended solids (MLSS) level was 3.36 g L^{-1} and the sludge volume index (SVI) was 148.8 mL g^{-1} in the SBR system. The glucose was added as carbon source, and the influent organic concentration was between 1000 and 2500 mg COD L^{-1}. The synthetic wastewater was composed of (mg L^{-1}): NH_4Cl (500); KH_2PO_4 (140); $CaCl_2$ (150); $MgCl_2$ (31); and $FeSO_4{\cdot}7H_2O$ (10). The influent pH was maintained at 7.0 ± 0.1 by adding 2 M $NaHCO_3$ or HCl solution. In addition, 2.5 mL of trace element solution was added into 50 L of influent, which contained (in mg L^{-1}): H_3BO_3 (50); $ZnCl_2$ (50); $CuCl_2$ (30); $MnSO_4{\cdot}H_2O$ (50); $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (50); $AlCl_3$ (50); $CoCl_2{\cdot}6H_2O$ (50); and $NiCl_2$ (50) (Ren et al., 2008).

2.3. DNA extraction, high-throughput sequencing and analysis

Granular sludge was collected at different salt concentrations: 0%, 3%, 6%, 8% and 9% for DNA extraction. Then, the sludge samples were stored at −80 °C. The 3S DNA Isolation Kit for Environmental Samples (Shenergy Biocolor, China) was used to extract DNA according to the protocol, after extraction, 1% agarose gels electrophoresis was used to examine the quality of DNA. The V3-V4 region of the 16s rRNA gene was amplified by the bacterial primers 338F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′), and marked by the reverse primer including a 6bp barcode (Zhao et al., 2016). The polymerase chain reaction (PCR) was performed in 50 μL mixtures containing 25 μL of 2 × taq PCR Mix, 22 μL of ddH_2O, 1 μL of each primer, and 1 μL of template DNA, and the PCR products were confirmed using electrophoresis through 1% w/w agarose gel. Amplicons were extracted from 2% agarose gels, purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, then quantified with QuantiFluor™-ST (Promega, USA). Purified amplicons were pooled in the equimolar mixture and paired-end sequenced (2 × 250) on an Illumina MiSeq platform at Major Bio-Pharm Technology CO., Ltd. (Shanghai, China) (Yan et al., 2016). After that, the processing of raw data was conducted as previous studies (Guo et al., 2015; Wang et al., 2016). Lastly, the raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Access Number: SRX2175355).

2.4. Analytical methods

The conventional indexes of COD, MLSS, mixed liquor volatile suspended solids (MLVSS), and SVI were analyzed according to the Standard Methods.

3. Results and discussion

3.1. Performance and formation of SAGS

The SBR system was operated in three different phases (Fig. 1), and the operational conditions of SBR system were listed in Table 1. The performance in each phase including MLSS, MLVSS, SVI and removal rate was described as follows:

Phase I (start-up): During the phase I, the influent COD concentration varied from 1000 to 1800 mg L^{-1} as the salinity increased from 0% to 3%, the system always showed a high COD removal rate of greater than 85%. In case of excessive loss of sludge, the system initially maintained the 30 min settling time, so that the MLSS and MLVSS increased significantly, and the sludge maintained the flocculent morphology. When salt concentration gradually increased from 2% to 3%, the settling time decreased from 30 min to 15 min to promote the formation of granular sludge. Moreover, anaerobic time (20 min) was introduced to inhibit the overgrowth of filamentous bacteria at 2% salt concentration, and with the increase of salinity the anaerobic time extended to 30 min, which was an effective way to decrease the disintegration of aerobic granular sludge (Long et al., 2014). The granular sludge emerged on day 32, soon after that MLSS and SVI were found to reduce considerably due to the wash out of floc sludge, which was associated to the reduce of settling time and the increase of wastewater density caused by salinity.

Phase II (acclimation under saline stress): The system was continued to operate at 4–6% saline stress, and the settling time further decreased

Table 1
Operating conditions and performance of aerobic granular sludge under different salinities.

Salinity (% w/v)	Time (d)	Influent COD (mg L^{-1})	Effluent COD (mg L^{-1})	Removal rate (%)	Setting time (min/cycle)	Anaerobic time (min/cycle)
0	0–14	1000	40	96	30	0
1	15–28	1200	36	97	30	0
2	29–42	1400	84	94	15	20
3	43–56	1800	216	88	15	20
4	57–70	2000	500	75	5	30
5	71–84	2200	770	65	5	30
6	85–98	2500	1150	54	3	30
7	99–112	2500	1175	53	3	30
8	113–126	2500	1275	49	3	30
9	126–140	2500	1250	50	3	30

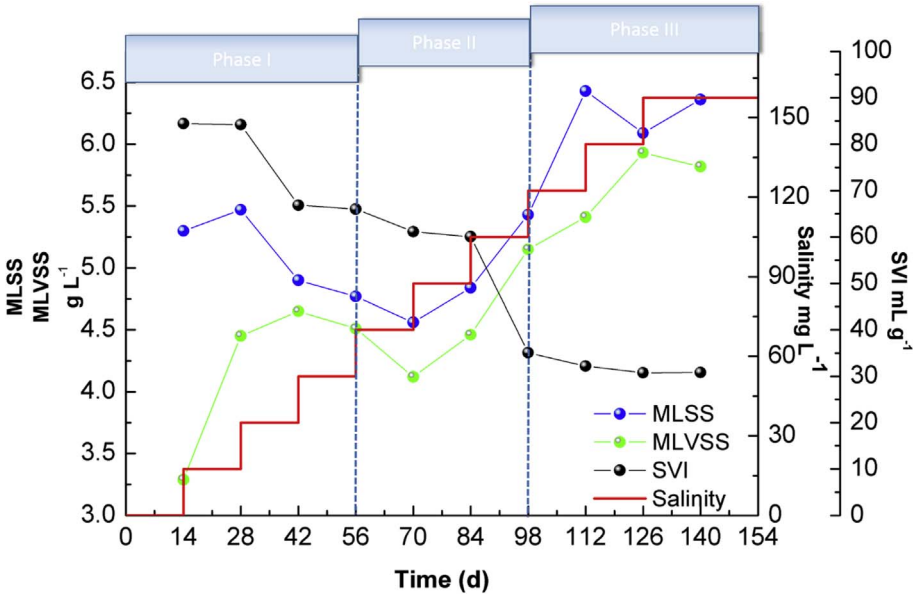

Fig. 1. Overall performance of the salt-tolerance granular sludge at different phases.

to 3–5 min. With the maturation of granular sludge, the MLSS and MLVSS showed little changed in this phase. While the salinity increased from 5% to 6%, SVI decreased and MLSS increased sharply. With continuous double pressure of salinity and settling time, the granular sludge dominated gradually, which accounted for more than 90% in the SBR system. Besides, the COD removal rate decreased from 88% to 54%. As previous reports (Yogalakshmi and Joseph, 2010; Zhao et al., 2016), the increase of salinity inhibited the microbial metabolism, thereby reduced the degradation ability of SAGS.

Phase III (elevated salt concentration): However, the salt stress increasing from 7% to 9% salinity had little influence on the removal of COD, the system could maintain about 50% removal rate. In this period, the MLSS and MLVSS continuously increased to 6.36 and 5.82 g L^{-1} respectively, the SVI had no obvious fluctuation and maintained at about 54 mL g^{-1}. The SAGS was observed to dominate in SBR system at 7–9% salinity.

3.2. Bacterial community analysis

The high-throughput sequencing technology was applied to explore the microbial community structure and characteristics of SAGS responding to hypersaline stress. The activated sludge were collected at 0%, 3%, 6%, 8% and 9% salt concentrations to characterize the microbial community, which were labeled as 0, 3, 6, 8 and 9, respectively. All samples were collected at the same operation conditions to reduce operating errors.

A total of 175,162 effective reads (36,697, 31,450, 31,843, 40,789 and 34,383 for 0%, 3%, 6%, 8% and 9%, respectively) were obtained by the MiSeq pyrosequencing from sludge samples at different salt concentration. Each sample was then normalized to 31,450 sequences, then conducted the downstream analyses to obtain 115(0%), 96(3%), 82(6%), 66(8%) and 54(9%) operational taxonomic units (OTUs) at 97% similarity threshold. Rarefaction curve (Fig. 2) was employed to standardize and compare the observed taxon richness among 6 samples and to verify the representativeness of collected samples (He et al., 2016). As shown in Fig. 2, all curves tended to be flat as sequence numbers increased, indicating that the sequence volume was reasonable to represent almost microbial community of six sludge samples. This could be also confirmed by the high coverage (Table 2).

In addition, the community richness was characterized by Chao values and abundance-based coverage estimation (ACE). The results showed that 0% sample had the highest species richness. With the

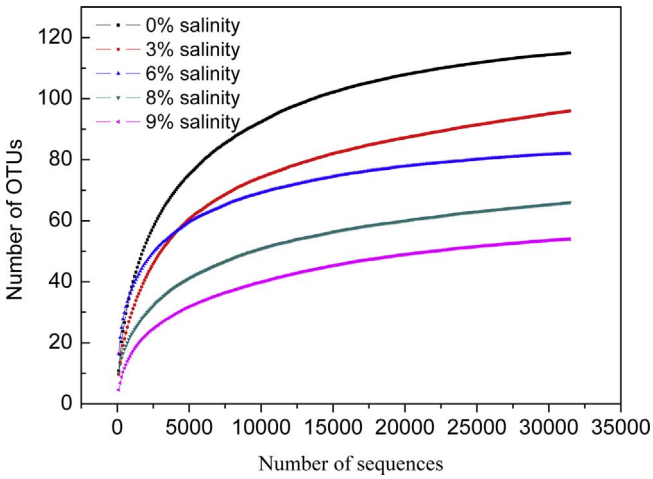

Fig. 2. Rarefaction curves of OTUs at a dissimilarity of 3% cutoff for different salinity samples.

increase of salt concentration, the species richness decreased obviously. It may be related to the selection pressure of high salinity, some bacteria were not suitable for high salt conditions, causing the reduction of community richness. Moreover, the Shannon and Simpson values were used to evaluate the community diversity (Li et al., 2016). Results showed that the 6% sample had the highest diversity, afterward, the diversity decreased sharply. The 6% salt concentration seemed to be a threshold for microbial diversity, which increased at 0–6% salinities, while decreased due to the higher salt concentration at 7–9% salinities.

Venn diagram depicted (Fig. 3) that only 6.05% of total OTUs were the shared OUTs among the six sludge samples, so that the variation of microbial structure happened along with elevated salinities. Some bacteria adapting to high salinity grew well and gradually predominated in the SBR system, while others without salt-tolerance capacity were gradually washed out or weakened with increasing salinity. Besides, the prosperity of some halophilic bacteria changed the diversity and richness of microbial community. In addition, the shared OTUs between adjacent samples 0% and 3%, 3% and 6%, 6% and 8%, 8% and 9% were 23.92%, 31.46%, 31.76% and 38.33%, respectively, indicating samples from closer salt concentration had more similarities in the microbial community composition, and the succession of the microbial community was a gradual process.

Table 2
Bacterial diversity of different salinity samples.

Sample	Level[a]	Sequences	OTUs	ACE[b]	Chao[b]	Shannon[c]	Simpson[d]	Coverage[e]
0	97%	31450	115	121	120	1.15	0.5879	0.999587
3		31450	96	112	119	1	0.6402	0.999332
6		31450	82	86	85	2.16	0.195	0.999746
8		31450	66	76	79	1.47	0.3813	0.999587
9		31450	54	60	60	0.42	0.851	0.999682

[a] Degree of similarity.
[b] Community richness. A higher number represented more richness.
[c] Community diversity. A higher number represented more diversity.
[d] Community diversity. A higher number represented less diversity.
[e] Sampling depth.

3.3. Dynamics and functional groups of the microbial community

The gene taxonomic analysis for six different salinity samples assigned to phylum, class and genus taxa levels were summarized at Fig. 4. As shown in Fig. 4a, the phylum *Proteobacteria* predominated in the whole operating process, which was similar to other microbial community from different environment areas, such as soil (Roesch et al., 2007) and sewage (Zhang et al., 2012). The second most dominant phylum was *Firmicutes* in six sludge samples. Moreover, the relative abundance of these two phyla was over 98%, and the *Proteobacteria* maintained over 80% abundance in all sludge samples. In addition, the relative abundance of *Firmicutes* increased at 0–6% salinity and decreased at 6–9% salinity, but the relative abundance of *Proteobacteria* appeared the opposite trend compared with the abundance of *Firmicutes*. The results showed that *Proteobacteria* had a wider range of salinity (0–9%) compared with the *Firmicutes* (0–6%) according to its high relative abundance of phylum taxa level in different salinity. It was noteworthy that the phyla *Proteobacteria* and *Firmicutes* were always detected in some adverse environmental conditions (Li et al., 2011; Zhang et al., 2016). For example, the phyla *Proteobacteria* and *Firmicutes* were detected and dominant in all antibiotic containing water samples (Li et al., 2011). *Proteobacteria* was also found to dominate in saline wastewater, and presented the increasing trend from initial 33.4% to 79.6% and 85.1% when salt concentrations increased from 0 to 50 g L^{-1} (Zhang et al., 2016).

The taxonomic analysis at the class level was summarized at Fig. 4b. The six main classes were detected in all samples, including *Gammaproteobacteria*, *Bacilli*, *Alphaproteobacteria*, *Clostridia*, *Epsilonproteobacteria* and *Actinobacteria* respectively. Notably, the *Gammaproteobacteria* had the highest abundance in class level and was always dominant at different salt stages. In addition, the *Bacilli* accumulated at 0–6% salinity and its highest abundance was 12.3% at 6% salinity, after that, the abundance of *Bacilli* decreased from 9.1% to 6.2% with the salinity increased from 8% to 9%. It was worth noting that *Epsilonproteobacteria* was 2.27% abundance at 0% salinity, while it was undetected at 3% salinity. The abundance of *Alphaproteobacteria* increased to 5.4% at 6% salinity and decreased sharply at 8–9% salinity. It may indicate that *Bacilli* and *Alphaproteobacteria* could tolerate salinity in some extent, when salinity increased more than the tolerance limit of these classes, the microbe may be weakened or be washed out from the SBR system, which was corresponding with the variation of community diversity (Table 2). And the *Gammaproteobacteria* seemed to have better

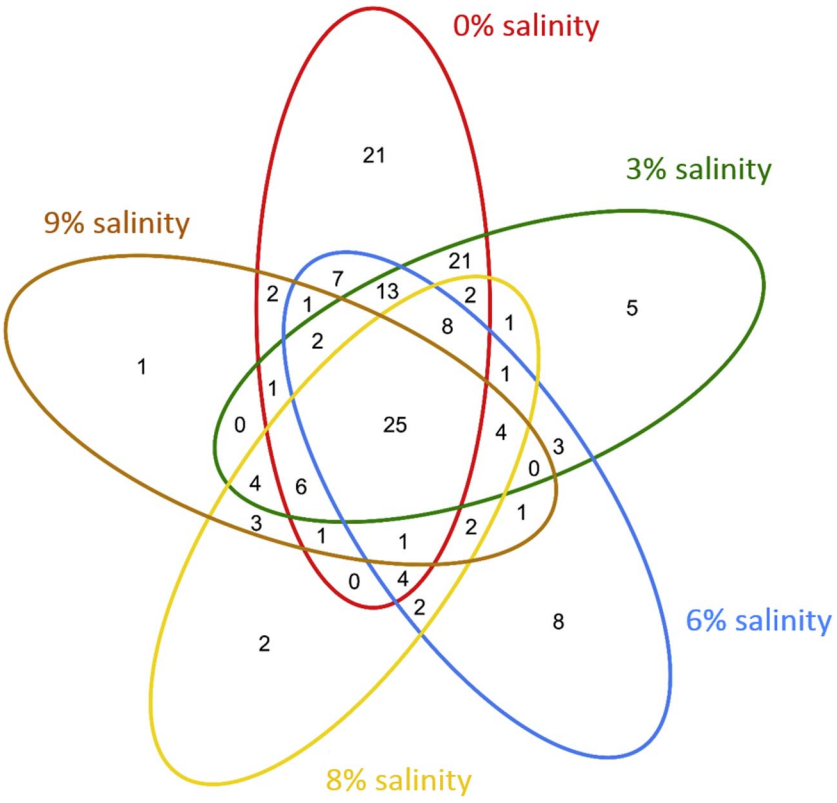

Fig. 3. Venn diagram for different salinity samples: different color represented different salinity of sludge samples.

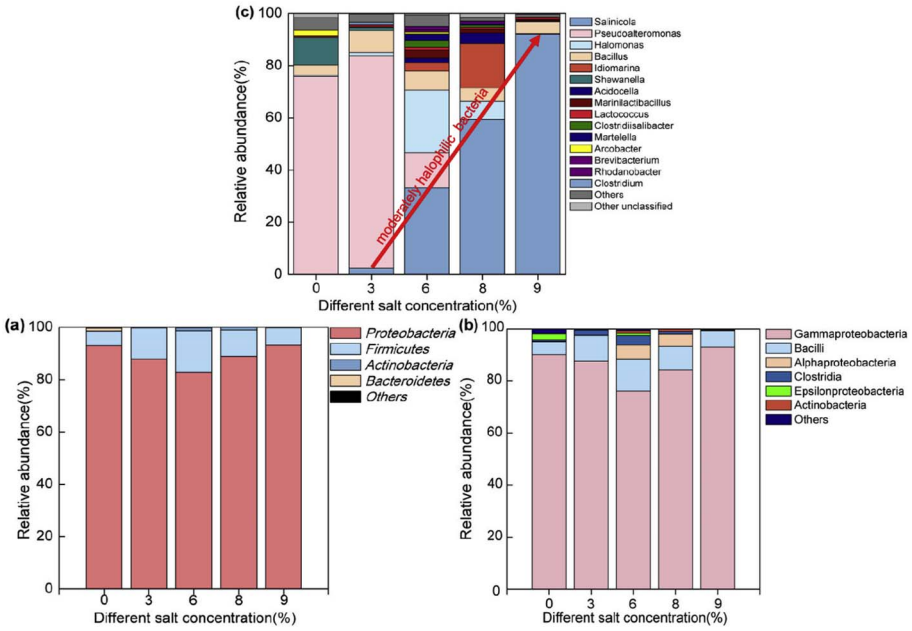

Fig. 4. Analysis of microbial diversity at different taxonomic level for different salinity samples: phylum (a); class (b); genus (c).

ability to tolerate higher salt stress compared with other main classes.

Fig. 4c clarified the taxonomic analysis of six samples at genus level, which could have more detailed information and more profound comprehension on microbial community succession with the elevated salt concentration in the SBR system. The dynamic variation of microbial distribution during the whole operating process could be clearly seem in Fig. 4c. The microbial community structures at elevated salinity were quite different at genus level, moreover, some genera showed an increasing or decreasing tendency with elevated salinity. For example, the *Pseudoalteromonas* has the highest abundance, 75.8%, at 0% salinity and was predominant in the SBR system. While the *Pseudoalteromonas* dropped sharply with the addition of salinity, even the abundance of *Pseudoalteromonas* decreased to 0.003% at 9% salt concentration, which could conclude that the *Pseudoalteromonas* was dramatically affected by higher salinity (8% and 9% salinity). On the contrary, the *Salinicola* was not detected at 0% salinity, while with the increasing salinity, the abundance of *Salinicola* gradually increased to 2.4%, 33.3%, 59.4% and 92% at 3%, 6%, 8% and 9% salinity respectively. And the *Salinicola* became the most dominant genus of SAGS at 9% salinity in the SBR system. This increasing dynamic change indicated that the *Salinicola* was capable to adapt the hypersaline stress and contributed a lot to granular sludge for the tolerance of salinity. In addition, the *Halomonas* appeared the similar increasing trend with the increase of salinity, and dominated in aerobic granular sludge, which was 24% relative abundance at 6% salt concentration. But with the enhanced salinity, the abundance of the *Halomonas* dropped from 6.83% to 0.1% at 8–9% salinity. The *Bacillus* was always existed in all sludge samples during the whole process with the increase of salinity, and the abundance of this genus was 3.88%, 8.38%, 7.34%, 5.2% and 4.7% respectively along with the increasing salinity, indicating that the *Bacillus* could adapt the wide range of salt concentration (0–9%). *Bacillus, Pseudoalteromonas, Halomonas* and other halophilic microorganisms were often isolated from different saline environments and some different strains even belonged to the same genus (Zhuang et al., 2010).

From the microbial functional groups perspective, the existence of some halophilic or halotolerant bacteria made aerobic granular sludge adapt to live and thrive in hypersaline stress. Moreover, saline conditions could stimulate bacteria produce more extracellular polymeric substances (EPS) to protect themselves from adverse environment. With the increase of salinity step by step, an aerobic granular sludge system dominated by moderately halophilic genera was gradually formed with increasing relative abundance of *Salinicola* and *Halomonas*. Besides, the *Salinicola* and *Halomonas* was the member of the family Halomonadaceae, which showed a remarkable versatility with respect to their salt tolerance (Vargas and Nieto, 2004). de la Haba et al. (2010) found that the *Salinicola socius* DSM 19940^T grew optimally in media containing 3–10% NaCl and *Halomonas salaria* DSM 18044^T grew optimally in media containing 10–20% NaCl. The phylogenetic tree (Fig. 5) showed that *Salinicola* and *Halomonas* constituted a coherent cluster, which indicated that these two genera were phylogenetically closely related and had same salt-tolerant function.

Moreover, some researchers studied the effect of salinity on microbial community at the range of 0–5% salt concentration, or detected the microbial community using two or three different salinity level of sludge samples (Ramos et al., 2015; Zhang et al., 2016; Zhao et al., 2016). In this study, the wider range of salinity level (0–9%) and the strategy of stepped increased of salinity provided more detailed microbial succession information of SAGS, and demonstrated the stepped increase of the relative abundance of *Salinicola* with the increase of salinity.

3.4. Formation mechanism of SAGS

The formation of salt-tolerant granular could be explained as following 3 mechanisms:(1) Crystal nucleus hypothesis considered the formation of granular sludge started with some nucleuses, which inoculated aerobic (anaerobic) granules, inorganic material or some inorganic salt into SBR system as a crystal nucleus, aerobic granular sludge will gradually form through microbial growth with the help of a nucleus (Long et al., 2014). The nuclei of granular activated carbon provided the supporting media for sludge to attach and enhance the morphological regularization of sludge (Tao et al., 2017). Verawaty et al. (2012) also found that flocs were observed to attach to the surface of the seeding granules, resulting in reduced biomass washout during granulation. In such experiment, the addition of anaerobic granules provided the sludge for strong support medium in the initial start-up phase. On the basis of the anaerobic nucleus, young granules gradually formed with the growth of microbes. (2) Selection pressure hypothesis could be separated as hydraulic selection pressure and biological selection pressure, which was widely used to optimize the operation parameters of reactor and to explain the formation of aerobic granular sludge. In such experiment, one selection pressure was the settling time,

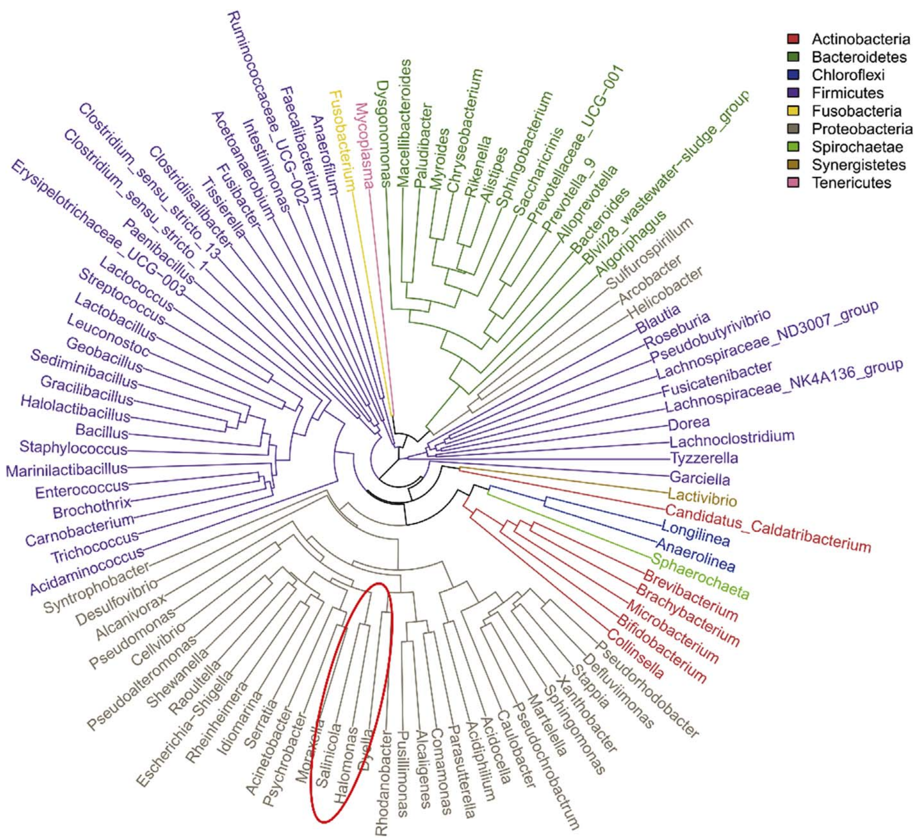

Fig. 5. Phylogenetic tree based on the relative abundance of the top of 100 OTUs in all sludge samples at genus level: different color represented different phylum levels of 100 OTUs.

which was considered as a successful strategy for the rapid start-up of aerobic granular reactors (Szabó et al., 2016). Su et al. (2013) also suggested that automatically adjusted of settling time was an optimum controlling strategy for granulation process. When settling time continuously decreased from 30 min to 3 min, young granules were pushed to adhere more focs and flocs were pushed to get together to obtain enough weight, so that the lighter flocs were washed out and the enough heavy young granules accumulated in the system. It was worth noting that the other selection pressure was salt concentration, which can be seen not only as hydraulic selection pressure but also as biological selection pressure. As a hydraulic selection pressure, with stepped increase of salt concentration from 0% to 9%, synthetic wastewater density correspondingly increased, resulting only bigger flocs or young granules could stay in the SBR system, which could be seen as the enhancement of the function of decreased settling time. Moussa et al. (2006) also found that the settling time were chosen to allow only granules to retain, and density played a similar role in systems under salt stress. As a biological selection pressure, elevated salinity pushed the SBR system to alter the microbial community to adapt hypersaline stress. Under high salt concentration ($>$ 1 wt% salinity), the increase of salinity resulted in an increase of the halotolerant bacteria, and the microbial community could express different tolerance levels to such a biological selection pressure of salinity (Lim et al., 2007). The result of salinity selection pressure was that some adapted bacteria was capable of salt tolerance, while other unadaptable bacteria was granularly washed out or weakened with increasing salinity. (3) As described by the DLVO theory, the double layer becomes compressed at high ionic strength, reducing the surface potential, but keeping the surface charge density constant (Esparza-Soto and Westerhoff, 2003). The increase of salt concentration from 0% to 9% resulted in compressing the double layers, reducing the total repulsive forces between different zoogleas, and improved the mature of young granules. In addition, the combination of electrostatic and hydrophobic interactions with the flocs increased the flock size (Moussa et al., 2006). Li et al. (2017) found that the addition of seawater significantly accelerated the granulation process compared with freshwater and a salinity-induced decrease in electrostatic charge on the surface of cells allowed sludge flocculation. Thereby, it was beneficial for the aggregation of flocs and the maturation of salt-tolerant granular sludge.

In summary, the strong support material of anaerobic granules stated the formation process of young granules. With the double selection pressure of settling time and salinity, young granules gradually accumulated in SBR system, and the halophilic microorganisms enriched as the corresponding to the high saline stress. Meanwhile, the aerobic granular sludge gradually transited from the young stage to mature stage with the combination of electrostatic and hydrophobic interactions. Finally, the salt-tolerant granular sludge was successfully cultivated in response to the hypersaline stress.

4. Conclusion

The salt-tolerance aerobic granular sludge dominated by moderately halophilic genera was successfully cultivated in SBR system, and it could tolerate 9% saline concentration. Crystal nucleus hypothesis, selection pressure hypothesis and compressed double electric layers hypothesis were used to explain the formation of salt-tolerant granular sludge. Notably, salinity contributed a lot to the formation and the microbial community structure of SAGS, while it declined the performance of SAGS system. Sequence analysis showed halophilic bacteria obviously increased corresponding to the increased salinity. In detail, *Salinicola and Halomonas* were gradually dominant and considered as the functional groups for the tolerance of hypersaline stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.07.154.

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