Image analysis as a tool to predict washout in sludge bed reactors through the quantification of microbial filamentous structures

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Abstract Image analysis techniques are applied to monitor the morphological changes in granular sludge, present in an EGSB reactor fed with oleic acid. Deterioration of granular sludge was monitored along the trial period by measuring the percentage of aggregates smaller than 1 mm (in terms of Feret diameter) either using the projected area or the number of aggregates. The ratio of total filaments length to cross-sectional area of aggregates defined as LfA, was applied to quantify the dispersion level of the granular sludge. LfA was sensitive to the sludge deterioration process and was able to indicate, with the anticipation of about 1 month, the most significant biomass washout episode that occurred in the trial period. A mechanism of filaments release, detachment and selective washout was proposed to explain the action of LfA from this viewpoint.
Keywords Long chain fatty acids (LCFA), image analysis, EGSB reactors, granular sludge stability

Introduction

When fed with some problematic substrates, the stability of granules-based anaerobic reactors, such as the UASB or EGSB reactors, is largely affected. Lipidic compounds are one of these problematic substrates. They are easily hydrolyzed to Long Chain Fatty Acids (LCFA), which adsorb onto the biomass, inducing granule disruption, flotation and washout (Hwu, 1997). This seems to occur at concentrations far below the toxicity limit, which might suggest that complete washout of granular sludge would occur prior to inhibition. Furthermore, the addition of calcium salts prevents LCFA inhibition to some extent, but does not prevent sludge flotation (Hanaki et al., 1981).

Usually the processes of granulation and granule-disintegration are linked to a macroscopic transformation of size and morphology that can be quantified by image analysis. Although some published works are mainly focused on size determinations (Dudley et al., 1993; Jeison and Chamy, 1998), morphological parameters such as fractal dimension (Bellouti et al., 1997), and quantification of bacterial morphotypes within anaerobic granules from transmission electron micrographs (Howgrave-Graham and Wallis, 1993), are examples of application of image analysis techniques to granular sludge characterization. In a previous work, the number and the total length of filaments present in anaerobic sludge when exposed to oleic acid shock loads was monitored by image analysis (Alves et al., 2001).

Bacterial filamentous forms play a key role in the process of granulation (Wiegant and de Man, 1986) being therefore expected to occur the release of these forms to the bulk medium in the process of granules disruption. The quantification of filaments and filaments to aggregates ratio can thus give important insights on process stability and can potentially be included in expert systems, as alert indicators, for supervision and control of high rate anaerobic wastewater treatment.

The aim of the present study was to develop and apply quantitative image analysis to monitor a granules deterioration process. Morphological parameters sensitive to the aggregation status of anaerobic sludge are proposed.

Materials and Methods

Experimental set-up and operation mode

An EGSB reactor with a volume of 10 liters was fed with increasing oleate concentrations between 2 and 8 g COD/l, at an HRT of 1 day. Table 1 summarizes the steady-state operating conditions and performance during the trial period.

Table 1 Operating conditions and performance of the EGSB reactor (mean± 95% confidence intervals)

Period	Time (d)	HRT (d)	Influent COD (g/l)	Influent oleate COD (g/l)	COD Removal efficiency (%)	Effluent VSS (g/l)	Methane production (l CH_4/(l.d))
I	0-70	1.01 (±0.01)	3.8 (±0.3)	1.9 (±0.2)	96.5 (±0.6)	0.38 (±0.07)	1.06 (±0.1)
II	70-119	1.01 (±0.01)	3.8 (±0.3)	3.8 (±0.3)	83.4 (±4.8)	0.85 (±0.22)	0.23 (±0.05)
II	119-162	1.01 (±0.01)	6.2 (±0.7)	6.2 (±0.7)	74.2 (±3.8)	1.96 (±0.43)	0.16 (±0.02)
IV	162-219	1.01 (±0.01)	8.2 (±0.5)	8.2 (±0.5)	68.8 (±3.4)	2.71 (±0.57)	0.15 (±0.02)

Temperature was kept at 37±1 ºC. A recycling rate of 14 l/d was applied.

Routine analysis

Routine reactor performance was monitored by determining influent and effluent total and soluble (centrifuged 10 min at 15 000 rpm) Chemical Oxygen Demand (COD), influent flow rate, methane production and Volatile Suspended Solids (VSS). COD and VSS were determined according to Standard Methods (APHA et al., 1989). Biogas flow rate was measured by a “Shinagawa Seiki DC-1C Dry test gas meter” (Shinagawa Corp., Tokyo, Japan). Methane content of the biogas was determined by gas chromatography using a Haysep Q (80-100 mesh) column (Chrompack, Middelburg, The Netherlands), with N2 carrier gas at 30 ml/min and a flame-ionisation detector. Temperatures of the injection port, column, and flame-ionisation detector were 120, 40 and 130 ºC, respectively. Biogas samples were analysed in triplicate and the results average expressed as percentage methane by calibration against standards of 50% (v/v) methane/air.

Seed Sludge and substrate

Granular sludge, obtained from an UASB treating a brewery effluent, was used as seed sludge. 1.6 l (20.2 g VSS/l) were inoculated. The inoculum was characterized in terms of size and morphological characteristics. The average equivalent diameter of the aggregates larger than 1 mm was 1.59±0.07 mm and the average equivalent diameter of the aggregates smaller than 1 mm was 0.184±0.008 mm. 46.5% of the total projected area accounted for aggregates larger than 1mm. The ratio between the total filament length and the total area of aggregates (LfA) was 37.7 mm^{-1}.

For the first 70 days, the substrate consisted of skim milk (50% COD) and sodium oleate (50% COD) diluted with tap water. From the day 70 on, the carbon source was exclusively composed by sodium oleate, supplemented with macro and micronutrients and $NaHCO_3$/l as described by Araya Kroff et al. (2004).

Sludge sampling and processing for image analysis

The sludge inside the reactor was segregated into two layers: a heavy bottom layer and a top floating layer, located in the top of the reactor, at liquid-gas interface. Sludge samples for image analysis were taken on days 70, 92, 119, 141, 162, 191, and 219 from both layers. For all the samples, the VSS content was determined. Special care was put on the dilution process. Biomass samples must be diluted for image analysis using an optimized dilution factor. Excessive dilution increases the number of objects detected, due to a search-error practice. If the dilution is insufficient, the objects will be overlaid. Experiments were done in order to determine the optimal dilution value for the different measurements performed (filaments, micro and macro-aggregates). The optimal dilution value was determined as the lowest dilution that enabled the maximum percentage of objects recognition. The percentage of recognition is the ratio between the area of objects that are completely inside the image and the total area of objects in the image, including those that are at the boundaries and cannot be completely recognized.

Image acquisition and analysis

Filament image acquisition was accomplished through phase contrast microscopy on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 100x magnification. Images used to quantify aggregates larger than 0.2 mm in equivalent diameter were acquired through visualisation on an Olympus SZ 40 stereo microscope (Olympus, Tokyo) with a 40x magnification. Images used to quantify aggregates smaller than 0.2 mm in equivalent diameter were acquired through visualisation on a Zeiss Axioscop microscope (Zeiss, Oberkochen) with a 100x magnification. All the images were digitised and saved with the help of a CCD AVC D5CE Sony video camera (Sony, Tokyo) and a DT 3155 Data Translation frame grabber (Data Translation, Marlboro), with a 768 x 576 pixel size in 8 bits (256 grey levels) by the Image Pro Plus (Media Cybernetics, Silver Spring) software package. 100 images in average per sample were acquired. Three programmes were developed for image analysis and processing which are detailed in Araya-Kroff et al. (2004) and Amaral (2003): 1) The Filament program that determines the total filament length present in images acquired through microscopic observation with a 100X magnification. The dispersed bulk filaments, but also those that are attached to an aggregate were quantified. 2) The Micro-aggregates program that determines the Feret diameter of aggregates smaller than 0.2 mm, also applied to images acquired through microscopic observation with 100X magnification, and 3) the Macro-aggregates program that determines the Feret diameter of aggregates larger than 0.2 mm, present in images acquired through visualization on a stereo microscope (40X magnification).

Batch experiments

Some of the samples characterized by image analysis were characterized for the amount of biomass-associated LCFA as well as their mineralization rate, in batch tests, as previously described by Pereira et al. (2002). Table 2 summarizes the obtained values.

Results and Discussion

The tentative monitoring of granules deterioration by the average equivalent diameter was unsuccessful. The aggregates were then classified using the minimal Feret Diameter (minFD) defined as the minimum distance between parallel tangents touching opposite sides of an object (Figure 1). Two size ranges were considered: smaller aggregates and large aggregates with minFD < 1 mm and minFD > 1 mm, respectively. Figure 2 shows the change of the percentage of small aggregates in terms of their projected area and number of particles. These results represent average values between the two segregated bottom and top layers of sludge.

Table 2. Maximum plateau and methane production rate obtained in the batch experiments for the biomass associated LCFA present in the bottom and top sludge at different moments of the reactor operation (Mean±SD).

Period	Maximum "plateau" (mg COD-CH_4/gVSS)		CH_4 production rate (mg COD-CH_4/(gVSS.day))	
	Bottom	Top	Bottom	Top
I (Day 70)	263±17	276±37	100±17	114±12
II (Day 119)	97±34	434±17	11±3	86±9
III (Day 162)	97±6	997±109	14±3	200±6
IV (Day 219)	51±3	100±11	3±1	4±1

It is evident that size reduction of granules occurred along the trial period, as the fraction of small aggregates increased steadily, whatever the method used for its determination.

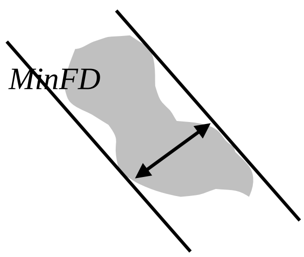


Figure 1 Definition of the Minimum Feret Diameter (minFD)

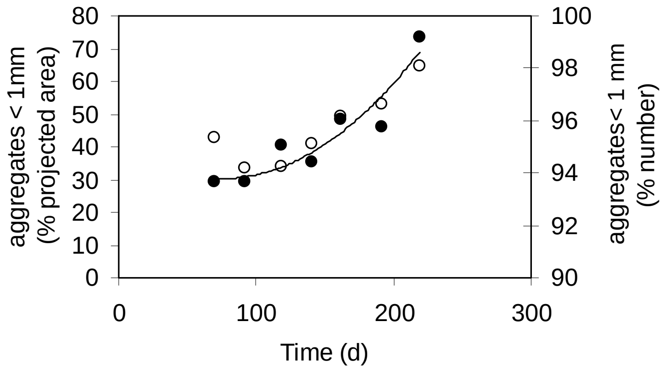
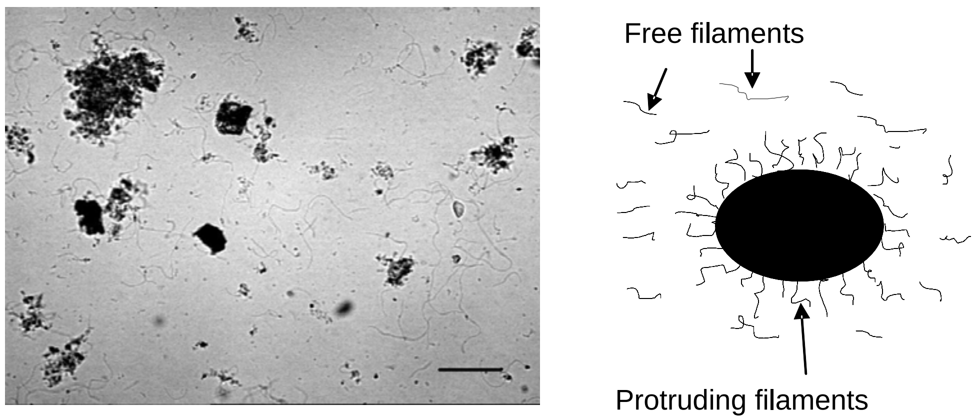


Figure 2 Time course of the ratio of aggregates < 1 mm determined by image analysis (o - % of number, • - % of projected area)

Filaments length was quantified and a parameter defined by the ratio between the filaments length, either free or protruding from dense biomass aggregates (Figure 3) was calculated along the operation for the bottom and top layers.



(a) (b)

Figure 3. (a) Microscopic image of aggregates and filaments (Bar represents 100 m). (b) Schematic representation of an aggregate and filaments quantified by the "filaments" programme.

This parameter, designed as Lfa was already applied to detect critical events in a granulation process (Araya-Kroff et al., 2004). Figure 4 presents LfA values as well as effluent VSS concentration along the trial period.

A morphological stratification occurred within the reactor, with a significantly higher filaments-to-aggregates ratio in the top than in the bottom layer, from the day 100 on. After day 140, the morphological structure in terms of filaments-to-aggregates ratio remained approximately invariant for both sections. An important aspect is the relative change of *LfA* and effluent VSS along the operation. It can be observed that, in the period between days 100 and 140, *LfA* in the top layer increased about 6 times, from 40 to 250 mm^{-1}. In the same time period the VSS increased moderately from a minimum of 0.81 to a maximum of 1.97 g/L with an average value of 1.3±0.5 g VSS/L. From the day 140 on, the *LfA* remained oscillating between 200 and 270 mm^{-1}, but the effluent VSS concentration showed the most significant increase, oscillating between a minimum of 1.4 g VSS/L and a maximum of 4.8 g VSS/L with an average value of 2.4±0.9 g VSS/L.

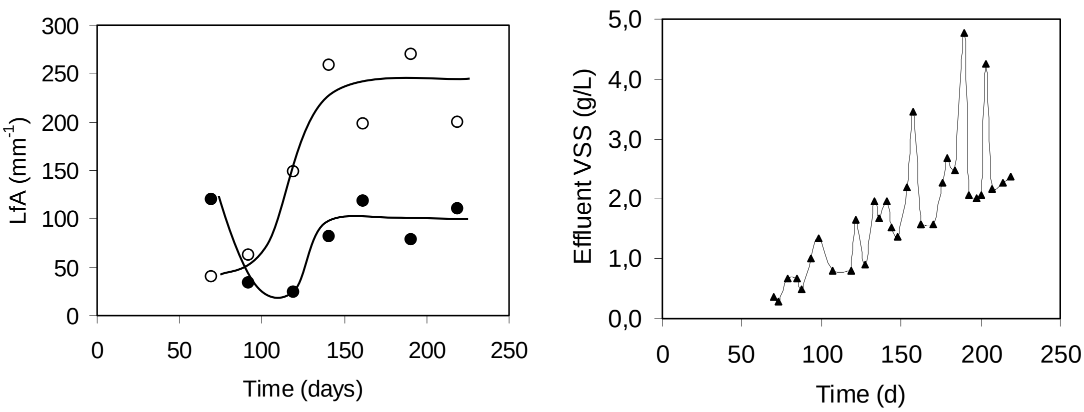


Figure 4 Time course of the ratio between the total free filament length and the total projected area of aggregates (*LfA*) in the bottom (•) and top (o) sludge layers and effluent VSS concentration.

Data from tables 1 and 2 allowed estimating that about 30% of VSS could be attributed to the accumulation of LCFA onto the biomass. Even after correction of the observed effluent VSS concentration a large biomass washout could be detected. *LfA* was, in this case, sensitive to the sludge deterioration process and was able to indicate, with the anticipation of about 1 month, the most significant biomass washout episode that occurred in the reactor operation. This potential capacity of *LfA* to predict washout events is of paramount importance from a practical viewpoint.

The release of filaments simultaneous with the fragmentation induced by the contact with oleic acid seems to be a reasonable explanation for the increase in *LfA* in the top sludge, between days 100 and 140. However, the evolution of filaments and small aggregates did not follow similar trends, during the operation of the reactor. Although the relative amount of small aggregates increased steadily all along the operating period (Figure 2), the ratio between filaments and aggregates (*LfA*), remained almost constant after day 140, which is likely because filaments, especially those that are not linked to any fragment of granule, could have been selectively washed out from the reactor. The small aggregates which can be fragments of granules that have still granular properties in terms of density may have been retained inside the reactor, notwithstanding the smaller size. The dynamic of fragmentation, filaments release and washout is described in the schematic chronogram of Figure 5.

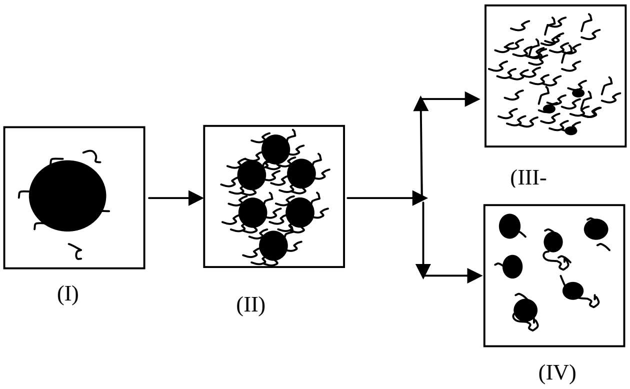


Figure 5 Schematic chronogram of filament release, fragmentation, and washout. I – granule, II – fragmentation and filaments release, increase in LfA, III – detachment and selective washout of filaments, IV – Permanence of small aggregates inside the reactor.

Conclusions

A morphological parameter relating the amount of free filaments and the projected area of aggregates (LfA) is proposed to monitor granular sludge stability in UASB/EGSB reactor. An example of application to an Oleic acid fed EGSB reactor is presented. There are hints that Lfa can indicate with some anticipation washout events in these reactors. A mechanism of filaments release, detachment and selective washout was proposed to explain the effect on LfA.

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