Characterisation by image analysis of anaerobic sludge under shock conditions

M. Alves,* A.J. Cavaleiro,* E.C. Ferreira,* A.L. Amaral,* M. Mota,* M. da Motta, H. Vivier** and M-N. Pons****

*Centro de Engenharia Biológica – IBQF, Universidade do Minho, 4710-057 Braga, Portugal (Fax: +351.253678986), (E-mail: *madalena.alves@deb.uminho.pt*)

**Laboratoire des Sciences du Génie Chimique, CNRS-ENSIC-INPL, BP 451, F-54001 Nancy cedex, France

Abstract In the present work the characterisation by image analysis of anaerobic biomass under organic and hydraulic shocks was performed. The digester was fed with a synthetic substrate, containing 50% oleic acid (as COD). Organic and hydraulic shocks were performed by stepwise increasing the substrate concentration or by reducing the hydraulic retention time. In both cases the organic loading rate changed from 6 to 30 kg COD/m^3.d. Hydraulic shock induced a fast decrease in the number of free filaments and in total filament length, which attained a minimum value 51 hours after beginning the shock. The initial filament values were not recovered 340 hours after the hydraulic shock. In the organic shock, the minimum values of these parameters were detected 200 hours after beginning the shock and initial values were recovered 840 hours after. During the hydraulic shock the methanogenic acetoclastic activity was directly correlated to the number and length of free filaments. This result suggests that filaments are predominantly acetoclastic bacteria, probably *Methanosaeta*.

Keywords Aggregates; anaerobic digestion; biomass; granulation; image analysis; shock conditions

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Introduction

In the last two decades, anaerobic digestion technology was significantly improved by the development of the sludge bed digesters, based on granular biomass. Upflow Anaerobic Sludge Blanket (UASB) reactors represent nowadays more than 65% of all anaerobic digesters installed for treating industrial wastewater. However, in spite of the existence of more than 900 UASB units operating all over the world, it is recognised that some basic mechanisms underlying granulation are still unclear. On the other hand, granular characteristics such as settling rate, size and mechanical resistance are frequently affected by operating conditions, resulting in irreversible granule disintegration.

Usually the processes of granulation and granule-disintegration are coupled with a macroscopic transformation of morphology. Several works refer to the characterisation of these morphological changes using image analysis, although most of them are mainly focused on size determinations (Bellouti *et al.*, 1997; Jeison and Chamy, 1998). It is accepted that filamentous organisms play a key role in the process of granulation, being responsible for the first nuclei of aggregated biomass. Systematic microscopic examinations have not been used so far to follow the granulation/disintegration process, because they are tedious and difficult to implement in a quantitative way. However, the use of automatic image analysis coupled to microscopic observations may overcome this problem.

In the present work, image analysis techniques were applied to the characterization of biomass from an anaerobic hybrid digester fed with an oleic acid based effluent, under shock loading conditions. It was possible to differentiate between aggregates and dispersed sludge, quantifying the number and the length of free filaments as well as the total and aggregated biomass. Due to inherent slow growth rate of the methane producing bacteria, a better knowledge of the dynamic growth of microbial populations during transient

conditions can contribute to the development of appropriate modifications in design and operation, leading to improvements in the stability of the system (DeLorme and Kapuscinski, 1990).

Oleic acid is a long chain fatty acid (LCFA) that exerts a toxic effect on both acetogens and methanogens. There is evidence that aggregated biomass is more resistant to LCFA toxicity than the suspended one (Hwu *et al.*, 1996). However granulation and/or granule stability is problematic for lipid-containing wastewaters (Sam-soon *et al.*, 1991; Hawkes *et al.*, 1995). From a thermodynamic viewpoint, biomass disintegration is predictable because at neutral pH, LCFA act as surfactants, lowering the surface tension. Consequently the aggregation of hydrophobic bacteria, like most acetogens (LCFA-degraders), is unfavourable (Daffonchio *et al.*, 1995).

Methods

Experimental set-up

The anaerobic reactor configuration was described in detail elsewhere (Alves *et al.*, 1998) and allowed the regular withdrawal of biomass with minimum operation disturbances. The reactor was a cylindrical tank constructed in PVC, with a diameter of 48 cm and a total volume of 86.8 L. In the central section 27 mini-bioreactors, with a total volume of 989 cm^3 each, were arranged in parallel constituting the support matrix. The support medium consisted of PVC Raschig rings of 21 mm in size (86 in each mini-bioreactor), and had a specific surface area of 230 m^2/m^3 and a porosity of 92.5 %. The effluent leaving the reactor entered a Plexiglas settler, and the settled biomass was recycled. The applied recirculation ratio was approximately 1. However in the case of the hydraulic shock the recirculation ratio was 0.2, otherwise the upflow velocity would be excessively high. Figure 1 represents a scheme of the experimental set-up.

Substrate and inoculum

The substrate was based on oleic acid (50% COD) with skim milk as co-substrate (50% COD). Macro and micronutrients were supplemented as previously described (Alves *et al.*,

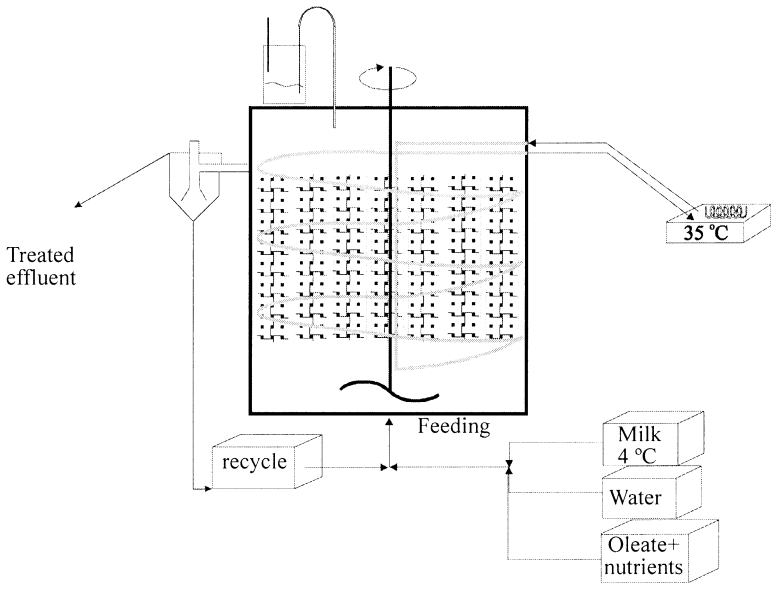

Figure 1 Schematic representation of the experimental set-up

1998). The inoculum was taken from a local municipal sludge anaerobic digester. A volume of 15 L of anaerobic sludge containing 10 g volatile suspended solids/L was inoculated in the reactor.

Operation mode

After the start-up, oleate was introduced as 50% of the total fed COD, on day 83. During the "pre shock conditions", the total COD was 4 g/L and the hydraulic residence time (HRT) was 16 hours. The first load shock – organic shock, was accomplished by increasing the substrate concentration to 20 gCOD/L, during four days, keeping the HRT and the relative proportion of skim milk and oleic acid. The second load shock – hydraulic shock, was accomplished by decreasing the HRT to 3.2 hours during 4 days, keeping the substrate concentration. Table 1 summarizes the operating conditions applied to the reactor during the trial period.

Biomass sampling

At different times during the shocks, the reactor was opened and some of the mini-bioreactors, randomly selected, were removed and replaced by new similar ones, containing biomass accumulated in the settler. The separation and quantification of adhered and entrapped fractions are described elsewhere. Biomass samples for image analysis were representative of the entrapped fraction which, on average, accounted for 75% of the total supported biomass. The samples had similar VSS concentrations in the range of 120–150 mg/L. After starting both shocks, biomass samples were taken at the times specified in Table 2.

Methanogenic activity and toxicity

Methanogenic activity tests were performed using the pressure transducer technique (Colleran *et al.*, 1992), which involves the monitoring of the pressure increase developed in sealed vials fed with non-gaseous substrates or pressure decrease in vials pressurised with

Table 1 Operating conditions applied to the reactor

Time (days)	Total influent COD (mg/L)	Oleate influent COD (mg/L)	HRT (hours)	Organic loading (kg COD/m^3 · day)	Remarks
0–16	2000	0	96	0.5	
16–34	2000	0	48	1	
34–54	4000	0	48	2	
54–83	4000	0	24	4	
83–102	4000	2000	24	4	Introduction of oleate
102–140	4000	2000	16	6	Pre-shock conditions
140–144	20000	10000	16	30	Organic shock
144–230	4000	2000	16	6	Pre-shock conditions
230–234	4000	2000	3.2	30	Hydraulic shock
234–286	4000	2000	16	6	Pre-shock conditions

Table 2 Times of biomass withdrawal after starting the organic and the hydraulic shocks

	Sampling time after starting the shocks (hours)											
Organic shock	0	4	11	14	25	33	49	63	80	95	145	840
Hydraulic shock	0	5	9	24	51	74	95	167	341	893		

gaseous substrates. Strict anaerobic conditions were maintained and no calcium or trace-nutrients were added. The same technique was used to perform the methanogenic toxicity tests (Colleran and Pistilli, 1994). The oleate concentrations ranged from 100 to 900 mg/L and 30 mM acetate was added to each vial. The fifty percent inhibition concentration (IC_{50}) was defined as the oleate concentration that caused a 50% relative activity loss.

Image analysis

Image Acquisition. Image acquisition was accomplished through the visualisation on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 400× magnification, and digitised with the help of a CCD AVC D5CE Sony camera (Sony, Tokyo) and a DT 3155 Data Translation frame grabber (Data Translation, Marlboro). The images were digitised with a 768×576 pixel size and 256 grey levels by the Image Pro Plus (Media Cybernetics, Silver Spring) software package.

The morphological parameters were determined by a programme developed in Cool language and run in the commercial software package Visilog 5.1 (les Ulis, France). The image analysis techniques here described were already applied to the morphological characterization of activated sludge flocs (Da Motta *et al.*, 1999), and consist of four major parts:

Image improvement and binarisation. A series of morphological openings followed by morphological closings are applied to obtain the background image. A histogram equalisation and a delineation are then applied to improve the image. The background image is afterwards subtracted from the later one. In order to binarise the image, a method based on the histogram variance is applied to determine the threshold level.

Filament/debris and flocs identification. A morphological opening (2 pixels size) and a logical subtraction from the binary image are applied. The resulting image contains only flocs. The filaments/debris image is obtained by subtracting the flocs image from the total binary image.

Filament identification. A labelling of the filament/debris image is then performed: the 2nd order moments and the area of the objects are determined. With these parameters, the programme is able to determine the Gyration radius (Gr):

$$G_r = \frac{\sqrt{M_{2X} + M_{2Y}}}{D_{eq} / 2} \tag{1}$$

where D_{eq} is the equivalent diameter $\left(D_{eq} = 2\sqrt{\text{Area} / \tau}\right)$ and, M_{2x} and M_{2y} are the 2nd order moments.

The objects that do not meet the Gyration radius and Area criteria (Gr >1, Area >200) are subsequently considered as debris and removed. The remaining objects are filaments. A morphological skeletonisation is then performed and the number and length of the objects (filaments) are determined.

Floc characterisation. A dilation, a hole-fill function and an erosion are then applied in order to fill possible holes in the flocs. Next, the objects lying in the image borders are discarded by a border-kill function. Morphological erosion and subsequent reconstruction followed by image labelling and area determination of the remaining objects (flocs) are the final steps of this stage.

Total biomass. The total biomass is given by the sum of the areas of the filaments and the flocs. Figure 2 represents examples of images to be processed by the software. Image 2b has a greater number of aggregated filaments forms than image 2a.

Results and discussion

The parameter total surface detected by the image analysis software was determined for each sample. For the organic shock the average value was 8582±385 μm^2 and for the hydraulic shock it was 8002±225 μm^2. These values indicate that the variation in biomass concentration, as detected by the image analysis system was about 2% for the samples from the hydraulic shock and 4.5% for the samples of the organic shock.

Figure 3 shows some steps of the image processing

Figures 4 a and b represent the number of filaments per image during the organic and the hydraulic shocks, respectively. During both shocks a decreasing oscillating behaviour was observed. In the organic shock a minimum value of 72% of the initial value was achieved 200 hours after the beginning, and after 840 hours, the initial value was recovered. However, in the hydraulic shock a minimum of 82% of the initial value was achieved 51 hours after the beginning, but there was no evidence of recovery improvement 340 hours after. This suggests that washout or aggregation of filaments occurred by suddenly increasing the concentration or the flow rate. However this effect was more persistent after the hydraulic shock. The decrease in the number of filaments was expected under shock load conditions. It is known that microbial filamentous forms prevail under low substrate concentration. On the other hand, the selection pressure imposed by the hydraulic shock which is the sum of the hydraulic loading rate and the gas loading rate could have induced the aggregation of biomass as suggested by Hulshoff Pol *et al.* (1988). This effect is visible by comparing Figures 2a and b.

This possible aggregation can, in part, justify the increase in resistance to the toxicity, expressed by the increase in the toxicity limit (IC_{50}) measured before and after the hydraulic shock (140±30 and 215±25 mg oleic acid/L, respectively). The same effect was not observed in the organic shock, where the toxicity limit decreased significantly between the beginning and the end of the shock (120±30 and 20±5 mg oleic acid/L, respectively).

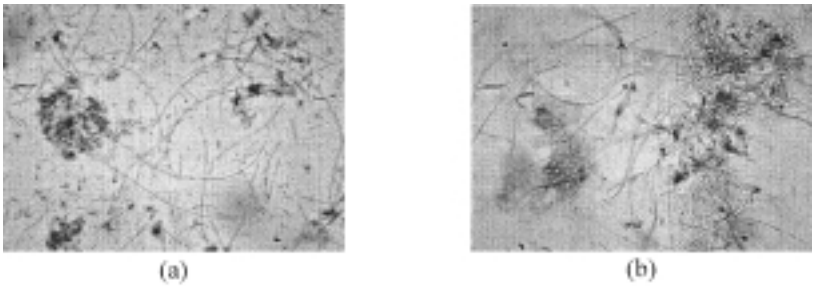

Figure 2 Images from two samples collected before (a) and 51 hours after (b) the beginning of hydraulic shock

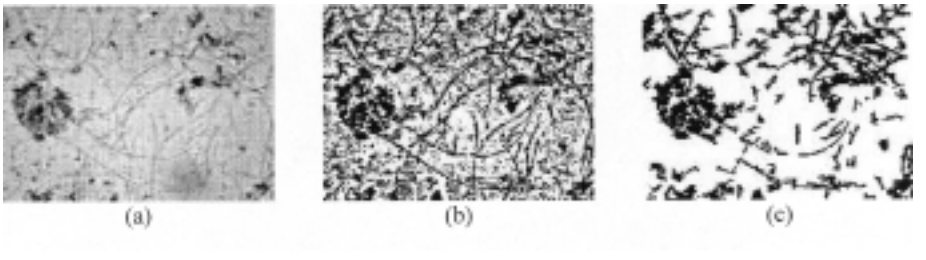

Figure 3 Images from the image processing .(a) Acquired image. (b) Image resulting from binarisation. (c) Final image containing flocs and filaments

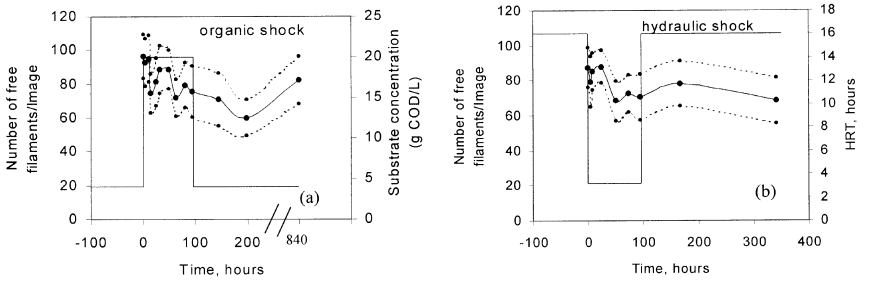

Figure 4 Number of free filaments per image. (a) organic shock. (b) hydraulic shock. The dashed lines represent the boundaries of standard deviation

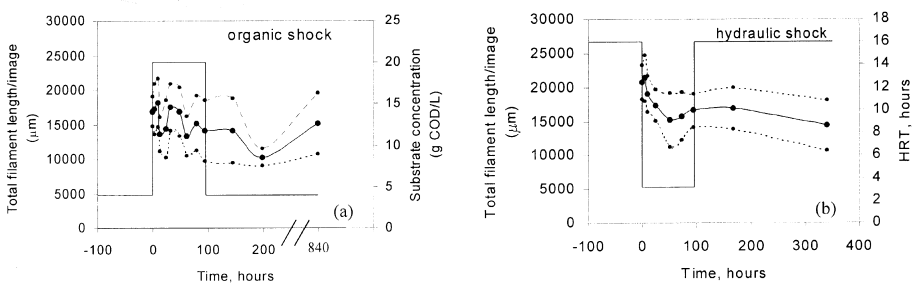

Figure 5 Average total filament length detected per image. (a) organic shock. (b) hydraulic shock. The dashed lines represent the boundaries of standard deviation

The average total filament length detected per image processed is represented in Figure 5. As obtained for the number of filaments, in the organic shock a minimum value was detected 200 hours after the beginning, representing 60% of the initial filament length. After 840 hours 90% of the initial value had been recovered.

As detected for the number of filaments, in the hydraulic shock a minimum of 73% of the initial filament length was achieved after 51 hours. No significant recovery was detected after 340 hours. At that time the filament length was only 70% of the initial value. The filament length can be related to the filament number, but also to the filament morphology. Filamentous microorganisms in anaerobic digesters can represent several different species of acidifiers, acetogens and methanogens. However one of the more well known filamentous methanogenic bacteria is the acetoclastic *Methanosaeta*. As reported by Wiegant (1988), this bacterium can have different morphologies from very long filaments (>1000 units long) to short filaments of 5–10 units long and is the dominant acetoclastic under low substrate concentration (Gujer and Zehnder, 1983). In the hydraulic shock, where the effluent acetate levels achieved a maximum value of 250 mg/l in comparison with 2200 mg/L in the organic shock, it was supposed that the detected filamentous forms could be predominantly due to the presence of the acetate splitting methanogen *Methanosaeta*. This was confirmed by the correlations obtained between the methanogenic acetoclastic activity and the number and length of filaments determined in the samples taken during the hydraulic shock (Figure 6). In the organic shock, as expected, there was no correlation between these parameters (Figure 7).

Conclusions

Hydraulic shock induced a fast decrease in the number of free filaments and in total filament length, which attained a minimum value 51 hours after beginning the shock. The initial

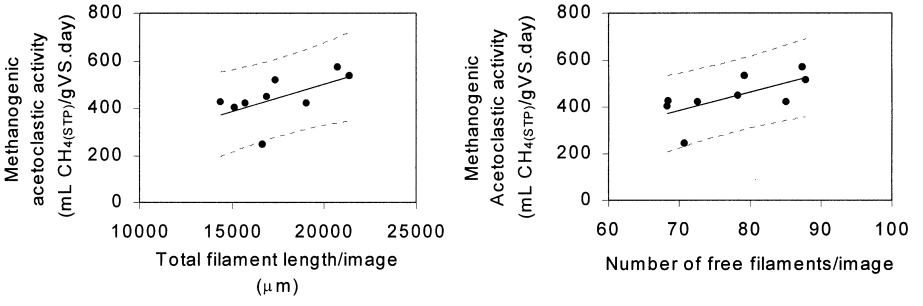

Figure 6 Correlation between (a) the total filament length and (b) the number of free filaments with the methanogenic acetoclastic activity during the hydraulic shock

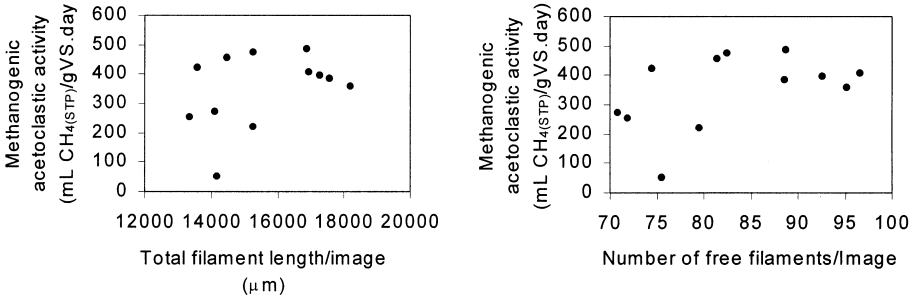

Figure 7 Total filament length (a) and number of free filaments (b) versus methanogenic acetoclastic activity during the organic shock

filament values were not recovered 340 hours after the hydraulic shock. In the organic shock, the minimum values of these parameters were detected 200 hours after beginning the shock and initial values were recovered 840 hours after. During the hydraulic shock the methanogenic acetoclastic activity was directly correlated to the number and length of free filaments suggesting that these filaments are predominantly acetoclastic bacteria, probably *Methanosaeta*. In the organic shock, where levels of acetate at the effluent were ten-fold those from the hydraulic shock, the acetoclastic activity was not correlated to the number and length of free filaments. From the current work it is expected that the developed image analysis techniques would be helpful to study early stages of anaerobic granulation.

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