The influence of settling time on the formation of aerobic granules

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Abstract Aerobic granular sludge, without the addition of carrier material, has only been reported in one suspended growth system, the Sequencing Batch Reactor (SBR) operated with short fill and settling periods. Recent studies have demonstrated that extracellular polysaccharides increased with the formation of aerobic granules, and that the shear force may stimulate production of these polysaccharides. In the study described herein, two SBRs were operated with the same shear force (air flow rate 275 L h^{-1}) and two different settling times (2 and 10 min). Only the reactor with 2 min settling formed completely granular sludge, although granules were present in both reactors. Community analysis using 16S rRNA PCR products and DGGE showed that the communities diverged quickly after reactor start-up. For samples taken at steady-state, the granular population was more stable and less diverse than the flocculent reactor. EPS extraction of samples using cation exchange resin yielded similar values for aerobic granular sludge and previously reported anaerobic granules. While differences in the protein and TOC content between the flocculent and granular reactors increased appreciably as the sludge became more granular, the protein to polysaccharide ratio was relatively constant. The experiment confirmed previous theories that short settling times in SBRs select for granular sludge. The settling time results in granular sludge having a higher EPS protein content and a less diverse but more stable population.
Keywords Aerobic granules; DGGE; extracellular polymeric substances; sequencing batch reactor; settling time

Introduction

The Sequencing Batch Reactor (SBR) combines the treatment steps of a cascade-continuous flow treatment plant, with multiple tanks for activated sludge and settling, into one reactor. In this way, the SBR can be used to build compact treatment facilities where land area is limited. However, the total number of SBRs required to treat a continuous waste stream sometimes minimizes this advantage. During the SBR cycles of react, settle, and draw, a continuous waste stream must be diverted to parallel SBRs or a holding tank. Therefore, the efficiency of these phases impacts the total treatment facility size. In recent years, much research has focused on reducing the settling time required for activated sludge flocs by forming dense flocs or by using biofilm reactors. Biofilm reactor systems allow the accumulation of biomass without the need for long settling times or high hydraulic residence times (Wilderer *et al*., 2002). The amount of active biomass in a reactor is limited by the surface available for bacteria to attach to and mass transfer within the biofilm itself. The maximum biomass surface area per volume is obtained when biofilms are grown into spherical granules, termed aerobic granular sludge (de Kreuk and van Loosdrecht, 2003).

Spontaneous aerobic granulation of suspended growth can be obtained in the SBR applying short fill periods. The causes and mechanism of granulation are not yet fully understood, but several important factors have been described in the literature. In order to form spherical, compact granules, the conversion of biodegradable substrate into inter-cellular stored

substrate must occur rapidly in the reactor. This is often described as a feast–famine regime, and can easily be applied in the SBR with short fill periods (McSwain *et al.*, 2003; de Kreuk *et al.*, 2003). Additionally, a short settling time has been utilized to select for fast settling flocs and granules, forcing the washout of less dense flocs and suspended organisms (Beun *et al.*, 1999, 2002). Tay *et al.* (2001a) operated four parallel SBRs with increasing aeration rates, showing that granules formed only in reactors with a superficial gas velocity greater than 1.2 cm sec^{-1}. In the same study, cellular polysaccharides were shown to increase with the aeration rate, leading to a discussion of the microbial processes responsible for cell aggregation in granules at the microscale.

Extracellular polymeric substances (EPS) are often defined as substances of biological origin that participate in the formation of microbial aggregates (Wingender *et al.*, 1999). The abbreviation "EPS" has often been expanded to extracellular polysaccharides or exopolysaccharides. However, EPS has been shown to be a rich matrix of polymers including polysaccharides, proteins, glycoproteins, nucleic acids, phospholipids, and humic acids. Since cells must expend energy to form the rich EPS matrix, the exact function of EPS has been explored. EPS is typically reported to aid in the formation of a gel-like network that keeps bacteria together in biofilms, causes the adherence of biofilms to surfaces, and protects bacteria against noxious environmental conditions (Wingender *et al.*, 1999). Because EPS are a major component of cell flocs and biofilms, they are hypothesized to play a central role in all types of biofilm formation. Granules can be described as a collection of self-immobilized cells into a somewhat spherical form and are considered to be a special case of biofilm growth (Grotenhuis *et al.*, 1991; El-Mamouni *et al.*, 1995; Beun *et al.*, 2002).

To address the microbial processes affected by short settling times in granule reactors, two SBRs were operated with the same superficial gas and two different settling times. During start-up, the bacterial community population dynamics were assessed using denaturing gradient gel electrophoreses (DGGE) to understand how settling time affected species selection. At steady-state, the EPS content and composition were measured to determine if the polysaccharide and protein content varied between flocculent and granular sludge produced under the same aeration rate.

Methods

Two 5 litre column-type SBRs were operated for six months. The reactors were shaped as a cylinder (height 100 cm, diameter 9 cm). They were aerated at a rate of 275 L h^{-1} (superficial gas velocity of 1.2 cm sec^{-1}) with a 50% volumetric exchange ratio. The reactors were inoculated with 5 L activated sludge (initial MLSS 2.5 g L^{-1}). The walls of the reactors were cleaned every two weeks. Both reactors were fed from a common feed of glucose and peptone with nutrients (similar to that used by Moy *et al.*, 2002) at a volumetric loading rate of 2.4 kg COD m^{-3} d^{-1} over 90 minutes of fill for six cycles per day (90 min static fill, 120 min react, 2 or 10 min settle, 15 min draw, 5 or 13 min idle). The only variation in operating strategy was the settling and idle times (see Table 1).

Mixed liquor and volatile suspended solids (MLSS and VSS), effluent solids and volatile solids (ESS and EVSS), and the sludge volume index (SVI) were measured according to APHA standard engineering methods (*Standard Methods*, 1998). Substrate removal was measured using Dr.Lange COD kits (following the colorimetric COD standard method). Endogenous specific oxygen uptake rate (SOUR) samples were collected from the end of react and aerated for at least two hours before the OUR measurement, whereas beginning and end of react OUR samples were measured immediately after sampling (*Standard Methods*, 1998; Manning, 1986). The development of flocs and granules was observed using a stereomicroscope (Leica Wild MPS 46/52), and images were obtained with an attached Kodak digital camera.

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Table 1 Operating strategy (variation of settling time to select for granules)

Cycle phase	Cycle times (min)	
	Reactor 1	Reactor 2
Static fill (fill, no aeration, no mixing)	90	90
React	120	120
Settle	10	2
Draw	15	15
Idle	5	13
Total cycle time	240	240

EPS extraction was determined for non-homogenized and homogenized samples. For both samples, approximately 0.5 g volatile solids (VS) were taken from each reactor at the end of the SBR cycle. VSS was measured at the time of sampling. The sample was centrifuged at 4°C, 10,000 rpm, for 15 min. The supernatant was collected to determine chemical composition of reactor wash, and samples were re-suspended in Milli-Q water and centrifuged again. For non-homogenized samples, the remaining pellet was resuspended in phosphate buffer (Frølund *et al.*, 1996) to a total volume of 100 mL. For homogenized samples, the pellet was resuspended in 40 mL phosphate buffer and divided into two aliquots. Each aliquot was homogenized for 10 min in a homogenizer (Janke and Kunkel, RW 20 DZM) at max rpm (980), and the two homogenized parts were combined with phosphate buffer to 100 mL total volume. Cation Exchange Resin Extraction: EPS extraction using a Dowex 50×8, Na^+ Form, cation exchange resin (Fluka) was performed with a 0.5 g VS:35 g CER ratio according to Frølund *et al.* (1996). The samples were stirred at 750 rpm for 4 h in the dark at 4°C. A blank with CER and phosphate buffer was also measured. Chemical composition: TOC was measured using a Elementar High TOC II machine. Protein analysis was performed with Bovine albumin serum standards, according to Lowry *et al.* (1951). Polysaccharide content was measured with glucose standards according to Dreywood *et al.* (1950). Cell lysis: Cell lysis was measured directly after EPS extraction using a glucose-6-phosphate dehydrogenase kit (Sigma 345-A) and was negligible (Frølund *et al.*, 1996).

For community analysis, reactor samples were homogenized, washed twice in 0.85% KCl and extracted using a Bio 101 DNA for soil extraction kit. 50 ng DNA was amplified using the 318F-GC clamp and 541R 16S rRNA primers as described in Muyzer *et al.* (1998) and Qiagen HotStart Taq Polymerase with recommended reagent mixes. PCR proceeded with 15 min activation at 94°C, 30 cycles each of 94°C denaturation (1 min), 55°C annealing (1 min), 72°C elongation (1 min), and a final step of 72°C for 10 min. 20 μL of product were loaded with 10 μL loading dye to a 8% acrylamide, 40–60% denaturing gel and run for 16 h at 100 V. DGGE gels were stained with Sybr Gold (Molecular Probes, Oregon, USA), and cluster analysis of gel lanes were performed using GelCompar II software (Applied Maths, Belgium).

Results and discussion

Both reactors began with the same amount of inoculum, but the MLSS of reactor 2 suddenly dropped as shown in Figure 1. Due to the difference in settling time, the washout of sludge during the first two weeks of operation was much greater for reactor 2 (2 min settling) (the MLSS dropped to 0.7 g L^{-1}) than for reactor 1 (10 min settling) (the MLSS began to increase immediately after start-up). After one week of operation, granules were observed in both reactors, and after 50 days of operation, both had granules of similar size and character, co-existing with flocs. On day 56 of operation, the SVIs of reactors 1 and 2 were 60 and 63 mL g^{-1}, respectively, and the settling time seemed to have no effect on granule

formation. However, after 80 days of operation, the reactors began to diverge in terms of sludge characteristics, MLSS, and SVI. For reactor 1, the granules always co-existed with flocculent sludge during one hundred days of steady-state operation (see Figure 1). For reactor 2, the MLSS began to increase while the size and number of granules in the reactor increased, and the flocs were continuously washed out with the short settling time. Based upon these measurements and microscopic observation, pseudo-steady-state was established after four months.

The steady-state values for MLSS, VSS, effluent SS, SVI, OUR, and COD removal are summarized in Table 2. It is clear that both reactors performed well in terms of COD removal and oxygen uptake rate. Complete COD removal was achieved within 30 min of the beginning of aeration after one week of operation, and all reactors had stable and complete COD removal (> 96%). The SOUR values were also similar during the SBR cycle (beginning and end of React phase) and for the endogenous rate. The reactors differed in the properties of the sludge, MLSS content, and settling characteristics. Most significantly, the granular reactor 2 developed an average MLSS of 8.8 g L^{-1} and an SVI of 47 mL g^{-1}, compared with an MLSS of 3.0 g L^{-1} and SVI of 115 mL g^{-1} for the flocculent reactor 1. The standard deviations for measurements are provided for all measurements taken after pseudo-steady-state conditions were reached (i.e., after three months of steady state data). The standard deviation is omitted for SOUR data taken during reactor start-up.

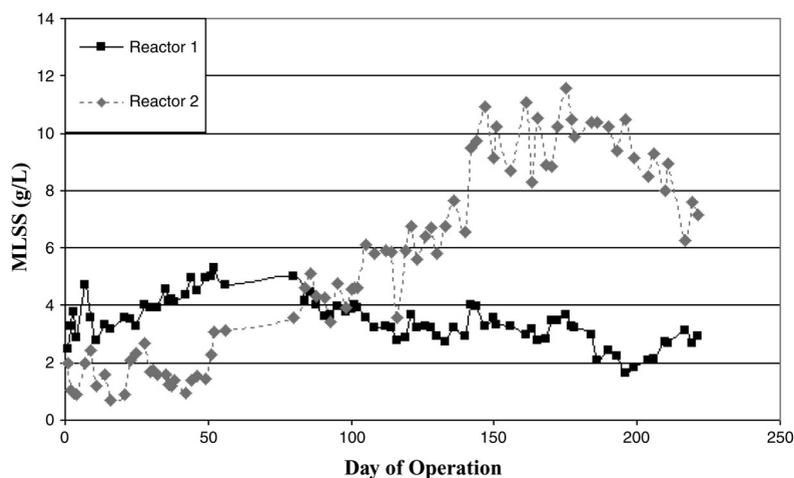Microscopic investigation was performed regularly during granule formation. In

Figure 1 MLSS over reactor operation

Table 2 Steady-state values for standard measurements (days 120 to 220 of operation)

Sludge properties	Reactor 1 (10 min settling)	Reactor 2 (2 min settling)
MLSS (g L^{-1})	3.0 ± 0.6	8.8 ± 1.7
VSS (g L^{-1})	2.7 ± 0.5	7.0 ± 1.5
SVI (mL g^{-1})	115 ± 36	47 ± 6
Effluent SS (mg L^{-1})	290 ± 170	170 ± 100
Reactor performance		
Endogenous SOUR*	12 ± 2	8 ± 3
Begin react SOUR	146	139
End react SOUR	18	11
COD removal (%)	96 ± 1	96 ± 1

* SOUR is reported in [mg O_2/(mg VSS*h)]

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Figures 2 and 3, the development of flocs and granules in each reactor is presented. On Day 7 of operation, small granules were present in both reactors. During the first two weeks of operation, there was appreciable washout of flocs in reactor 2 due to the short settling time, and the image shows that the MLSS was low. On day 52 of operation, both reactors were similar in terms of SVI and granule content, but they afterwards diverged until pseudo-steady-state was reached. Reactor 1 stabilized with flocs co-existing with granules, where-as reactor 2 accumulated large granules with small granules and flocs as secondary structures. The difference in final sludge structure is due to the difference in settling times.

The only difference in reactors 1 and 2 was the settling time. It has been hypothesized that short settling times select for fast-settling flocs and granules, enhancing granule formation (Beun *et al.*, 2002). Since granules formed in both reactors, the current experiment shows that short settling times are only necessary to select for predominantly granular sludge. The settling time may influence two microbial processes: (1) microbial selection and diversity through washout of slow settlers and (2) the production of EPS to aid in cell adhesion.

To address the effect of washout on species selection, genetic fingerprints of the reactor communities over time were compared using PCR-DGGE of 16s rDNA (shown in Figure 4). The first four lanes for each reactor show the population dynamics during the first month of operation. By day 28, it is clear that some dominant bands, that were common to both reactors, lose their intensity. Likewise, differences arise between the dominant bands in reactors 1 and 2 (represented with arrows). The last two lanes for each reactor show samples taken from steady-state operation, 60 days apart. These lanes represent the stability and diversity of steady-state sludge. The normalized densitograms were produced using image analysis, and these are presented in Figure 5. The gray line represents day 160, and the black line represents day 220. The similarity of the four lanes was calculated, and the matrix is presented in Table 3. Steady-state samples from the flocculent reactor 1 were more diverse than the granular reactor 2, and the community showed less long term stability (only 57% for reactor 1 steady-state). Reactor 2 selected for four main bacterial species, and the genetic steady-state fingerprints were 96% similar. Overall, the communities from R1 and R2 were only 19% and 50% similar on days 160 and 220, respectively. This clearly shows that settling time causes differences in species selection and community stability.

To investigate the physical-chemical differences in flocs versus granules, EPS of steady-state samples were extracted and characterized. The total TOC content for each reactor is presented in Figure 6, for both non-homogenized and homogenized sludge samples. Homogenization greatly aids the EPS extraction from granular sludge samples, since
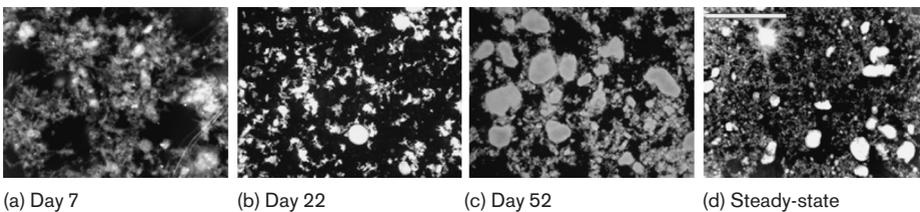

(a) Day 7　　　　　　(b) Day 22　　　　　　(c) Day 52　　　　　　(d) Steady-state

Figure 2 Development of flocs and granules in reactor 1 (scale bar at steady-state equals 5 mm)

(a) Day 7　　　　　　(b) Day 22　　　　　　(c) Day 52　　　　　　(d) Steady-state
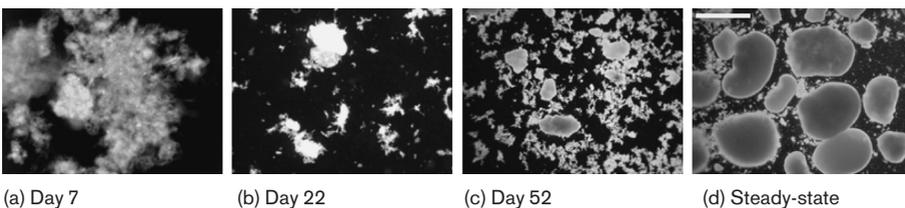
Figure 3 Development of granules in reactor 2 (scale bar at steady-state equals 5 mm)　　**199**

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Figure 4 DGGE gel showing samples from R1 and 2 taken over time. Arrows indicate differences in dominant bands during start-up; boxes indicate dominant bands at steady-state

Table 3 % similarity based on Pearson correlation of DGGE fingerprints (Figure 3) from steady-state samples

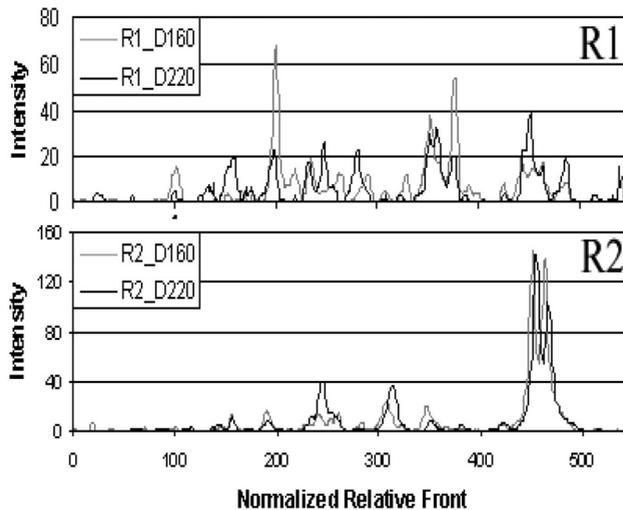|	R1 D160	R1 D220	R2 D160	R2 D220
R1 D160	100			
R1 D220	57	100		
R2 D160	19	49	100	
R2 D220	18	50	96	100

Figure 5 Normalized densitograms of steady-state DGGE patterns

granules can be very large (>1 mm) and have a surface to volume limitation for the cation exchange resin. As can be seen by the TOC data in Figure 5, there is a 33% increase in TOC content with homogenization for the flocculent reactor 1 and a 344% increase for the granular reactor 2. Due to the dependency of EPS extraction from granules on homogenization, homogenized samples will be compared. The EPS extracted from reactor 2 had a total TOC content of 69 mg C/g SS, while EPS from Reactor 1 had a much lower TOC content of 44 mg C/g SS. Figure 5 also contains error bars for the TOC values. These error bars represent the deviation of the method and were calculated from the separate extraction of triplicate samples.

Proteins and polysaccharides were measured in the EPS extracts of reactors 1 and 2, and the protein to polysaccharide ratio was calculated. The data are presented in Figure 6. The protein to polysaccharide (PN/PS) ratio was similar for both reactors (6.6 and 6.7, respectively), while the polysaccharide and protein content were both higher for reactor 2 than reactor 1. However, the increase in protein is greatest (23 mg protein/g SS vs. 3.3 mg polysaccharide/g SS), indicating that proteins contributed most to a higher TOC content in the EPS extract. The error bars in Figure 6 indicate the EPS extraction deviation for each measured (based on a triplicate extraction).

The results for EPS content differ strikingly from those reported by Tay *et al.* (2001a). For an SBR operated with a superficial gas velocity of 1.2 cm sec^{-1} (the same as used in the current experiment) and 5 minutes settling, Tay *et al.* (2001b) reported a polysaccharide to protein (PS/PN) ratio of 10. In previous experiments, the polysaccharide content was hypothesized to contribute more to the granule structure and stability than the protein content. However, in the current experiment, the protein content was much higher than the polysaccharide content, and a protein to polysaccharide (PN/PS) ratio was calculated to be 7 for both reactors 1 and 2. The differences in the two studies may be explained by the extraction methods used. Tay *et al.* (2001) used a heat and alkaline extraction, while the current study employed a cation exchange resin and homogenization. The data leads to two different conclusions for aerobic granular sludge formed under identical shear force.

A brief review of the EPS literature shows that the EPS extraction method greatly affects the reported composition of EPS from various sources. However, extraction using Dowex cation exchange resin is reported to cause less cell lysis than alkaline or heating methods (Nielsen and Jahn, 1999), which reduces the amount of contamination from inter-cellular proteins and carbohydrates (Frølund *et al.*, 1996). The values for EPS composition obtained in this experiment correlate well with values previously reported for a variety of sludges, including anaerobic granular sludge, in which proteins outnumbered carbohydrates. A brief comparison of sludge types, extraction method, and EPS content is
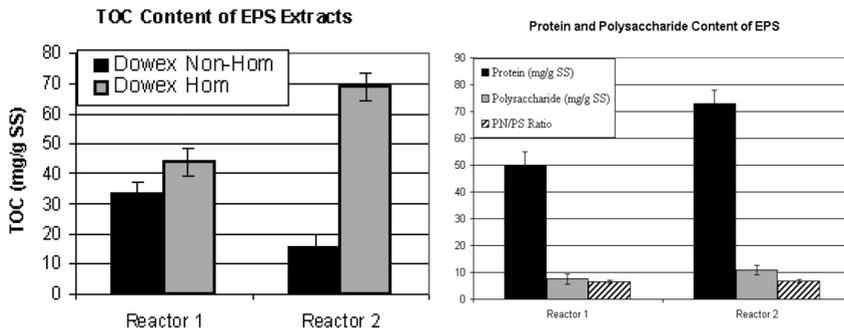

Figure 6 (a) TOC Content of EPS extracts (non-homogenized and homogenized samples); (b) protein and polysaccharide content of homogenized EPS extracts

Table 4 EPS composition from various sludges, using different extraction procedures

EPS extraction method	Sludge type	Protein (mg/g VS)	Carbohydrate (mg/g SS)	Reference
Heating 70°C	UASB	80	13	Schmidt and Ahring, 1994
Heating 80°C	Activated sludge	121	8	Frølund *et al.*, 1996
NaOH (pH 11)	Activated sludge	96	22	Frølund *et al.*, 1996
Dowex/shear	Activated sludge	243	48	Frølund *et al.*, 1996
Dowex/shear	Sewer biofilm	154	12	Jahn and Nielsen, 1995
Dowex/shear	Anaerobic granules	140	41	Batstone and Keller, 2001

presented in Table 4. Further investigation for the influence of EPS extraction method on the EPS protein and polysaccharide content in aerobic granular sludge is needed.

Conclusions

Short settling times in the SBR cycle select for fast-settling granules. Although granules formed in both reactors with a high shear force, only reactor 2 with 2 min settling became completely granular. The initial mass wash-out and continual removal of flocs affects species selection during start-up and produces a less diverse but more stable population. The EPS content of sludge from reactors 1 and 2 indicates that the TOC and protein content of EPS increases appreciably with granulation. This conclusion differs from previous studies, which found polysaccharides to be more critical to granule structure and stability than proteins. Overall, short settling times produce differences in species selection and EPS content in order to form granular sludge. Further research should investigate the EPS production by different species to determine if certain species are necessary for EPS aided granule formation. The influence of the EPS extraction method on the measured protein and polysaccharide content must also be discerned to allow correct interpretation of EPS data.

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