

How does shear affect aggregation in granular sludge sequencing batch reactors? Relations between shear, hydrophobicity, and extracellular polymeric substances

E. Dulekgurgen, N. Artan, D. Orhon and P. A. Wilderer

ABSTRACT

The objective was to provide an answer to “how to grow/survive in aggregative physiology” through evaluating the relation between physical stress and observed biomass characteristics. For that, a lab-scale sequencing batch reactor was operated at an anaerobic-aerobic mode and under altered hydraulic selection pressures of settling time (10–1 min) and hydrodynamic shear rates due to mechanical mixing (15.5–12.0 cm/s) and/or aeration (1.76–0.24 cm/s). Main physical stress experienced by the biomass was mechanical mixing, which resulted in extreme shearing conditions at the first operational stage (days 1–86), during which first granules formed but settling properties deteriorated and biomass was almost totally washed out. After relaxing the overall shear stress at the second stage, biomass formation accelerated, settling properties enhanced and granulation proceeded (days 86–136), until disturbance of the process at the last month of operation (days 136–163). Aggregative physiology-related parameters, being cell surface hydrophobicity and extracellular polymeric substances (EPS), followed increasing trends parallel to the progress of granulation, and then decreased upon disturbance of the process. There was an increase in the EPS production also during the first stage under extreme shear, while a substantial amount of biomass was present in the system. A direct correlation was also found between %hydrophobicity and EPS-composition expressed as ExoPN/ExoPS.

Key words | aggregation, extracellular polymeric substances, granular biomass, hydrophobicity, shear

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INTRODUCTION

In their review on factors influencing microbial aggregation, Bossier & Verstraete (1996) mention that there is neither a direct evidence showing that environmental stress signals instigate genetic rearrangements for aggregation, nor any solid information substantiating the link, which came later in 2000 (Davey & O'Toole 2000), between external stimuli and metabolic variations resulting in microbial adhesion/aggregation. Nonetheless, they draw attention to the likelihood of physical and/or chemical stress factors to trigger microbial responses resulting in a shift from dispersive- to aggregative-physiology. Consequently, it is

reasonable to state that keeping close proximity, in other words colonization and further aggregation, may provide a relatively safe growth environment for microorganisms, where they are rather protected from adverse effects of environmental conditions. Microorganisms are able to alter their surface characteristics in the direction of decreasing the repulsive forces between cells to enhance cell-to-cell interaction or decreasing their surface charges to promote self-immobilization for transitioning from a dispersive- to an adhesive/aggregative-physiology, which offers a niche of protection. Such an alteration translates into an elevated

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cell surface hydrophobicity promoting aggregation and eventually granulation into compact and durable structures. In fact, several researchers confirmed that progress of granulation is usually accompanied by an increase in hydrophobicity (Tay *et al.* 2001a; Liu & Tay 2002, 2004; Qin *et al.* 2004). Moreover, microorganisms, if not in a dispersed physiological state, are embedded, together with the neighboring cells, in a slimy matrix of extracellular polymeric substances (EPS), constituting mainly of water and biopolymers like ExoPS (extracellular polysaccharides) and ExoPN (extracellular proteins), as well as some other macromolecules of microbial-origin, and some entrapped non-cellular organic or inorganic materials. This three-dimensional, gel-like, highly hydrated, adhesive, and usually charged matrix with both hydrophobic- and hydrophilic-sites mediates the change in the negative surface charges of dispersed cells, serves for decreasing the repulsive forces between them and increasing their proximity, bridging them physico-chemically, and eventually contributes to adhesion and aggregation and offers a microenvironment sheltered from external stress conditions. Hydrodynamic shear is a good example of physical stress experienced by microorganisms in activated sludge systems and correlation between application of a relatively high shear-in terms of superficial upflow air velocity-and initiation, formation, and stability of more compact and denser biofilms, as well as aerobic and anaerobic granules, is well demonstrated in several studies (Liu & Tay 2002). Moreover, several researchers reported an increase in the EPS production coinciding with application of reasonably high shear rates in aerobic granular biomass systems (Tay *et al.* 2001b; 2004; Liu & Tay 2002). Knowledge from fundamental and applied research continues to accumulate, supporting the effort to answer three questions of “who, why, and how to grow–survive in adhesive/aggregative physiology”, both from a microbiological and from an engineering stand-point. The current study aims to contribute to this effort by trying to provide an answer to the question of “how”. For that, the scope was framed as determining the relation between the extent of shear applied to the biomass in a lab-scale reactor and the observed biomass characteristics, in particular cell surface hydrophobicity and EPS; the two regarded as the macro-scale measures of aggregative-physiology.

MATERIALS AND METHODS

Experimental set-up and reactor operation

The lab-scale sequencing batch reactor (SBR) had an internal diameter of 19 cm, initial volume of 3 L, working volume of 6 L (50% exchange ratio), working height of 22.5 cm, and minimum settling height of 11 cm. The reactor was seeded with a floccular activated sludge (3,400 mg MLSS/L, SVI of 78 mL/g, average particle diameter: 0.10 ± 0.01 mm) collected from a full-scale conventional WWTP (Garching, Germany) and fed with an influent containing 800 mg COD/L and 44 mg PO₄-P/L. Acetate was used as the sole carbon source at a daily COD load of 1.6 kg/m³.d. Feeding was under anaerobic conditions and only for 5 min at the head of each cycle, and the system was operated at an anaerobic-aerobic sequential mode of 6 h per cycle and 4 cycles per day. Phases during a typical cycle (i.e., after day 86) were as follows: 5 min feeding, 2 h anaerobic phase followed by 3 h 45 min aerobic phase, 1 min settling, 5 min effluent withdrawal and 4 min idle phase. Biomass wastage was only due to effluent withdrawal and no additional sludge extraction was employed. pH was monitored and maintained at 7.0–7.2.

Hydraulic selection pressure parameters

During the start-up period (days 1–22), settling time (TS) was kept as 10 min to ease acclimation and to avoid extensive biomass washout. Then, TS was gradually decreased and superficial up-flow air velocity, thus shear due to aeration (v_{Sair}) was gradually increased. Between days 1–86, mechanical mixing was applied both during the anaerobic and aerobic periods. After day 86, shear due to mechanical mixing (v_{SMix}) was decreased, first by limiting mechanical mixing only to the anaerobic phase, then by decreasing the speed of the stirrer. Applied changes and resulting hydraulic selection pressure levels are given in Table 1.

Measurements

Mixed liquor suspended solids (MLSS, in duplicates), and SVI₃₀ measurements were carried out according to the

Table 1 | Settling time (T_S), minimum settling velocity (v_{\min}), and shear components in the system

Days*	T_S min	Mixing* rpm	Q_i L/h†	Q_{air} L/h	v_{\min} cm/min	v_{SMix} cm/s*	v_{Sair} cm/s
1–22	10	310	15,810	250	1.1	15.5	0.24
22–35	5	310	15,810	250	2.2	15.5	0.24
35–42	5	310	15,810	500	2.2	15.5	0.49
42–64	3	310	15,810	500	3.7	15.5	0.49
64–77	1	310	15,810	500	11.0	15.5	0.49
77–86	1	310	15,810	1,800	11.0	15.5	1.76
86*–95	1	310	15,810	1,800	11.0	15.5	1.76
95–127	1	240	12,240	1,800	11.0	12.0	1.76
127–136	1	240	12,240	500	11.0	12.0	0.49
136–163	1	240	12,240	500	11.0	12.0	0.49

*Mechanical mixing was applied both at the anaerobic and aerobic periods (days 1–86), then limited to the anaerobic period (days 86–163).

†Pump discharge (input) of the mechanical mixer calculated for a 4-blades pitched (32°) impeller with a flow number of 0.85 and diameter of 0.1 cm.

Standard Methods for the Examination of Water and Wastewater (1998) in grab samples collected right before the end of aeration. Changes in macro-structure of the biomass were followed by examining the samples with a stereomicroscope (Leica Wild MPS 46/52, Vienna, Austria), and conducting particle size measurements on the digital images captured with a camera (Kodak E995) mounted on the microscope. Average granule diameter for each sample (aver. d_{gra} ; fictive value representing the arithmetic average of the mean diameters of all particles in a sample) was determined by image analyses (ImagePro Plus, V4.0, Media Cybernetics). Mixed liquor samples were subjected to %hydrophobicity measurements by the MATH (microbial adhesion to hydrocarbons) assay (Rosenberg *et al.* 1980). To isolate the loosely- and tightly-bound biomass-EPS, samples were subjected to EPS extraction with a cation exchange resin (DOWEX 50x8: strongly acidic, Na^+ form, 20–50 mesh size; Fluka 44445, Sigma-Aldrich) via a slightly modified version of the protocol proposed by Frolund *et al.* (1996). The “anthrone method” (Gerhardt *et al.* 1994) was used for isolation and measurement of the total carbohydrates of extractable-EPS in the samples treated with DOWEX resin (ExoPS), and that of soluble EPS and/or of any other origin in the supernatants. Amount of proteins associated with the corresponding fractions (i.e., ExoPN) were determined by the “Lowry Assay” (Lowry *et al.* 1951), recommended for

determining the amount of proteins in biological samples (Gerhardt *et al.* 1994).

RESULTS

The reactor was operated for a total of 163 days. Values of hydraulic selection pressure parameters – settling time (T_S) and superficial up-flow air velocity (v_{Sair}) – were gradually altered one at a time to help initiate and enhance granulation and to evaluate their influence on the process. Shear due to mechanical mixing (v_{SMix}) was also taken into account as a component of external physical stress, while determining the level of shear experienced in the system. Progress of granulation was monitored by following the changes in biomass concentration (MLSS), settling properties (SVI), and size of granules (aver. d_{gra}). Changes in cell surface hydrophobicity, as well as in ExoPS and ExoPN fractions of the EPS were followed up during reactor operation. Results are as follows:

Hydraulic selection pressures

Decreasing the T_S gradually from 10 to 5, 3, and 1 min resulted in increased operationally-set minimum settling velocities (v_{\min}) in the system (Table 1). Yet, the highest v_{\min} was 11 cm/min even when T_S was as low as 1 min (days 64–163). Increasing the airflow rate from 250 to 500 and

1,800 L/h on days 35 and 77, caused the aeration-related shear rate (v_{SAir}) to increase from 0.24 to 0.49 and 1.76 cm/s, respectively (Table 1). Applying mechanical mixing at a speed of 310 rpm resulted in a mixing-related shear rate (v_{SMix}) as high as 15.5 cm/s. This value decreased to 12.0 cm/s when the speed of the stirrer was decreased to 240 rpm on day 95. As mentioned previously, mechanical mixing was applied both during the anaerobic and aerobic periods for the first 3 months. Hence for that period, shear realized in the system during the anaerobic phase (v_{SAner}) was due to mechanical mixing (v_{SMix}), and that realized during the subsequent aerobic phase (v_{SAer}) was due both to mechanical mixing and aeration (v_{SAir}). After day 86, mechanical mixing was limited only to the anaerobic phase, thus shear during the aerobic phase was only due to aeration. Varying levels of shear rates are given in Figure 1, panel a, where v_{STR} represents the fictitious overall shear rate roughly estimated for the total react time from the values calculated for the anaerobic and aerobic phases and taking into account the duration of these periods and normalizing to the total react time. Although decreased after day 95, shear during the anaerobic phase was significantly high at all times. Between days 1–86, shear during the aerobic phase was also significant due to mechanical mixing, and small increases were because of increased airflow rates. On the other hand, after day 86,

there was a dramatic decrease in the shear valid during the aerobic phase, as well as in the fictitious overall shear. This dramatic change was the result of avoiding mechanical mixing during aeration. The decrease in overall shear (v_{STR}) on day 95 was due to decreasing the speed of the stirrer and that on day 127 was due to decreasing the airflow rate. As apparent from the figure, there were two main stages of reactor operation with regard to the extent of shear realized in the system: Stage-I (days 1–86) with extreme conditions and Stage-II (days 86–163) with decreased conditions.

Biomass growth, settling properties, and progress of granulation

Biomass concentration, settling properties, and progress of granulation (in terms of changes in size of granules), monitored for 163 days are presented in Figure 1, panel b. After a significant washout on the first day, MLSS in the system first increased from 1,155 to 4,664 mg/L, then decreased back to 1,752 mg/L during the start-up period (days 1–22) and SVI increased to 285 mL/g. Decreasing the settling time to 5 and 3 min on days 22 and 42, respectively, did not cause a significant change in MLSS and biomass concentration fluctuated in a narrow range of 1,100–1,800 mg/L between days 22–64. First granules (0.16 ± 0.06 mm) became apparent after the first month of

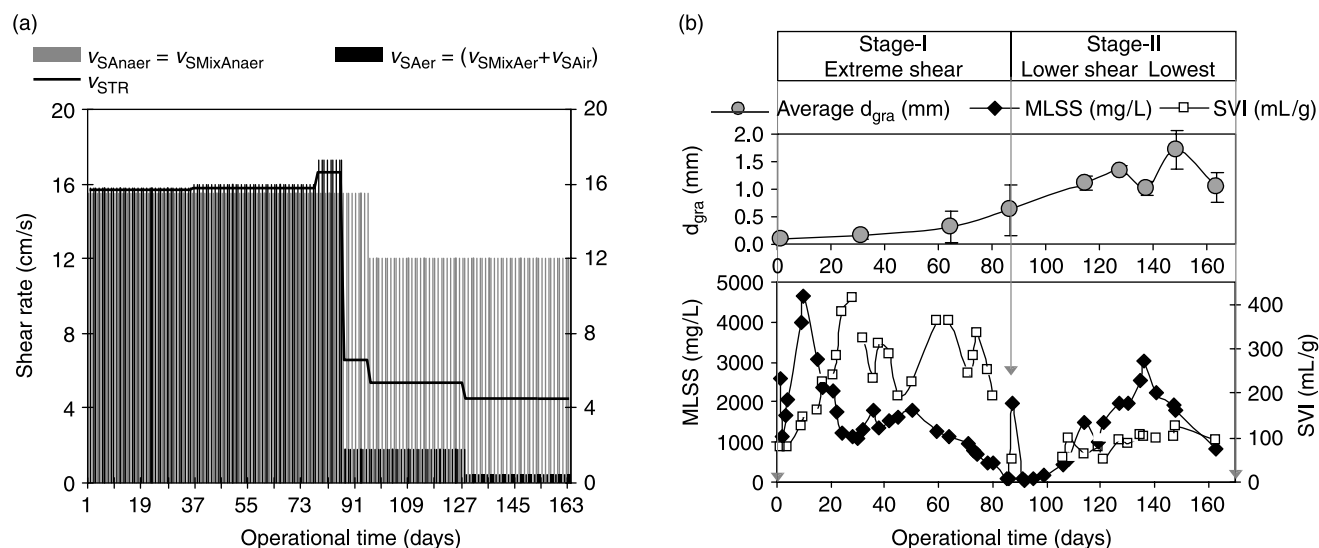


Figure 1 | Panel (a): shear rates at the anaerobic (v_{SAner}) and aerobic (v_{SAer}) phases due to mechanical mixing (v_{SMix}) and/or aeration (v_{SAir}), and fictitious overall shear rate (v_{STR}). Panel (b): biomass concentration (MLSS), settling properties (SVI), and size of granules (aver. d_{gra}).

operation (Figure 1, panel b). Yet, upon decreasing the T_S to 1 min on day 64, the amount of biomass retained in the system decreased continuously and dropped down to 80 mg MLSS/L on day 86. Although granules with average dgra of 0.32 ± 0.28 mm and 0.62 ± 0.46 mm were present in the system at the respective days, floccular biomass was formed and the reactor content became soupy, with a slimy biomass and a turbid supernatant. Meanwhile, SVI values fluctuated between 190–416 mL/g and measurements became impossible after day 80. The sudden increase in MLSS on day 87 was due to addition of a floccular biomass collected from the aeration (nitrification) tank of a full-scale BNR plant (East Munich, Germany) to the system. Yet, MLSS immediately dropped back to its previous level. Upon start of the second operational stage (with decreased shear), biomass formation speeded up and MLSS increased from 94 to 3,000 mg/L between days 95 and 136. SVI values were stable around 70 mL/g and biomass had a granular structure with increasing granule diameter (Figure 1, panel b). During the last month of operation, MLSS gradually decreased down to 850 mg/L. SVI was constant around 100 mL/g and granule size fluctuated between 1.01 and 1.71 mm.

Cell surface hydrophobicity

Results of cell surface hydrophobicity measurements are given in Figure 2. The first value measured was 45% on day 73, when the system was under extreme shearing conditions. In less than 2 weeks, cell surface hydrophobicity decreased to <1% (day 85). During the second stage of operation under decreased shearing conditions, %hydro-

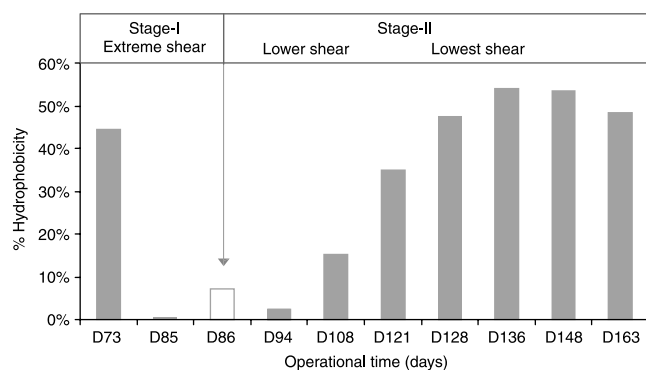


Figure 2 | Changes in the hydrophobic character of the biomass during reactor operation.

phobicity values followed a steadily increasing trend. The increase stopped after day 136 and a relatively small decrease (from 53.5% to 48.3%) was recorded during the last 2 weeks of operation (Figure 2). Note that, the value for day 86 plotted on Figure 2 represents the %hydrophobicity value of the mixture comprised of the biomass already present in the reactor and the floccular BNR biomass added to the system. Comparing the values recorded on days 85 and 86, it is apparent that the value measured as 7.2% on day 86 was mostly due to the %hydrophobicity of the added biomass.

Extracellular polymeric substances (EPS)

The amount of loosely and tightly bound biomass-EPS was represented by the amounts of the two main components of EPS: extracellular polysaccharides (ExoPS) and extracellular proteins (ExoPN). Results are given in Figure 3 (panel a) as mg glucose/L and mg BSA/L, respectively; BSA (bovine serum albumin) being the model protein for measurements. During the first 2.5 months of operation under extreme shearing conditions (days 1–74), there was a steady increase in the EPS production as evident from the increasing ExoPS and ExoPN values. This was followed by a significant decrease in the amount of both components towards the end of the first stage. Values measured on day 85 were as low as 26 mg glucose/L and 107 mg BSA/L. Note that the values plotted on the figure for day 86 represent the ExoPS and ExoPN concentrations of the mixture comprised of the biomass already present in the reactor and the BNR biomass added to the system. At the beginning of the second stage (with decreased shear), ExoPS and ExoPN concentrations remained the same (days 85–108, excluding day 86), then increased significantly in two weeks (day 121). The amount of ExoPS continued to increase until day 148, whereas the amount of ExoPN gradually decreased between days 121 and 148. During the last two weeks of operation (days 148–163), a significant decrease was recorded for both parameters, with a more pronounced decline in ExoPN (Figure 3, panel a). In addition to the absolute values of the two EPS components, variations in the composition of the EPS-matrix expressed as changes in the amount of ExoPN relative to that of ExoPS are also plotted in Figure 3, panel b. ExoPN/ExoPS ratio followed

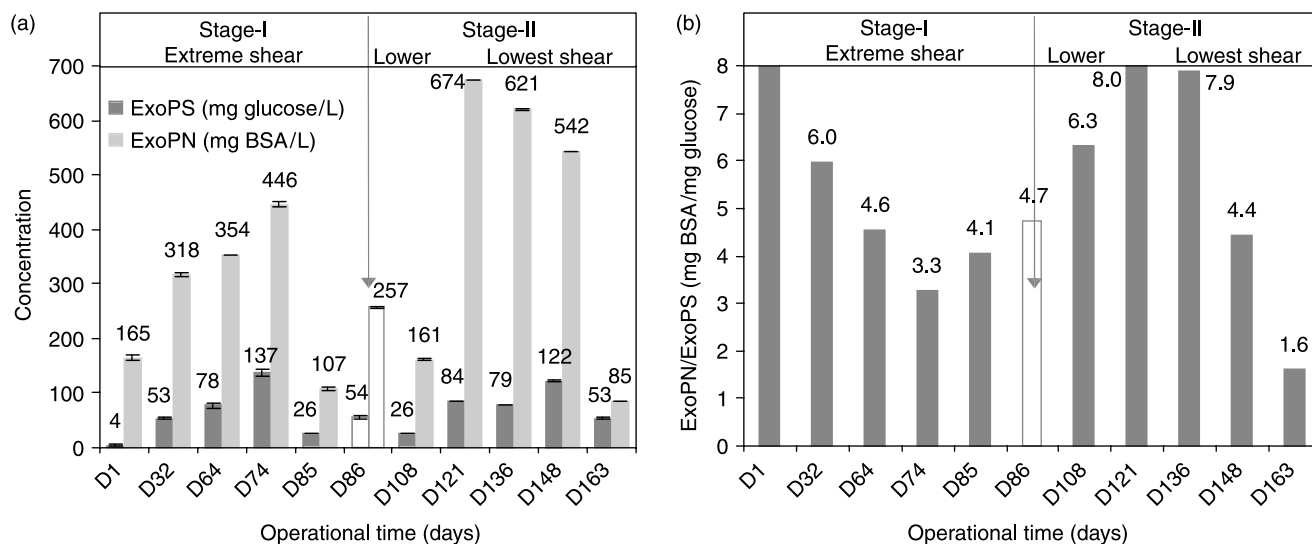


Figure 3 | Changes in (a) EPS production in terms of absolute values of ExoPS and ExoPN, and (b) in EPS composition in terms of ExoPN/ExoPS ratio.

a decreasing trend during the first operational stage with extreme shear, then an increasing trend between days 85–136 at the second stage with decreased shear. A dramatic decrease was recorded towards the end of reactor operation. Note that, the ExoPN/ExoPS trend observed at the second operational stage was the same as that observed for %hydrophobicity at the same period.

DISCUSSION

Physical stress and aerobic granulation

A low settling time is considered as one of the components of hydraulic selection pressure in favor of aerobic granulation. Yet, even when T_S was decreased to as low as 1 min in this study, the highest v_{min} was 11 cm/min due to reactor geometry (working height to diameter ratio: 1.2). This value was much lower than ≥ 20 cm/min; a value reported in many cases to be sufficient for a successful granulation in aerobic systems (Beun *et al.* 2002; de Kreuk & van Loosdrecht 2004; McSwain *et al.* 2004; Wang *et al.* 2004). Thus, the hydraulic selection pressure in terms of v_{min} was considered to be relaxed in the system at all times. In contrast, shearing conditions were at significant levels during the entire operational time even without considering the impact of particle-to-particle collision. Wang *et al.*

(2004) reported that a minimum of 0.87 cm/s of superficial upflow air velocity was required to support granulation in their system. Tay *et al.* (2001a) reported a minimum of 1.2 cm/s within the same context. In many cases, a value ≥ 2 –2.5 cm/s is considered to be sufficient for providing the shear to facilitate aerobic granulation and provide structural stability (Tay *et al.* 2001a; Beun *et al.* 2002; Tay *et al.* 2004; Zheng *et al.* 2005). The highest superficial upflow air velocity, in other words aeration-related shear rate (v_{Sair}) applied to the system in this study was 1.76 cm/s (days 77–127) and this value was close to those reported in the literature. On the other hand, shear rates due to mechanical mixing were one order of magnitude higher than that due to aeration. Hence, rather than the settling time, the main hydraulic selection pressure exerted on the system was determined to be the hydrodynamic shear stress, with a relatively small contribution from aeration (v_{Sair} , max. of 1.76 cm/s) and a major contribution from mechanical mixing (v_{SMix} ; min. of 12.0 cm/s). During the first operational stage (days 1–86), the system was under extreme shearing conditions since mechanical mixing was applied both at the anaerobic and the aerobic periods. At the second stage (days 86–163), extreme conditions were prevented by limiting mechanical mixing only to the anaerobic phase and the overall shear was further relaxed by decreasing the speed of the stirrer (day 95) and the airflow rate (day 127).

However, shear was still at a considerable level even when those applied during the anaerobic and aerobic phases were lowest towards the end of reactor operation (days 127–163).

At the start-up, T_S was kept relatively high to allow for acclimation and to avoid extensive biomass washout. Yet, decreasing MLSS and increasing SVI values indicated not only that the floccular inoculum started to be washed out but also that the settling properties of the remaining biomass continuously deteriorated under extreme shearing conditions. First granules became apparent in 30 days upon start and were present for the next 20 days, though they became unstable and diluted from the system afterwards. After around 3 months of operation under extreme shear, almost no biomass was left inside the reactor. These observations indicated that the extreme shearing conditions applied at the first operational stage (days 1–86) had an adverse impact on the granulation process, primarily preventing the initiation of granulation, causing deterioration of the biomass characteristics, and resulting in extensive washout. The strategy of decreasing the settling time to impose an indirect selective pressure on the biomass in the direction of discarding the slowly settling flocks and retaining only the particles with a presumably aggregative physiology and with settling velocities higher than v_{\min} , resulted only in a temporary positive impact which remained negligible when compared to the adverse impact of extreme shear. The amount of suspended solids in the effluent (ESS) monitored during the first stage (data not shown) revealed that extreme conditions had also a negative impact on the effluent quality. Significantly high ESS values (aver. 170 mg/L) were suggested to be the consequence of extreme shear instigating disintegration of biomass aggregates or sloughing of particles from granules' surface, eventually causing small particles with settling velocities lower than the operationally-set v_{\min} appear in the effluent.

It was only after decreasing the overall shear by applying the above mentioned strategies at the second stage that a significant granular biomass formation was achieved. Increasing MLSS and decreasing SVI values, parallel to the increasing trend in average size of granules suggested that providing a relaxation in the overall shear promoted initiation of aerobic granulation and progress of

the process until day 136. After that time point, SVI remained fairly constant, yet decreasing MLSS (Figure 1, panel b) and increasing ESS values (data not shown) suggested a disturbance in the granulation process during the last month of operation with lowest shear. This deterioration was not apparent from the size of granules but from the change in granule morphology, as revealed by a decrease in average shape factor values (from 0.80 on day 127 to 0.76 and 0.60 on days 137 and 148, respectively) at the final episode, due to occurrence of filamentous outgrowths on granules' surfaces. This eventually resulted in loss of granule stability, formation of some floccular biomass, washout of biomass, and deterioration of the effluent quality towards the end. The difference between the final episode with lowest shear and the previous period was the applied airflow rate. Decreasing the airflow rate from 1,800 to 500 L/h on day 127 had a minor impact on the extent of overall shear, since the dominant shear causing factor was mechanical mixing. Yet, this operational change was speculated to result in a decline in the dissolved oxygen (DO) level; a condition known to favor proliferation of fast-growing organisms like the filamentous ones, when combined with the concomitant presence of a carbon source to support growth (Wanner 1994). In fact, researchers reported occurrence of filamentous organisms on granules' surfaces and loss of granule stability under low DO conditions (Mosquera-Corral *et al.* 2005). Although not definite, since no DO data was available for the final episode of this study but the COD removal at the anaerobic phase through conversion of acetate to intracellular C-storage products was determined to be low (<21%) implying the presence of acetate at the aerobic phase, it was possible to extrapolate the reasons and consequences of the changes observed in biomass characteristics at the last month of operation to those stated in the studies mentioned above.

Physical stress and aggregative physiology-related parameters

Increase in the hydrophobic character of the biomass was determined to be clearly parallel to the progress of granulation; the latter being directly influenced by the extent of the physical stress (in terms of shearing

conditions) applied to the system. The direct correlation between increasing %hydrophobicity values and progress of granulation observed during the second operational stage is in well agreement with the observations reported in the aerobic granulation literature (Tay *et al.* 2001a; Liu & Tay 2002, 2004; Qin *et al.* 2004). It was only after disturbance of the granulation process and loss of granule stability towards the end of reactor operation that cell surface hydrophobicity values also decreased to some extent (days 136–163). Rate of increase in %hydrophobicity between days 108–128 was 33%, whereas rate of decrease between days 136–163 was only 6%, suggesting a more pronounced (positive) change in the hydrophobic character upon initiation and progress of granulation, and a less significant (negative) impact upon disturbance of the process and loss of granule stability. Finally, from the observations attained in this study it was possible to state that the biomass experiencing a considerable physical stress – i.e., extreme shear at the first stage or decreased but still significant shear at the second stage – tends to grow into an aggregative physiology through increasing its cell surface hydrophobicity, yet severe conditions should be avoided primarily to prevent total biomass loss.

Similar to the case described above for %hydrophobicity, EPS production expressed in terms of ExoPS and ExoPN concentrations also increased upon initiation and during progress of granulation at the second operational stage with relatively relaxed -yet still significant- shearing conditions, and then decreased upon disturbance of granulation. These observations are also in line with those reported in the literature (Tay *et al.* 2001b, 2004; Liu & Tay 2002). Moreover, increase in both parameters during the first stage was interpreted as a consequence of extreme shear, forcing the biomass to raise its EPS production for enhancing its aggregative properties and be retained in the system. There was a significant decrease both in the ExoPS and ExoPN concentrations, as well as in %hydrophobicity values at the end of the first operational stage and this was attributed to the fact that the system was under the most severe physical stress conditions, which resulted in almost total biomass washout prior to the start of the second stage.

The EPS-composition fluctuated significantly in time and in relation with the changes in level of applied shear.

Continuous decrease in the ExoPN/ExoPS ratio, in other words the increase in the relative amount of ExoPS during the first operational stage, was interpreted as a metabolic strategy of the biomass to deal with extreme shearing conditions and be retained in the system. Relation between the EPS-composition and progress of granulation determined in this study was an increasing ExoPN/ExoPS trend parallel to progress of granulation during the second operational stage with decreased shear, and a decreasing trend upon disturbance of the process. This relation was the opposite of those reported by several researchers (Tay *et al.* 2001a,b; Liu & Tay 2002; Qin *et al.* 2004), but was in agreement with the observations reported by McSwain *et al.* (2005a,b), who attributed the discrepancy between their results and the others in the literature to application of different EPS-extraction methods in the compared studies. Yet, although the same extraction procedure as by McSwain and her colleagues was applied in a parallel study with two other SBRs run together with that evaluated in this study, those results were also contradictory (data not shown here). Thus, it was not possible to attribute the observed differences only to the difference in the employed extraction procedures. Consequently, it was concluded that both ExoPS and ExoPN (as absolute values) are useful parameters for monitoring and confirming the progress of granulation, but the EPS-composition, expressed either as ExoPN/ExoPS or ExoPS/ExoPN, may not be as accurately informative as the absolute values of the individual parameters. The latter is considered to be consistent with the fact that the EPS-matrix is a lump-sum of different extracellular biopolymers and molecules, which are produced in different compositions and in different relative amounts by the aggregative biomass consisting of different microbial populations.

In several studies, cell surface characteristics -in particular cell surface hydrophobicity- are stated to be closely related with the EPS produced by the biomass where microorganisms are tightly packed and embedded. In this study, changes in the composition of the EPS-matrix (as ExoPN/ExoPS) were also determined to be parallel to variations in cell surface hydrophobicity, with both parameters following increasing trends during the progress of granulation at the second operational stage and then decreasing upon disturbance of the process. This directly

proportional relation can be explained to some extent, considering the data by Higgins & Novak (1997), who reported that the total amount of amino acids contributing to the hydrophobic portion of the ExoPN fraction extracted from a full-scale WWTP was 50% higher than that contributing to the hydrophilic part. Assuming that the hydrophobic portion of the ExoPN produced by the granular biomass in this study was higher than the hydrophilic portion, the increase in %hydrophobicity could be the result of higher ExoPN production; the latter also translating into higher ExoPN/ExoPS ratios. Results indicated that upon disturbance of granulation at the final episode, the impact was much more influential on the composition of the EPS-matrix than on the hydrophobic character of the biomass.

CONCLUSION

Extreme shear, due to mechanical mixing both at the anaerobic and aerobic periods at the first operational stage, had an adverse impact on the granulation, caused deterioration of biomass characteristics and resulted in extensive washout. Decreasing the settling time to impose an indirect selective pressure in the direction of discarding the slowly settling flocks and retaining the particles with a presumably aggregative physiology and with high settling velocities, resulted only in a temporary positive effect, which remained negligible when compared to the adverse impact of extreme shear. Relaxing the overall shear stress at the second stage promoted initiation and progress of granulation until the process was disturbed upon decreasing the airflow rate. The consequence of the latter might be a low DO level; a condition known to result in the kinetic selection of fast-growers like filaments, when combined with the presence of a readily biodegradable carbon source. The biomass experiencing a considerable physical stress – significantly high shear rates – grew into an aggregative physiology through increasing its cell surface hydrophobicity. The change in the hydrophobic character of the biomass upon initiation and progress of granulation was more pronounced (positive) than that (negative) upon loss of granule stability. Note that, severe conditions should be avoided primarily to prevent total biomass washout. Increased EPS production was concluded to contribute to the aggregative-physiology and

eventually to granulation at the second stage. The relation between the EPS-composition and progress of granulation determined in this study (increasing ExoPN/ExoPS) was the opposite of those reported in many cases, but in line with some others and it was not possible to attribute the observed difference only to the employed extraction procedures. The definite outcome of the EPS measurements was that ExoPN, but not ExoPS, was the major component of biomass-EPS under all conditions, as also stated by Henze (2007). Finally, variations in the EPS-composition were parallel to those in the hydrophobic character of the biomass, yet disturbance of granulation was more influential on the former.

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