Characteristics of Developed Granules Containing Phototrophic Aerobic Bacteria for Minimizing Carbon Dioxide Emission

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Abstract
Aerobic wastewater treatment has contributed to the greenhouse gases (GHG) emission in the atmosphere, which can cause global warming. The GHG consists of six major gases particularly carbon dioxide (CO_2). Existing biological system of the wastewater treatment needs to be reviewed in order to minimize the emission of GHG especially CO_2. Some findings had shown that photosynthetic bacteria can be employed for CO_2 utilization during the wastewater treatment processes. The aim of this study was to characterize phototrophic microbial granule in order to minimize CO_2. Synthetic wastewater was used throughout this study to obtain the aerobic granules. A 3-L bioreactor phototrophic Sequencing Batch Reactor (SBR_P) was applied to produce phototrophic aerobic granular sludge (AGS_P) and the biomass concentration increased from 3 to $14gL^{-1}$. Such growth has resulted in a maximum settling velocity of $40mh^{-1}$ with granule average size of ~ 2.0mm. The high settling velocity was found to be attributed by the smooth, compact, and regular characteristics of the aerobic granules. High magnification microscopic analysis revealed that AGS_P was dominated by cocci-shaped bacteria embedded within the extracellular polymeric substances (EPS). Detailed observation on the structure of the AGS_P showed the presence of 30µm of cavity to allow nutrients and gas exchanges within the aerobic granule. Scanning Electron Microscope-Energy-Dispersive X-ray (SEM-EDX) examination showed AGS_P composed of different types of inorganic and organic compounds. AGS_P achieved 92% of CO_2 reduction and 84% of chemical oxygen demand (COD) removal.

Keywords: Characterization, carbon dioxide, phototrophic aerobic granular sludge (AGSp), Scanning electron microscope - energy-dispersive X-ray spectroscopy (SEM-EDX)

Introduction
The early discovery of global warming was first reported in 1896 by Svante Arrhenius, a Swedish physicist who came to the theory that carbon dioxide (CO_2) contributes to the greenhouse effect and long term climate change (Farrah, 2013). Currently, global warming is the most highlighted subjects in the environmental issues. This climate change is normally related to greenhouse gases (GHG) emissions from anthropogenic activities like the combustion of fossil fuel used for power generation, transportation, and industrial processes (Chen, 2008). Inefficient wastewater treatment results in excessive production of carbon dioxide (CO_2) and methane (CH_4) continuously which will over time period contribute to global warming.

Although the contributing effect of the CO_2 released from wastewater is considered as natural process and only 3.4% of total global anthropogenic GHG emissions (Bogner et al., 2008; Fytili and Zabaniotou, 2008), with higher population to be served by modern wastewater treatment plants, the increasing trend of CO_2 emissions may intensify the future atmospheric CO_2 concentration more and may affect future global warming trend, which has been speculated to rise up to year 2030 (Akashi et al., 2010). Apparently, immediate response to minimise CO_2

emissions from wastewater treatment is required to counter the challenge of future global warming. Various approaches have been studied and implemented recently to minimize GHG emissions (Jacob-Lopes et al., 2010; Morath, 2010; Jarvis et al., 2009; Rosso and Stenstrom, 2008). Hence, proper wastewater treatment process that can minimize CO_2 emissions into the atmosphere is needed.

Aerobic granulation technology is a promising aerobic wastewater treatment system that can overcome the limitations in conventional activated sludge system. This technology provides an excellent characteristic of sludge in granular form with compact structure, good settling rate, high biomass concentration, and simultaneous removal of organic matter, nitrogen and phosphorus Sue et al., 2012). Aerobic granules cultivated via the aerobic granulation process is a form of biofilm in which various microorganisms including photosynthetic bacteria adhere to each other (self-immobilisation) to form a compact structure, which is good for solid-liquid separation in wastewater.

The role of photosynthetic bacteria in the aerobic granules is important for the CO_2 sequestration within the microsystem of wastewater. This will minimize the emission of CO_2 to the environment. Microbial communities in aerobic granules have been shown to be highly distinct from activated sludge, even within a single reactor system. Recent studies imply the importance of gaining an understanding of the functions of microbial communities, as population diversity alone may not be adequate in determining the microbial characteristics. This study focuses on the characterizations of photosynthetic microbial granules developed to minimize the CO_2 emission. The presence of photosynthetic bacteria was determined, the change in morphologies, biomass concentration and settling properties were investigated during growth of the bacteria.

Methodology

Experimental set-up

The bio-granulation of activated sludge was performed in a three litre transparent double-jacketed laboratory scale bioreactor specifically designed to be operated for 24 hour continuously as illustrated in Figure 1. The temperature was kept at 30°C by circulating water from a thermostat water bath around the bioreactor. The pH was not controlled but monitored as indicator of photosynthetic reaction. Air was supplied through immersed diffuser from the air pump and maintained at ± 4L/min. The shape of bioreactor was customized to be slender to allow maximum aeration surface. The feeding of nutrients was supplied at the bottom of the reactor to allow maximum nutrient distribution. The effluent discharge point was designed at midpoint of the vertical length of the bioreactor to collect the supernatant of treated wastewater. Sampling point was set to be at the upper level in order to collect mixed sludge during aeration. Headspace gas was trapped and connected to the CO_2 detector mounted on the bioreactor.

The bioreactor was operated as an SBR configured with a complete cycle length of three hour and each cycle consist of 5 process operation phases as shown in Table 1 and Figure 2, which can be categorized into two metabolic phases (aerobic and anaerobic). Digital timers programmed according to appropriate period control the configuration of operation phases as shown in Table 1. The bioreactor was also provided with 9.9 W/m^3 light (12 hour diurnal variation) source purposely for the cultivation of FP-AGS.

Table 1 Operation phases for biogranulation process

Description	Duration (min)	Time (min)	Aeration	Metabolic Phase
Aeration	65	0-65	On	Aerobe
Settling	5	65-70	Off	Aerobe
Discharge	5	70-75	Off	Aerobe
Feeding	10	75-85	Off	Aerobe
Idle	95	85-180	Off	Anaerobic

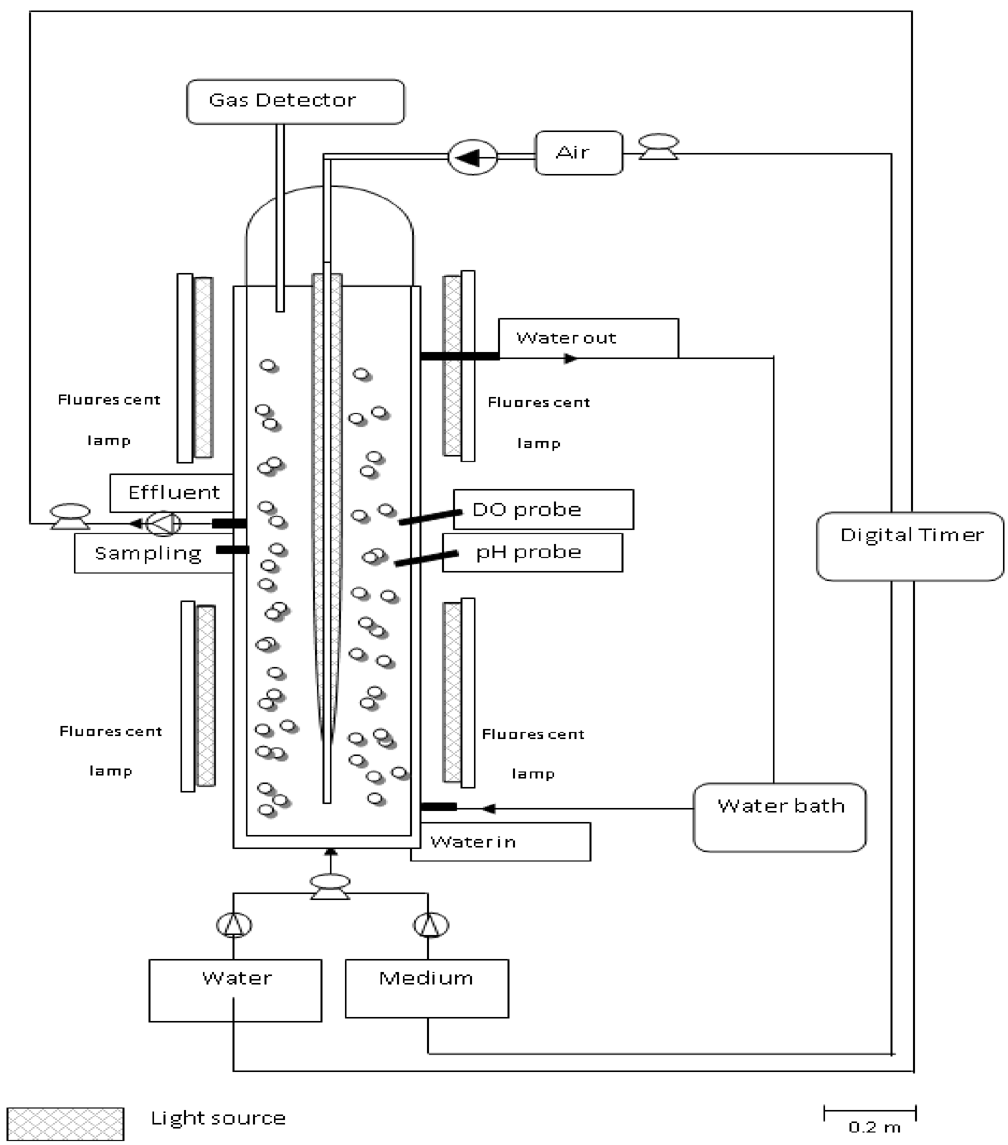


Figure 1 Schematic diagram of bioreactor used for biogranulation process

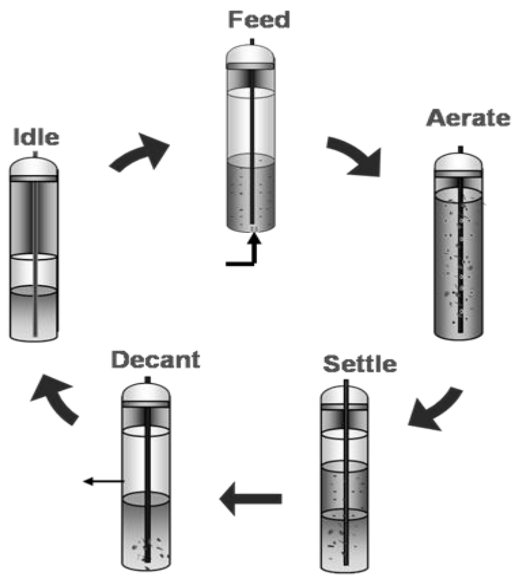


Figure 2 The routine operation phases in the bioreactor

Feeding

Synthetic wastewater was used as nutrients feeding for the biogranulation process. It was shown in a previous study that AGS development was very good in synthetic wastewater compared to actual wastewater (Nor-Anuar *et al*., 2007). In every feed, 150ml of medium organic (O) and nutrient (N) were added to the reactor together with 1200 ml of distilled water. The chemical composition of synthetic wastewater used is as shown in **Table 2**. Aerobic seed sludge obtained from an aeration tank of a local municipal wastewater treatment plant at Bandar Baru Kulai, Johor, Malaysia which was operated with an anaerobic-aerobic biological nutrient removal configuration. For the start-up batch, approximately 1.5L of seed sludge was used resulting in initial mixed liquor suspended solids (MLSS) of ±3000 mgL-1 in the bioreactor.

Table 2. Composition of the synthetic wastewater

Medium	**Composition**
Medium O	65.1 mM CH3COONa
	3.7mM $MgSO_4.7H_2O$
	4.8 mM KCl
	35.2 mM NH_4Cl
Medium N	2.2 mM K_2HPO_4
	4.4 mM KH_2PO_4
	10 ml L^{-1} Trace Element solution
Water	1200 ml distilled water

Analytical Methods

Measurement of mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) were performed using standard methods for the examination of water and wastewater (APHA, 2005). The settling velocity was measured by recording the time consumed for individual granule to reach the bottom from a 30cm height of a measuring cylinder. Light microscope (Leica BM E, Germany) and Stereo microscope (Leica M165 C, Germany) equipped with PAX-IT Image Analysis Software (U.S.A) was used to obtain digital images of peripheral morphology and size of granules. For SEM analysis, granule was fixed with 2.5% (w/v) glutaraldehyde in phosphate buffer for 2 hour before dehydrated through a fraction series of water-ethanol solution (10%, 25%, 50%, 75%, 90%,100%) and subjected to critical drying. Dried granule was then sputter-coated with gold particles before analyzed under SEM. The mineral composition analysis was performed by scanning electron microscope coupled with energy disperse X-ray spectroscopy (SEM-EDX) (Zeiss Supra 35VP, Germany). Meanwhile, throughout the biogranulation process the CO_2 biofixation rate will be quantified using CO_2 sensor (Air Quality Meter, EA80) attached to the headspace of the bioreactor in order to verify the ability of photosynthesis aerobic granules to absorb CO_2 in wastewater.

Results and Discussion

Characteristics phototrophic aerobic granular sludge of AGS$_P$

In this study, aerobic granules were successfully cultivated under phototrophic conditions. The granules developed in this study was called phototrophic aerobic granular sludge (AGS$_P$). The use of the term "*photosynthetic*" is based on the presence of photosynthetic bacterial community that occurred in the cultivated aerobic granules. The presence of the photosynthetic bacteria was confirmed at the end of this experiment by using molecular biology method. The confirmation of the existence of this bacterial community is very important to validate the existence of photosynthesis for carbon sequestration process in the bioreactor system. This is because all photosynthetic bacteria are phototrophic, but not all phototrophic bacteria are photosynthetic as observed by Bryant and Frigaard (2006). The granules cultivated from the SBR$_P$ are as illustrated in Figure 3.

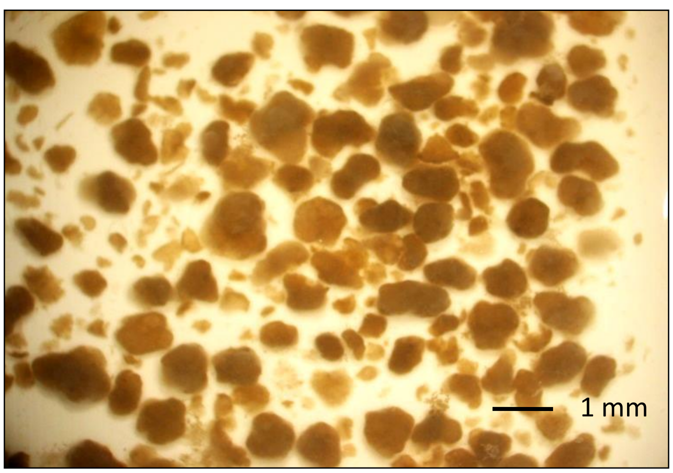


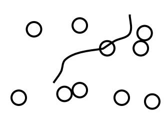
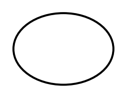
Figure 3. The AGS$_P$ developed in this study

The MLSS obtained from an extended aeration activated sludge plant was 3 gL^{-1} to start the operation of the bioreactors. In extended aeration system, a typical low value of MLSS ranges between 3 - 6 gL^{-1} (Karia and Christian, 2006; Peavy *et al*., 1985). Typically, extended aeration system process in wastewater treatment is developed for the minimisation of excessive sludge disposals and eliminatation of primary sedimentation treatment by maintaining low concentration of MLSS (Wang *et al*., 2004b; Weiner and Matthews, 2003). In extended aeration system, the low biomass concentration is due to endogenous decay of the sludge mass, where sludge biomass is enhanced through low food/high microorganisms ratio (F/M ratio) (0.2 kg BOD_5/kg VSS d) (Metcalf and Eddy, 2003) in order to maximise substrate consumption by microorganisms during aerobic starvation phase (Lin and Lee, 2007).

In SBR system applied in the present study, sludge granulation has exponentially increased in term of biomass concentration of the seed sludge from the extended aeration activated sludge plant to form AGS and AGS_P. During the SBR operation, a higher F/M ratio was induced in the feeding phase (0.5 kg COD/kg VSS d) to promote microbial growth as claimed by Aznah *et al*., (2008).

It was suggested that application of higher F/M ratio in wastewater treatment system is favourable for the growth of floc-forming bacteria (Wang *et al*., 2009b). Significantly, the increased F/M ratio applied in both AGS and AGS_P has resulted in flocculation of seed sludge to form aerobic granules due to log-phase growth of microorganisms. Additionally, the idle period applied for the formation of AGS_P has resulted in higher biomass concentration compared to the AGS. Hence, the growth state of the seed sludge, AGS, and AGS_P during the development period of the granules were determined as highlighted in **Table 3**.

Table 3. Growth stages of seed sludge, AGS_P

Wastewater treatment	**Sample from extended aeration activated sludge**	**SBR**
Characteristics	Seed sludge	AGS_P
Growth phase	Endogenous	Log-growth
SRT	High	High
F/M ratio	High	High → Low
Morphology		

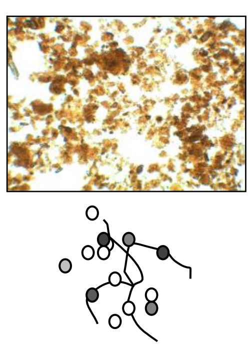
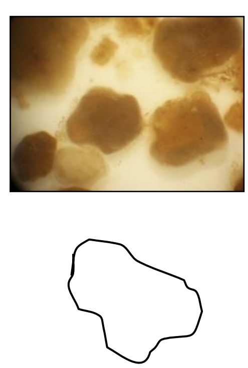
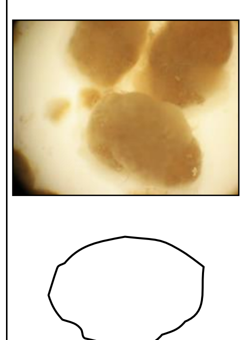
Previously, it was determined that aerobic granulation system could operate at wide range of F/M ratio (0.5 - 2.4 kg COD/kg VSS d) (Thanh *et al*., 2009). It is a practice that F/M ratio is not taken as a critical parameter for the formation of aerobic granules. Significantly, aerobic granulation involves the association of various factors that have been discussed in Section 2.4.4. The inter-relation of these factors has significantly contributed to the compact structure of aerobic granules.

In the present study, after over one month of bioreactor operation, AGS_P with an average size of 1.5 mm and biomass concentration of 15 gMLSS/L was formed in the bioreactor. The physical characteristics of the AGS_P and AGS obtained are presented in Table 4.3. Throughout the process, the physical morphology of seed sludge has changed gradually from flocs to granules. The evolution of seed sludge from flocs to granules was achieved due to the interactions between interparticle bridging under a condition of turbulent flow mixing (Sunil *et al*., 2008). In between the mixing, initiation of a starvation phase that involved an episode of lack of oxygen in the system has enhanced the interactions of negatively-charged particles for granulation (Li *et al*., 2006). Throughout the present study, not only changes discovered on the shape and size, the colour of granular sludge also changed from dark brown to yellowish brown.

In term of structural stability, seed sludge possessed irregular loose structures and were not stable. The flocs were easily broken up into small pieces when exposed to mixing or shear force during aeration at the initial stage of granulation. Subsequently, a large part of the biomass was washed out and left a small volume of dense biomass in the bioreactor. A settling period of 30 min was applied during the initial seven days to prevent washout of the biomass from the bioreactor as suggested by Liu and Tay (2007). From day-7 to day-14, the settling time was kept at 15 min, and from day-14 until the end of the granulation, the settling time was kept at five min in order to provide short HRT (Nor-Anuar *et al*., 2007). Therefore, this will accumulate the aerobic granules with high settling velocity. When the washout biomass fraction had been recycled into the reactor, it aggregated with the retained biomass and increased the SVI. A new granulation eventually occurred at this stage. The routine of recycling the washout fraction into the reactor was carried out continuously until the steady state of granulation had been achieved. At this stage, a large number of particles with large diameter were present. Mature granules were observed after 35 days of granulation.

The results obtained from the study showed that it is possible to obtain aerobic mature granular sludge in the SBR after only 35 days of operation. In most studies, aerobic granules were harvested at longer period: 60 days (Yu et al., 2010; Hu et al., 2005), 78 days (Bao et al., 2009), 80 days (Adav et al., 2009), and 90 days (Quan et al., 2010; Nor-Anuar et al., 2007). The longest harvest period for aerobic granules reported so far is 450 days (Wang et al., 2010). The fastest aerobic granules cultivated is 14 days (Li et al., 2005). Recently, Xu et al. (2010) have successfully obtained aerobic granules within 25 days of granulation. Technically, by controlling the bioreactor condition, assorted forms of aerobic granules can be produced under different structural features and hydrodynamic properties (Xiao et al., 2008).

Table 4. Physical characteristics of AGS_P and AGS developed at 30°C using synthetic wastewater compared with seed sludge

Characteristics	**Seed sludge**	**AGS**	**AGS_P**
MLSS (g/L)	3	8.2	14
MLVSS (g/L)	1	8	13
Diameter (mm), ø	< 0.2	0.5 - 1.0	0.5 - 1.3
Average Size (mm), ø Size Fractions (%): <0.2 0.2-0.4 0.4-0.6	< 0.2 10 8.5 6	1.1 - 2.0 13 6 16	1.5 - 2.0 35 29.4 30.4
Settling velocity (mh^{-1})	5	6 - 34	18 - 40
Density ($gTSSL^{-1}$), ρ	N.D	135	151
SVI (mLg^{-1})	190	38	26
Shape	Dispersed 	Irregular 	Spherical 

Biomass Concentration

Monitoring of biomass accumulation throughout the sludge granulation is important to ensure any increase in the biomass concentration. In this study, the biomass concentration of AGS_P was compared with the one of AGS. The profiles of biomass concentration throughout the granulation process for both AGS_P and AGS are shown in Figure 4.

The initial concentrations of biomass were at 3.0 gMLSS/L for both types of aerobic granules. After four days of reaction in the SBR_P, an increase of 5.5 gMLSS/L in the biomass concentration was observed for the AGS_P. However, for the AGS, the biomass concentration remained at the same level at ~3 gMLSS/L. After ten days of reaction, the biomass concentration of AGS_P increased further to 8.8 gMLSS/L. However, the growth of the AGS was still very low during the first 10 days. After that, the growth took off from about 3.7 gMLSS/L to about 7.1 gMLSS/L at 19 days with a growth rate of 0.4 gMLSS/L.

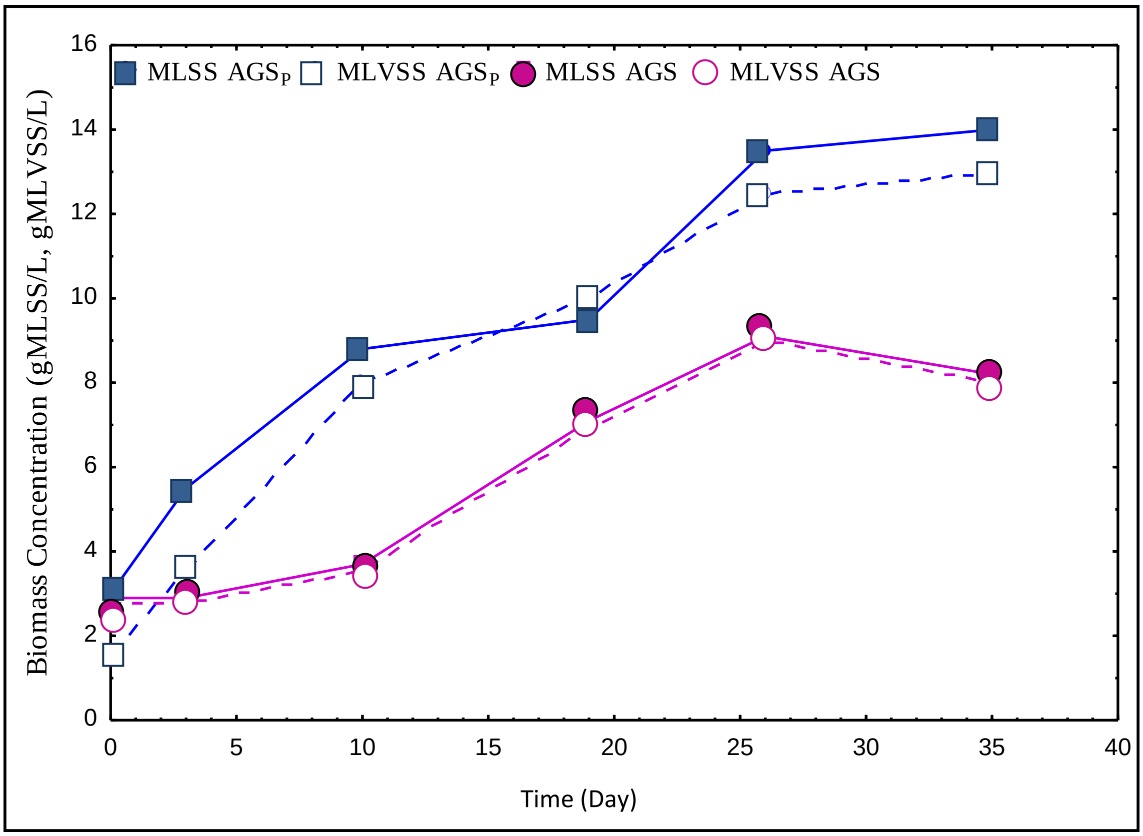


Figure 4. Biomass concentrations throughout the granulation process of AGS_P

On day-19, the biomass concentration of AGS 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). Most importantly, under this condition, the stability of the granules for long-term operation was enhanced. Moreover, the slowdown due to the disintegration of aerobic granules where limitation of oxygen transfer occurred as a result of competition between autotrophic and heterotrophic growth as suggested by Wan *et al*. (2009). This would have caused the breakdown and separation of aerobic granules into smaller pieces. In addition, the lower biomass production of AGS compared to the AGS_P was significantly due to conversion of substrate to CO_2 and H_2O during respiration, where higher CO_2 production in AGS culture elucidates the lower biomass yield of aerobic granules as suggested by Liu (2008). Consequently, it was found that AGS produced higher CO_2 concentration than the AGS_P as discussed in Section 4.6.2.

The disintegrated aerobic granules were inclined to be removed via washout from the SBR_P system and only smaller portion of heavier and smaller mature granules was discarded. 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; Liu *et al*., 2004b; Hulshoff *et al*., 2004). 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 facilitate the cell-to-cell interaction to take place. Significantly, this phase can be distinguished within day-10 to day-20. As a result, stable flocs with compact microbial structure were formed at the end of this maturing stage.

On day-26, the biomass concentration of AGS_P had increased sharply to 13.5 gMLSS/L. 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 aerobic granules morphology appeared as dark brown with an additional structure of yellowish brown flocs patches fixed firmly on the granules surface. Over time, all the aerobic granules were transformed into yellowish brown structure, indicating that the facultative anaerobic microorganisms gradually became inadequate. Thus the aerobic microorganisms dominated at the outer part of the aerobic granules. This phenomenon was discovered and well documented by Hu *et al*. (2005).

A significant decrease of the biomass concentration of AGS was seen on day-35 of granulation. This was due to granules disintegration, similar to what occurred earlier during the granulation of AGS_P. This profile suggests that the same behaviour followed the event as experienced by AGS_P in between day-10 to day-18. 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 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 the 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 suggested 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. Both the MLVSS concentrations of AGS_P and AGS increased substantially in both bioreactors: SBR_P and CAgsBio, indicating biomass enrichment during sludge granulation. Based on the biomass concentration monitoring and microscopic observations done in this study, a mechanism for the formation of aerobic granules in SBR_P by acclimatising activated sludge in phototrophic condition may be proposed in the following section. 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. Then the flocs and particles from the disintegrated granules recombined under aerobic conditions, and finally the granules grew larger, resulting in the formation of mature AGS_P as also observed by Sturm *et al*., (2008) and Hu *et al*., (2005). The development of AGS followed the same manner but a longer time was needed due to various physicochemical circumstances.

The results from the present study revealed clearly that it is possible to cultivate aerobic granules in SBR under phototrophic condition. Besides that, the granules produced also provide acceptable and satisfactory outcome over the AGS. The idle phase of 35 to 40 minutes 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 $SBR_{P.}$ This is in agreement with the studies by Liu and Tay (2008) and Li *et al*., (2006). With the high biomass concentration over the AGS, it is supposed that the phototrophic facultative anaerobic bacteria have comfortably resided in the inner part of granules (de-Kreuk, 2006). It is obvious that a shorter starvation time leads to the production of lower biomass concentration of AGS and the difference of the formation time is also significant, also observed by Pijuan *et al*., (2009) and Li *et al*., (2006).

Settling Velocity of the Granules

Improvement of sludge settling velocity in wastewater treatment is important in preventing sludge bulking (Janczukowicz *et al*., 2001). Compared to a conventional activated sludge system, aerobic granulation technology offers aerobic granules with excellent settleability and compact microbial structure.

Different environmental conditions, particularly different phototrophic conditions, produced significantly different settling behaviours of aerobic granules in the two SBRs; SBR_P and CAgsBio. After over one month of granulation, the study on settling velocity indicated that floc particles and granules settled at rates ranging from less than 5 m/h to greater than 70 m/h due to the increasing biomass concentration and granule sizes as discussed previously in Sections 4.5.1 and 4.5.2. The average settling velocities of the AGS_P and AGS are 23 and 19 m/h, respectively. The density obtained for AGS was lower than the AGS_P with 135 $gTSSL^{-1}$. The compactness of the granule showed a density of 151 $gTSSL^{-1}$, which is higher than the AGS. The settling behaviours of AGS_P and AGS are presented in **Figure 5**.

During the experiment, the settling velocity of the AGSP increased from 21 to 75 m/h. Meanwhile, the settling velocity of the AGS increased from 20 to 40 m/h, which is 1.9 times smaller than the settling velocity of the AGSP. The high settling velocity possessed by the developed AGSP enabled the granules to avoid from being washed out from the SBRP during the decanting phase. Such conditions have caused more AGSP to remain in the reactor as a result of good settling properties.

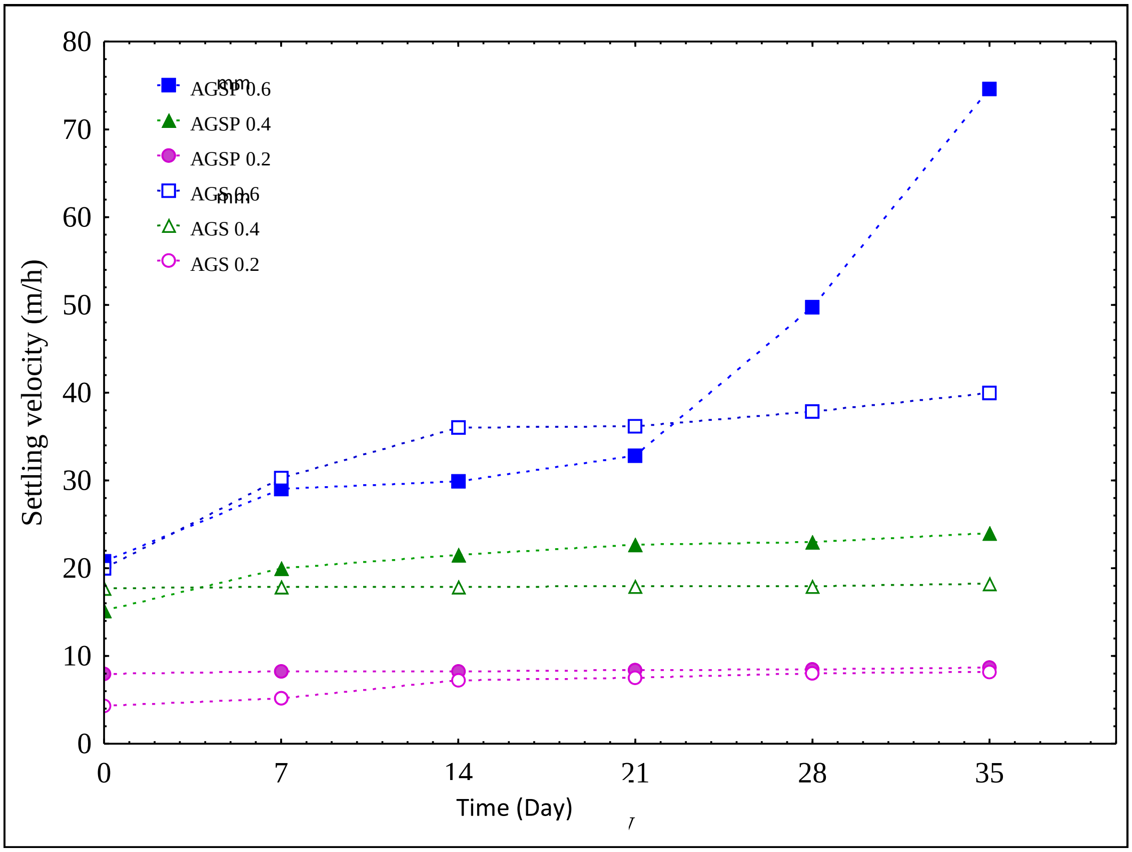


Figure 4.9 The settling velocity of AGS_P and AGS

The settling velocity obtained as shown in Table 5 in the present study was almost 1.5 times greater than the settling velocity of aerobic granules reported by Juang *et al*. (2010). Variability in granule size, settling velocity, and granulation period were observed. This showed that one granule size may be different from another when exposed to different environment and operations. This is because granulation is influenced by reactor design and operating conditions such as seed sludge, substrate composition, organic loading rate, feeding strategy, hydrodynamics, settling time, exchange ratio and aeration intensity (Wang *et al*., 2004a; Li *et al*., 2006; de-Kreuk *et al*., 2007; Yuan *et al*., 2008; Adav *et al*., 2009; Lee *et al*., 2010; Juang *et al*., 2010).

Significantly, the increase of settling velocity is also related to the changes in the SVI value in SBR_P and CAgsBio. Figure 4.10 shows the SVI profile of AGS_P and AGS together with settling velocity. The SVI value of AGS_P improved from 72 to 29 mg/L within 35 days of experiment signifying the good settling properties of the granules which is an important criteria in wastewater treatment plant operation.

For the granulation of AGS, the reactor operation was conducted based on the study by Nor-Anuar et al. (2007). In the present study, between day-3 and day-10, the SVI value of AGS increased, indicating the release of small particles from granules of all sizes in the reactor. A similar observation was also made by Pijuan et al. (2009). The SVI value of AGS decreased irregularly along the period of the study. This was due to the reactor operation cycle in CAgSBio, where a short starvation phase was included in the cultivation process of AGS. If the starvation period is made too short, the granules are less efficient in performing biochemical metabolisms and in maintaining their morphological structure as also observed by Pijuan et al. (2009). In addition, Li et al. (2006) have suggested that the starvation phase is very important in the initial phase of aerobic granulation prior to the shear force reactions.

Table 5. Settling velocity obtained in this study and other studies

Reference	Type of wastewaters	Average granules size (mm)	Average settling velocity (mh^{-1})	Granulation (days)
Beun *et al*. (1999)	Domestic	1.9 - 4.6	12 - 24	70
Toh *et al*. (2003)	Domestic	0.2 - 5.0	36 - 81	21
Kong *et al*. (2009)	Domestic	1.7	39 - 42	91
Lee *et al*. (2010)	Domestic	2.3	50	60
Juang *et al*. (2010)	Domestic	1.9 - 2.3	14 - 15	216
Nor-Anuar *et al*. (2008)	Domestic	1.1	10 - 30	90
This study:				
AGS	Domestic	1.0 - 2.0	18 - 24	35
AGS_P	Domestic	1.5 - 2.0	22 - 75	35

The higher settling velocity and lower SVI value of the mature AGS_P as compared to previous reports by other studies indicate that the formation of the granules under phototrophic condition with longer starvation period would favour better settling properties of the granules. The cultivated AGS_P in the present study also appeared to be more compact in structure with smoother outer surface than the AGS.

Extracellular polymeric substances (EPS)

In this study, observation via SEM on mature AGS_P showed domination of cocci-shaped bacteria (size ~ 200 nm) that are tightly linked and embedded via cohesive structure, i.e. EPS as shown in Figure 4.19. Significantly, distribution of EPS over the entire aerobic granules is very important (D'Abzac *et al*., 2010). It was found that EPS comprises of α-amylase and β-amylase, which are the main polysaccharides supply for bacterial growth during starvation, where poysaccharides hydrolisis was found to occur at the edge of the cavities that are connected to each other via a biomass wall, which eventually live a hollow core within the aerobic granules as a result of nutrient deficiency (Lee *et al*., 2008).

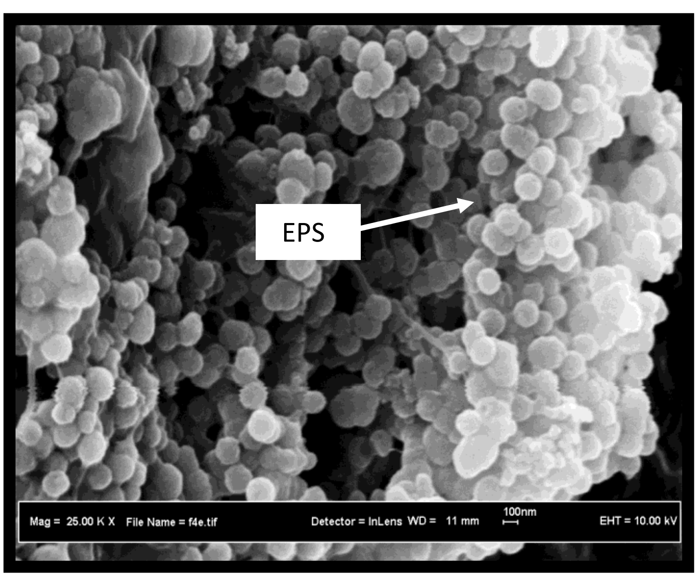


Figure 6. Domination of cocci shaped bacteria observed on AGS_P via SEM examination. Detailed inspection on the bacterial colonies revealed that the bacteria are embedded through a mesh of EPS filaments of alginate-like gel and clustered within the EPS sheath (Magnifications: 25,000x)

From the aerobic granulation point of view, EPS is the key element to flocculation and granulation by structuring and consolidating not only bacteria to bacteria but also bacteria to inorganic or organic matter (Hulshoff et al., 2004). EPS also can provide protective shield to the microbial cells against shock loading and toxic compound in wastewater (Tay et al., 2005).

Apparently, from the microbiological perspective, the microbial communities in both AGS_P and AGS successfully allocated themselves for maximum accessibility towards nutrients, which helped them to increase survivality and stability. However, from the aerobic granulation point of view, AGS_P exhibited better granular structure compared to the AGS. Therefore, this observation inteprets the better performance of AGS_P in previous study, i.e. granule size development and settling velocity.

The Elemental Composition of AGS_P

The morphology of an aerobic granule is significantly associated with the accumulation of organic or inorganic compounds by the microorganisms in the aerobic granules. Material analysis performed by SEM-EDX showed there are differences in the composition of inorganic and organic compounds in AGS_P and AGS. Table 4.7 displays the mineral content of AGS_P and AGS analysed by SEM-EDX.

Table 6. Composition of inorganic and organic element of AGS_P and AGS

Elements	**Composition, Wt %**
Carbon, C	63.0
Oxygen, O	15.0 – 16.0
Magnesium, Mg	4.0
Sodium, Na	0.7 – 1.2
Aluminium, Al	0 – 1.0
Silicon, Si	0 – 1.0
Chlorine, Cl	4.0 – 5.0
Potassium, K	8.0 – 10.0
Calcium, Ca	2.0 – 3.0
Iron, Fe	ND

*ND – Not detected

Many different inorganic cations were present in both types of aerobic granules. The concentration differed from one granule to another depending on how the seed sludge was treated. Concentrations of all elements present on the granules also depended on the association of one genus to another and the physicochemical conditions. In this study, the physicochemical environment of biogranulation in term of light intensity was found to strongly affect the concentration of the mineral contents in the AGS_P.

Minerals attachment on aerobic granules surfaces is highly dependent on surface area, surface potential and surface energy (Piirtola et al., 1999). The presence of minerals can be seen by the formation of precipitates. Calcium may form calcium salts Ca(PO4)2, Ca4H(PO4)3, and

Ca5(PO4)3(OH). Other minerals form various precipitates such as ferrihydrite (ferric oxyhydroxide), goethite (α-FeO(OH)), hematite (Fe2O3), jarosite (KFe3(OH6)(SO4)2), lepidocrocrite (γ-FeO(OH), magnethite (Fe2O3) and strengite (hydrated iron phosphate) (Juang et al., 2010).

From the SEM-EDX analysis in the present study, ten types of elements were detected. They were identified as, carbon (C), oxygen (O), magnesium (Mg), sodium (Na), aluminium (Al), silicone (Si), chlorine (Cl), potassium (K), calcium (Ca), and iron (Fe). Ca2+, K+, and Mg2+ were found to be higher in the AGSP compared to the AGS. These elements are highly associated with the EPS matrix composition. According to a study conducted by Piirtola et al., (1999), high settleability of activated sludge often relates to high Ca/Mg-ratio in mineral materials, which increases the ratio of monovalent to divalent cations. This confirms their role in assemblage and maintenance of the granule structure and density of the granules. The structural integrity of granules is highly due to EPS that binds inorganic ions for bridging of organic compounds to give strong and dense characteristics (Flemming et al., 2001).

Ca2+ is induced by EPS through cation binding and it provides mineral nucleation sites (Dupraz et al., 2009). Ca2+ is essential in forming the backbone and it makes the architecture structure of aerobic granules (Ren et al., 2008). Ca2+ binds to proteins, and this makes bacteria adhere to surfaces.

Carbon dioxide (CO_2) biofixation reduction

At normal condition, the concentration of CO_2 in ambient air is approximately at 400 ppm (Sidek *et al*., 2009). However, in immobilised form of bacteria in wastewater, there are many growth environmental factors especially CO_2, which is often the factor limiting anaerobic bacterial growth due to the 3D structure of aerobic granules. Kaya *et al*. (1996) suggested that high concentration of CO_2 (1500 ppm) is favorable for strict anaerobic growth. Since the development of aerobic granules involves intermittent anaerobic and aerobic growth, a feasible CO_2 concentration, which was lower than 1500 ppm was provided in the bioreactors to avoid detrimental effect on the aerobic bacterial growth. Hence, a CO_2 concentration of 500 ppm was decided to be applied in the bioreactors system.

In the present study, as shown in Figure 7, when air was supplied with the concentration of CO_2:500 ppm, AGS_P has constantly maintained the CO_2 concentration at ~ 495 ppm during the aeration phase. However, after the termination of aeration process, the CO_2 concentration has subsequently decreased to ~ 490 ppm and dropped drastically to ~ 460 ppm after 85 minutes of anaerobic phase. Then, the CO_2 concentration remained at ~ 460 ppm until the end of the experiment as observed in Figure 7. Additionally, an extra of 40 min was extended at the end of each experiment in order to observe feasible changes in the CO_2 concentration.

Based on present investigation in this study, the declining profile of the CO2 concentration indicated the consequence of photosynthetic bacteria metabolism, which occured in the AGSP. Significantly, the genetic information of a number of photosynthetic bacteria was succesfully obtained in Section 4.5. Moreover, formation of calcium carbonate precipitates was also detected previously in Figure 4.27. Hence, it is suggested that the decline in CO2 concentration produced by AGSP was a result of increasing CO2 biofixation carried out by photosynthetic bacteria that immobilised in the aerobic granules. Noticeably, previous studies have shown that CO2 biofixation process can be visibly presented by the products of photosynthetic metabolism, i.e. formation of EPS, precipitates of chemical species such as carbonates and bicarbonates (Jacob-Lopes et al., 2008a; Jacob-Lopes et al., 2008b; Lee et al., 2006).

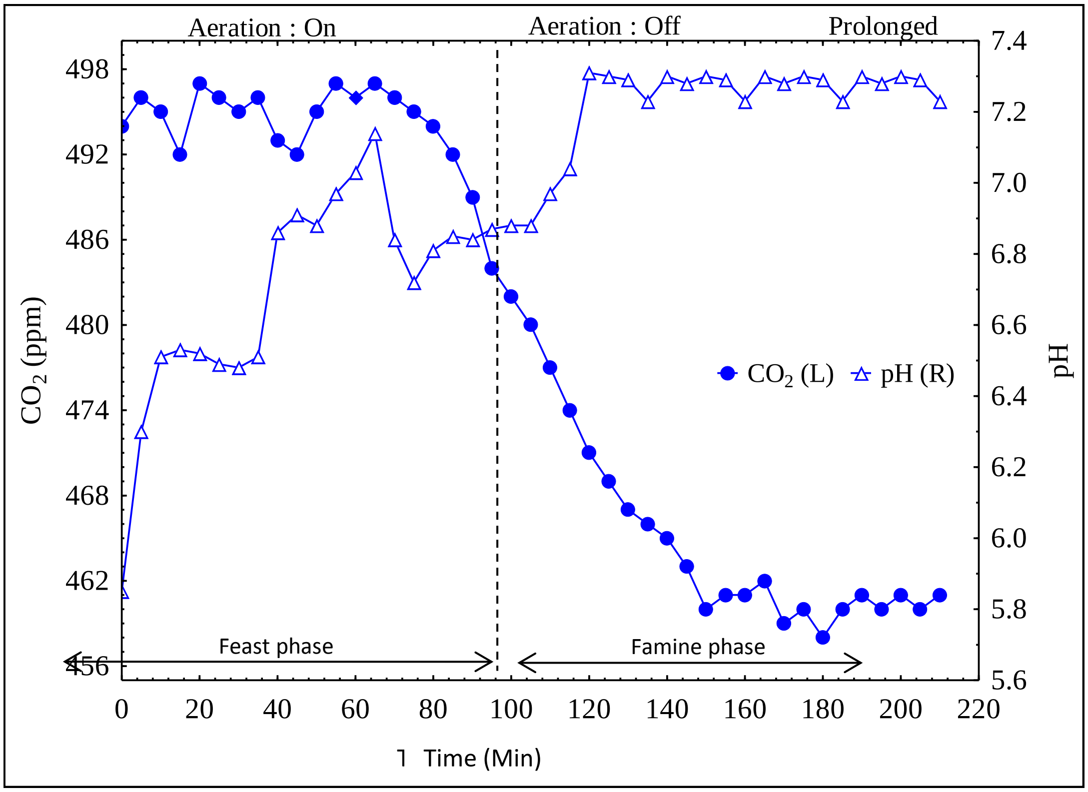


Figure 7. CO_2 production profile by AGS_P

In the present study, it was also determined that the pH value in the SBR_P has increased throughout the treatment. The changes of the pH value was a result of completion of nitrification process as suggested by Guo *et al*. (2009). However, when photosynthetic bacterial metabolism takes into account, the significant changes of pH value is highly due to production of hydroxide (H^+) catalysed by carbon anhydrase enzyme in the bacterial cell during bioconversion of bicarbonate ions to supply CO_2 for photosynthetic reaction as described by Jacob-Lopes *et al*. (2008). The potential mechanism occurred in the photosynthetic reaction that increased the pH value in the present study was also due to the activity of RubisCo enzyme as suggested by Zuber (1986). The enzyme activity is pH-dependent, in which the activity increased with the increase in pH as shown by Zuber (1986).

For quantification of CO_2 production in AGS granulation in the present study, it was determined that CO_2 was continuously produced during the first hour of treatment process as shown in Figure 4.41(b). According to Martins *et al*. (2011), CO_2 is highly produced during the feast phase of aerobic granulation process. However, in this study, it was observed that along the first hour of AGS granulation, the concentration of CO_2 has reduced by 84%. Significantly, the decrease in CO_2 concentration was due to low bicarbonate content in the feed solution to serve for anaerobic growth as suggested by Martins *et al*. (2011). Therefore, in the present study, it is suggested that the anaerobic bacteria occurred in the AGS have accumulated CO_2 from the outside of the cell to serve their anaerobic metabolism in the aerobic granules as mentioned by Madigan *et al*. (2000).

Conclusion

This study highlights that aerobic granules cultivated under different physicochemical condition especially phototrophic condition possessed distinct morphological and biochemical characteristics. The morphology and chemical composition of the mineral fraction of the granules surface depends on the association of diversity of microorganisms, and environment in the SBR. The chemical composition also imposed the biomass concentration, size distribution and settling velocity of the two types of granules. It was found that the phototrophically cultivated aerobic granules, FP-AGS possessed higher settling velocity. Consequently, the minerals identified in both types of granules have dissimilar concentration. High magnification microscopic analysis revealed that AGSP was dominated by cocci-shaped bacteria embedded within the extracellular polymeric substances (EPS). Detailed observation on the structure of the AGSP showed the presence of 30µm of cavity to allow nutrients and gas exchanges within the aerobic granule. Scanning Electron Microscope-Energy-Dispersive X-ray (SEM-EDX) examination showed AGSP composed of different types of inorganic and organic compounds. The CO_2 production in the SBRP decreased from 495 to 460 ppm due to the presence of photosynthetic bacteria discovered in the AGSP. This shows that CO_2 biofixation can be performed facultatively by photosynthetic bacteria in an SBR based on the nomenclature of microbial species obtained.

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