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Comparison of Ca^{2+} and Mg^{2+} enhancing aerobic granulation in SBR

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ABSTRACT

Two sequencing batch reactors (SBRs) were operated to investigate the effect of Ca^{2+} and Mg^{2+} augmentation on aerobic granulation. Reactor R1 was augmented with Ca^{2+} at 40 mg/L, while Mg^{2+} was added to the reactor R2 with 40 mg/L. Results showed that the reactor R1 had a faster granulation process compared with R2, and the mature granules in R1 showed better physical characteristics. However, the mature granules in R2 had the higher production yield of polysaccharides and proteins, and aerobic granules in R2 experienced a faster substrate biodegradation. Microbial and genetic characteristics in mature granules were analyzed using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) techniques. The results revealed that Mg^{2+} addition led to higher microbial diversity in mature granules. In addition, an uncultured bacterium (AB447697) was major specie in R1, and *β-proteobacterium* was dominant in R2. It can be concluded that Ca^{2+} had an important effect on physical properties of aerobic granules, while Mg^{2+} played a key role on biological properties during the sludge granulation.

1. Introduction

Aerobic granulation technology has become attractive in wastewater treatment, which is a new and promising approach to overcome the principal weaknesses of the activated sludge process. Previous results showed that aerobic granules had compact and strong microbial structure, good settling properties, high biomass retention and so on [1–3]. Thus aerobic granulation technology would have great potential in treating a wide variety of wastewater.

A brief review showed that aerobic granulation is not a function of microbiological groups but that of reactor operating conditions and factors, e.g. aerobic starvation, shear force, divalent metal ions and so on [4–8]. In these factors, divalent metal ions had been proved to play an important role in the process of aerobic granulation, especially Ca^{2+} and Mg^{2+} which are widely recognized to enhance the sludge granulation in the sequencing batch reactor [7–9]. Augmentation with Ca^{2+} significantly decreased the time to cultivate aerobic granules from 32 days to 16 days, and Ca^{2+}-fed granules showed better settling and had higher polysaccharide contents [9]. Compared with aerobic granules without Ca^{2+} addition, the Ca^{2+}-fed granules had more rigid structure and a higher strength [8]. Li et al. found that addition Mg^{2+} in reactor significantly decreased the sludge granulation time from 32 days to 18 days [7]. However, the information about the different effect of Ca^{2+} and Mg^{2+} on sludge granulation and distinct characteristics of granules with Ca^{2+} or Mg^{2+} addition is still sparse.

It was reported that Ca^{2+} augmentation from 40 to 100 mg/L could stimulate the formation of aerobic granules during the start-up process [8,9]. The addition of a lower content Mg^{2+} was even more favorable for the formation and growth of granular sludge [7]. Thus, we selected 40 mg/L Ca^{2+} and Mg^{2+} addition to investigate the effect of metal ions on aerobic granulation. The purpose of this study was to find the different effects of Ca^{2+} and Mg^{2+} on aerobic granulation and granules performances. It was expected that the results derived from this study would be useful for the cultivation of aerobic granules in SBR.

2. Materials and methods

2.1. Experimental setup and SBR operation

Experiments were conducted in two column reactors (60 cm in height and 22 cm in diameter) with a working volume of 12 L, and were operated as sequencing batch reactors (R1 and R2) for aerobic granulation. The influent was added from the top of reactor, and the effluent was discharged at 10 cm above the reactor bottom with a volumetric exchange ratio of 75%. Aerobic granules were developed in SBR reactors by stepwise reducing settling time. In two reactors, the cycle time was set to about 5 h. Each cycle consisted of 1 min of feeding, 280 min of aeration, 1–10 min of settling, and 5 min of decanting. The settling time was 10 min at the beginning, and then

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it was reduced gradually from 10 to 1 min during aerobic granulation. Air was introduced through a diffuser at the reactor bottom by air pump and the airflow rate was controlled by a gas-flow controller. Superficial gas velocity and dissolved oxygen concentration were 1.2 cm/s and 4–6 mg/L, respectively. The temperature of the reactor was maintained at $24 \pm 1\,^{\circ}C$ using a ribbon heater and a temperature controller.

Activated sludge taken from a local municipal wastewater treatment plant (Wenchang Municipal Wastewater Treatment Plant, Harbin, China) was used as the seed sludge for the reactors at a respective initial sludge concentration of 2300 mg/L in mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) of 1620 mg/L in two reactors. The ratio value of MLVSS/MLSS was about 70%. The seed sludge was grayish brown in color and had a sludge volume index (SVI_{30}) of 92 mL/g.

Reactor R1 was augmented with $CaCl_2$ at 40 mg/L Ca^{2+}, while $MgSO_4{\cdot}7H_2O$ was added to the reactor R2 with 40 mg/L Mg^{2+}. A synthetic wastewater with the following composition was used: the influent glucose concentration measured as chemical oxygen demand (COD) was 580 mg/L at the beginning, and then it was increased from 580 to 660 mg/L after 50 cycles for enhancing aerobic granulation. NH_4Cl 125 mg/L, KH_2PO_4 25 mg/L, $NaHCO_3$ 250 mg/L, trace element solution 1.0 ml/L. The composition of the trace element solution was $FeCl_3$ 20 mg/L, $CuSO_4$ 50 mg/L, $MnSO_4{\cdot}H_2O$ 50 mg/L, $CoCl_2{\cdot}6H_2O$ 50 mg/L, KCl 18 mg/L, $AlCl_3$ 15 mg/L, $ZnSO_4{\cdot}7H_2O$ 30 mg/L, H_3BO_3 40 mg/L, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 30 mg/L was fixed at 500 mg/L.

2.2. Analytical methods

Measurement of COD, ammonium-nitrogen, total phosphorus, mixed liquor suspended solids, and sludge volume index (SVI) were performed using standard methods [10]. Extracellular polymeric substances (EPSs) in aerobic granules were extracted by heating-centrifugation extraction method [11]. A phenol–sulfuric acid method was used to quantify polysaccharides in EPS [12]. The amount of protein in extracted EPS was determined using the modified Lowry method [13]. The various sizes of aerobic granules were collected through the wet sieving method, and the granules samples taken from 10 cm above the reactor bottom [14]. The settling velocity was measured by recording the time taken for the granules to fall from a certain height in a measuring cylinder [15]. Physical strength of aerobic granules could be expressed as the integrity coefficient (%), which is defined as the ratio of residual granules to the total weight of granules sludge after 5 min of shaking at 200 rpm on a platform shaker [16]. Photographs of the granules were taken with a digital camera (Canon IXUS 8601S, Japan). Moisture content (%) = (the total sludge weight − dry sludge weight)/the total sludge weight. Specific gravity (g/cm^3) = sludge mass/distilled water mass (the equal volume). Dissolved oxygen (DO) was measured in the reactors, using an oxygen electrode (HANNA, HI 98186).

2.3. Analysis of the structure of microbial community

The biomass for bacterial population analysis was sampled from the reactor on cycle 120th. Specific bacterial primer GC-338 and a reverse universal primer 518 supplied by Shanghai Songon Biology Engineering Technology & Services Co. Ltd. (China), were used in this study to amplify bacterial 16S rDNA. The nucleotide sequence of the primers was as follows: primer GC-BSF: 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCC TACGGGAGGCAGCAG-3′; primer 518: 5′-ATTACCGCGG CTGC TGG-3′. Genomic DNA extraction and PCR conditions were the same as described previously except that the annealing temperature of the touch down PCR was 55 °C [17]. PCR products were verified in 1% agarose gel. DGGE analysis of PCR products was performed with a Bio-Rad D-Code System (Bio-Rad Laboratories, Mississauga, Ontario, Canada). PCR samples were concentrated and 300 ng were loaded onto a 8% (w/v) polyacrylamide gel containing a 30–60% gradient of denaturant (80% denaturant correspond to 5.6 M urea and 32% (v/v) deionized formamide). Bands of interest were reamplified, purified and sequenced using the Gel Recovery Purification Kit (Watson Biotechnologies Inc., Shanghai, China) according to the manufacturer's instruction. The DNA sequences were determined using the chain termination method in an ABI 3730 stretch sequencing system by a commercial service (Sangon, China), and submitted for comparison to GenBank database using BLAST algorithms.

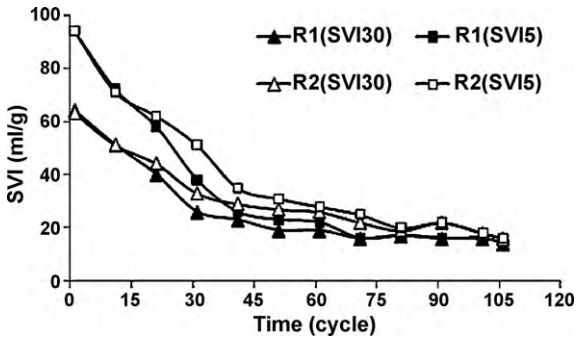

Fig. 1. Variation of SVI along with aerobic granulation in R1 and R2.

3. Results and discussion

3.1. Formation of aerobic granules

The seed sludge was firstly activated through aeration with tap water for 3 days (aeration without substrate feeding), and then the activated sludge was inoculated into R1 and R2 reactors [18]. The SVI_{30} value of seed sludge was 92 mL/g, and it decreased to about 64 mL/g after 3 days.

In the process of aerobic granulation, after 10 cycles of operation, the activated sludge was still in the form of sludge flocs, and the performance of sludge did not show much difference in two reactors. However, as showed in Fig. 1, the SVI_{30} value of sludge decreased to about 51 in R1 and R2, which indicated that the settling property was improved in two reactors. So the settling time decreased to 5 min at this stage. On cycle 16th, aerobic granules were firstly observed in R1 with Ca^{2+} augmentation, and the color of sludge changed to yellow. However, aerobic granules did not be observed in R2 with Mg^{2+} augmentation until cycle 30th. From then on, the number of aerobic granules increased, and its size increased gradually as well.

It is difficult to identify the steady state in the process of aerobic granulation because the granulation is a gradual process from flocs sludge to granules. Liu and Tay [5] indicated that sludge granulation was achieved when the difference between SVI_5 (the measurement time was 5 min) and SVI_{30} of the same sludge is lower than 10%, and a clear shape of the granular sludge was observed. SVI_5 and SVI_{30} of both reactors were showed in Fig. 1. In R1, the difference between SVI_5 and SVI_{30} was lower than 10% after cycle 41th, while it occurred on cycle 51th in R2. Thus, according to the definition from the Ref. [5], aerobic granulation was achieved in reactor R1 after 41 cycles, but 51 cycles in R2. Due to the flocs sludge still existing in two reactors in this phase, the settling time was further decreased to 1 min in order to remove activated flocs sludge. The complete granulation (defined as that no flocs could observe in reactor, and a granules size smaller than 0.3 mm was below 5%) was reached at cycles 88th in R1, but a little flocs still be found in reactor R2 at this stage. After 100 cycles of operating, the com-

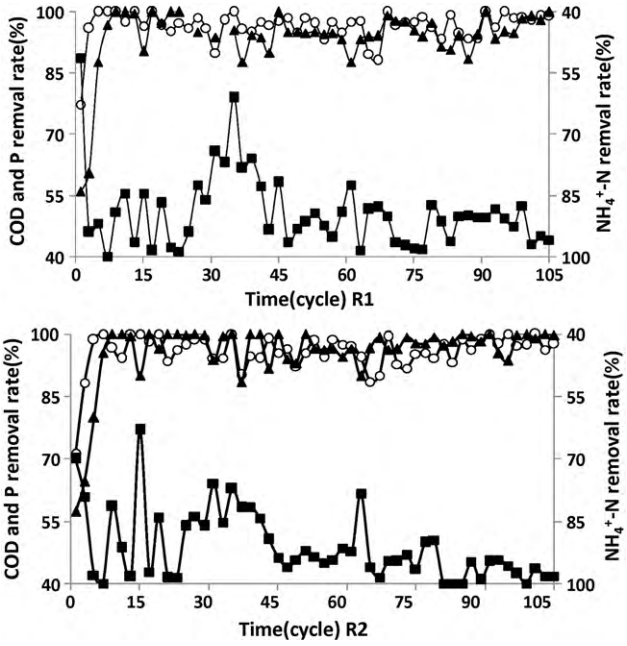

Fig. 2. Changes of COD, NH_4^+-N, P removal during granulation in R1 and R2. (▲) COD removal efficiency, (■) NH_4^+-N removal efficiency and (○) P removal efficiency.

plete granulation was reached in reactor R2. The result showed that aerobic granulation with Ca^{2+} augmentation had a little faster rate compared with Mg^{2+} augmentation.

As showed in Fig. 1, the settling ability of aerobic granules with Ca^{2+} augmentation was almost similar with Mg^{2+} augmentation. The mature granules with both metal ions additions showed good settling property. The COD, NH_4^+–N and P removal during the granulation process were shown in Fig. 2. The COD removal was similar in the two reactors, and was kept above 94%, and the NH_4^+–N and P removal in R2 ($98 \pm 1.6\%$, $99.6 \pm 0.5\%$) was a little higher than that in R1 ($92 \pm 3.5\%$, $97 \pm 1.6\%$) after the complete granulation.

3.2. Granules morphology and size distributions

The morphology and size distributions of mature granules in two reactors were revealed in Figs. 3 and 4. The volume percentage of mature granules in two reactors with a size smaller than 0.3 mm was below 3%, which showed that the granules were dominant in two reactors. The morphology analysis also indicated that aerobic granules were fully achieved, and no flocs were found in two reactors. However, it could be found that different divalent metal ions augmentation resulted in different morphological features and

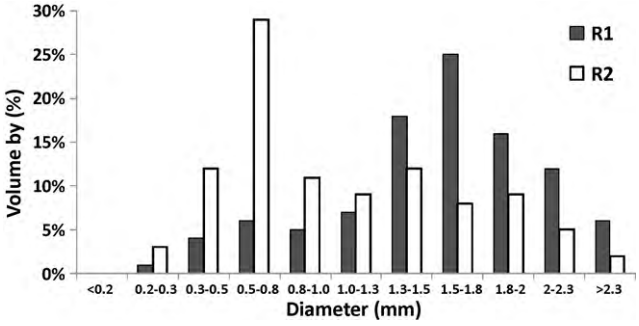

Fig. 4. Size distributions of mature granules in R1 and R2 on cycle 105th.

size distributions of mature granules. Aerobic granules in R1 had a regular and compact appearance while the granules in R2 had a loose structure. Moreover, approximately 60% of the granules in R1 were in the range of 1.3–2 mm, and the granules size in the range of 0.2–1 mm was less than 16%. By contrast, approximately 61% of the granules fell in the size range of 0.3–1.3 mm in R2. The similar results could be found in other researches [7,8]. Thus, Ca^{2+} augmentation could achieve a larger size of granules than Mg^{2+}, and also it could improve the strength of structure of aerobic granules. Compared with seed sludge, the color of sludge was changed after granulation augmented with Ca^{2+} and Mg^{2+}. The color of mature granules was yellow in R1, and it was white in R2. This result implies that the shift of microbial population could occur during the granulation, and the microbial community structure would be different in two reactors.

3.3. Physical and chemical characteristics of mature granules

The samples of aerobic granules were taken from the two reactors at cycle 110th. The physical and chemical characteristics were analyzed in terms of MLSS, MLVSS, SVI, settling velocity, specific gravity, moisture content, EPS and physical strength of mature granules, and the results were summarized in Table 1.

The ratio of MLVSS to MLSS of the granules with Ca^{2+} augmentation was about 84%, and lower than Mg^{2+} augmentation. In the process of sludge granulation, two SBRs were operated under the same conditions, so the different of two reactors could be attributed to divalent metal ions in reactors. The settling velocity of mature granules in reactor was 75 ± 17 m/h (R1) and 63 ± 16 m/h (R2), respectively, and this result was similar to the SVI_5 of mature granules in two reactors. Thus it could found that the divalent metal ions had a little influence on the settling capacity of aerobic granules.

Moisture content, specific gravity and physical strength are the important parameters on physical characteristics of the granules.

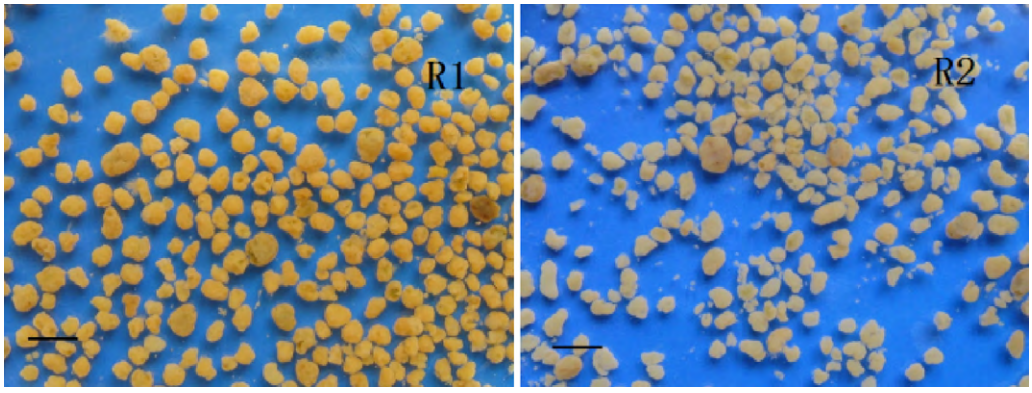

Fig. 3. Granules developed in R1 and R2 after 105 cycles operation (bar = 5 mm).

Table 1
Characteristics of aerobic granules in two reactors after 110 cycles.

	Seed sludge	R1 (Ca^{2+})	R2 (Mg^{2+})
MLVSS/MLSS (%)	70%	84%	95%
Settling velocity (m/h)	<10	75 ± 17	63 ± 16
Specific gravity (g/cm^3)	1.003	1.045	1.024
Moisture content (%)	>99	96.5	97.2
Integrity coefficient (%)	–	97.2	93.7
Proteins (mg/g VSS)	36.4	42.3	67.2
Polysaccharides (mg/g VSS)	11.7	21.3	22.7

As illustrated in Table 1, the physical characteristics of mature granules in R1 were better than in R2, and this indicated that the Ca-rich granules had highly dense and compact structure which was benefit the biomass retaining property and solid–liquid separation in the reactor.

Extracellular polymetric substance (EPS) are widely recognized to play a key role in the process of aerobic granulation, and EPS is mainly composed of polysaccharide (PS) and protein (PN) [19,20]. Compared with the seed sludge, the production yields of polysaccharide (PS) and protein (PN) were increased (Table 1) in both two reactors. After the complete granulation of sludge in the reactors, PS content was increased to 21.3 mg/g VSS from initial 11.7 mg/g VSS in R1, while it was rose to 22.7 mg/g VSS in R2. In addition, PN content was increased to 42.3 mg/g VSS in R1 and 67.2 mg/g VSS in R2 from 36.4 mg/g VSS in seed sludge. During aerobic granulation, a number of operational conditions (e.g. pH, DO, MLVSS) affect the production yield of PS and PN. However, in our research the two reactors were run under the same conditions, so the different of EPS between two reactors should be attributed to divalent metal ions augmentation. Moreover, adding Mg^{2+} to the reactors had a more positive effect on EPS production than adding Ca^{2+}.

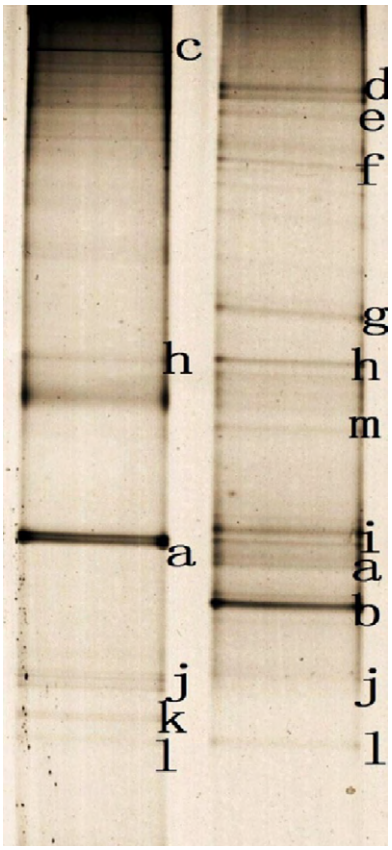

Fig. 5. The DGGE profiles of mature granules in R1 (left) and R2 (right) on cycle 120th.

3.4. The kinetics of substrate degradation

The substrate degradation kinetics by aerobic granules is assumed to follow Monod equation as

$$q = \frac{q_{\max} S}{K + S} \quad (1)$$

where q = specific substrate degradation rate (gCOD/gVSS-h), S = substrate concentration (g/L); $q_{\max}$ is the maximum specific substrate degradation rate, and K is apparent half rate constant (mg COD/L). The values of $q_{\max}$ and K can be obtained by the Lineweaver–Burk plot of the inverse of the specific rate ($1/q$) versus of the substrate concentration ($1/S$).

$$\frac{1}{q} = \frac{K}{q_{\max} S} + \frac{1}{q_{\max}} \quad (2)$$

The specific substrate degradation rate q can be estimated from substrate degradation time course experiment as

$$q = \frac{S_0 - S_e}{X_t} \quad (3)$$

where S_0 is the start concentration of the substrate, S_e is the substrate concentration after time t, X is the concentration of granular biomass (g SS/L) at time t. During a short time test, the change of the biomass concentration is ignored.

Insert the Eq. (3) into (2) gives:

$$\frac{X_t}{S0 - Se} = \frac{K}{q_{\max}} \frac{1}{S_e} + \frac{1}{q_{\max}} \quad (4)$$

The experimental data were obtained by monitoring COD concentration in two reactors every 1 h, and operational parameters kept the same in two reactors. Meanwhile, according to Eq. (4), plotting $X_t/S_0 - S_e$ as y, $1/S_e$ as x to calculate K and $q_{\max}$. Therefore the kinetic equation of aerobic granules under different divalent metal ions can be derived, as shown following: R1 was $q = 0.18S/(13.69 + S)$, R2 was $q = 0.26S/(16.23 + S)$. It could be found that aerobic granules in R2 experienced a faster substrate biodegradation. Aerobic granules, were cultured by Mg^{2+} augmentation, would be favorable for substrate biodegradation.

3.5. Microbial and genetic characteristics

The bacterial species were detected by isolation of DNA from the granules and then by performing a DGGE analysis of amplified 16S rRNA gene. Approximately 13 dominant species were present in R1 and R2 (Fig. 5 and Table 2), and the different divalent metal ions augmentation led to the different microbial population distributions of mature granules. It could be found that the number of dominant DGGE bands was about 11 in R2, while only 6 in R1. This result may be related to two reasons. Firstly, the size of aerobic granules with Ca^{2+} addition was larger, so it could affect mass transfer limitation in the granules, thereby lead to a low diversity. The researches had indicated that an increase in granule size resulted in the Ca^{2+} accumulation in granules, and finally led to a reduced granule bioactivity [8]. Secondly, Mg^{2+} addition would be beneficial to the species diversity by stimulating growth of bacteria in system [15,20]. Thus aerobic granules with Mg^{2+} contained with relatively higher microbial diversity. In addition, uncultured bacterium (AB447697) was major specie in R1, and *β-proteobacterium* was dominant in R2. This result indicated that divalent metal ions addition could lead to the alteration of dominant species in activated sludge system.

Table 2
Sequence analysis and species identification of selected DGGE bands for the granules in two reactors (the band names are shown in the schematic DGGE profiles in Fig. 5).

Band	Closest relatives (accession no.)	Identity (%)	Sequence (bp)
a	Uncultured bacterium (AB447697)	94	1446
b	Uncultured beta proteobacterium (AY823971)	99	1462
c	Uncultured gamma proteobacterium (EU434903)	100	747
d	Uncultured geobacteraceae bacterium (EF669073)	98	551
e	Azospira sp. (GQ183355)	99	658
f	Uncultured bacterium (EU285323)	95	465
g	Uncultured bacterium (AB158718)	97	155
h	Nitrosomonas sp. (FM997833)	99	440
i	Aeromonas hydrophila strain (EF669478)	90	1454
j	Uncultured bacterium (AB487476)	97	1335
k	Anaerofilum sp. (FJ823903)	92	1502
l	Uncultured bacterium (AB106418)	93	194
m	Uncultured bacterium (AB205989)	96	564

3.6. Mechanism analysis of aerobic granulation

The previous research indicated that the divalent metal ions, especially Ca^{2+} and Mg^{2+} played an important role in the process of aerobic granulation [7–9]. Ca^{2+} and Mg^{2+} addition resulted in faster granulation process and shorter starting-up period. However, compared with aerobic granules in R1 and R2, it could be found that both divalent metal ions showed different effect on the granulation process, and also led to the different characteristics of mature granules in SBRs.

In this paper, specific gravity of aerobic granules in R1 was 1.045 g/cm^3, compared with 1.024 g/cm^3 in R2. The MLVSS/MLSS ratio of sludge in R1 was lower than in R2. Therefore, Ca^{2+} enhanced the sludge granulation process through three ways: (1) adding Ca^{2+} in the reactor can neutralize the negative charge on the surface of bacteria, and promote the sludge granulation [21]; (2) Ca^{2+} could bind to the negatively charged groups on bacterial surfaces and extracellular polymeric substances (EPS), and act as a bridge to interconnect these components, and then form EPS–Ca^{2+}–EPS cross-linkage [8,22]; (3) Ca^{2+} could be used as a core-induced ($CaCO_3$) to accelerate microbial aggregation, and increase the physical strength of mature granules [23].

However, Mg^{2+} had a weak effect on the structure of granules, and could not play the role for bridging function and core-induced. It might imply that Mg^{2+} enhanced the sludge granulation process through the biochemical function. Enzyme is a special active protein, and it had a positive effect on the microbial activity. Mg^{2+} had been proved to play an important activator role on the enzyme, and stimulated enzyme reactions associated with a synthesis of cell materials [24]. In our research, we found that protein content was extremely increased in aerobic granules with Mg^{2+} augmentation. With Ca^{2+}augmentation, the aerobic granules produced a larger amount of polysaccharides without corresponding increase in protein [9]. However, the granules augmented with Mg^{2+} showed higher amounts of both polysaccharides and protein content. The higher EPS content could enhance various microbial species to form stable granules, so the granules would contain microbial communities with a relatively higher diversity. As a result, the aerobic granules with Mg^{2+} augmentation showed a faster substrate biodegradation rate compared with Ca^{2+} augmentation.

4. Conclusions

Aerobic granulation with Ca^{2+} augmentation was grown a little faster than with Mg^{2+} augmentation. After aerobic granules reached to steady stage, the mature granules augmented with Ca^{2+} showed superior physical characteristics than augmented with Mg^{2+}. However, microbial community of the mature granules augmented with Mg^{2+} had relatively higher diversity, and showed a little faster substrate biodegradation rate. In addition, adding Mg^{2+} had a more positive effect on EPS production of aerobic granules than adding Ca^{2+}. Therefore, it could be concluded that Ca^{2+} and Mg^{2+} played a different role in aerobic granulation.

Acknowledgments

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