Investigating the influence of elongated anaerobic feeding strategy on aerobic sludge granulation and characteristics in sequencing batch reactor

S. Sadri Moghaddam and M. R. Alavi Moghaddam

ABSTRACT

In this research, two sequencing batch reactors (R1 and R2) were operated with different feeding strategies to investigate the effects of elongated anaerobic feeding mode on the granulation process. For this purpose, R1 was operated in a short-feed strategy (5 min) as a reference, whereas an extended anaerobic feeding of 85 min was applied in R2. Results showed that aerobic granules formed in R1 were denser and more uniform with lower sludge volume index than those formed in R2. Investigation of tightly bound extracellular polymeric substances (TB-EPS) showed that aerobic granules in R1 produced lower amounts of TB-EPS than those in R2. This was due to the bigger and more compact granules cultivated in R1 in comparison with looser structure granules in R2 with higher flocculent sludge percentage. The relative hydrophobicity of granules in both reactors was increased with culture time and reached about 63 and 65% at day 70 for R1 and R2, respectively. Moreover, no significant correlation between protein/polysaccharide ratio and hydrophobicity was found, which showed that protein in loosely bound extracellular polymer substances was not the predominant hydrophobic component. Considering the outcomes of this study, it can be concluded that the elongated anaerobic feeding strategy was unfavourable for cultivating aerobic granules.

Key words | aerobic granules, extracellular polymer substances (EPS), feeding strategy, hydrophobicity, sequencing batch reactor (SBR)

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INTRODUCTION

Aerobic granulation of sludge is a novel biotechnological process that has the potential to become a major biological wastewater treatment technology in degradation and removal of organic material (Sheng *et al.* 2010a). Over the past two decades, aerobic biological granulation technology has shown to be useful for the treatment of sewage or industrial wastewater under various environmental and operational factors such as shear force and dissolved oxygen (DO) (McSwain & Irvine 2008), substrate composition (Sun *et al.* 2006), organic loading rate (Gao *et al.* 2011; Zhang *et al.* 2011) and settling time (Gao *et al.* 2011). Despite these extensive efforts, one of the main challenges still facing researchers is the influence of feeding strategy on aerobic granulation.

McSwain *et al.* (2004) utilised intermittent feeding for the purpose of aerobic granule formation. They found that the structural properties and content of filamentous organisms were clearly dependent on a high feast condition and only the reactor with dump fill formed compact and stable granules. They concluded that intermittent feeding affects the selection and growth of filamentous organisms and has a critical role in granule structure and composition. On the other hand, Rocktäschel's research group compared two different anaerobic feeding strategies (an anaerobic plug flow operation vs a fast influent step followed by an anaerobic mixing phase) to optimise the development and performance of aerobic granules. They found that anaerobic mixing has advantages compared to a plug flow regime. The growth of granules was more stable and resulted in an optimal balance of heterotrophic and autotrophic growth of microorganisms under anaerobic mixing conditions (Rocktäschel *et al.* 2013).

In this study, the effect of a long anaerobic feeding period was investigated regarding aerobic granule formation

doi: 10.2166/wst.2014.209

and properties. For this purpose, a conventional short feed of 5 min was applied in the first reactor (R1) as a reference, while the other reactor, namely R2, ran with an extended anaerobic feeding of 85 min. This study focuses on settling properties of granules, paying attention to the role of extracellular polymer substances (EPS) and hydrophobicity on granule formation and characteristics. Development of aerobic granules was also followed by microscopic observations as well as particle size distribution measurement. Moreover, the effect of increase of influent chemical oxygen demand (COD) on the properties of cultivated granules was investigated.

MATERIALS AND METHODS

Experimental set-up

Experiments were conducted in two sequencing batch reactors (50 cm in height and 14 cm in diameter) with an effective volume of 5 L to cultivate aerobic granules. These reactors (R1 and R2) were inoculated with activated sludge taken from the sludge return line of a local municipal wastewater treatment plant (Zargandeh) located in Tehran, Iran. The reactors were operated in successive cycles of 6 h (360 min). The feeding period for R1 was 5 min and feeding of R2 was extended to 85 min in anaerobic conditions using a peristaltic pump (Heidolph, PD 5201, Germany). The reaction phase started with an anaerobic phase of 85 min, followed by an aerobic phase for 264 min. Mixing was applied during the anaerobic condition using a shaft with three propellers connected to a motor with a speed of 90 rpm. For both reactors, the mixing started 5 min after the beginning of the cycle. In other words, mixing was not applied during the first 5 min of anaerobic fill in the reactors (R1 and R2). The sludge settling time was reduced gradually from 20 to 5 min in the first 9 days of operation (adaptation period) and effluent withdrawal was set at 1 min. The volume exchange ratio and aeration rate of the reactors was 64% and 5.5 L/min, respectively. The composition of synthetic wastewater used in this study with initial COD of 700 mg/L was as follows: glucose (328 mg/L), sodium acetate (447 mg/L), urea (75 mg/L), KH_2PO_4 (14.75 mg/L), K_2HPO_4 (20.45 mg/L), $MgSO_4 \cdot 7H_2O$ (35 mg/L) and $CaCl_2$ (65 mg/L). The organic loading rate was 2.8 kg/m^3 d (initial COD of 700 mg/L; four cycles per day) during the first 38 days of operation, for both reactors. Then, it was suddenly increased to 4 kg/m^3 d (initial COD of 1,000 mg/L; four cycles per day) at day 38 and held constant over the remaining period of operation in both reactors.

Analysis

The COD, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI) were measured using *Standard Methods for the Examination of Water and Wastewater* (APHA 1998), and the observations of aggregate morphology were carried out by light microscopy (Hund Wetzlar S200, Germany) using ×10 binocular objective (×100 total magnification). The loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) were extracted using extraction methods from Guo *et al.* (2011) with some modifications. For each sample, about 10 mL of sludge was centrifuged at 2,500 rpm for 5 min and washed once with 0.9% NaCl solution. The supernatant was centrifuged at 6,000 rpm for 15 min and then filtered through 0.45 μm filter for LB-EPS analysis. After discarding the supernatant, the precipitate was re-suspended in 0.9% NaCl solution. The suspension was extracted by ultrasound at 90 W for 5 min in an ice bath before the chemical extraction was applied. After that, the sample was re-suspended in 10 mL EPS extraction buffer (8.5% NaCl and 2% EDTA, pH 8) and kept at 4 °C for 3 h. The supernatant was centrifuged at 6,000 rpm for 15 min and then filtered through a 0.45 μm filter. Subsequently, the sample was transferred to a clean tube for TB-EPS analysis. The protein content was examined using the Lowry method (Lowry *et al.* 1951) and the polysaccharide content was determined by the phenol–sulphuric acid method (Dubois *et al.* 1956). To increase the accuracy of the data, each experiment was repeated three times and the presented results are the average values with standard deviation of 1.89–4.38%.

Particle size distribution was determined by a sieving method from Laguna *et al.* (1999) using a series of standard sieves with aperture size of 0.3, 0.5 and 1 mm. The sludge with diameter (d) lower than 0.3 mm was defined as floc sludge, whereas the other sludge ranges ($d > 0.3$ mm) were regarded as granules. The granulation rate was calculated as the ratio of MLSS concentration for aerobic granules with $d > 0.3$ mm to total MLSS of sample.

The hydrophobicity of the granules was measured according to the method of Kim & Jang (2006) using a hydrocarbon (n-hexane). The procedure was as follows: a 25 mL sample was centrifuged once with PUM (phosphate, urea, magnesium sulfate) buffer (2.5 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$) at 2,500 rpm for 5 min. Then, the supernatant was re-suspended in 25 mL *n*-hexane in a separating funnel for 5 min. After 10 min, when the two phases had separated completely, the aqueous phase was transferred to other glassware. The relative hydrophobicity was calculated by the following equation:

$$\text{Relative hydrophobicity (\%)} = 100 \times [1 - (S_e/S_i)]$$

where S_e and S_i are the MLSS concentration in aqueous phase after emulsification and total MLSS concentration of sample, respectively. Each experiment was repeated at least twice and the results given are the average values. The standard deviation for suspendibility measurement was less than 5%.

RESULTS AND DISCUSSION

General observations

The SVI_{30} is a parameter of sludge settling ability over 30 minutes, which was measured in R1 and R2 during the operational period. Regarding R1, as the sludge flocs were transformed into granules, the SVI_{30} decreased from an initial value of 90 mL/g to approximately 40 mL/g and remained in the range of 40–65 mL/g in the last days of operation. The settling ability of the aerobic granules in R2 became poorer under the elongated feeding and SVI_{30} values gradually increased to 318 mL/g at day 70. A sharp increase of SVI_{30} was observed during the last 16 days of operation, which contributed to the appearance of filamentous structures with poorer settling ability.

As revealed from microscopic observations (Figure 1(a)), granular sludge in R1 appeared to be more compact in structure and each granule can be regarded as an independent microbial ecological system. Compared with R1, the granules formed in R2 showed loose structure morphology and filamentous microorganism in their structures (Figure 1(b)). Results showed that the looser structure of cultivated granules in R2 was influenced by the increase of influent COD after day 38 and transited to the unstable granules owing to filamentous microorganism appearance, while the compact granules in R1 were resistant to the increase of the organic loading rate from 2.8 to 4 kg/m^3 d. In addition, investigation of substrate (COD) removal efficiency showed that aerobic granules cultivated in both reactors are so efficient that they can remove more than 94% of influent COD all the time.

EPS components and characteristics

EPS are known to play an important role in the formation of microbial aggregates, adhesion to surfaces and flocculation (Sheng *et al.* 2010b), as well as in the building and maintenance of the structural integrity of aerobic granules during wastewater treatment (Tu *et al.* 2012). The bound EPS exhibit a dynamic double-layer structure, which can be classified as LB-EPS and TB-EPS (Tu *et al.* 2012). In this research, variations of both LB-EPS and TB-EPS during the operational period were measured and are presented in Figure 2. As shown, no apparent change was observed in the LB-EPS of aerobic granules formed in R1 even after the increase of influent COD. However, after day 35, a small increase in LB-EPS of aerobic granules in R2 was observed, which resulted in the formation of loose-structure granules with poorer settling ability.

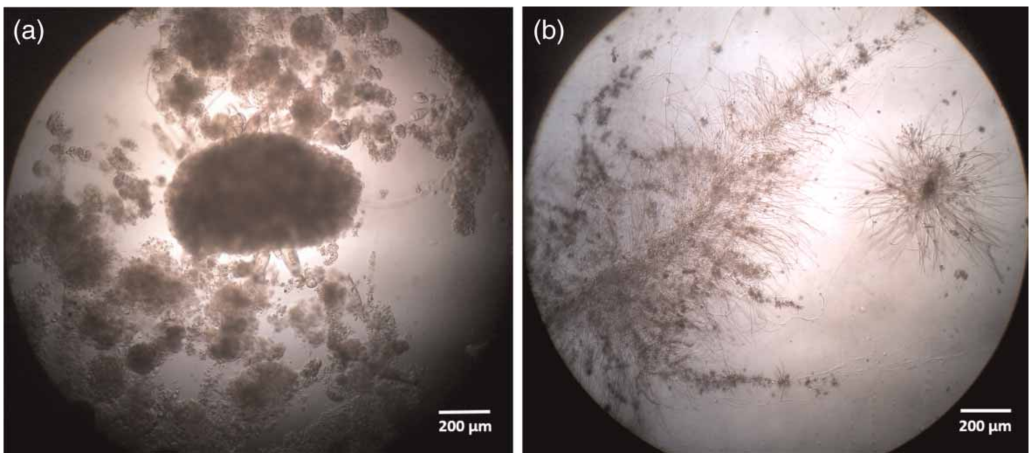

Figure 1 | Microscopic observations of aerobic granules in (a) R1 and (b) R2 at day 55.

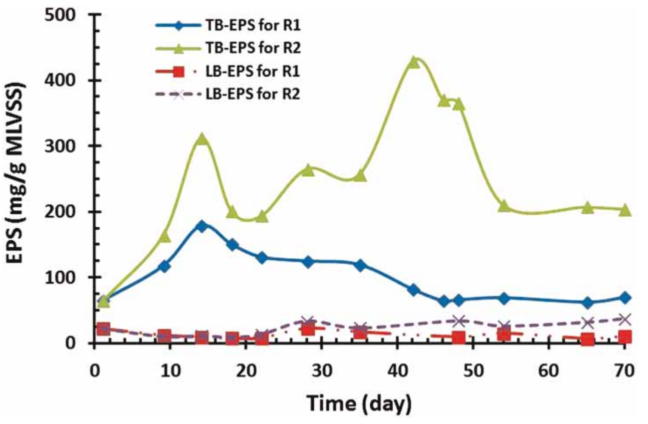

Figure 2 | Changes in LB-EPS and TB-EPS in R1 and R2.

A sharp increase in TB-EPS amounts during the first 14 days of operation, when the first granules were developed in both reactors, was attributed to the granulation stage. Along with the maturation of granules in R1, the TB-EPS value decreased and finally reached about 69 mg/g MLVSS at day 70.

The decrease of the amount of TB-EPS in R1 may be attributed to the feast–famine condition. The profiles of effluent COD, pH and DO were measured to determine the feast and famine period of the reactors during an operational cycle and are presented in Figure 3. As shown, the effluent COD concentration decreased to almost negligible value in only 110 min for R1 and 150 min for R2. Therefore, the famine period (starvation phase) in R1 was longer due to the short-feed strategy, while the smaller substrate load during the long feeding phase in R2 led to a longer feast condition. The longer starvation in R1 resulted in the consumption of EPS as carbon and energy sources to maintain self-survival owing to nutrient deficiency in the deep area of granules, which was also proposed by Wang *et al.* (2010).

TB-EPS content in R2 increased with time and reached its maximum value (428 mg/g MLVSS) at day 42. As shown, a sudden increase of TB-EPS from day 35 to 42 was observed, which contributed to the increase of influent COD. However, the TB-EPS amounts subsequently decreased when the granules gradually transited to the filamentous ones and were maintained in the range of 203–210 mg/g MLVSS. This finding is in agreement with that observed by Li *et al.* (2010) who reported that the filamentous microorganisms produced lower EPS concentrations.

Aerobic granules in R1, which were denser and more regularly spherical shaped in comparison with R2, produced lower TB-EPS. Therefore, the production of higher TB-EPS quantity by aerobic granules in R2 did not guarantee the structural integrity of granules. Concerning the obtained results, it can be concluded that increasing or decreasing of EPS in the granulation process is not a key factor, and it is important that EPS excreted by microorganisms can be used in response to the external environment and to protect the microorganisms under unfriendly circumstances. Urbain *et al.* (1993) similarly found that the presence of a large amount of EPS has negative effects on sludge flocculation. Moreover, Li *et al.* (2006) reported that a reasonable amount of EPS should be controlled to form and maintain aerobic granules.

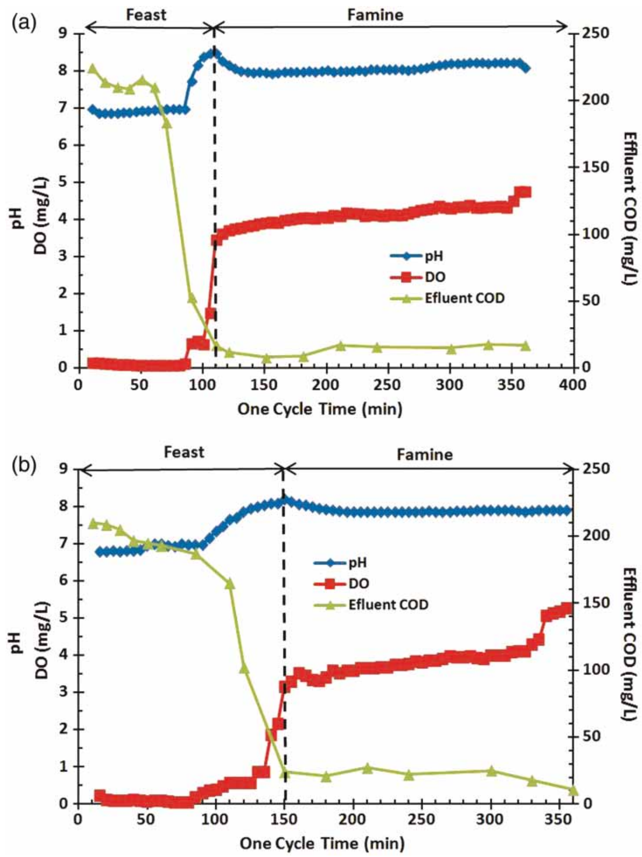

Figure 3 | Effluent COD, pH and DO profiles for (a) R1 and (b) R2 during an operational cycle.

Hydrophobicity

Hydrophobicity of the bacterial cell surface plays an important role in the self-immobilisation and attachment of cells to a surface (Zita & Hermansson 1997). The changes in cell surface hydrophobicity are presented in Figure 4. As shown, no significant difference was observed in the relative

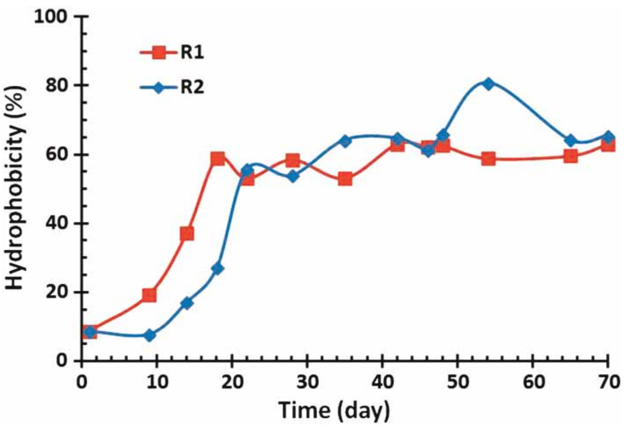

Figure 4 | Variation of cell hydrophobicity at different culture times.

hydrophobicity of cultivated granules in the reactors. The hydrophobicity of granules in both reactors quickly increased in the first days of operation (indicating that the cultivated granules are more hydrophobic than seeding sludge) and reached about 59 (at day 18) and 56% (at day 22) for R1 and R2, respectively. Over the remaining period of operation, the value of hydrophobicity remained almost constant. Results also showed that the hydrophobicity was not very sensitive to the changes in the influent COD especially in R1. Liu *et al.* (2003) also found that organic loading rate in the ranges of 1.5–9 kg COD/m^3 d did not influence the cell hydrophobicity of granules (Liu *et al.* 2003).

Some evidence suggests that EPS are a direct factor that contributes to cell hydrophobicity. In particular, extracellular protein contributes more to cell hydrophobicity, and lower protein/polysaccharide in LB-EPS leads to poorer hydrophobicity (Chen *et al.* 2010). Concerning our results, no significant correlation between protein/polysaccharide ratio for LB-EPS and hydrophobicity was found, while R^2 was 0.033 and 0.449 for R1 and R2, respectively. The results suggest that protein in EPS was not the predominant hydrophobic component. Therefore, other EPS components such as amino acids may play an important role in hydrophobicity and cellular surface properties of aerobic granules, and this remains to be researched.

Particle size distribution and granulation rate

Development of aerobic granules was followed by particle size distribution measurement (Figure 5(a)) as well as granulation rate (the ratio of MLSS concentration for granules with $d > 0.3$ mm to total MLSS of sample) in both reactors (Figure 5(b)). As shown in Figure 5(a), about 90% of the seed sludge size was less than 0.3 mm. The sludge size did not increase significantly during the first 31 days of operation. After that, the size of granules increased quickly and the proportion of large granules with particle diameter above 1 mm reached about 34 (R1) and 37% (R2) at day 65. The granulation rate in both reactors was increased with time and finally reached 80 and 69% in R1 and R2,

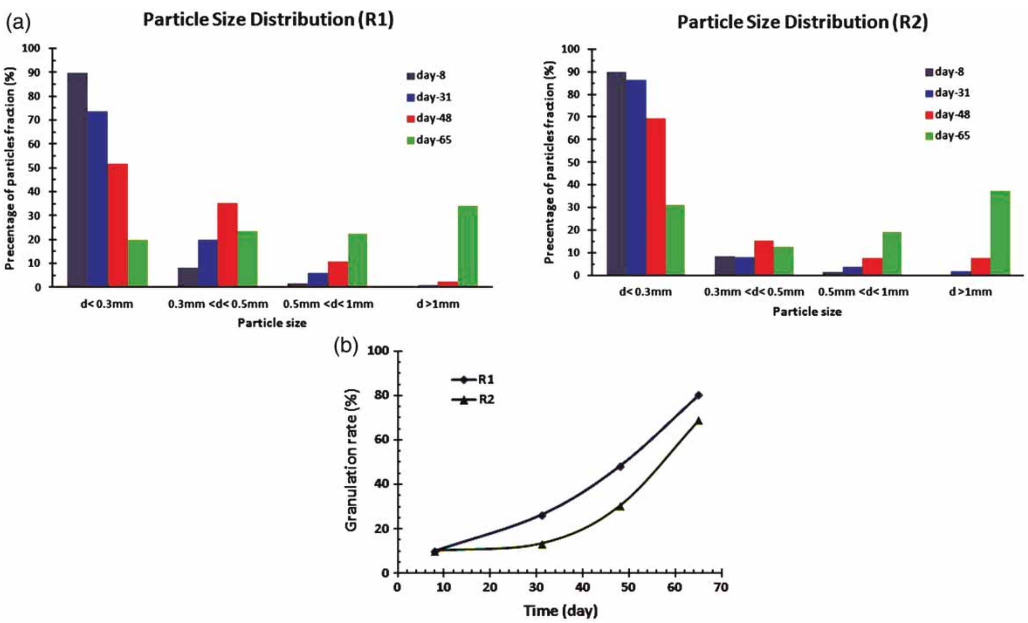

Figure 5 | Changes in (a) particle size distribution and (b) granulation rate in R1 and R2 during the operational period.

respectively (Figure 5(b)). However, the granulation rate in R1 was always higher than in R2, indicating the higher fluffy-like flocculent sludge content of R2. The higher flocculent sludge percentage in R2 could be another reason for the higher TB-EPS release into the mixed liquor. Similarly, Wang *et al*. (2010) reported that flocculent sludge has higher EPS release than granular sludge especially in large granules with diameter >0.45 mm.

The increase of granule size together with the compactness of structure in R1 led to a serious oxygen transfer resistance in granules and a possible anaerobic zone in the core of large-sized aerobic granules, as similarly reported by Zheng *et al*. (2006). Therefore, facultative microorganisms in the inner part of granules gradually showed anaerobic characteristics which resulted in producing lower amounts of TB-EPS. This observation is consistent with Forster's (1991) findings that aerobic microorganisms produced much more EPS than anaerobic ones. Moreover, Li *et al*. (2006) found that the decrease of EPS concentration could be due to suppressed production of EPS caused by the increase of granule size and occurrence of anaerobic metabolism of facultative organisms.

CONCLUSIONS

The main results of this study are summarised as follows:

- The aerobic granules cultivated in R1 (with short-feed strategy) showed regular and compact structure with good settling ability, whereas the aerobic granules under elongated anaerobic feeding in R2 were unstable and transited to filamentous ones in the last days of operation.
- The aerobic granules in R2 produced much more TB-EPS, while occurrence of facultative microorganisms with anaerobic characteristics in the core of big and compact granules in R1 led to the release of lower TB-EPS. Moreover, the longer starvation in R1 (due to the short-feed strategy) resulted in the consumption of TB-EPS as carbon and energy sources.
- The aerobic granular sludge cultivated in both reactors was more hydrophobic than the seed flocculent sludge. The cell surface hydrophobicity of granules was not related to protein/polysaccharide ratio and it seems that other EPS components besides protein might favour the hydrophobicity.
- Looser structure of cultivated granules in R2 was influenced by the increase of influent COD and transited to the filamentous granules, while the compact granules in R1 were resistant to the increase of organic loading rate from 2.8 to 4 kg/m^3 d.

As a result, the elongated anaerobic feeding strategy was unfavourable for cultivating aerobic granules, although more than 94% of influent COD could be removed all the time.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the Amirkabir University of Technology (AUT) for their funds and financial support to provide the research materials and equipment.

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First received 24 December 2013; accepted in revised form 15 April 2014. Available online 7 May 2014