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A proposed aerobic granules size development scheme for aerobic granulation process

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HIGHLIGHTS

- We propose illustrative scheme for mechanism of aerobic granule size development.
- Proposed scheme delineates the formation of aerobic granule size development.
- Different types of aerobic granules follows similar size development scheme.
- Stability of granules was enhanced through idle phase in SBR cycle time.
- Mature granules favour further aggregation with newly augmented sludge flocs.

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ABSTRACT

Aerobic granulation is increasingly used in wastewater treatment due to its unique physical properties and microbial functionalities. Granule size defines the physical properties of granules based on biomass accumulation. This study aims to determine the profile of size development under two physicochemical conditions. Two identical bioreactors namely R_{np} and R_p were operated under non-phototrophic and phototrophic conditions, respectively. An illustrative scheme was developed to comprehend the mechanism of size development that delineates the granular size throughout the granulation. Observations on granules' size variation have shown that activated sludge revolutionised into the form of aerobic granules through the increase of biomass concentration in bioreactors which also determined the changes of granule size. Both reactors demonstrated that size transformed in a similar trend when tested with and without illumination. Thus, different types of aerobic granules may increase in size in the same way as recommended in the aerobic granule size development scheme.

1. Introduction

Aerobic granulation is a combination of chemical engineering and biological processes to develop a compact form of activated sludge with high settleability properties. Transformation of seeded activated sludge into the form of aerobic granular sludge in a sequencing batch reactor (SBR) is determined by biomass increment in a granulation reactor system. Increase of biomass concentration is a result of dynamic microorganisms association and aggregation of minerals to form dense, strong, and spherical biomass aggregates (Simoes et al., 2009). According to Toh et al. (2003), the biomass distribution pattern may justify the changing physical properties of a granule as they change in size. Toh et al. (2003) further explained that as size increased, parameters such as the settling velocity, total and biomass densities may also

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increase although not in parallel with the size increment whilst the granule strength, specific hydrophobicity and sludge volume index (SVI) may decreased accordingly.

Aerobic granular sludge has been successfully cultivated in size ranging between 0.02 mm (Liu et al., 2005) to 9.0 mm (Wang et al., 2004). In average, aerobic granular sludge is commonly featured in diameters of between 0.1 mm and 3.0 mm (Li et al., 2008; Verawaty et al., 2013; Liu et al., 2012; Abdullah et al., 2013; Li et al., 2014). When compared to bioflocs, aerobic granules also featured a well-defined appearance and shape.

Granule size, although understudied, verifies the changes in physiological and physical performances of granules. Granule size may limit mass transport and diffusion due to the porosity of the structures which decreased with size increment. Porosity of granules also determined nutrient accessibility as well as seepage of unwanted products. These conditions are size-dependent which would impact the microenvironment of granules. Hence, granule size is an important factor in shaping the physical performance and determining the characteristics of aerobic granules.

Generally, it is well-understood that the size of granules which increases throughout the aerobic granulation process is related to the SBR operational conditions. Operational parameters such as substrate loading, dissolved oxygen, settling time, shear stress and so forth may affect the performance of aerobic granular sludge which is directly indicated through the morphology and structure of granules. For example, an increase in shear stress which largely controls the aerobic granulation system produced granules in the range of 0.28–0.35 mm with good sludge volume index (SVI) value (Tay et al., 2002).

However, understanding on the transition phase where smaller sludge particles aggregate with medium size granules, and medium size granules combined together to form larger size granules seems lacking. This knowledge is critical to ensure successful granulation as biomass retention in aerobic granulation environment is influenced by granule biomass density which is associated with the size of granules.

This study aims to investigate the profile of the granule size distribution during granulation period under phototrophic condition supplied by continuous illumination which supports the growth of photosynthetic microorganisms. The knowledge and understanding of illumination might be useful for aerobic granulation development under phototrophic conditions for several wastewater conditions. A size development scheme was developed to explain the phenomenon of granule formation. The knowledge and understanding of granule size would be useful for granule size-range that selects for optimal aerobic granular sludge system.

2. Methods

2.1. Reactor set-up and operations

Two identical reactors with internal diameters of 65 mm and effective height to diameter (H/D) ratio of 17 namely R_{np} and R_p, were setup and operated under non-phototrophic and phototrophic conditions at a working volume of 3 L each, respectively. Each reactor was equipped with an influent feeding tube located at the bottom of the reactor, an effluent port located at mid-height of the reactor yielding a volumetric exchange rate (VER) of 50% and sampling ports for mixed liquor and granular sludge samplings.

A constant aeration was supplied by means of air bubble diffusers similar to Abdullah et al. (2013) through compressed air distribution at a flow rate of 4 L/min to promote the formation of fine bubbles to ensure that the sludge was homogenised. Dissolved oxygen (DO) pattern in the reactor was continuously monitored during aeration period of SBR cycles. The pH and temperature were monitored using a pH meter (Orion 2 Star Benchtop pH). These probes were located at midpoint height of both reactors. Illumination to R_p was provided under constant supply of light/dark cycles of 16/8 h using 3 6 ft Phillips Cool white tubes and 3 6 ft Phillips warm white tubes placed around reactor R_p. Incoming light energy was measured at 70.9 μmol m^2 s^{-1} using a LI-250 Light meter connected to LI-192 Quantum sensor (LI-COR, Lincoln, USA).

The reactors were operated in successive SBR cycles for a continuous operation of 24 h. The operational period and phases of the SBRs in granulation of aerobic granular sludge under phototrophic condition (AGSp) and non-phototrophic condition (AGS_{np}) is shown in Table 1.

2.2. Wastewater preparation

Synthetic wastewater was fed from the bottom of both R_p and R_{np}, respectively. For each cycle, 150 ml of medium organic (O) and nutrient (N) were added to the reactors together with 1200 ml of distilled water. The COD-load with medium was 1.6 g COD/L/d and the COD/N ratio was 8.3. The composition of synthetic wastewater in this study is given in Table 2.

2.3. Experimental procedures

The formation of aerobic granules was closely monitored using scanning electron microscopy (FESEM-Zeiss Supra 35 VPFESEM). Prior to gold sputter coating, the sampled granules were left to dry at room temperature (Biorad Polaron Divisions SM Coating System) as previously explained in Abdullah et al. (2013).

The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were determined according to Standard Methods 2540-E (APHA, 2005). The morphological and structural observations of granules were measured by using an image analysis system (PAX-ITv6, ARC PAX-CAM). Prior to microscopic examinations, the granules were distributed based on sizes using a mesh sieve (0.2, 0.4, and 0.6 mm) to differentiate granules into 3different categories; small, medium, and large size granules.

3. Results and discussion

3.1. Biomass concentrations

In this study, AGS_{np} refers to aerobic granular sludge developed under non-phototrophic conditions while AGS_p are granules cultivated under phototrophic conditions. The biomass concentrations of AGS_{np} was compared with the one of AGS_p. The profiles of biomass concentrations throughout the granulation process for both AGS_p and AGS_{np} are shown in Fig. 1. The initial concentrations of biomass were at 3 gMLSS/L for both types of aerobic granules. After 4 days of reaction in the R_p, an increase of 5.5 gMLSS/L in the biomass concentration was observed for the AGS_p. However,

Table 1
The SBR operations for aerobic granulation of AGS_P and AGS_{np}, respectively.

Bioreactor operation phase	AGS_p	AGS_{np} (Nor-Anuar et al., 2007)
	Time (min)	Time (min)
Aeration	120	110
Settling	5	5
Discharge	5	5
Feeding	10	60
Idle	35–40	0
Total cycles per day	8	8

Table 2
Composition of the synthetic wastewater for cultivation of aerobic granules.

Medium	Composition*	Remarks
Medium O	65.1 mM NaAc 3.7 mM $MgSO_4 \cdot 7H_2O$ 4.8 mM KCl	COD-load = 1.6 g L^{-1} d
Medium N	35.2 mM NH_4Cl 2.2 mM K_2HPO_4 4.4 mM KH_2PO_4	COD/N ratio = 8.3 P-conc = 20 mg
Trace elements (10 ml L^{-1})	1 g $FeSO_4 \cdot 7H_2O$ 3 g $CuSO_4 \cdot 5H_2O$ 3 g $CoCl_2 \cdot 6H_2O$ 16.4 g $CaCl_2 \cdot 2H_2O$ 10.1 g $MnCl_2 \cdot 4H_2O$ 4.5 g $ZnSO_4 \cdot 7H_2O$ 100 g EDTA	L^{-1}
Water	1200 ml distilled water	Total influent 1500 ml per cycle

[*] Source: Nor-Anuar et al. (2007).

for the AGS_{np}, the biomass concentration remained at the same level at ~3 gMLSS/L. After 10 days of reaction, the biomass concentration of AGS_p increased further to 8.8 gMLSS/L. However, the growth of the AGS_{np} was still very low during the first 10 days. Then, the growth took off from about 3.7 gMLSS/L to about 7.1 gMLSS/L at 19 days at a growth rate of 0.4 gMLSS/L.

On day-19 of experiments onwards, the biomass concentration of AGS_{np} continued to grow in slower rate. At this stage, the “window” phase appeared in order to allow biological adjustment of the granulation conditions to ensure successful development of granules as suggested by Lee et al. (2010). From microbiological point of view, window phase is a period of time that allows the viable microorganisms to complement the enzymes for resynthesizes of the essential metabolites prior to microbial exponential growth. Most importantly, under this condition, the stability of the granules for long-term operation was enhanced. Moreover, the slowdown is due to the disintegration of aerobic granules where limitation of oxygen transfer occurred as a result of competition between autotrophic and heterotrophic growth which eventually led to deficiency of denitrification at the core of the granule (Wan et al., 2009). This would presumably caused the breakdown and separation of aerobic granules into smaller fragments.

In addition, the lower biomass production of AGS_{np} compared to the AGS_p could be due to conversion of substrate to CO_2 and H_2O during respiration, in which higher CO_2 production in AGS_{np} culture elucidates the lower biomass yield of aerobic granules as suggested by Liu and Tay (2008).

The disintegrated aerobic granules were removed alongside effluent washout from the R_p system consisting of a small amount of mature granules. Some studies claimed that the disintegrated granules may play a role of nucleus for the new granulation cycle (Lee et al., 2010; Hu et al., 2005). As series of new granulation cycles occur, the disintegrated small size mature granules have undergone a maturing stage in order to allow for the formation and establishment of substances that facilitates the cell-to-cell interactions. In this study, maturing stage can be distinguished within day-10–day-20 of overall experiments. As a result, stable flocs with compact microbial structure were formed at the end of this maturing stage.

On day-26, the biomass concentrations of AGS_p increased sharply to 13.5 gMLSS/L (Fig. 1). At this stage, mature aerobic granules were seen to have supplementary structure formed on the surface of the granules due to new flocs attachment. The morphology of aerobic granules appeared as dark brown colored with an additional structure of yellowish brown floc patches fixed firmly on the granules surface. Over time, all the aerobic granules were transformed into yellowish brown structures, indicating that the facultative anaerobic microorganisms have gradually become inadequate. Thus the aerobic microorganisms dominated the outer part of the aerobic granules. This phenomenon was discovered and well documented by Hu et al. (2005).

In addition, unusually mature AGS_p with sizes larger than 5 mm were unexpectedly discovered from the bed of reactor R_p which was operated under phototrophic conditions, having the capabilities to sustain the structural characteristics even after 90 days of prolonged granulation. Despite the large size, the AGS_p featured an oval asymmetrical-shaped representation with brownish color and a well-defined outer surface. New flocs tend to affix onto the AGS_p, enveloping the outer surface of the mature granules (Fig. SM 1-b). Based on microscopic examinations, the mature

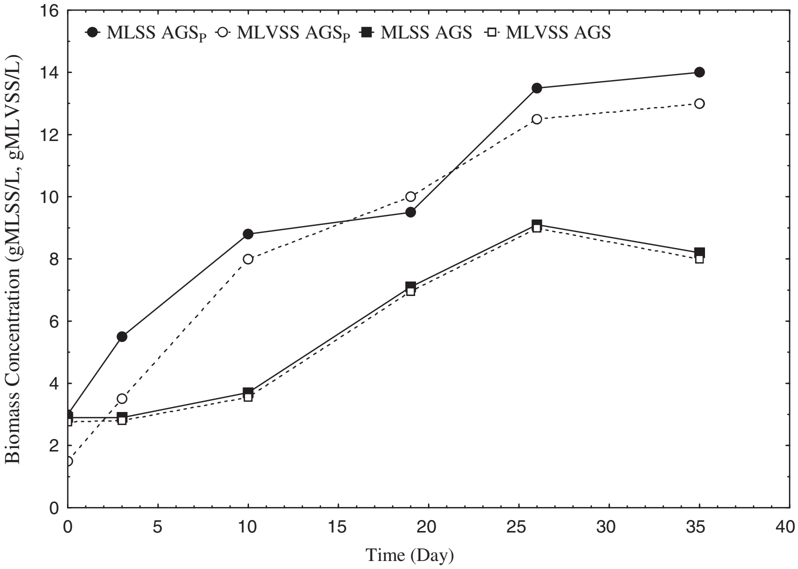

Fig. 1. Biomass concentrations profile of AGS_p and AGS_{np} indicates increasing biomass enrichment throughout the granulation process. AGS_p granules facilitate higher settling ability compared to AGS_{np}. (○) MLSS AGS_P; (●) MLVSS AGS_P; (□) MLSS AGS_{np}; (■) MLVSS AGS_{np}.

AGS$_{np}$ differed by 2 mm when compared to mature AGS$_p$ which reached larger size of up to 5 mm (Fig. SM 1-a), indicative of different morphology and structural characteristics of granules cultivated at different phototrophic conditions. The common sizes for aerobic granules ranges in between 2 mm and 4 mm as previously reported by Liu et al. Besides, the mature AGS$_p$ also favoured further aggregation with newly augmented sludge flocs to attach on the granule surface and formed irregular shape of aerobic granules. Although AGS$_{np}$ was able to expand the granule size to 5.0 mm, the ideal operational size-range for optimal performance and economically effective aerobic granules for SBR treatment should be in between 1.0 mm and 3.0 mm (Toh et al., 2003). Song et al. (2010) suggested that mature aerobic granules can be treated as seed sludge to serve as substratum for new episode of granulation. These granules have been applied and demonstrated good performance in bioremediation such as biodegradation of dye and heavy metals (Muda et al., 2010; Sun et al., 2010, 2008a,b).

A significant decrease of the biomass concentration of AGS$_{np}$ was seen on day-35 of experiments. This was due to granules disintegration, similar to what occurred earlier during the granulation of AGS$_p$. Microscopic and visual observations indicated that mature granules disintegration resulted from cleaving of cracked granules' structure. Cleavage of these granules revealed the internal structure which consisted of a central core of bacterium-encased cavities surrounded by the presence of a large diversity of bacterial morphotypes. Disintegration of granules was observed upon cleaving of granules' structure followed by splitting of the mature granules into smaller fragments (Fig. SM 2). This profile suggests that similar behaviour followed the event as experienced by AGS$_p$ in between day-10 and day-18 of experiments. Related phenomenon has been found in another study by Liu et al. (2012). As the AGS possessed late disintegration phase, this may also suggest that a longer period is needed to develop the granules. This hypothesis was regarded previously by Nor-Anuar et al. (2007), describing that aerobic granular sludge (AGS) had been cultivated within 90 days of granulation.

As for AGS$_p$, the increase of biomass concentration was slowed down again for the second time. Thereafter, no significant change of the granules occurred. At this stage, the aerobic microorganisms began to grow, and the total suspended solids increased and reached the highest value of 14 gMLSS/L. As granulation is a continuous process that involves evolution of suspended flocs to compact aggregates and then to compact granules, it is difficult to determine the turning point when aerobic granules are significantly enlarged. Thus, it was assumed that granulation was completed when clear boundary and regular shape of granules formed as described by Liu et al. (2008).

Overall, all the AGS$_p$ granules formed were in a stable, regular, dense, and compact state in which facilitate good settling ability compared to AGS$_{np}$. Both the MLVSS concentrations of AGS$_p$ and AGS$_{np}$ increased substantially in both bioreactors namely R$_p$ and R$_{np}$, indicating biomass enrichment during sludge granulation, respectively. Based on the biomass concentration monitoring and microscopic observations in this study, a mechanism for the formation of aerobic granules in R$_p$ by acclimatising activated sludge in phototrophic condition may be proposed in the following section as the seed sludge underwent a series of morphological and physical changes.

Initially, the seed sludge developed into "stable flocs" after inoculation. After a while, the number of phototrophic facultative anaerobic bacteria increased and congregated at the interior part of the granules together with the less capable and low oxygen tolerant bacteria and allowed the most capable, high oxygen tolerant and aerobic bacteria to reside on the outer side of the granules. Then the granules tended to disintegrate to form irregular, small flocs and particles. The flocs and particles from the disintegrated granules recombined under aerobic conditions allowing the granules to increase in size, resulting in the formation of mature AGS$_p$ as previously observed by Sturm and Irvine (2008) and Hu et al. (2005). The development of AGS$_{np}$ followed the same manner but a longer time was needed due to various physicochemical circumstances.

The results from this study reveals the possibility to cultivate aerobic granules in SBR under phototrophic condition. Besides that, the granules produced in R$_p$ also provide acceptable and satisfactory outcome over the AGS$_{np}$. The idle phase of 35–40 min applied on the bioreactor configuration during granulation also indicates that longer starvation time is required to form AGS$_p$ with high biomass concentration in the R$_p$. This is in agreement with the studies by Liu and Tay (2004a,b) and Li et al. (2006). With the high biomass concentration over the AGS$_{np}$, it is supposed that the phototrophic facultative anaerobic bacteria have comfortably resided in the inner part of granules. It is obvious that a shorter starvation time leads to the production of lower biomass concentration of AGS$_p$ and the difference of the formation time is also significant, also observed by Pijuan et al. (2009) and Li et al. (2006).

3.2. Size development and distribution of granules

Fig. 2 gives the profiles of size distributions of aerobic granules in both R$_p$ and R$_{np}$, respectively. Both the AGS$_p$ and AGS$_{np}$ were developed from sludge that possessed unstable, loose, and bulky flocs with an average size of less than 0.4 mm. Over time, episodes of granulation were observed for both AGS$_p$ and AGS$_{np}$ throughout the experimental period, and both exhibited similar size distribution schemes. Small size flocs were diminished, and they aggregated each other to form larger size of aerobic granules. The behaviour of the decreasing fraction of the small size flocs (AGS$_p$:AGS$_{np}$; 0.2–0.4 mm) was clearly shown from the size distribution profile as shown in Fig. 2. The decreasing number of flocs was presumably due to the formation of larger granules of between 0.4 and 0.6 mm.

On day-10 of experiments, an increased size of AGS$_{np}$ was observed. Despite the size increment, well-defined AGS$_{np}$ has not formed yet but remained as flocs which featured a net-like loose structure that was easily washed-out, which confirms the observation made earlier by Liu and Tay (2006). Some flocs were larger than the opening gap on the sieve, which has caused them to

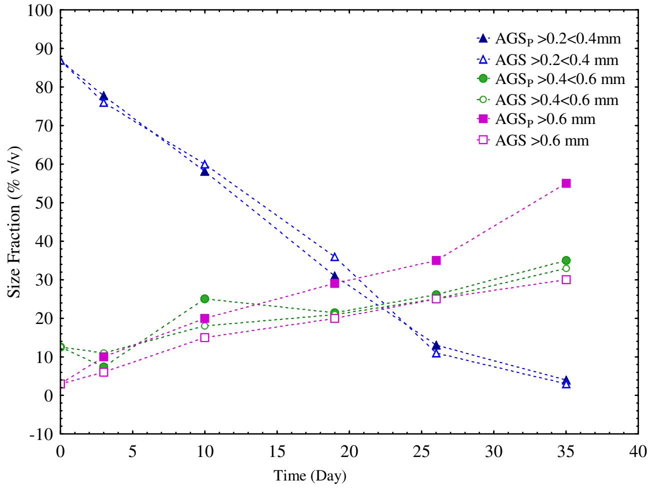

Fig. 2. Aerobic granule size distribution in SBR$_{np}$ and SBR$_p$. (●) AGS$_p$ > 0.4 < 0.6 mm; (○) AGS$_{np}$ > 0.4 < 0.6 mm; (■) AGS$_p$ > 0.6 mm; (□) AGS$_{np}$ > 0.6 mm; (▲) AGS$_p$ > 0.2 < 0.4 mm; (△) AGS$_{np}$ > 0.2 < 0.4 mm.

remain on the sieve. Therefore, the remaining flocs on the sieve were counted for the size distribution determination.

On day-19, the net-like structure flocs were seen to form more stable flocs with smoother external morphology. At this stage, both types of granules uniformly formed in both R$_p$ and R$_{np}$, but the granules' structure remained ill-defined. The size fraction volume showed that AGS$_p$ possessed higher percentage than the AGS$_{np}$ for the granules size >0.6 mm. This was due to longer period needed for AGS$_{np}$ to increase the biomass concentration. Similar phenomenon was also observed by Wang et al. (2008) where concentration of inoculated seed sludge did not show significant change within 24 days for granule sizes of larger than 0.6 mm. However, the biomass concentration showed rapid increase between day-24 and day-45, and stabilised between day-45 and day-70 of experiments.

There was only a small difference of 0.2 mm between the AGS$_p$ and AGS$_{np}$ indicative of not much difference on the size of granules cultivated in both reactors. The existing 0.4–0.6 mm size fraction granules fused with the small size fraction granules (>0.2 < 0.4 mm) to form >0.6 mm granules. As a result of these mechanisms, the net change of the 0.4–0.6 mm size flocs was negligible [Flocs were formed from the smaller sizes, but flocs were also combined with smaller flocs to form the large ones (>0.6 mm)].

Gradually, on day-26 of experiments, the AGS$_p$ size fraction volume continued to increase more and eventually reached over 50% of the total volume for >0.6 mm of granule size. At this point, the AGS$_p$ had around 1.5 times higher size fraction ratio than the AGS$_{np}$. Throughout these experimental observations, an illustrative scheme was developed to show the mechanism of aerobic granule size development as shown in Fig. 3. The proposed scheme gives a simple overview on the phenomenon of aerobic granule size development in order to provide simple concept of aerobic granulation process.

The transformation from bulky seed sludge to AGS$_p$ in the R$_p$ has resulted in granule sizes of between 0.5 and 5 mm. The average granule sizes were between 1.5 and 2 mm. In R$_p$, the granule sizes were between 0.5 mm and 1.2 mm. The average granule sizes were 1–2 mm, which are similar to the AGS$_{np}$ cultivated in previous study conducted by Nor-Anuar et al. (2007). Based on the "theory of sedimentation", the sizes obtained in this study for AGS$_p$ and AGS$_{np}$ are considered as granules. The theory has assigned the minimum diameter of bioparticles, which is defined as granule diameter of at least 0.34 mm (Bhunia and Ghangrekar, 2007).

The sizes of the cultivated granules in this study were in the common range frequently observed for aerobic granules. Zheng and Yu (2007) have categorised aerobic granule sizes into three groups: 0.2–0.6 mm (small), 0.6–0.9 mm (medium), and 0.9–1.5 mm (large). Verawaty et al., 2013 had cultivated small, medium, and large size fractions of aerobic granules with the size of 425 mm, 900 mm, and 1125 mm. In this case, the AGS$_p$ and AGS$_{np}$ can be categorised as large size granules (Zheng and Yu, 2007). In most cases, however, aerobic granules range between as low as 0.02 mm (Liu et al., 2005) to as high as 9.0 mm (Wang et al., 2004). The largest aerobic granules reported were 10 mm in diameter (Liu et al., 2010). In the study, the 10 mm granules were specially developed in order to study the mechanism of stability and disintegration purposes. Therefore, the size range of granular sludge is wider in an SBR system than in a conventional activated sludge system.

Generally, larger size granules are prone to mixing that may cause disintegration and small size granules tend to have low settleability. For those reasons, the significant magnitude of aerobic granulation should not be only restricted on the increase of granule size. In this case, size does not matter. The indication of a successful granulation is preferably based on the compactness and the settling ability of the granules. However, in the cultivation of aerobic granules it is important to keep the aeration rate at a controlled level in order to provide appropriate shear force for mixing for

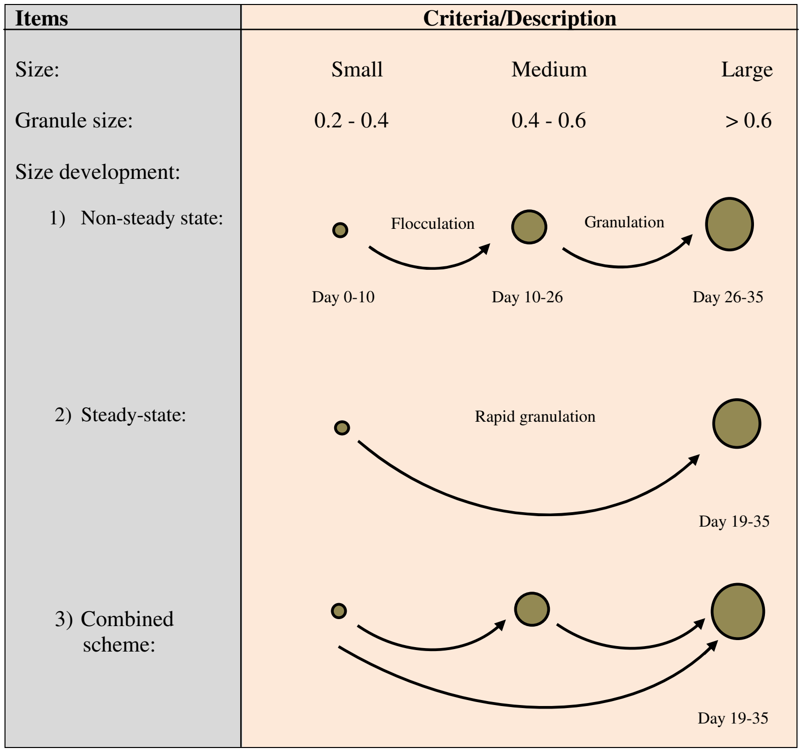

Fig. 3. Proposed aerobic granules size development scheme.

the prevention of the development of too large or too small aerobic granule size (Liu and Tay, 2004a,b). Hu et al. (2005) have suggested that granule size is an eminent factor in designing the physical performance and characteristics of aerobic granules. The limitation of mass transfer and diffusion are influenced by the porosity in the interior part of the granular structures, and increases along with an increase of size and age.

The increase in the size of AGS_P was presumably due to the increase of biomass concentration, which leads to inhabitancy of facultative anaerobic microorganisms that have growth preference in the inner part of granules, and assemblage of minerals forming precipitates that build and strengthen the structure of the granules (Juang et al., 2010; D'Abzac et al., 2010; Tay et al., 2002; Liu et al., 2003). For example, SEM-EDX analysis revealed that solid particles of mineral fractions such as $CaCO_3$ and $CaOH(PO_5)$ are formed and bound within the EPS surrounding the aerobic granules (D'Abzac et al., 2010). Furthermore, the minerals also coalesce with various metallic elements, i.e. Al, Fe, Cu, Mn) to dedicate strong structure to the aerobic granules. Juang et al., 2010 suggested that calcium and/or iron precipitates in the granule interior have enhanced the structural stability of aerobic granules.

4. Conclusion

The granule size development is a key parameter that describes the biomass concentrations during aerobic granulation. This study gives simplified understanding on the formation of aerobic granules based on granule size transformation. Different types of aerobic granules formation follows similar size development scheme despite differing physicochemical conditions. Aerobic granules may increase in size in the same way as recommended in the aerobic granule size development scheme with or without illumination. This study reveals the possibility to cultivate aerobic granules in SBR under phototrophic condition.

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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.2015.01.062.

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