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Special Article

# Granulation Process and Mechanism of Aerobic Granular Sludge under Salt Stress in a Sequencing Batch Reactor

# Proceso de granulación y mecanismo de lodo granular aeróbico bajo estrés salino en un reactor de secuenciación por lotes

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**Abstract**

The formation and characteristics of aerobic granular sludge (AGS) under different operational conditions in a sequencing batch reactor (SBR), designed to treat Mustard tuber wastewater (MTW, characterized as saline wastewater), had been investigated in this study. Morphology and structure during granulation were determined using a microscope with a digital camera and scanning electron microscope (SEM). Granules formed in the reactors could be classified as zoogloea granules with a clear boundary outline and filamentous granules with mycelia bestrewing boundary. Zoogloea granules, cultivated in reactor R1 and R2, was with higher density than filamentous granules, cultivated in reactor R3, and consequently had a higher settling velocity. Results showed that divalent metal ions such as $Ca^{2+}$ and $Mg^{2+}$ with phosphate in inflow could transform into precipitates, serving as crystal nucleus and carriers for granulation. Moreover, appropriate organic loading, hydrodynamic shear and salt-stress selection can induce moderate growth of filamentous bacteria to act as granulation backbone and consequently granulation process under salt stress was a result together with crystal nucleus, filamentous bacteria, and extracellular polymeric substances (EPS), which could be affected by salinity-shifting strategies and dosage of aluminum salt coagulant.

**Keywords**: aerobic granular sludge (AGS), salt stress, sequencing batch reactor (SBR), filamentous, extracellular polymeric substances (EPS).

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# Introduction

The type of pollutants in wastewater can determine the selection of wastewater treatment technology. Wastewaters from industrial sources may contain both organic matter and inorganic pollutants. Saline wastewater, rich in salt (mainly known as NaCl) and nutrients, are often discharged from food-processing, leather and oil industry *(Lefebvre* and *Moletta 2006)*. Biological treatment of high salinity wastewater, if feasible, would be relatively simple in processing flow, cost-effective in running and without farther pollution to the environment compared to physicochemical process. However, treating this wastewater can be more challenging, owing to plasmolysation of cells, inhibition of bioactivity, such as salt stress to microorganisms, and inhibition of some enzyme activity *(Rene et al. 2008)*. In spite of this, salt-tolerant activated sludge acclimation is accessible *(Aloui et al. 2009, Lefebvre and Moletta 2006)*. It has been reported that microbes attached growth have a higher tolerance capability than dispersed at high salt concentrations, and alternative bio-treatment systems for removing nutrients from saline effluents are increasingly the focus of research *(Aloui et al. 2009)*.

The aerobic granular sludge process, a promising prospect in the biological treatment, could cut the investment and operational costs as well as space requirements *(Liu et al. 2010, Zhu et al. 2013)*. Comparing with suspended activated sludge process, more outstanding advantages were confirmed, such as microbial community structure, favorable settling characteristics, high concentrations of biomass *(Chen and Lee 2015, Morales et al. 2012, Zhu et al. 2013)*, and tolerable to incoming shocks and medium toxic environment *(Adav et al. 2010, Zhu et al. 2013)*.

Besides, studies into applications of granular sludge technology in treating industrial wastewater have been advocated by some previous researchers *(Adav et al. 2008a, Rosman et al. 2014)*. These distinct characteristics approve that aerobic granular technology may become a promising alternative method for activated sludge process and has good applied prospects in treating saline and nutrients-rich wastewaters. Aerobic granules under high salt stress in SBR system exhibited a good stability and pollutants removal performance in treating saline wastewater *(Li et al. 2010, Moussavi et al. 2010, Taheria et al. 2012, Wan et al. 2014)*, and it is even observed that aerobic granules are more slippery and regular in appearance under high salinity *(Li and Wang 2008)*.

Mustard tuber wastewater (MTW) is a typical food-processing effluent, characterized by high-strength dissolved organic matters and high salinity *(Chai and Kang 2012)*. Many published studies treating saline effluent, mainly based on laboratory scale reactors, and focused on biofilm attached growth on surfaces of support materials and microbial fuel cells *(Chai and Kang 2012, Guo et al. 2013, Guo et al. 2015)*. Aerated granules sludge (AGS) could be regarded as a specific form of biofilm which is commonly developed by aggregation of a variety of microorganisms based on the microbiological point of view *(Ren et al. 2010)*. However, very few studies about the application of the AGS system for the treatment of MTW was reported.

The high concentration of divalent metal ions in MTW, such as $Ca^{2+}$ and $Mg^{2+}$ could enhance the granulation *(Jiang et al. 2003, Li et al. 2009, Yu et al. 2001)* and in the self-aggregation of microflora, since extracellular polymeric substances (EPS) are prone to link multi-valent metals *(Rudd et al. 1984)*. Furthermore, MTW has a high proportion of dissolved biodegradable substrate, which could promote the growth of filamentous organisms *(Liu* and *Liu 2006)*. In fact, filamentous organisms tend to exist in a variety of aerobic granular sludge, but at various levels *(Lee et al. 2010)*. It has been widely accepted that low-levels and moderate-levels growth of filamentous bacteria do not cause sludge bulking, on the contrary it could instead help entangle each other with mycelium under appropriate operational conditions, and thus build up the backbone of granules, which can stabilize the granule structure by binding material *(Lee et al. 2010, Liu* and *Liu 2006, Li et al. 2010)*. Therefore, maintaining moderate-filamentous growth might be supporting granulation but is unlikely to be the only key strategy to cultivate and maintain stable aerobic granular sludge.

Hence, aerobic granules appear to be an ideal process for MTW treatment. In this study, the granulation process of aerobic sludge in a sequencing batch reactor (SBR) operating with synthetic saline wastewater (salinity of 3%, calculated by NaCl) was studied. The research focused on the influence of salt-stress on the formation of AGS, the role of breeding filamentous involved in aerobic granulation by improving organic loading, and the strategy to induce filamentous microorganisms in moderate-levels growth. In addition, the variation of morphological structure in/on AGS and mechanism involved in their granulation under salt stress were investigated. A good understanding of the formation of AGS and its characterization would be helpful for developing rapid granulation strategy of activated sludge under high salt stress and thus promoting the application of aerobic granulation technology. Moreover, this study could contribute to the development of AGS-based systems for engineering application in treating MTW characterized by high-strength and high-salinity.

# Materials and Methods

## SBR System

Three duplicate column-type sequencing batch reactors (SBRs), labeled by R1, R2, and R3, respectively, were applied to granulate aerobic sludge in this study, where the effective volume, internal diameter and height of each reactor were 1.9 L, 0.048 m and 1.05 m, respectively. Bottom aeration of the reactor column was supplied by an air pump and microporous diffuser. The reactors were supplied with different airflow rate to meet the dissolved oxygen (DO) concentration and hydraulic shear force in a different experimental period during the aeration phase, and a gas flowmeter was used to control the air flow. The three columns were operated at the controlled temperature, and the water temperature was (30±1)°C. The reactor was operating for 12 or 24 h per cycle at a water drainage ratio of 50%, including 2 min influent (via the reactor top), 3 min discharge, aeration, and sedimentation for the remaining time, in which, the time of aeration and sedimentation was adjustable according to operating condition. The experiment included two stages, namely the stage without salt (Period I) and the stage of salinity lifting (Period II). Detailed information about reactor operation was shown in Table 1.

**Table 1.** Detailed experimental conditions of the reactor system.

| Index | Period I | Period II | | | | | |
|---|---|---|---|---|---|---|---|
| Duration (d) | 0-21 | 21-28 | 28-35 | 35-45 | 45-56 | 56-63 | 63-73 |
| Running time per cycle (h) | 12 | 12 | 12 | 12 | 12 | 12 | 24 |
| Setting time (min) | 5~10 | 5 | 5 | 5 | 5 | 5 | 5 |
| Organic loading (kg COD $m^{-3}$ $d^{-1}$) | 1.5 | 2.0 | 2.5 | 3.5 | 4.5 | 3.6 | 2.25 |
| Airflow rate (L $min^{-1}$) | 3 | 4 | 5 | 7 | 8 | 9 | 9 |

Simulated saline wastewater was employed as the influent of reactors. Its compositions were as follow: 0-30 g of sodium chloride (0-3% as salinity), 1415-4245 mg of glucose (1500-4500 mg $L^{-1}$ as chemical oxygen demand (COD) basis), 92.0-122.7 mg of $KH_2PO_4$ (21-28 mg $L^{-1}$ as $PO_4^{3-}$-P basis), 381.6-858.8 mg of $NH_4Cl$ (100-225 mg $L^{-1}$ as $NH_4^+$-N basis), 225.7-1333.3 mg of $MgSO_4 \bullet 7H_2O$ (22-130 mg $L^{-1}$ as $Mg^{2+}$ basis), 49.9-277 mg of $CaCl_2$ (18-100 mg $L^{-1}$ as $Ca^{2+}$ basis) and 0.1 mL of trace elements solution (*Kishida et al. 2006*). The pH of influent before dosing was controlled below 6.0 to avoid the precipitation among multi-valent metals, phosphate, and ammonium. The pH of the reactor throughout each initial stage of cycles was kept constant at 8.0 ± 0.5 by dosing a suitable amount of sodium bicarbonate ($NaHCO_3$). Meanwhile, poly aluminum chloride (PAC), as an aluminum salt coagulant, with a dosage of 20 mg $L^{-1}$ as Al basis was added into the influent of reactor R1 and R2 during day 21 to 38 to investigate whether aluminum ions ($Al^{3+}$) can accelerate the granulation of aerobic sludge.

## Inoculation of Aerobic Granules

Activated sludge with dark brownness and flocculent from sludge returning tank of Chongqing Wastewater Treatment Plant (with the $A^2$/O process), Chongqing, China, was used as inoculum sludge. Before inoculation, activated sludge was cultivated in aerobic condition without any substance feeding for several days to inhibit the activity of hydrophilic bacteria, which would be difficult to connect with sludge flocs in contrast with the hydrophobic counterpart *(Lee et al. 2010)*. Besides, hydrophobic bacteria were abundant in the inoculum sludge, which could accelerate aerobic granulation and then exhibit excellent settling property *(Wilen et al. 2004)*. After activated sludge pre-treated and experienced the endogenous respiration, the initial concentration of the mixed liquor suspended solids (MLSS) in the reactors was approximately 5000 mg $L^{-1}$, and the ration of mixed liquor volatile suspended solids (MLVSS) to MLSS for the seed sludge was 69.8 ± 0.2%. The pre-treated sludge had good settling property and poor bioactivity, sludge volume index (SVI) was 20 mL $g^{-1}$ and dehydrogenase activity (DHA) was only 1.59 ug TF $g^{-1}$ SS $h^{-1}$ because of microbes in endogenous respiration without sufficient substrates.

## Strategies to Increase Salinity

In this study, salt content in reactors was adjusted by feeding different salty influent to investigate the influence of the increasing salt stress on granulation and characteristic of sludge. The concentration of sodium chloride in each reactor was augmented stepwise by the added substrate as shown in Figure 1.

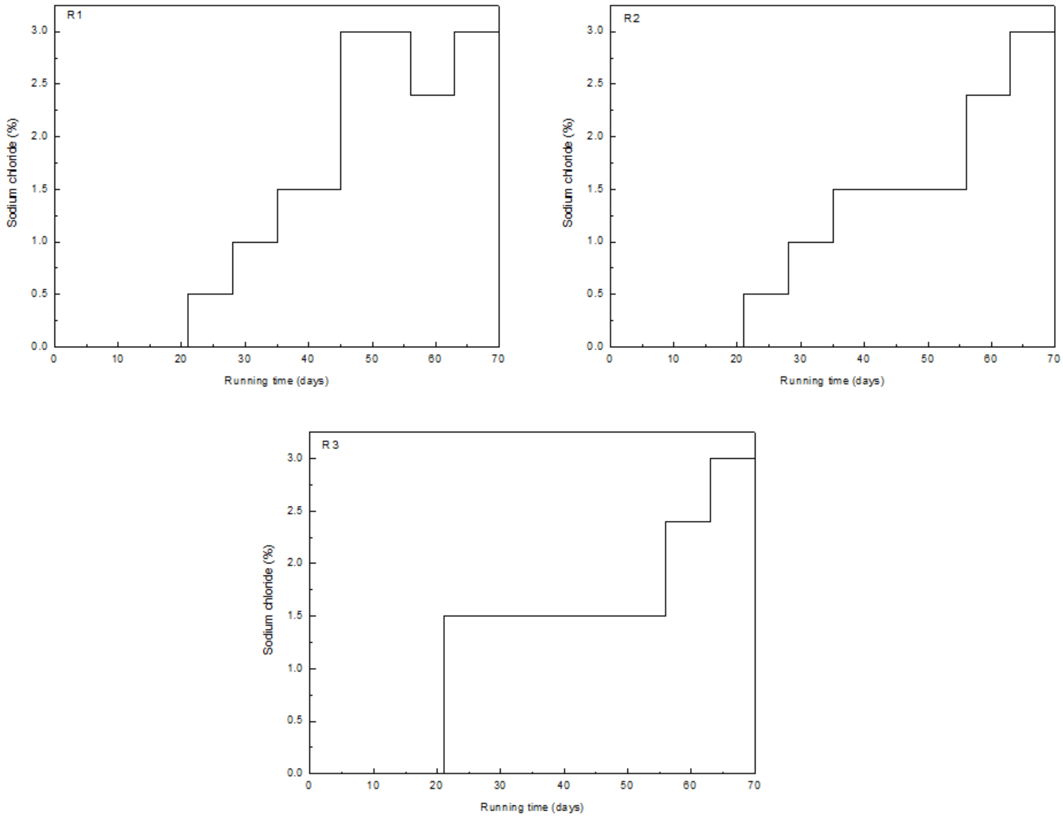


**Figure 1.** Augment of sodium chloride in the substrate of each reactor.

## Analytical Methods

**Morphologic Observation**. Shape images of granular were attained using microscope BA210 (Nikon, Japan) and digital camera. Images of particles were randomly selected with a total particle number more than 500 and then measured in length and width for the size distribution analysis. The granules sub-sampled at -80°C were taken out from the refrigerator to thaw in room temperature for the subsequent analysis with a scanning electron microscope (SEM), and fixed in 2.5% glutaraldehyde for 4 h at 4°C, and then washed using phosphate buffer solution (PBS). Furthermore, the washed samples were stored in 1% osmic acid overnight at 4°C, and rewashed with PBS and then frozen using liquid nitrogen. Before SEM image taking (S3400N, Hitachi, Japan), the stored samples were dehydrated by placing in 30%, 50%, 70%, and 100% ethanol stepwise and then coated with gold.

**Particle size distribution (PSD)**. The diameter of each particle was calculated by Sauter's Formula with length and width, as follows.

$$D = \frac{\sum (ab^2)^{\frac{1}{3}}}{n}$$

where, *D* means a diameter of each particle, calculated in mm; *n* means the number of particles; *a* and *b* mean the length and width of each particle respectively, calculated in mm. The results were then classified according to their particle size.

**EPS.** EPS was extracted from the granules by using cation exchange resin (CER) technique according to *Forlund et al. (1996)*. Granular samples were collected by centrifugation at 2000 rpm for 15 min, and then the compressed settling was washed twice with 0.1 M NaCl solution. Whereafter, the sludge settling was re-suspended to a prescribed volume and the solution was transferred to an extraction beaker, immediately adding the CER (strongly acidic styrene type-001×7, $Na^+$ form, pretreated with 0.1 M NaCl and 0.1 M NaOH for a pH of 7.0) with a dosage of 60 g $g^{-1}$ SS. These CER/sludge suspensions were then mixed for 12 h at 500 rpm, and subsequently, the suspensions were standing for 3 min to separate CER and sludge suspensions. In the end, the EPS were harvested by centrifugation at 12000 rpm and 4°C for 30 min to eliminate residual sludge components. After centrifugation, the supernatants were filtrated through 0.22-mm acetate cellulose membranes and finally, the filtrates were collected for chemical analysis of the EPS fraction.

Extracellular proteins (PN) in the extracted EPS were adopted a modified Lowry method using folin-ciocalteau phenol reagent with bovine serum albumin as standard *(Forlund et al. 1996)*. Extracellular polysaccharides (PS) were determined by using the anthrone-sulfuric acid method with glucose as standard *(Laurentin* and *Edwards 2003)*.

**Other Analyses.** Sample analysis included COD, ammonium-N ($NH_4^+$-N), MLVSS and MLSS, all according to Standard Methods for the Examination of Water and Wastewater *(APHA 2005)*. MLSS content was measured by oven drying of the sample at 105°C for 1 h, whereas MLVSS was measured by ashing the dry sample at 550°C in a muffle for 15 min. DHA of AGS was determined according to the iodonitrotetrazolium chloride method *(Sebiomo et al. 2011)*. The oxygen content of mixed liquor was determined with a DO meter (HQ40d, HACH, USA). pH was measured by a pH meter (SENSION2, HACH, USA).

The physical characteristics of sludge (including SVI and granular strength) during granulation of activate sludge in the SBR columns


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were analyzed. Settling performance of granules was evaluated in the aspect of SVI. The SVI was implemented according to the procedure described by *de Kreuk et al. (2005)*. The integrity coefficient (IC), which is defined as the ratio of residual particles to the total weight of granules after 5 min of shaking at 200 rpm on an orbital platform shaker *(Rosman et al. 2014)*, can be used to indirectly express as granular strength, and it also has an influence on granular compactness and bioactivity in the reactor.

# Results and Discussion

## Aerobic Granular Sludge Formation under Salt Stress and MorphologyCharacteristic

The morphology-evolution images of AGS, shown in Figure 2, during different formation stages were obtained by microscope and digital camera. Under microscopic examination, the morphology of initial seed sludge was fluffy, irregular and loose-structure. The sludge color gradually changed from dark brown to yellowish brown at the end of the experimental period.

In the initial stage of granulation (Period I), the loose flocs have easily broken into small pieces. After SBR reactors started, the sludge settling performance gradually became worse due to the high start-up loading and its poor activity, in where the DHA of inoculated sludge was only 1.59 ug TF $g^{-1}$ SS $h^{-1}$. In the initial of operation, MLSS in the three reactors was 4952 mg $L^{-1}$ and the SVI of inoculated sludge was 20 mL $g^{-1}$. Variations of MLSS and SVI are shown in Figure 3. Initially, the biomass was loose and bulked easily, and then flocs-like sludge gradually disappeared. MLSS decreased sharply with flocs washing out, and the SVI increased to around 200 mL $g^{-1}$ in the first-week operation. During the next two weeks, the settling property of particles was improved gradually, and when the settling time was shortened to 5 min, the color of sludge appearance became from dark brown to yellowish brown. This is because that the washout of flocculated sludge can be commonly facilitated when settling time is shortened and small aggregates from the reactor and retained only well-settled granules *(Adav et al. 2008a, Long et al. 2014)*, which is often referred to hydrodynamic selection pressure for granulation. Meanwhile, we occasionally observed that a few small particles appeared in all reactors and then gradually disappeared, and thus sampled some sludge to evaluate the granular strength. Results showed that IC value of samples was under than 10%, which indicated that the small particles were not strong enough to resist the mechanical collision and fluid shear stress resulting in the loss and disintegration of particles. The main components in EPS of all granules showed a similar trend, in where the extracellular proteins significantly increased, whereas the extracellular polysaccharides decreased, and thus the PN/PS ratio increased (Figure 4), which indicated the importance of the carbohydrates for promoting the cohesion and adhesion of cells during initial granules formed. Meanwhile, proteins existed in negative charge as pH of the reacting system controlled around 8.0, which can cross-bridge divalent metal ions (such as $Ca^{2+}$ and $Mg^{2+}$) and EPS to promote microbes in cell-cell aggregation, causing the loose granules generally compacted and then accumulated. Furthermore, metal ions transferred to minerals with phosphate acting as a nucleus, which results in the accumulation of bacteria and the formation of biological mass. After 21-days continuous operation, the appearance of granular nuclei in each reactor have indicated the initial formation of AGS was achieved, meanwhile, the settling properties and biological activity of sludge were gradually improved, thereafter the granules considerably developed. After the end of Period-I experiments, The SVI of reactor R1, R2 and R3 decreased to 30.7, 30.2 and 27.8 mL $g^{-1}$ respectively, and DHA were 50.91, 39.76 and 47.05 ug TF $g^{-1}$ SS $h^{-1}$ correspondingly. As aforementioned, the reactors were mainly dominated by flocculent sludge with good settling properties and some granules, but AGS were irregular in appearance (Figure 2b) and low granular strength (the IC values of granules in three reactors were only between 30% to 40%).

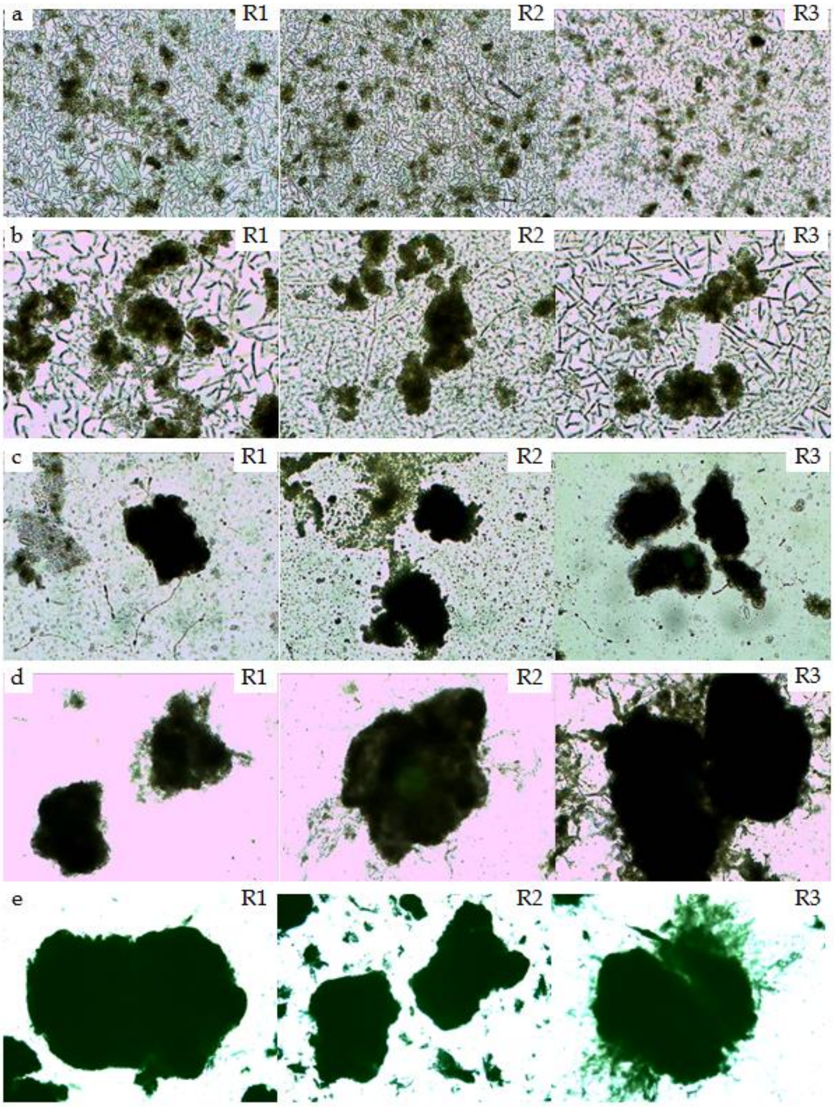


**Figure 2.** Photographs of the sludge after the following days in each SBRs: (a) 2 days (×100), (b) 19 days (×100), (c) 30 days (×100), (d) 47 days (×100), (e) 67 days (×40).

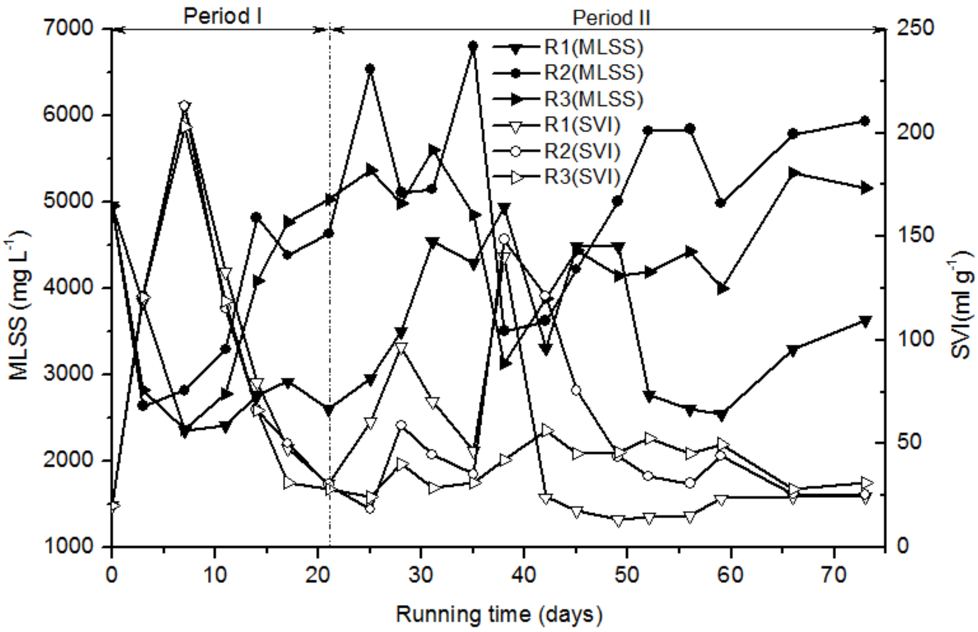


**Figure 3.** Variations of MLSS and SVI of the sludge in R1, R2, and R3.

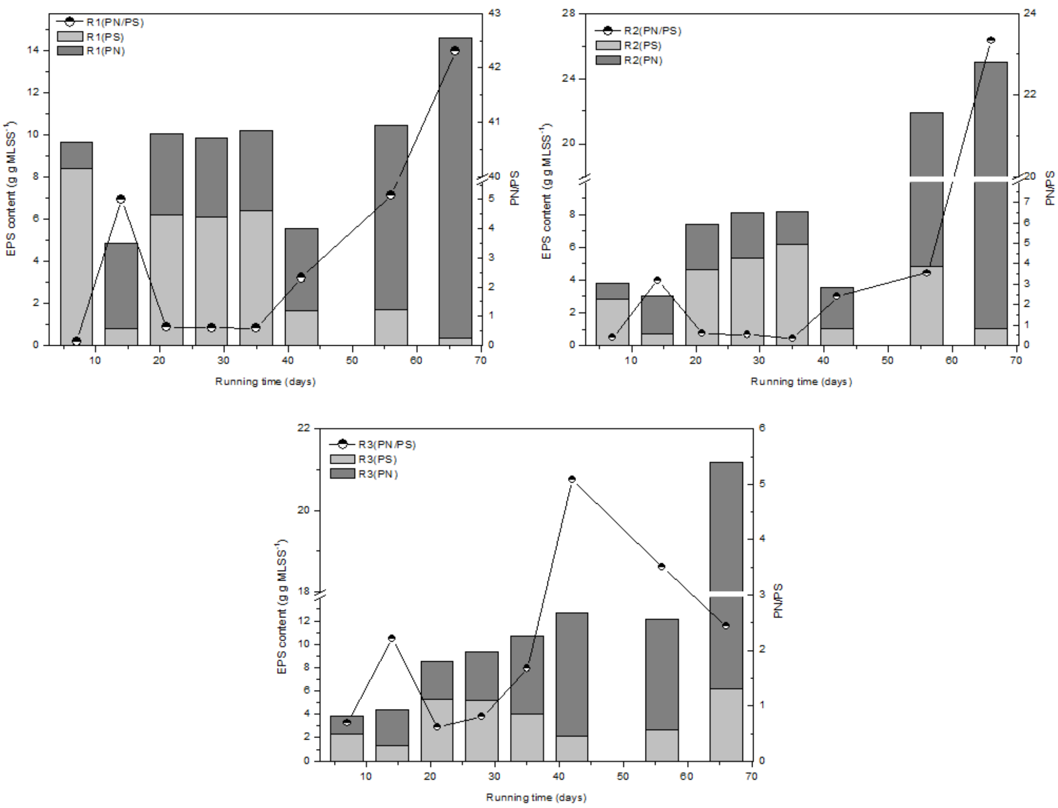


**Figure 4.** The variation of EPS content and ratio of PN to PS in granules.

In the subsequent Period II, the settling properties became worse gradually because of the adverse impact of a sudden increase of salinity on microbial species. Simultaneously, in a high and quick shifting sodium chloride content distributed system, granules were subjected to larger buoyancy, and the selection pressure was strengthened. Afterward, flocs-like sludge and granules with poor settling properties were continuously discharged from reactors, which result in the rise of SVI in reactor R1 and R2 to 96.9 and 58.7 mL $g^{-1}$, respectively (Figure 3). However, SVI in reactor R3 remained a relatively stable scale and biomass increased gradually, based on which can safely conclude that under initial high (1.5% of NaCl) and stable salinity, microbes had enough time to adapt and grow. It was reviewed that hydrodynamic shear would densify the granular sludge but have no impact on EPS content and compositions *(Di Iaconi et al. 2006)*, and the flocs would become compacter under a high shear force which induces the biomass aggregates to secrete more exopolysaccharides *(Dulekgurgen et al. 2008)*. Therefore, from 21st day, the aerating flux of reactors with the airflow rate of 3 L $min^{-1}$ was raised to improve the hydrodynamic shear force. Under a higher airflow rate, exopolysaccharides contents rapidly increased in all granules, which means more polysaccharide contents were secreted by bio-aggregates. And PS contents sharply increased to 6.2, 4.6 and 5.3 g $g^{-1}$ MLSS, respectively, while the PN contents remained small changes at the end of the 2nd cycle in the 21st day (Figure 4). Meanwhile, with a dosage of 20 mg $L^{-1}$ PAC as Al basis added in the influent of reactor R1 and R2, the cross-bridging of $Al^{3+}$ promoted bacterial self-immobilization to microbial aggregates, and then accelerated the granulation of aerobic sludge. Consequently, the settling properties of granules in reactor R1 and R2 were really not affected by the shocking of salinity in the following period, and the SVI values were under 30 mL $g^{-1}$ (Figure 3). Also, as shown in Figure 4, the addition of PAC had no direct effect on the extracellular proteins but promoted the production of extracellular polysaccharides. It was proved that extracellular polysaccharides could improve both cohesion and adhesion of cells and play an important role in maintaining the structural integrity of biofilms and granular sludge *(Liu et al. 2004, Tay, Liu and Liu 2001)*. The formation and stability of AGS would be enforced by polysaccharides since it could constitute a strong and sticky configuration during activated sludge cultivation *(Liu et al. 2004, Ren et al. 2008)*, and IC of granules in reactor R2 and R3 can achieve around 75%, higher than reactor R1 with 58%. Therefore, PAC addition played an active effect in the aerobic granulation process.

It should be noted that for NaCl <5 g $L^{-1}$, most of the microbial community were environmentally resistant *(Salvado et al. 2001)*. Along with the adaptation of microorganisms on salinity and the continuous appearance of halophilic microbes, the increase of EPS contents under


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higher salinity in the reactor played an important role in the granulation. And together with the impact of compressing the double electrode layer generated between the positively charged cationic ions, such as $Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$, and the negatively charged bacterial surface and EPS, acting as a bridge between bacteria, sludge flocs gradually became more dense and compact through bacterial self-immobilization and inter-granular binding, and then constantly grew up with filamentous twining.

By this time, it was considered that the evolution of inoculum sludge from flocculent to granular sludge was accomplished as a result of the interactions between inter-particle bridging process among EPS, bacterial cells and ion *(Sheng et al. 2010)*. Subsequently, the shape of small particles became more regular and its size gradually increased during the following weeks, whereas more flocs washed out from the reactor, resulting in the cumulation of the AGS with high settling rate. Some granules were sampled from each reactor and the morphology of which was observed on day 30. It was found that all particle size in reactors had increased, in which, the least granules in reactor R1, the most granules with uniform PSD in reactor R2, whereas irregularly shaped granules with maximum different PSD in reactor R3, and granular strength reached up to 80%. There was still a few flocs in each reactor, surrounding which a semitransparent floccule was EPS (Figure 2 c). The structure of EPS was yet loose, whereas the floccule was in a tendency of aggregating to the center of the flocs. Filamentous bulking broke out in reactor R1 and R2 on day 38, and the gradual disintegration of granules followed. The SVI of reactor R1 and R2 achieved up to 140 mL $g^{-1}$. In that case, it was reasonable to conclude that a dosage of $Al^{3+}$ can clog the pore interior granules, which result in aggravating substrate transfer resistance and inhibiting heterotrophic bacteria activity in granules, and metabolic blocking of exopolysaccharide synthesis (showed as a decrease of PS contents in Figure 4) owing to the clogging of granular porous prevented microbial aggregation *(Yang et al. 2004)*. Furthermore, filamentous bulking or viscosity bulking occurred easily under the condition of high organic loading, and under such condition, the organisms residing in the granules would consume the matrix EPS excessively and slash the density of the granules, perhaps even leading to the cells' autolysis *(Zhang* and *Zhang 2013)*. Consequently, this would result in a loosened structure and bad settling ability. *Weissbodr et al. (2012)* asserted that washout was a selective process of microbial while zoogloea enriched in their dense particles and filamentous dominated in granules with a loose structure. Therefore, some measures, such as stopping the dosage of $Al^{3+}$, increasing hydrodynamic shear stress, and reducing organic loading gradually from day 56, were adopted to control the filamentous bulking, and then the bulking phenomenon of reactor R2



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had been relieved. Under given conditions, high hydrodynamic stress could reduce substrate transfer resistance into the granules *(Lee et al. 2010)*, and enhance the activity of the inner microorganisms and avoid the cavities caused by cell autolysis. Meanwhile, maintaining high hydrodynamic shear stress can crush filamentous granules and then discharged from reactors. Additionally, it can induce microbes to secrete more EPS, which can work as a matrix for the granulation process at moderate levels, accelerating the granulation process of sludge, making the broken granules recover gradually, and finally making the granules evolve from fluffy inoculum to compact granules. With different salt stress selective pressure, mature and stable granules were achieved under salinity of 3% after 70-day cultivation. The yellowish-brown granules had good settling properties, with SVI of 24.8, 25.3, and 31.0 mL $g^{-1}$, respectively. In addition, the granules had a relatively higher value of biomass density and IC value was 95%, 98%, and 90%, respectively. It was observed that the granules in reactor R1 and R2 had a clear boundary outline in a round and a dense structure, with the maximum particle size of 3 and 2 mm respectively. While granules in reactor R3 manifested as filamentous granules with loose structure and filamentous mycelia bestrewing boundary due to the fast growth of particles and influence of mass transfer, and the maximum particle size of which can reach up to 6 mm, as shown in Figure 5.


**Figure 5.** Morphology of mature granules, R1 (a), R2 (b), R3 (c).

Comparing the bioactivity of granules in these reactors, the particle size of particles in reactor R1 was smaller than R2, but DHA in R2 was up to 48.91 ug TF $g^{-1}$ SS $h^{-1}$ was nearly double that figure in reactor R1 with 26.42 ug TF $g^{-1}$ SS $h^{-1}$ due to the more developed pore structure and the higher bioactivity. Moreover, the loosened granules in reactor R3 was covered with mycelia, and the bioactivity of which was the worst of the three reactors, and DHA was only 19.02 ug TF $g^{-1}$ SS $h^{-1}$.

Although it is not the objective of this paper to discuss the treatment efficiency of granules, simple descriptive statistics of the concentrations of organic matter and ammonia nitrogen during the overall mature granules period were conducted in order to allow a general view of the system's performance. Mean concentration of the influent COD and $NH_4^+$-N for the reactors was kept at 4500 and 225 mg $L^{-1}$ during mature granules formation, and AGS showed high removal performance. The COD removal efficiencies of reactor R1, R2 and R3 can reach up to 92.5%, 95.1%, and 90.2%, and the corresponding removal rates of $NH_4^+$-N achieved to 59.8%, 67.1%, and 65.6%.

## PSD and Average Particle Size

There was a vast difference in PSD of AGS cultivated under different operational conditions (Figure 6). The particle size of granules in reactor R2 was small, in which granules with the particle size less than 1 mm and the ranged between 1 to 2 mm occupied 58.4% and 39.7% respectively, and the average particle size was only 0.9 mm. In contrast, the particle size of granules in reactor R1 and R2, with an average diameter of 1.4 mm and 1.7 mm, was relatively larger, wherein with the diameter approximately 1-2 mm predominated, and occupied 70.5% and 64.4% respectively, and ranged between 2 to 3mm was 15.1% and 24.9% respectively. The PSD of granules in each reactor was fitted with normal distribution function, and it was found that the PSD of all reactors accorded with the normal distribution law, and fitting coefficient values ($R^2$) were all more than 0.9, especially which of reactor R1 was up to 0.96. At the same time, it was demonstrated that the average particle size of mature granules was positively correlated with dispersion. It can safely draw a conclusion that filamentous bacteria dominating in reactor R3 twined around each other to form fluffy filamentous granules surrounding with mycelium. And it was unlikely to maintain a stable structure and tend to expansion and even hydrolysis of aerobic granule core due to the poor stability. Thereafter, granules developed in dispersive growth, resulting in the maximum dispersion of 0.705 in reactor R3. By contrast, bacterial granules in reactor R2 predominated with zoogloea can aggregate with EPS, and manifested in good stability, resulting in a minimum dispersion of 0.377.



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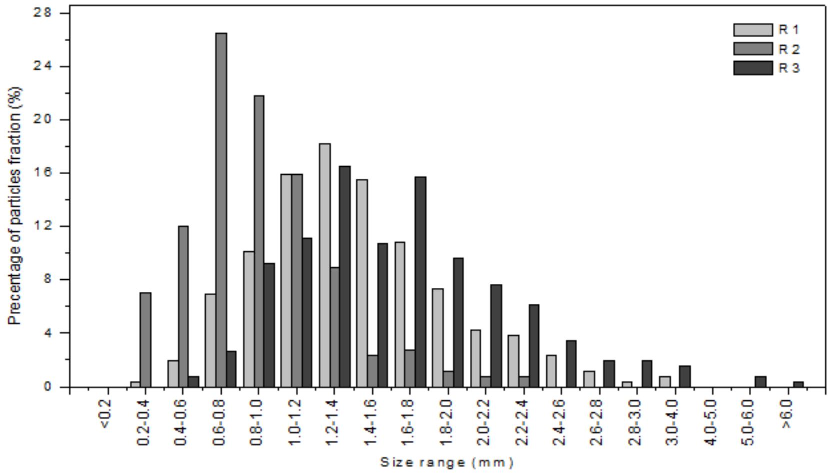


**Figure 6.** Size distributions of aerobic granules of each reactor.

## Mechanism of Formation of Aerobic Granules under Salt Stress

Although there are many hypotheses for the formation of AGS, granulation mechanism of aerobic granules is still unclear. It is now widely accepted that the complex formation of AGS is the result of various mechanisms involved in cell-to-cell immobilization *(Qin et al. 2004)*, extracellular polymer *(Liu and Tay 2004)*, filamentous bacteria *(Beun and Hendriks 1999)*, cell surface hydrophobicity *(Liu and Tay 2004, Qin et al. 2004)* and nucleation hypothesis *(Liu and Tay 2004)*. However, there is no unified, reliable aerobic granules cultivation mode, because abundant factors can affect aerobic sludge granulation, which makes it difficult to control and predict the set-up process for AGS.

According to many researches, the formation of AGS, in fact, can be regarded as an aggregation of various microorganisms with cell-cell interaction from the microbial perspective *(Ren et al. 2010)*, a phenomenon denoted as quorum sensing (QS). QS is a means of intercellular communication, and it can affect gene expression and physiological behavior of an entire microflora to adjust the changing environment *(Shrout and Nerenberg 2012)*, and the involvement of QS in aerobic granulation has been well characterized and confirmed *(Li et al. 2014, Xiong and Liu 2012, Zhang et al. 2011)*. As aforementioned, it is reasonable to conclude that that variation of cultural conditions can directly affect the microbial attachment and composition of microbial community with different signaling molecules secreted by bacteria, and then result in aerobic granulation. The external morphological characters and internal structures of the aerobic granules were further inspected using SEM. It showed that the mature granules in three reactors with the SEM had different morphology under cultural conditions of different strategies to augment salt stress and adding $Al^{3+}$ or not. A carefully inspect revealed that intra-granules showed different microbial structure, as shown in Figure 7.

Granules in reactor R1 consisted of zoogloea, filamentous and bacillus bacteria adhering to the surface of filamentous bacteria. In contrast, granules in reactor R2 were mainly dominated by zoogloea, while filamentous predominated in granules of reactor R3. It was safely hypothesized that halotolerant and halophilic bacteria with high biological activity easily dominated in reactor under the condition of initial salt-stress selection at low concentration, and these preponderant microbes can secrete a relatively high quantity of EPS (Figure 4), which can bond bacterial cells and other particulates into an aggregation generating the precursor of a particle *(Liu et al. 2004)*. But excessive EPS can block inner cores of the granules which acting as mass transfer channels. And the resistance of mass transfer of granular sludge in reactor R1 could shape an anaerobic core to motivate bioactivities of anaerobic strains *(Zheng et al. 2006)*, which can cause the hydrolysis of anaerobic granule core, hence result in multiple cavities of intra-granules caused by cell autolysis (Figure 7 a). For organic loading rate, the higher the rate, the better to the growth of heterotrophic microbes, such as the breeding of filamentous bacteria, which conduced to irregular frames *(Moy et al. 2002)*, and further hindered the granulation. Whereas overgrowth of filamentous bacteria can be controlled by high hydrodynamic stress and reducing organic loading gradually at high salinity (2.4-3.0%), in particular, fluctuation of salinity could effectively induce moderate-levels of filamentous growth, which could play a role in intertwining and connection with zoogloea during granulation. Meanwhile, the wrapping and adherence of zoogloea can provide more stable and favorable ecological conditions for filamentous maintaining the structural integrity in granules formation. Therefore, filamentous and zoogloea formed a special symbiotic relationship in granular sludge of reactor R1 at appropriate cultivation conditions.

The dosage of $Al^{3+}$ appeared to be a major cause of filamentous sludge bulking in aerobic granulation of reactor R2 and improved the aggregation of floc-like sludge through cell-cell bridging. However, $Al^{3+}$ can transform to chemical precipitate with high content phosphate, which can result in a clogging of granular core and then affect the mass transfer, meanwhile filamentous cannot excess intra-granules to intertwine and connect aggregations, instead of outgrowth on the surface of particles freely, and finally caused filamentous granular bulking in reactor R2. After adopted some corresponding measures,


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filamentous bacteria adhering to zoogloea and sediments clogging in granular cores were washed out from granules gradually. In addition, the high hydrodynamic shear force can drive microbes to secrete more EPS to promote granulation, improve microbial cell surface hydrophobicity *(Liu et al. 2003)*, and densify and compact the granules. The increase of EPS in Fig.4 from 56 days had confirmed the conclusion. Ultimately, the final matured aerobic granules dominated by zoogloea with a clear boundary outline and good settling properties were achieved. While filamentous microorganism appeared to be predominated microbes on high content initial salt-stress selection, result in a formation of filamentous granules. When the quantity of extracellular polysaccharides (decrease trend in Figure 4) was not sufficient to maintain granular structure, it will lead to a disintegration of filamentous particles and filamentous bulking.

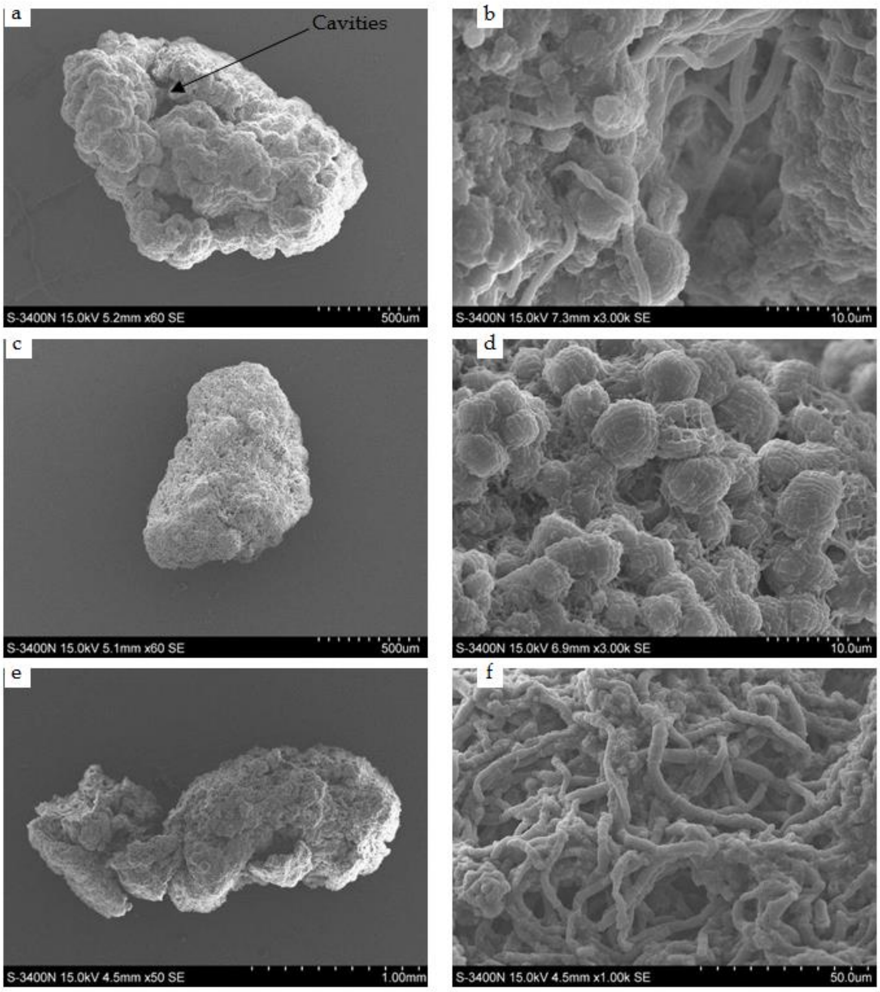


**Figure 7.** Image of granule, its surface, and inner part for each reactor, R1 (a, b), R2 (c, d), R3 (e, f).

Studies intend to examine the mechanisms that may involve in the granulation processes. In general, two models were proposed on granulation for AGS *(Li et al. 2010)*. The first model suggests that microbe-to-microbe contact form aggregates by physical forces, and initial attraction to shape into aggregates by physical, chemical or biochemical forces, and then EPS bind each other to make the aggregates become stronger, allowing physical enmeshment, consequently the final compact granules formed under hydrodynamic stress. The second model states that filamentous bacteria intertangle and thus form the backbone of particles. As in Figure 7, microbe-to-microbe adhered and aggregated by EPS binding in reactor R2, and granular backbone formed by filamentous microbes entangling each other in reactor R3. In contrast, how the inner granular structure found in reactor R1 formed could be proposed that filamentous bacteria accessed to inner granules and entangled with zoogloea to form granules based on cell-to-cell attachment through EPS enmeshing. Therefore, we can propose that in this research, two steps involved in the granulation under high salt stress were as follow:

1. Under unsalted condition, positive charged ions such as $Ca^{2+}$ and $Mg^{2+}$ combine with some bacteria as well as with negatively charged EPS to form high polymer bridged linkage biomacromolecule, which can provide crystal nucleus for aerobic granulation;

2. Under subsequent salt-shifting conditions, granules would develop mainly complied with the first model under low initial salt-stress selection, while the development of granules would comply with the second model under high initial salt-stress selection.

# Conclusion

Inoculated with activated sludge when reactor adapted different operational conditions, aerobic granulation was realized within 70 days in three pilot scale SBRs fed with simulated saline wastewater. The reactors had good performance for COD and $NH_4^+$-N removal when the mean concentration of the influent COD and $NH_4^+$-N for the reactors was kept at 4500 and 225 mg $L^{-1}$. The COD removal efficiencies of reactor R1, R2, and R3 can reach up to 92.5%, 95.1%, and 90.2%, and the corresponding removal rates of $NH_4^+$-N achieved to 59.8%, 67.1%, and 65.6%.

Compact and stable aerobic granules dominated by zoogloea with an excellent settling ability and a clear boundary outline were successfully cultivated in SBR system on low initial salinity, while filamentous granular sludge with irregular, loose structure and mycelia on outer layer was formed when given high initial salinity. Divalent metal ions such as $Ca^{2+}$ and $Mg^{2+}$ can transfer to minerals with phosphate in simulated MTW, then, it served as crystal nucleus and carriers for granulation. Appropriate organic loading, hydrodynamic shear, and salt-stress selection can induce filamentous bacteria on moderate growth to act as granulation backbone. Aerobic granulation process under salt stress was a result together with crystal nucleus, filamentous bacteria, and EPS, which can be affected by salinity-shifting strategies and dosage of aluminum salt coagulant. On low initial salt-stress selection, aerobic granules of reactor R1 cultivated were predominated by cell-to-cell aggregation with EPS bridging and filamentous intertwining and connection, and the average particle size was 1.4 mm without dosage of $Al^{3+}$, whereas adding coagulant can shorten granular shape on an average particle size of 0.9 mm without any filamentous microbes in granules of reactor R2, and then develop more pore structure with higher bioactivity of 26.42 ug TF $g^{-1}$ SS $h^{-1}$, which compared to the double that figure in reactor R1. By contrast, moderate-level filamentous microorganisms induced can entangle each other acting as the backbone of granules and thus form filamentous granules in reactor R3 with average particle size of 1.7 mm and high bioactivity of 19.02 ug TF $g^{-1}$ SS $h^{-1}$ on high initial salt-stress selection.

Further researches are necessary with the focus on biological issues like how to control QS to reduce the EPS production and alter its component by bacteria under salt stress and to promote and maintain filamentous spatial structures.

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