Characterisation by image analysis of anaerobic sludge from two EGSB reactors treating oleic acid: automatic detection of granules disintegration

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ABSTRACT

Suspended and granular sludge from two expanded granular sludge bed (EGSB) reactors fed with oleic acid at organic loads between 2 and 8 kg COD/m^3.day was characterised by image analysis. The number, and the average length of free filaments in the dispersed fraction of biomass, and the morphologic changes of granular sludge when exposed to increasing oleic acid concentrations were quantified. Two methods to distinguish fine and non-fine solids by image analysis were validated by a method based on the ratio fine VSS/total VSS. Granules disintegration was detected by these methods. The adsorption of LCFA onto the sludge could be related to the morphology of the non-fine aggregates and to the number of free filaments. The number of free filaments and the average free filament length was significantly higher in the suspended than in the granular sludge. The degradation rate of the adsorbed LCFA decreased sharply as the size of fine aggregates increased, possibly due to internal diffusion limitations of intermediates such as acetate and products such as methane.

KEYWORDS

EGSB; granular sludge; image analysis; oleic acid; suspended sludge

INTRODUCTION

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 of microbial aggregates. It is accepted that filamentous organisms play a key role in the process of granulation, being responsible for the first nuclei of aggregated biomass. However, the behaviour of filaments during a process of granules disintegration is not well known. 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 a previous work, image analysis was used to follow the number and the total length of filaments present in sludge when exposed to oleic acid shock loads (Alves *et al*., 2000).

Oleic acid is toxic to the methanogenic bacteria and adsorb onto the biomass, provoking sludge flotation. Furthermore, from a thermodynamic viewpoint, disintegration of granules is predictable, when in contact with this compound, because at neutral pH, long chain fatty acids (LCFA) act as surfactants, lowering the surface tension. Consequently the aggregation of hydrophobic bacteria, like most of acetogens (LCFA-degraders), is unfavourable (Daffonchio *et al*., 1995).

In the present work, suspended and granular sludge from two expanded granular sludge bed (EGSB) reactors fed with a synthetic effluent containing oleic acid as the sole carbon source was characterised. The number, the total and the average length of free filaments in the dispersed fraction of biomass, and the morphologic changes of granular sludge when exposed to increasing oleic acid concentrations were quantified, by image analysis.

METHODS

Biomass

Biomass was sampled from two EGSB reactors. One of the reactors was inoculated with granular sludge (RI) and the other reactor was inoculated with suspended sludge (RII). The operation of these reactors is described in a companion paper (Pereira *et al*., 2001). The influent oleate concentration increased from 2 to 8 g COD/l and the HRT was set at 1 day. For both reactors, the biomass was segregated in two layers, a bottom layer where practically no substrate was adsorbed, and the top layer, which exhibited large amounts of adsorbed substrate, as described elsewhere (Pereira *et al*., 2001).

Table 1. Operating conditions applied to RI and RII

Time (d)	HRT (d)	Influent COD (g/l)	Influent oleate COD (g/l)
0-70	1.01±0.01	3.8±0.3	1.9±0.2
70-119	1.01±0.01	3.8±0.3	3.8±0.3
119-162	1.01±0.01	6.2±0.7	6.2±0.7
162-219	1.01±0.01	8.2±0.5	8.2±0.5

Sludge samples for image analysis were taken on days 70, 92, 119, 141, 162, 191, and 219. For all the samples, the Volatile Suspended Solids (VSS) content was determined. Sludge from RI was also characterised by the % of fine solids, determined by the ratio between the VSS that could be removed using a syringe equipped with a 20 Gx1″ needle, and the total VSS. The breadth of the largest particle that could enter the needle was set at 1 mm and was determined by image analysis using the minimal Feret diameter (*minFD*). This procedure allowed to divide all samples in two categories: fine solids which were all particles with a *minFD* smaller than 1 mm and non fine solids which had a *minFD* larger than 1 mm.

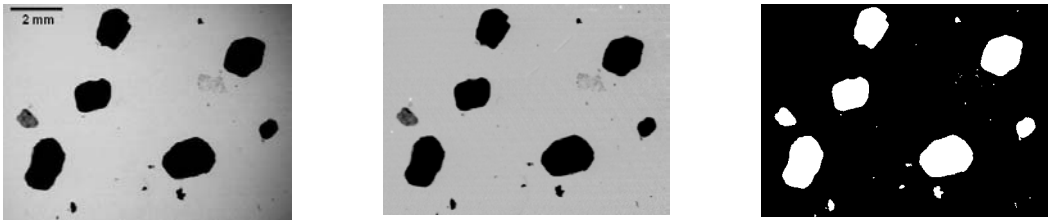
Image Analysis

Image Acquisition. Image acquisition of the filamentous bacteria was accomplished through the visualisation on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 100x magnification. Images from flocs were acquired in an Olympus SZ40 stereomicroscope with a 15x magnification. Images were digitised with a CCD AVC D5CE camera (Sony, Tokyo) and a DT 3155 frame grabber (Data Translation, Marlboro), with a 768 x 576 pixel size and 256 grey levels using the ImagePro Plus (Media Cybernetics, Silver Spring) software package. Size and morphological parameters were determined by means of two programmes developed in Matlab 5.3 (The Mathworks, USA).

Filaments programme. The number of filaments and the average filament length were determined by this programme. First the original image is enhanced by means of a Mexican-hat filter (Russ, 1995), followed by a background homogenisation, Wiener filtering and histogram equalisation. Subsequently, the image is binarised by a defined threshold. In order to identify the filaments skeletonisation and a 10 pixels length end-point removal is performed.

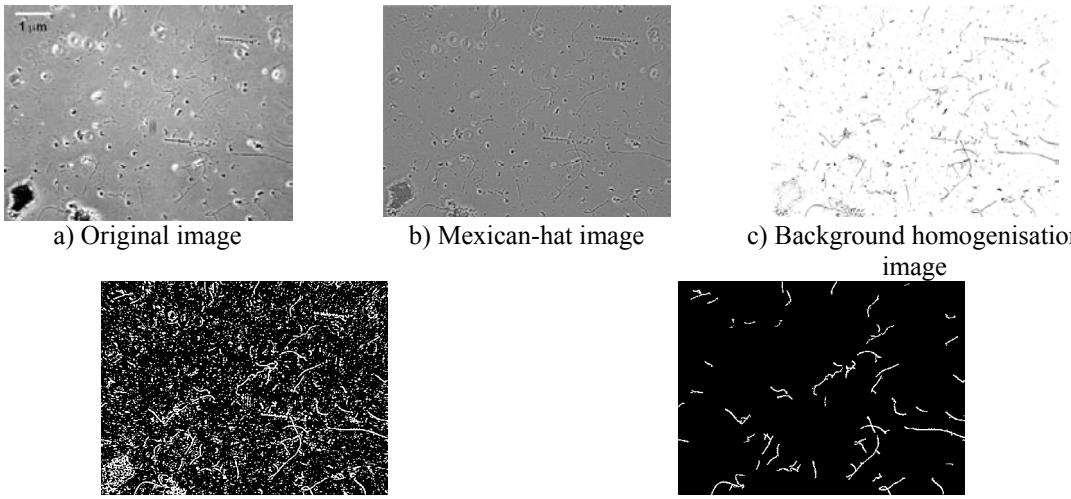
Floc programme. This programme was applied only to the granular sludge. The background from the original image is subtracted and then the image is binarised by a previously defined threshold. In the binarised image only the objects larger than 5x5 pixels are treated, discarding the debris present in the image. The particle size was evaluated by its equivalent diameter (D_{eq}), calculated from its projected area (A) by $D_{eq} = 2\sqrt{A/\pi}$, and by its minimum Feret diameter (*minFD*). *minFD* is the minimum distance between parallel tangents touching opposite sides of an object. The particle morphology was assessed by its roundness (Russ, 1995), defined as the ratio of the particle projected area to the area of the disk with the same convex perimeter (i.e. the perimeter of the smallest convex object that fits the object's boundaries).

Figure 1 and 2 present examples of images from the floc and filament programmes.



a) Original image b) Background subtracted image c) Binary image

Figure 1. Floc programme images.



a) Original image b) Mexican-hat image c) Background homogenisation image

d) First binary image e) Final binary image

Figure 2. Filament programme images.

RESULTS AND DISCUSSION

Figure 3 represents the results obtained from the filament programme.

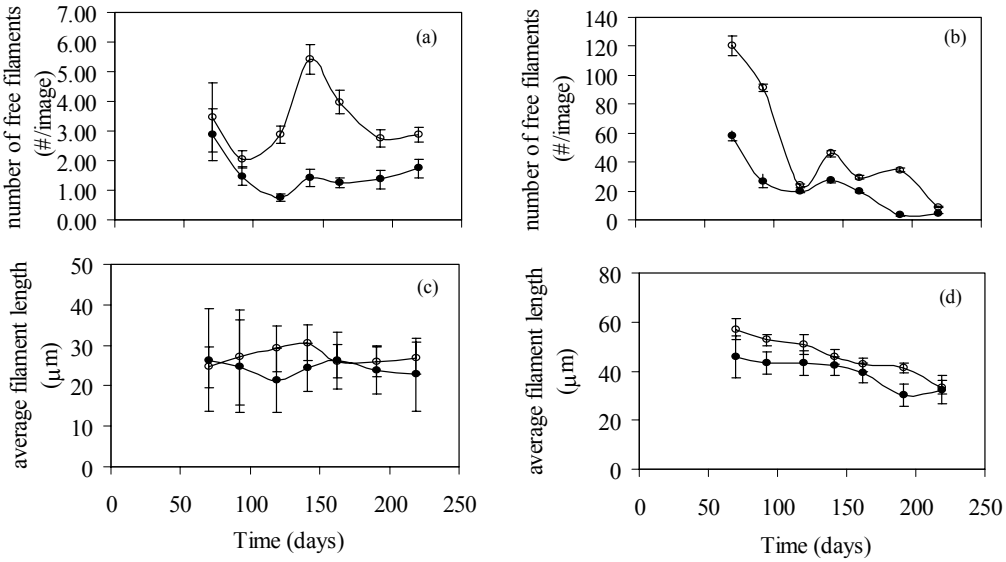


Figure 3. Number of free filaments in RI (a) and RII (b) sludges. Average filament length in RI (c) and RII (d) sludges. Bars represent the 95% confidence interval. (•) bottom, (o) top.

The number of filaments is per VSS concentration unit. As expected, the number of filaments was significantly higher in the suspended (RII) than in the granular sludge (RI). This parameter can be related to the aggregation state of biomass. In the top section of both reactors the free filament concentration was higher than in the bottom section, suggesting that floating sludge was more dispersed than settled sludge. The significant increase of the number of free filaments in the granular sludge around the day 150 is possibly related to the disintegration of granular sludge. The subsequent decrease can be due to the washout of this

dispersed fraction of biomass. The average filament length decreased significantly in the bottom and top sludge of RII, but in RI the changes were no significant. The average free filament length in RI was significantly lower than in RII.

The morphological characterisation of granular sludge was affected by the morphology of the fine solids as they represented more than 90% in terms of number of particles and between 26% and 77% in terms of total detected area of particles (Figure 4 a). This figure clearly shows that granule disintegration occurred along the trial period, as the fraction of fine solids increased steadily, whatever the method used for its determination. A good correlation was obtained between the ratio fine VSS/total VSS and the ratio fine area/total area determined by image analysis (Figure 4 b).

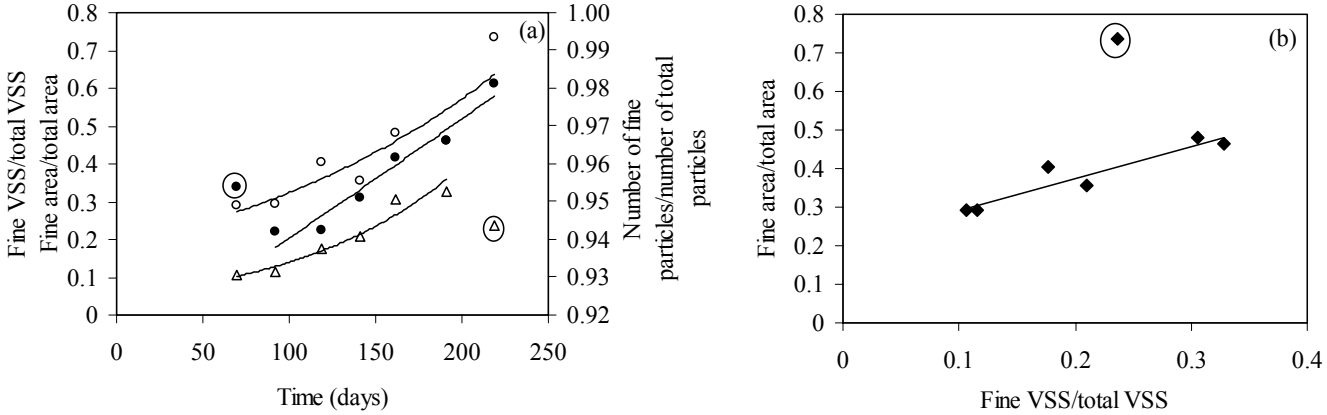


Figure 4. (a) Time course of the ratio of fine solids determined by weight (△-fine VSS/total VSS) and determined by image analysis (o fine area/total area; • number of fine particles/number of total particles). (b) Correlation between a method of image analysis and the method of weighting for the determination of the ratio of fine solids. Marked points were not taken into account for the correlations.

Figure 5 shows the variations of the morphology with the minimal Feret diameter for all the particles examined (≈ 24000) during the run with the granular reactor. The largest particles (*minFD* > 1 mm) are compact but slightly elongated, with an average roundness of 0.77. The standard deviation is slightly larger for the particles at the top (0.08) than at the bottom (0.07). The smallest particles have also an average roundness of 0.77 but with larger standard deviations (0.14).

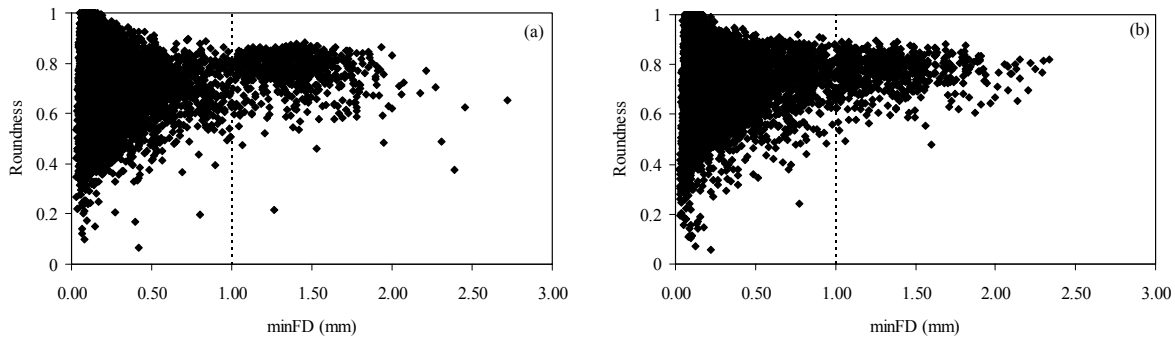


Figure 5. Scatter plots of the roundness versus the minimal Feret diameter for the granular reactor (a) top (b) bottom.

As the oleate loading rate increased, more substrate could adsorb onto the sludge. The amount of adsorbed substrate, which was mainly composed of palmitic acid, was determined in a companion paper by the methane production rate obtained in batch vials without any added carbon source (Pereira *et al*., 2001).

Figure 6(a) and (b) represents the effect of the amount of adsorbed substrate on the average equivalent diameter of the non-fine solids and on the number of free filaments, respectively. In the bottom sludge the maximum amount of adsorbed LCFA was about one fourth the one determined in the top sludge. It is accepted that adsorption of LCFA induces biomass flotation (Hwu, 1997) and consequently higher amounts of adsorbed substrate were expected in the top sludge. In the bottom sludge, the size of the non-fine aggregates increased with the amount of adsorbed substrate. However, when the amount of adsorbed LCFA increased more, the sludge migrated to the top layer and aggregates became smaller. It is understandable that

smaller aggregates were located at the top section and adsorbed more LCFA. Density of these aggregates will be lower than density of the bottom aggregates. It is however difficult to prove that reduction in size is due to the accumulation of adsorbed LCFA, though an effect of disintegration by excess of biogas production or due to thermodynamic reasons could be justified (Daffonchio *et al*., 1995). On the other hand, the number of free filaments (per unit of VSS concentration) increased also with the amount of adsorbed LCFA, suggesting that granular disintegration was simultaneous with a release of filamentous organisms to the medium. However, by comparing figures 6 (a) and (b) it can be seen that only a slightly increase in the filament length is related to the decrease in the average equivalent diameter of the non-fine particles. A sharp increase in the number of free filaments was detected when the average equivalent diameter of the aggregates was still increasing.

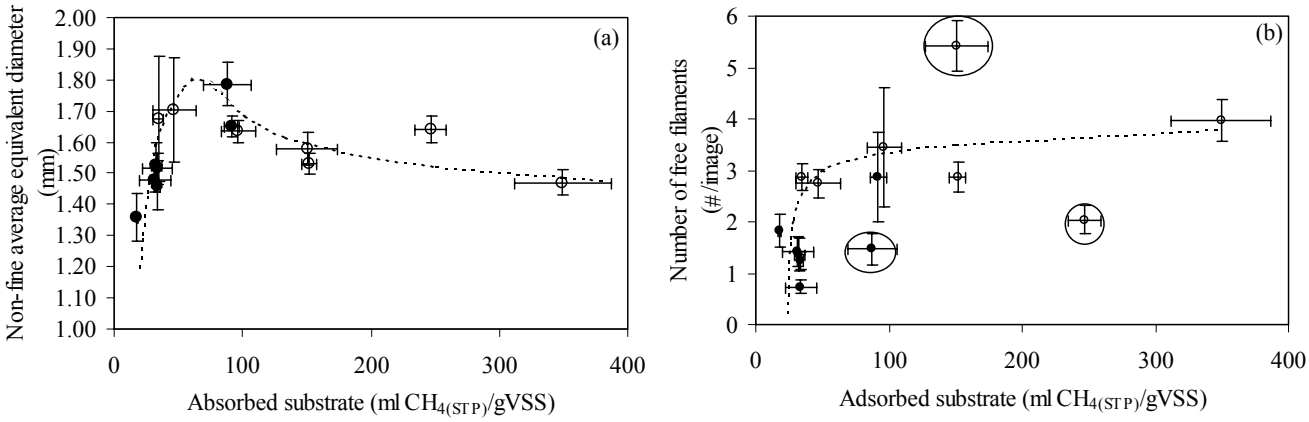


Figure 6. Effect of the adsorbed substrate on the average equivalent diameter of non-fine aggregates (a) and on the number of free filaments detected (b). (•) bottom, (o) top.

The effect of the diameter of fine solids on the degradation rate of the adsorbed LCFA and on the number of free filaments can be observed in figures 7 (a) and (b), respectively.

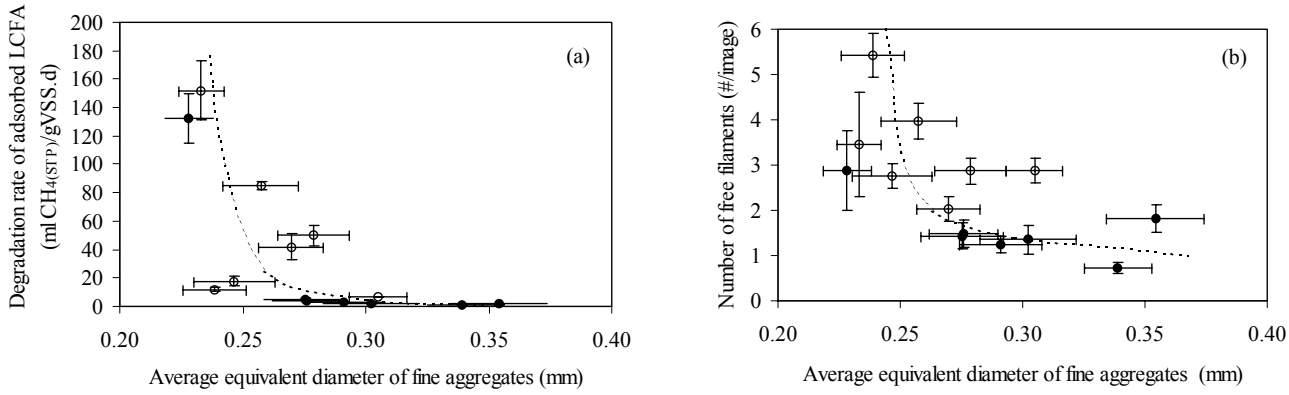


Figure 7. Influence of the average equivalent diameter of fine solids on the degradation of adsorbed substrate (a) and on the number of free filaments (b). (•) bottom, (o) top.

Fine solids represent a significant fraction of aggregates either in terms of number or in terms of detected area (Figure 4 a). A sharp decrease on the degradation rate of the adsorbed substrate (measured in batch vials through the methane production – Pereira *et al*., 2001) was observed when the size of fine aggregates increased. This strong effect can be explained by internal diffusion limitations. Anaerobic microbial consortia can be structured in a well-organised granular sludge taking advantage of the close proximity between syntrophic partners. Higher syntrophic activities are usually detected in granules and biofilms than in dispersed sludge (Alves *et al*., 1999). However, other trophic groups in the consortium do not take any advantage in terms of access of substrate. For instance acetoclastic bacteria that are responsible for about 70% of the total methane production are usually located in the inner core of the aggregates and consequently suffer from substrate unavailability, in the case of bigger aggregates. When a LCFA such as palmitate is degraded through the β-oxidation mechanism, acetate is produced and should diffuse through the particle in

order to reach the acetoclastic trophic group. On the other hand, methane produced should be released from the particles and this will also be easier in small aggregates.

Other interesting aspect is the decreasing trend between the average equivalent diameter of the fine particles and the number of free filaments (Figure 7 b). This behaviour is similar to the observed previously with the non-fine solids (Figure 6). As the size of particles decreases, the number of free filaments increases, suggesting that, in consequence a release of filaments to the bulk medium occurred.

CONCLUSIONS

The number of free filaments was significantly higher in the suspended (RII) than in the granular sludge (RI) from two EGSB reactors fed with oleic acid at load between 2 and 8 kg COD/m^3.day. The average free filament length in RI was significantly lower than in RII. The morphological characterisation of granular sludge was affected by the morphology of the fine solids (minimum Feret diameter < 1 mm) as they represented more than 90% in terms of number of particles and between 26% and 77% in terms of total detected area of particles. Two methods to distinguish fine and non-fine solids by image analysis were validated by a method based on the ratio fine VSS/total VSS. Granules disintegration was detected by these methods along the operation of the EGSB reactor inoculated with granular sludge. The adsorption of LCFA onto the sludge could be related to the morphology of the non-fine aggregates and to the number of free filaments. The aggregates located in the bottom layer of the reactor were bigger and their diameter increased with the amount of adsorbed substrate until a threshold value of about 80 ml CH_4/gVSS. When more LCFA was adsorbed, a migration of aggregates towards the top layer was observed with a simultaneous decrease in size, possibly due to granules disintegration. The degradation of the adsorbed LCFA decreased sharply as the size of fine aggregates increased, possibly due to internal diffusion limitations of intermediates such as acetate and products such as methane. Image analysis could give valuable information about morphology and aggregation status of anaerobic sludge.

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