Title	The quorum-sensing effect of aerobic granules on bacterial adhesion, biofilm formation, and sludge granulation
Author(s)	Ren, TT; Yu, HQ; Li, XY
Citation	Applied Microbiology And Biotechnology, 2010, v. 88 n. 3, p. 789-797
Issued Date	2010
URL	http://hdl.handle.net/10722/124559
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Submitted to: *Applied Microbiology and Biotechnology* (**AMB-10-20291**)

Date: July 22, 2010

The Quorum Sensing Effect of Aerobic Granules on Bacterial Adhesion, Biofilm Formation, and Sludge Granulation

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Abstract

Quorum sensing (QS) through signal chemical molecules is known to be essential to bacterial adhesion and biofilm formation. In this study, the QS ability of aerobic granules - a special form of biofilms used for biological wastewater treatment - was investigated and compared with that of conventional activated sludge flocs. A novel sectional membrane bioreactor (MBR) was used together with a flow-cell to evaluate the possible influence of signal chemicals produced by the source sludge on the growth mode of bacterial cells. The results demonstrate the apparent production of QS chemicals from granules and its impact on initial cell attachment and granule formation. When granules were used as the signal producing biomass, the attached-growth mode was dominant for the free cells, and the biofilm formation rate in the flow-cell was about 10 times faster than in cases which used activated sludge as the signal source biomass. In addition, the intracellular extract from mature granules significantly accelerated the sludge granulation process. It is argued that the production and expression of QS signal chemicals from granules and granule precursors might have induced the gene expression of bacteria in suspension for attached-growth rather than suspended-growth, leading to granule formation and its stable structure.

Keywords: Aerobic granules; biofilm; biological wastewater treatment; granulation; quorum sensing; signal chemical molecules.

Introduction

Aerobic granules are a new form of microbial aggregates that are cultivated in sequencing batch reactors (SBRs) used for biological wastewater treatment (Beun et al. 1999). Compared to conventional activated sludge, the compact structure of aerobic granules confer on granular sludge a high degree of settleability that allows for rapid sludge-effluent separation, an elevated level of biomass concentration, and a greater organic loading capability (Tay et al. 2005). The aerobic sludge system is particularly attractive for the treatment of high-strength and/or toxic organic wastewater (Jiang et al. 2002; Jiang et al. 2004). Thus, aerobic sludge granulation has the potential to be developed as one of the next-generation biological wastewater treatment technologies.

Nonetheless, effective aerobic granulation strategies and the underlying mechanism of granule formation remain issues that require further investigation. Aerobic granules are considered a special form of bacterial biofilm growth in suspension (Tay et al. 2005; Yang et al., 2008; Li and Li 2009). For the development and maintenance of a biofilm structure, a wide range of genetic determinants are required to regulate bacterial sub-populations for different functions (Malik et al. 2003). One of the regulatory mechanisms that plays a significant role in coordinating biofilm formation is intercellular signaling, or quorum sensing (QS) (Pearson et al. 1994; Parsek and Greenberg 2005). The quorum sensing ability of bacteria functions through the secretion and detection of autoinducer molecules, or signal chemicals, that accumulate in a cell density-dependent manner (Davey and O'toole, 2000; Hall-Stoodley et al., 2004; Velicer, 2003; Bassler and Losick 2006). When the signal molecule concentrations reach a threshold level, QS cells respond to induce gene expression for the mediation of specific functions and growth behavior (Miller et al., 2002; West et al., 2006). Traits under QS control include biosurfactant synthesis (Schuster and Greenberg 2006),

extracellular polymer production (Davies et al. 1998), and surface attachment (Miller and Bassler, 2001, Dunne 2002).

The QS effect of biofilm bacteria on cell adhesion and biofilm formation and structure has been well characterized. Signal chemical molecules released by the bacteria of biofilm induce attached-growth rather than suspended-growth in bacterial cells, which plays a crucial role in biofilm growth (Pratten et al., 2001; Beenken et al., 2003; Bakker et al. 2004). However, given the biofilm feature of aerobic granules, the potential quorum sensing ability of granular sludge and its effect on granule growth are issues that require examination. In the present study, the quorum sensing ability of aerobic granules was investigated and compared with that of activated sludge flocs. A sectional membrane bioreactor (MBR) was employed in connection to a flow-cell to evaluate the production of QS chemical molecules from the source sludge and the effect of the signal chemicals on the growth mode of bacterial cells. In addition, the influence of intercellular substances extracted from granular sludge on the bacterial granulation process was tested. The aim of the experimental study was to reveal the QS ability of aerobic granules and gauge the importance of QS chemicals in granule formation and growth.

Materials and Methods

Cultivation of aerobic granules and activated sludge flocs

Activated sludge (AS) and granular sludge (GS) were cultivated using two laboratory sequencing batch reactors, SBR1 and SBR2, respectively. Each SBR column was 6 cm in diameter and 80 cm in height with a working volume of 2.4 L. Activated sludge from a full-scale sewage treatment plant (Stanley Sewage Treatment Works, Hong Kong) was used as the seed sludge for inoculation of the reactors. Both SBRs were fed with the same synthetic

wastewater consisting of 0.5 g L^{-1} glucose as the carbon source and the following main nutrients: NH_4Cl-0.20 g L^{-1}, $MgSO_4 \cdot 7H_2O$-0.13 g L^{-1}, K_2HPO_4-1.65 g L^{-1}, KH_2PO_4-1.35 g L^{-1}, and the micronutrients described by Tay et al. (2005). Aeration was supplied from the bottom of the reactors and both SBRs were operated in 4-h cycles and 6 cycles per day. Different sludge discharge strategies were applied to the two reactors: for SBR1, small and slow-settling sludge flocs were discharged with the supernatant during the settling phase, and for SBR2, mixed sludge suspension was discharged during the aeration phase (Li and Li 2009). As a result, granulation was achieved and granular sludge produced in SBR1, while no granules were formed and typical AS flocs were maintained in SBR2 (Figure 1).

Test on the quorum sensing effect of different types of biomass sludge (AS and GS) using a sectional membrane bioreactor and a flow-cell

A sectional membrane bioreactor was fabricated to investigate the production and quorum sensing effect of signal chemical molecules potentially arising from granular sludge. This novel MBR setup was rectangular in shape, had a working volume of around 900 mL, and was 18 cm in length, 3 cm in width, and 18 cm deep (Figure 2). The reactor was divided lengthwise into three sections - section 1, section 2, and section 3 - with lengths of 12, 3, and 3 cm, respectively. Two pieces of flat-sheet microfiltration (MF) membrane (0.2 μm, Mill-Q SP, Millipore) were used to separate the three sections. The source biomass, either the aerobic granules used for SBR1 or the activated sludge flocs from SBR2, was placed in section 1 to function as the potential QS signal chemical producer. Section 2 was used to grow bacterial cells in a suspension that would act as the chemical signal receiver. Section 3 was free of biomass, and water was pumped continuously from this section back to section 1 to force a water flux through the membranes from MBR section 1 to sections 2 and 3. The use of membrane filters ensured the isolation of microorganisms in the designated sections, while

the water flow between the sections could transport chemical molecules from one section to another.

During a test on the release and function of the signal chemicals, the sludge in MBR section 1 was fed twice a day by replacing the supernatant with a concentrated substrate solution that had a chemical oxygen demand (COD) concentration of 4000 mg L^{-1}. The biomass had a mixed liquor suspended sludge (MLSS) concentration of around 5000 mg/L, a food-to-microorganism (F/M) ratio set at 0.22 g COD g^{-1} SS d^{-1}, and a solution pH of about 7.2. Fine-bubble aeration was conducted at a flow rate of 1.0 L min^{-1} through a diffuser at the bottom of section 1. The putative signal receivers inoculated in MBR section 2 were bacterial cells in either the GS supernatant from SBR1 or the AS supernatant from SBR2. The supernatants were obtained after 6 h of sludge sedimentation and the bacteria in section 2 had an initial concentration of about 10^8 cells mL^{-1}. The water was recirculated by pumping from section 3 to section 1 at a rate of around 40 mL h^{-1}, resulting in a hydraulic retention time (HRT) of 15 h for section 1 (working volume = 600 mL). The solids retention time (SRT) for the biomass sludge in section 1 was controlled at around 20 d. During the experimental test with the MBR, the growth mode of bacterial cells in section 2 was evaluated using a flow-cell (FC) apparatus.

The flow-cell consisted of a flow channel with dimensions of 20×5×1 mm. The setup was placed horizontally and the flow channel was covered with a microscopic glass slide (Christensen et al. 1999; Foster and Kolenbrander 2004). The bacterial suspension in MBR section 2 was circulated by pumping through the flow channel at a constant velocity of 0.2 mm s^{-1} as suggested by Sternberg et al. (1999). The FC cover slide could be readily retrieved for examination of bacterial growth on the slide. For each MBR-FC test, the cover slide was removed and analyzed under a microscope every 6 h or so in the first day and every day thereafter. The amount and rate of bacterial adhesion, or biofilm formation, on the FC cover

slide would signify the importance of attached-growth to the bacteria in the suspension. More specifically, the attachment of more bacteria to the slide at a faster rate would indicate the dominance of the attached-growth mode for the cells in section 2, while the attachment of fewer bacteria to the slide at a slower rate would suggest the primacy of the suspended-growth mode among the cells.

Bacterial cell attachment and biofilm formation on the FC cover slide were analyzed under a confocal laser scanning microscope (CLSM) (LSM 5 Pascal, Zeiss, Jena, Germany). A fluorescent dye, SYTO9 (25 μM, Molecular Probe, Eugene, OR), was used as a probe to stain all microbes. For each examination, a drop of the dye was used to stain a small circular area (~3 mm in diameter) on the slide, which was then incubated in a moisture chamber in the dark at room temperature for 20 min. After the staining process was complete, the stained area was rinsed gently three times with filtered phosphate buffered saline (PBS) to remove any unbound probes. The stained cells were observed under the microscope on a lighting channel with excitation and emission for SYTO9 (488 nm, BP 515-530). The bacterial cells attached to the slide were countable before biofilm formation and the cells in the CLSM images were enumerated using an image analysis system (analySIS 3.1, Olympus Soft Imaging Solutions, Germany).

Effect of the cellular substances extracted from granular sludge on the bacterial granulation process

The effect of cellular signaling substances from the GS biomass on the formation of granules was also investigated with column bioreactors. Both extracellular and intracellular materials were regularly extracted from the mature granules of SBR1 using a two-step extraction method. To extract the extracellular substances, 120 mL of settled granular sludge was thoroughly homogenized using a mechanical blender. Bacteria and solids in the

suspension were removed by centrifugation at 10,000 rpm for 15 min and the supernatant was filtered through a 0.2 μm membrane. Filtrate of about 200 mL was obtained as the GS extracellular extract. Subsequently, the sludge pellet after centrifugation was again suspended into deionized water and homogenized in the blender. The suspension was then treated by an ultrasonic homogenizer (4710 Series, Cole Parmer, Chicago, USA) at an acoustic intensity of 5 W mL^{-1} and a frequency of 20 kHz for 30 min to break up bacterial cells. The mixture was centrifuged at 10,000 rpm for 15 min and the supernatant was collected and filtrated with a 0.2 μm membrane. Filtrate of about 150 mL was obtained as the GS intracellular extract. Both the GS extracellular and intracellular extract solutions were stored at 4˚C in a refrigerator before use.

Three identical small cylinder columns (H 30 cm × D 3.6 cm with a working volume of 200 mL), R1, R2, and R3, were used as batch column reactors for the sludge granulation tests. Air was supplied from the bottom of the reactors at a flow rate of 1.0 L min^{-1} during the aeration phase. The seed-activated sludge flocs collected from the full-scale sewage treatment plant (Stanley Sewage Treatment Works, Hong Kong) were inoculated in the three comparative reactors at an initial MLSS concentration of around 2.0 g L^{-1}. The sequential operating cycle for each reactor was 8 hr and the reactor was refilled with the substrate solution described previously after the effluent was withdrawn. The organic loading rate for the three batch reactors was maintained at 0.5 g COD L^{-1} d^{-1}. $NaHCO_3$ was added to the feed to maintain the solution pH in the reactors at between 7.0 and 7.5. The reactors were operated at room temperature and the water temperature was 23-25˚C.

The three column reactors were operated in the same manner in terms of substrate feeding, effluent withdrawal, and biomass discharge (Sheng et al. 2010). After a pre-determined period of sedimentation, e.g. 10 min, the effluent and the slow-settling sludge were withdrawn from the middle point of each column, resulting in a volumetric exchange

ratio of 50% per cycle for each reactor. By adjusting the sedimentation time, the ratio of sludge discharge from each reactor was kept at about 10% every day. The main difference between the three reactors lay in the addition of cellular materials from mature granules. The GS extracellular extract solution was dosed into the R2 suspension twice a day at 33 mL d^{-1} and the GS intracellular extract solution was dosed into the R3 suspension twice a day at 22 mL d^{-1}. No GS substance was added to R1, which was used as a control. Sludge samples were taken from the reactors and analyzed for particle size and morphology under a stereomicroscope (S8 APO, Leica, Cambridge, UK) equipped with a digital camera (EC3, Leica, Cambridge, UK). The size of the sludge flocs and granules was determined using a computer-based image analysis system (analySIS 3.1, Olympus Soft Imaging Solutions, Germany).

The COD concentration, sludge MLSS concentration, and effluent suspended solids (ESS) were measured in accordance with the Standard Methods (APHA 1998). The total organic carbon concentration was determined by a TOC analyzer (IL550, HACH-Lachat, Milwaukee, WI, USA).

Results

Influence of the signal chemical molecules produced by granular sludge on the growth behavior of bacterial cells

In the sectional MBR, a sludge mixture was grown in section 1 as the potential QS signal producer and a diluted bacterial suspension was introduced to section 2 as a putative signal receiver. Either the mature aerobic granules from SBR1 or the activated sludge flocs from SBR2 were tested as the source sludge, while either the cells in the GS supernatant from SBR1 or the cells in the AS supernatant from SBR2 were used as the signal receivers. There

were a total of four combinations of the source sludge in MBR section 1 and the bacterial suspensions placed in MBR section 2: (a) GS+GScell - granular sludge as the source biomass and the cells from the GS supernatant as the receivers; (b) GS+AScell - granular sludge and the cells from the AS supernatant; (c) AS+GScell - activated sludge flocs and the GS cell suspension; and (d) AS+AScell - activated sludge flocs and the AS cell suspension. The test on each combination was repeated three times, and each test lasted for up to 7 days. The MBR-FC test focused on the attachment and growth of bacteria on the cover slide of the flow-cell connected to MBR section 2.

The number of cells grown on the surface of the slide could be counted quite accurately in the early phase of the test before the biofilm formation. The results were rather repeatable for all of the four source sludge and bacterial suspension combinations. The mean value and standard deviation of the cell counts from the three repeated tests on each combination are presented (Figure 3). After the first day, the GS+GScell combination yielded the highest cell adhesion density of 10^6 cm^{-2} on the FC cover slide, followed by the GS+AScell combination with a cell density of 4×10^5 cm^{-2}. When activated sludge was used as the source biomass, a lower cell adhesion density of 10^5 cm^{-2} was observed for the AS+GScell combination and hardly any cell adhesion was found for the AS+AScell case (Figure 3). The comparison between the different test results demonstrates the significant influence of the source biomass in MBR section 1 on the growth behavior of free bacteria in MBR section 2 and the flow cell. The attached-growth mode appeared to be more important and dominant for the free bacteria in the tests when mature granules were used as the signal source sludge than it did when activated sludge was used as the signal producer.

A significant difference in the rate of biofilm formation on the FC cover slide was observed for the different test cases (Figure 4). The figure shows the representative images of the examined areas on the FC slide. Each of the images is typical for the stained area on the

slide observed under the microscope after a specified test period. According to microscopic examinations, cell attachment on the slide occurred earlier in the tests for which granular sludge, rather than activated sludge, was placed in MBR section 1. Subsequently, biofilm formation and growth on the FC slide was much faster in the tests with the granular sludge used as the source biomass than in those with activated sludge used. While the FC slide was fully covered by a thick biofilm in the tests of the GS+GScell and GS+AScell combinations, hardly any biofilm formation was observed on the slide for the AS+AScell test. The different biofilm growth rates on the FC slide evidenced the impact of the source biomass in MBR section 1 on the growth behavior of bacteria in section 2. When aerobic granules were used as the source sludge, more of the cells in section 2 exhibited an attached-growth mode than the cases when activated sludge flocs were used as the source biomass. It is apparent that the granular sludge produced signal chemical molecules that induced the attached-growth of bacteria in section 2.

Effect of GS cellular substances on the bacterial granulation process

Although conditions in the three column batch reactors were otherwise identical, with the same seed biomass and operating protocol being used, a different cellular substance was added to each reactor. As a result, granulation was achieved at rather different rates in the three reactors. Aerobic granules formed most rapidly in R3, followed by R2, while granule formation was much slower in R1 (Figure 5). With the addition of the GS intracellular extract to R3, a large amount of granules with a mean size of around 0.5 mm were produced after only 5 days. Following addition of the GS extracellular extract to R2, a few granules were observed after 10 days. No granules were found until 20 days in R1, the control to which no GS cellular extract was dosed. Comparison of these results suggests that the sludge granulation process could be greatly accelerated by the addition of cellular extracts from

mature granules, and by the intracellular substances in particular.

Granulation can be achieved through the selective discharge of small and slow-settling sludge flocs (Li and Li 2009; Ren et al. 2009; Sheng et al. 2010). In comparison with the strategy of enhanced sludge washout (de Kreuk and van Loosdrecht, 2004; Tay et al. 2005), the method used in the present study with a controlled sludge discharge ratio at 10% led to slower granule formation. However, dosing with the GS intracellular substances was effective in accelerating the sludge granulation process. The GS cellular extract is likely to have contained chemical molecules that exerted the apparent QS effect on the growth mode of bacteria in R3. More specifically, the signal chemicals could induce the gene expression of the bacteria under the attached-growth mode, which in turn facilitated the formation of aerobic granules and the maintenance of granular structures. The results also suggest that the granule formation process can be greatly enhanced by increasing the intensity of the chemical signals in the early startup stage. A prior study has reported that adding mature granules to seed-activated sludge is an effective strategy for rapid aerobic granulation (Liu et al. 2005).

Discussion

In the sectional MBR with separate membranes, microorganisms were prevented from being transported between different sections and only chemical molecules were permitted to move from one section to another. Flow-cell test results showed that when mature granules were used as the signal-producing biomass, the attached-growth mode was the more important growth mode among the cells acting as the signal receivers. In contrast, in the tests which used activated sludge flocs as the signal source biomass, hardly any cell adhesion and biofilm growth were observed for the signal-receiving bacteria. Aerobic granules are known to be a special formation of biofilm growth (Tay et al. 2005; Ren et al. 2008; Li and Li 2009). The

quorum sensing ability of bacteria is believed to play a crucial role in the coordination of biofilm formation and structure (Pearson et al. 1994). As a means of cell-cell communication involving autoinducers, quorum sensing is achieved through the secretion and detection of signal chemical molecules (Bassler and Losick 2006). The present experimental results suggest the quorum sensing ability of bacteria in aerobic granules. The granular sludge in MBR section 1 apparently released signal chemical molecules that were received by the free bacteria in section 2. The QS molecules produced by the granules induced the attached-growth of bacteria with a high adhesion capability, leading to initial cell attachment and subsequent biofilm growth on the FC cover slide.

Aerobic granulation in a reactor likely involves the transformation of bacteria from the suspended-growth mode to the attached-growth mode. For the signal chemical molecules that regulate the potential of cell adhesion, different autoinducers have been reported for different species. Many Gram-negative bacteria produce N-acy-L-homoserine lactones (AHL) as signal molecules. In contrast, most Gram-positive bacteria use modified peptides as signal molecules (Parsek and Greenberg 2005). Valle et al. (2004) showed that AHLs affect the microbial community dynamics and growth behavior in an industrial wastewater treatment system. In general, AHL-mediated gene expression is the most thoroughly characterized bacterial intercellular signaling mechanism (Whitehead et al. 2001). The quorum sensing function of biofilm bacteria depends on a multitude of factors such as biofilm structure, plasmid transfer, and virulence, all of which have been shown to be AHL-dependent to various degrees (Valle et al. 2004). Therefore, AHLs could be the signal molecules that induce the QS effect of granular sludge on biofilm formation and bacterial granulation.

Chemical analysis also was conducted in the present study on the AS and GS cellular extracts using an ultra performance liquid chromatography (UPLC) system equipped with a triple-quadrupole mass spectrometer (MS) (Waters). The cellular extract samples were

processed with ethyl acetate before injection into the UPLC. Based on the MS detection, an m/z peak at 102 has been identified for the GS cellular extract. The fragment at m/z 102 is likely for the homoserine lactone group according to previous analytical analysis (Shaw et al. 1997, Michels et al. 2000), and the chemical fragment detected can be related to AHLs. Details of the UPLC-MS analysis and results are provided in Supplementary Material. The MS peak signifying the possible AHL fragment is found only for the GS extracts but not for the AS extracts, and it is much more significant for the GS intracellular extract. The UPLC-MS results would also suggest the potential role of AHL-mediated QS in the granule formation observed in the bioreactors dosed with GS extracts. However, it should be noted that the chemical analysis results may not be complete and conclusive. There is a wide variety of QS signal chemicals according to the literature (Fuqua et al. 2001; Irie and Parsek 2008). The possible detection of AHLs does not exclude the presence and function of other potential QS-related substances produced by aerobic granules.

The present study demonstrates the likelihood of the production of QS signal chemicals by granular sludge. Aerobic granules are a special formation of biofilm growth of bacteria in the attached-growth mode. The extracts taken from mature granules, particularly the intracellular substances, significantly accelerated the aerobic granulation process. The intracellular extract of aerobic granules appeared to contain signal chemical molecules that affected the growth behavior of bacterial cells in the suspension. It is argued that the signal substances produced by the granular sludge might have exerted the quorum sensing effect and induced attached-growth, rather than suspended-growth, among bacteria in the reactor. Thus, the production and function of QS signal molecules from granules and/or granule precursors can be essential to the initiation of bacterial granulation and the maintenance of granular structures.

Acknowledgements

This research was supported by grant HKU7144/E07 from the Research Grants Council (RGC) of the Hong Kong SAR Government and grant 50828802 from the Natural Science Foundation of China. The technical assistance of Mr. Keith C.H. Wong is highly appreciated.

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Figure legends

Figure 1. Photographic images of (a) seed-activated sludge from a full-scale sewage treatment plant, (b) aerobic granules produced in SBR1, and (c) activated sludge flocs formed in SBR2.

Figure 2. Schematic of the sectional membrane bioreactor connected to a flow-cell to test bacterial growth behavior.

Figure 3. Cell adhesion rates on the FC cover slide for different MBR test cases: (a) GS+GScell, (b) GS+AScell, (c) AS+GScell, and (d) AS+AScell.

Figure 4. Biofilm formation and growth on the FC cover slide after day 2, 5, 6, and 7 (from left to right) for different MBR test cases: (a) GS+GScell, (b) GS+AScell, (c) AS+GScell, and (d) AS+AScell.

Figure 5. Comparison between reactors R1 (control), R2 (dosed with the GS extracellular extract), and R3 (dosed with the GS intracellular extract) in the evolution of the size and morphology of the sludge after day 1, 7, and 15.

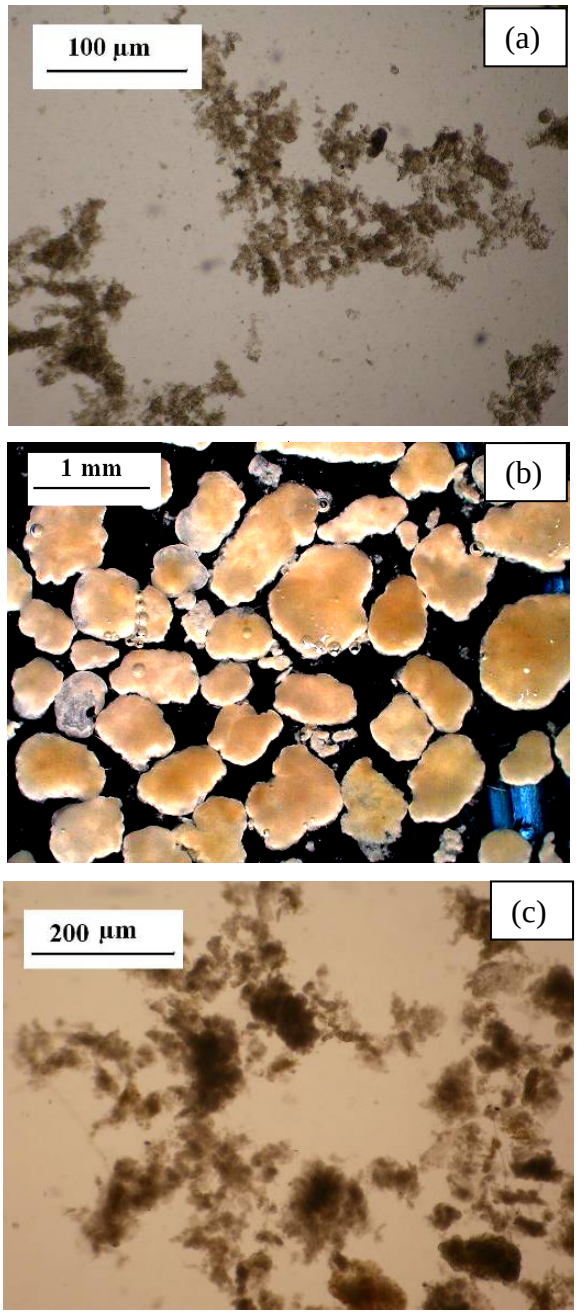

Figure 1. Photographic images of (a) seed-activated sludge from a full-scale sewage treatment plant, (b) aerobic granules produced in SBR1, and (c) activated sludge flocs formed in SBR2.

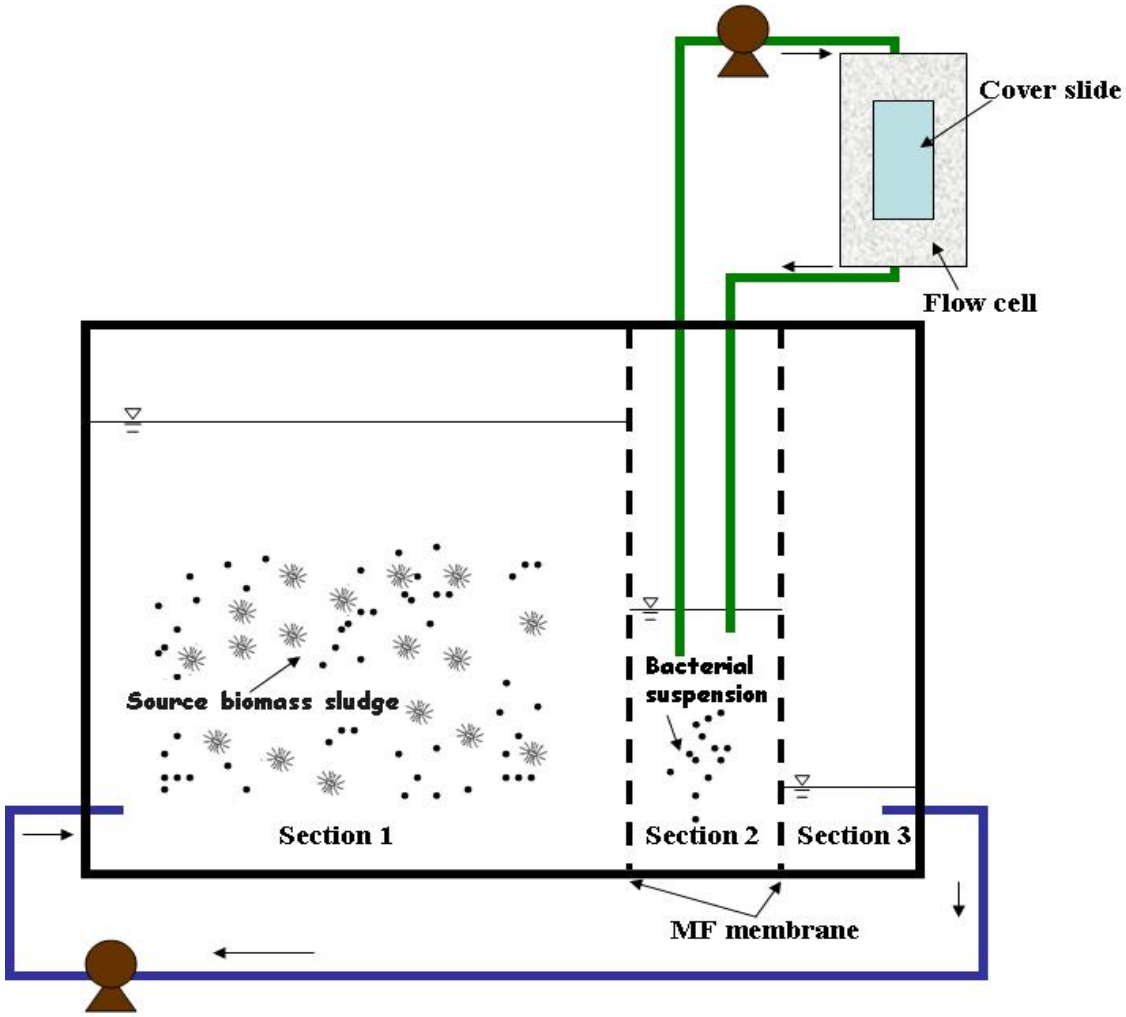

Figure 2. Schematic of the sectional membrane bioreactor connected to a flow-cell to test bacterial growth behavior.

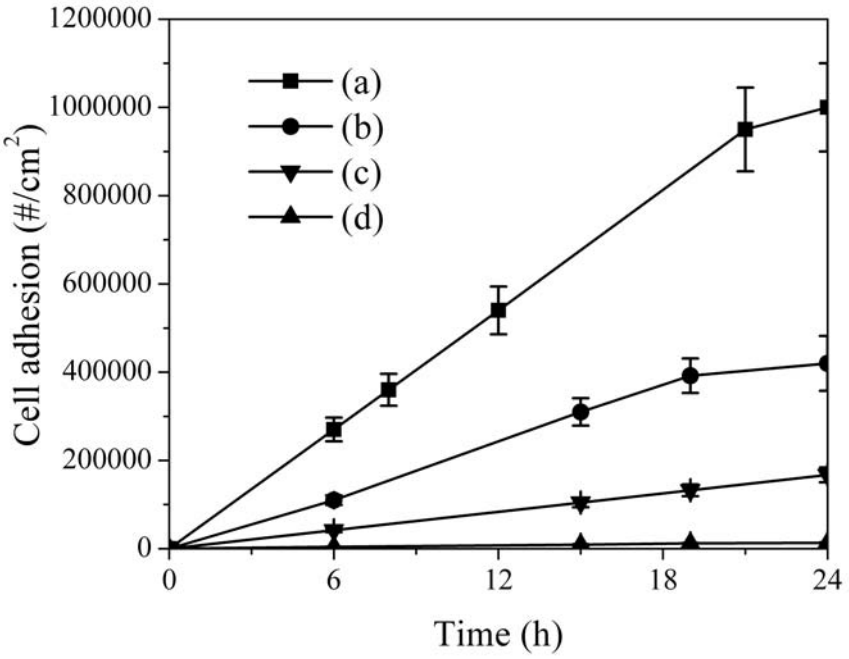

Figure 3. Cell adhesion rates on the FC cover slide for different MBR test cases: (a) GS+GScell, (b) GS+AScell, (c) AS+GScell, and (d) AS+AScell.

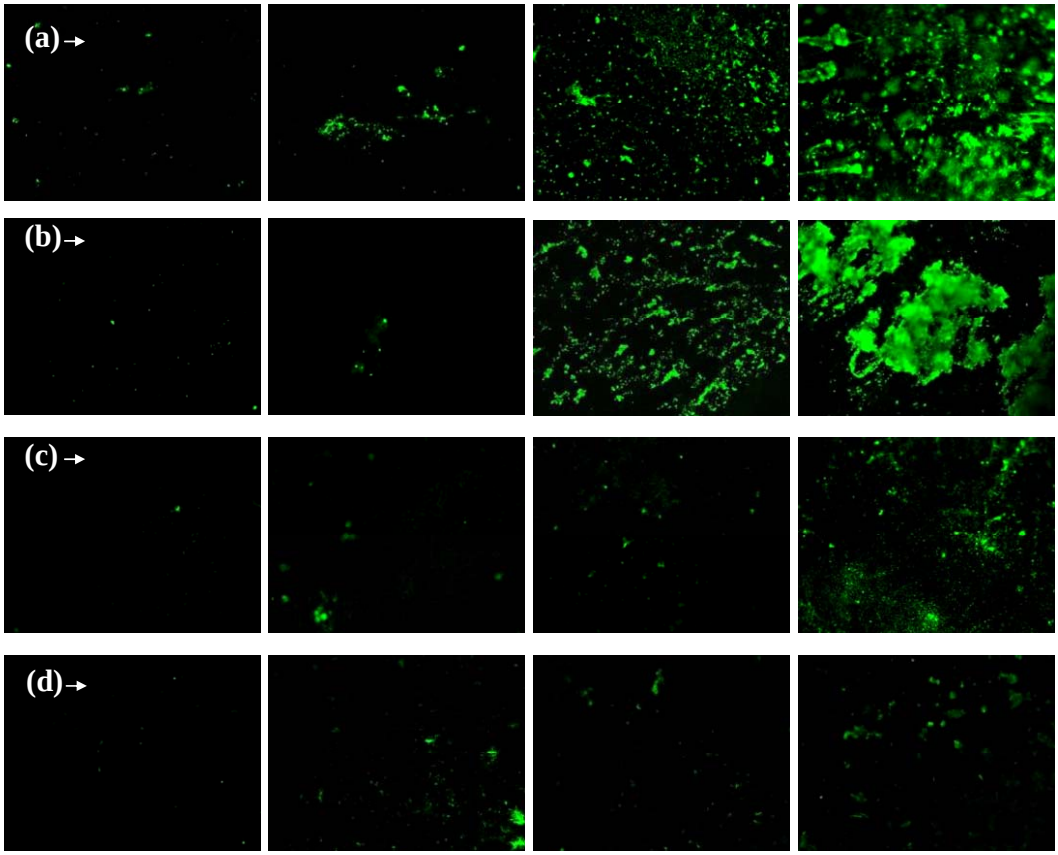

Figure 4. Biofilm formation and growth on the FC cover slide after day 2, 5, 6, and 7 (from left to right) for different MBR test cases: (a) GS+GScell, (b) GS+AScell, (c) AS+GScell, and (d) AS+AScell.

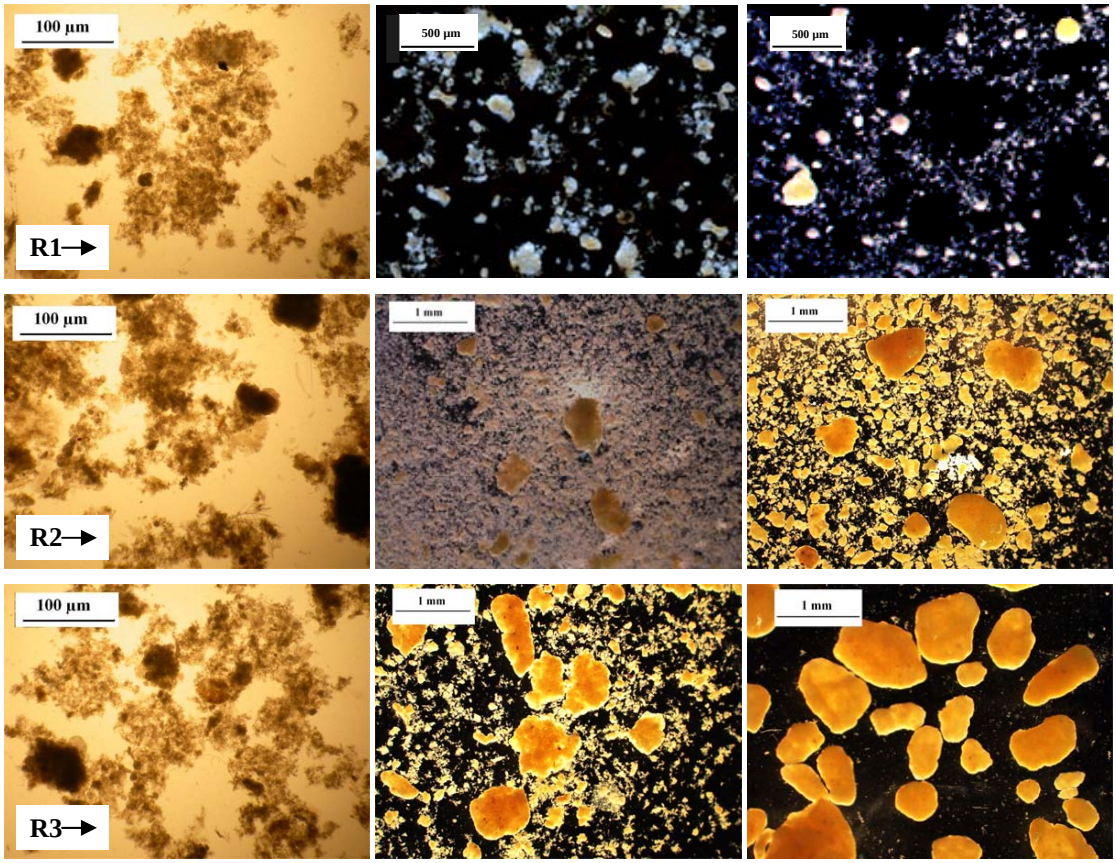

Figure 5. Comparison between reactors R1 (control), R2 (dosed with the GS extracellular extract), and R3 (dosed with the GS intracellular extract) in the evolution of the size and morphology of the sludge after day 1, 7, and 15.