

Image analysis, methanogenic activity measurements, and molecular biological techniques to monitor granular sludge from an EGSB reactor fed with oleic acid

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Abstract Morphological changes in anaerobic granular sludge fed with increasing loads of oleic acid were quantified by image analysis. The combination of this technique with data on the accumulation of adsorbed long chain fatty acid and with the molecular characterization of microbial community gave insight into the mechanisms of sludge disintegration, flotation and washout. It was found that the bacterial domain was more affected than the archaeal domain during this process. However, no acetoclastic activity and only a residual hydrogenotrophic activity were detected in the sludge at the end of the operation.

Keywords EGSB reactor; granular sludge; image analysis; long chain fatty acids; methanogenic activity; molecular techniques

Introduction

The use of 16S rDNA-based methods employing Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer and Smalla, 1998), molecular cloning and sequencing (Godon *et al.*, 1997; Wu *et al.*, 2001) can give insight into the microbial composition and diversity in complex communities, such as granular sludge or biofilms. Furthermore, when combined with morphological, and/or physiological monitoring techniques, they can provide important information to improve the understanding on the role and dynamics of these communities and their effect on reactor performance (Verstraete *et al.*, 1996; Oude Elferink *et al.*, 1998; Santegoeds *et al.*, 1999). In anaerobic treatment of effluents with high lipid content, the application of sludge bed reactors is problematic because, besides the acute toxicity towards the acetogenic and methanogenic bacteria, long chain fatty acids (LCFA) resulting from lipids hydrolysis adsorb onto the biomass and provoke sludge flotation and subsequent washout. Granular sludge disintegrates and becomes encapsulated by a whitish, light and gelatinous mass. From a thermodynamic viewpoint, disintegration of granules is predictable because at neutral pH, LCFA act as surfactants, lowering the surface tension, and hindering the aggregation of hydrophobic bacteria, like most acetogens (LCFA-degraders) (Hwu *et al.*, 1997).

The aim of the present work was to monitor the granular sludge from an EGSB reactor fed with increasing loads of oleic acid, using an integrated approach combining image analysis, methanogenic activity measurements and molecular biological techniques to quantify changes in the granular sludge at morphological, physiological and microbial levels. Oleic acid was chosen as a model because it is generally, one of the more abundant LCFA in wastewaters as well as one of the more toxic.

Materials and methods

A 10 l EGSB reactor inoculated with 1.6 l of granular sludge (20.2 g VSS/l) obtained from an UASB treating brewery effluent located in Oporto, was operated with increasing oleate concentrations between 2 and 8 g COD/l at a constant hydraulic retention time of 1 day. The operation of this reactor is described by Pereira *et al.* (2002) and Table 1 summarises the steady-state values of the operating conditions and performance data for each period. During the first 70 days the feed was composed of skim milk (50% COD) and oleic acid (50% COD). From day 70 onwards, the carbon source was exclusively composed of oleic acid. Macro- and micronutrients with a composition described elsewhere (Alves *et al.*, 2001) were added.

During reactor operation the biomass was segregated into two layers, located at the bottom (heavy sludge) and at the top (floating sludge) of the reactor. On days 70, 92, 119, 141, 162, 191 and 219 samples from the bottom and top layers were collected and characterized in terms of amount of adsorbed substrate and the corresponding potential maximum degradation rate measured in batch experiments. Image analysis was applied to the same samples in order to quantify the size of the aggregates and the number and total length of free filamentous bacterial forms. The biomass samples collected at the end of each applied oleate loading rate (days 70, 119, 162, 219) were also analyzed by molecular techniques in order to monitor changes in microbial diversity along the operation.

Batch experiments

All the sludge samples collected from the reactor were washed and centrifuged (4000 rpm, 10 min) twice with anaerobic basal medium and were incubated in batch vials of 25 ml at 37°C, 150 rpm under strict anaerobic conditions, without any added substrate. The methane production due to the degradation of the adsorbed substrate was followed by measuring the pressure developed in each vial, using a hand-held pressure transducer capable of measuring a pressure variation of two bar (0 to ± 202.6 kPa) over an output range of -200 to $+200$ mv and a minimum detectable variation of 0.005 bar corresponding to 0.05 ml biogas in 10 ml headspace (Colleran *et al.*, 1992). The anaerobic basal medium used in all the batch experiments was composed of cysteine-HCl (0.5 g/l) and sodium bicarbonate (3 g/l), the pH was adjusted to 7.0–7.2 and was prepared under strict anaerobic conditions. No calcium or trace-nutrients were added. The initial maximum methane production rate and the maximum values achieved (indirect measure of the amount of adsorbed substrate) were determined for each vial. All the batch experiments were performed in triplicate assays. Methanogenic activity tests were also performed using this technique.

Image analysis

Image acquisition for the quantification of free filamentous bacteria was accomplished through the visualization on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 100 \times magnification. Images for the quantification of morphological parameters in the aggregates were acquired in an Olympus SZ40 stereomicroscope with a 15 \times magnification.

Table 1 Steady-state operating conditions and performance (mean \pm 95% confidence intervals)

Period	Time (days)	Influent COD (g/l)	COD removal efficiency (%)	Methane production (ICH ₄ /day)
1	0–70	3.8 \pm 0.3	96.5 \pm 0.6	10.6 \pm 1.0
2	70–119	3.8 \pm 0.3	83.4 \pm 4.8	2.3 \pm 0.5
3	119–162	6.2 \pm 0.7	74.2 \pm 3.8	1.6 \pm 0.2
4	162–219	8.2 \pm 0.5	68.8 \pm 3.4	1.5 \pm 0.2

COD– Chemical Oxygen Demand.

Images were digitised with a CCD AVC D5CE camera (Sony, Tokyo) and a DT 3155 frame grabber (Data Translation, Marlboro, MA, USA), with a 768×576 pixel size and 256 grey levels using the ImagePro Plus (Media Cybernetics, Silver Spring, MD, USA) software package. Morphological parameters were determined by means of two programs developed in Matlab 5.3 (The Mathworks, USA):

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

Floc program. The background from the original image was subtracted and then a previously defined threshold binarised the image. In the binarised image only the objects larger than 5×5 pixels were treated, discarding the debris present in the image. The particle size was evaluated by its equivalent diameter (Deq), calculated from its projected area (A) and by its minimum Feret diameter (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).

Microbial community analysis

Total DNA was extracted as described by Harmsen *et al.* (1995) and the 16S rRNA-genes were amplified by Polymerase Chain Reaction (PCR) using a Taq DNA polymerase kit (Life Technologies, Gaithersburg, MD.). Complete bacterial 16S rDNA was selectively amplified for cloning and sequencing using 7-f (5'-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3') and 1510-r (5'-ACGG(C/T)TACCTTGTACGACTT-3') primers (Lane, 1991). For DGGE a specific region of eubacteria 16S rDNA (V6-V8 region) was amplified using 968-GC-f (5'-CGCCCCGGGGCGCGCCCCGGGGCGGGGGCACGGGGGAACGCGAGAACCTTAC-3') and 1401-r (5'-CGGTGTGTACAAGACCC-3') primers (Nübel *et al.*, 1996). For archaea, primers A109-f (5'-AC(G/T)GCTCAGTAACAGTAACACGT-3') (Grosskopf *et al.*, 1998) and 1510-r were used for complete 16S rDNA amplification and A109(T)-f (original Grosskopf *et al.*, 1998, third nucleotide changed into T only, Hans G.H.J. Heilig, personal communication) and 515-GC-r (5'-CGCCCCGGGGCGCGCCCCGGGGCGGGGGCACGGGCTGCTGGCAC-3') (Lane, 1991) for V2-V4 region amplification for DGGE use. All primers were purchased from MWG-Biotech (Ebersberg, Germany).

DGGE analysis of the amplicons was done as described by Zoetendal *et al.* (2001). Denaturant gradients from 35 to 50% for bacterial amplicon separation and from 30 to 45% for the archaeal ones, were used. DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Molecular Analyst 1.12 software package (BioRad, Hercules, CA, USA). Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient (Häne *et al.*, 1993). Community shifts were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

Cloning and sequencing with the Sp6 primer (5'-GATTTAGGTGACACTATAG-3') (MWG-Biotech, Ebersberg, Germany), was done as described by Heilig *et al.* (2002). Similarity search of the partial 16S rDNA sequences derived from the sludge clones was

performed using the NCBI sequence search service available from the internet (<http://www.ncbi.nlm.nih.gov/blast/>).

Results and discussion

Figure 1 summarises the results obtained in the batch experiments. The granular sludge present in the top layer exhibited higher amounts of adsorbed substrate attaining a maximum value of 997 ± 109 mg COD-CH₄/gVSS on day 162, when oleate was fed at 6 kg COD/m³.day. Methane production rate from the adsorbed substrate was also maximal under these conditions (200 ± 6 mg COD-CH₄/gVSS.day).

For the oleate organic load of 8 kgCOD/m³.day, a clear inhibition of the adsorbed substrate degradation was found as a lag-phase of 300 h preceded the initial methane production (Pereira *et al.*, 2002). A clear decrease was observed in both the amount of adsorbed substrate effectively degraded (100 ± 11 mg COD-CH₄/gVSS) and the corresponding methane formation rate (11 ± 3 mg COD-CH₄/gVSS.day) in batch assays. The excessive accumulation of non-degraded substrate adsorbed onto the biomass can hinder the transfer of gaseous products, and thus cause a delay in the initial methane production as well as a reduction of the methane production rate. The same effect can, in part, explain the significant reduction in the methanogenic activity measured in the granular inoculum and in the final sludge. The sludge present at the end of the operation exhibited only a low hydrogenotrophic activity, which was less than 5% of the value exhibited by the granular inoculum and no detectable activity on the other substrates (Table 2). It should also be noted that, if the acetoclastic activity is rate limiting, the measurement of the methanogenic activity in the presence of propionate, butyrate and ethanol, indirect methanogenic substrates, can be underestimated.

Image analysis provided information about the morphology and aggregation status of the granular sludge. Along the trial period, the fraction of fine aggregates (with a minimal Feret diameter smaller than 1 mm) increased steadily from 27% to 76%, in terms of

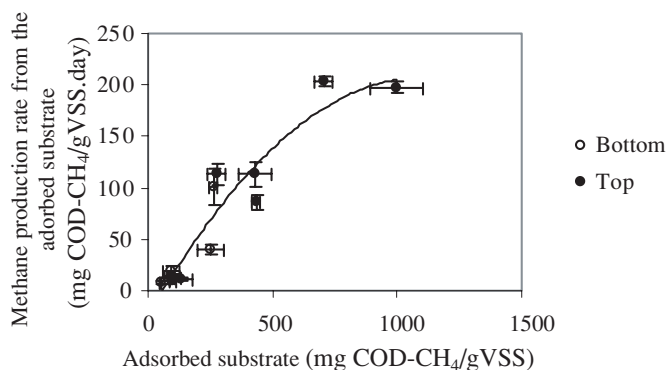


Figure 1 Effect of the amount of adsorbed substrate on the methane production rate

Table 2 Methanogenic activity for the granular sludge (mean \pm standard deviation of triplicates)

	Granular inoculum	Final sludge (end of operation)
Methanogenic activity in presence of:	(mlCH ₄ (STP)/gVSS.day)	
Acetate	327 \pm 11	(n.d.)
Propionate	160 \pm 10	(n.d.)
Butyrate	(n.d.)	(n.d.)
Ethanol	514 \pm 94	(n.d.)
H ₂ /CO ₂	597 \pm 16	27.8 \pm 1.1

STP – standard temperature and pressure conditions; n.d. – not detected

projected area, clearly indicating the occurrence of granule disintegration. The morphology of the non-fine aggregates could be related to the adsorption of LCFA onto the sludge (Table 3). In the bottom sludge, the size of the non-fine aggregates increased with the amount of adsorbed substrate to a maximum value of about 250 mg COD-CH₄/gVSS, corresponding to a diameter of 1.79 mm. On the other hand, in the top sludge which in general exhibited a significantly higher amount of adsorbed substrate, a decreasing trend of the size of the non-fine aggregates was observed with increasing amounts of adsorbed substrate. This suggests that when more LCFA was adsorbed, a migration of aggregates towards the top layer will occur with a simultaneous decrease in size, possibly due to disintegration.

The number of free filaments (per unit of VSS concentration) also increased with the amount of adsorbed LCFA, suggesting that granular disintegration was simultaneous with a release of filamentous organisms to the medium (Amaral *et al.*, 2001). Concerning the fine aggregates (<1 mm), it was observed that when their size increased in the range of 0.2–0.3 mm the degradation rate of the adsorbed LCFA decreased sharply from 434 to 13 mg COD-CH₄/gVSS.day, possibly due to internal diffusion limitations of intermediates such as acetate and products such as methane. This result highlights the advantage of using suspended/dispersed biomass over aggregated biomass towards the methanization of LCFA, as reported before (Pereira *et al.*, 2002).

Microbial community analysis seemed to be in accordance with the granular disintegration quantified by image analysis, as a clear shift in the bacterial consortium (usually located in the outer layer of granules) was observed with the increase in the toxicant fed to the reactor. This suggests a selective washout of acetogenic bacteria. The comparison of DGGE band-patterns revealed a decrease in the similarity indices between the bottom and top from 86.8, in the first operation period, to 56.7 in the last operation period (oleate load of 8 kg COD/m³.day) (Table 4 (I)). At the end of the operation (period 4), the similarity index between bottom sludge and the inoculum was 42.8, whereas between the top sludge and the inoculum it was 17.3. This suggests that a higher shift in the community structure occurred in the top than in the bottom sludge. Furthermore, in the top sludge, the increase in the oleate loading rate from 6 to 8 kg COD/m³.day caused a large shift in the community structure (the similarity index between the top sludge at the end of period 3 and 4 was of only 14.9). This significant shift in the microbial structure of the top sludge could include the loss of microorganism particularly important in the methanisation of LCFA and thus explain the differences in the methane production capacity from the adsorbed substrate exhibited by the sludge at the end of both periods, in the batch experiments.

The selective washout of acetogenic bacteria during the operation can also be related to the absence of methanogenic activity with propionate, butyrate and ethanol exhibited by the granular sludge at the end of operation (Table 1). For the archaeal consortium no significant shift in the granular community patterns was detected (Table 4 (II)), which can be

Table 3 Amount of adsorbed substrate and equivalent diameter of non-fine aggregates present in the bottom and top sludge (mean \pm standard deviation)

Bottom sludge		Top sludge	
Adsorbed substrate (mgCOD-CH ₄ /gVSS)	Non-fine average equivalent diameter (mm)	Adsorbed substrate (mgCOD-CH ₄ /gVSS)	Non-fine average equivalent diameter (mm)
51.1 \pm 2.9	1.36 \pm 0.08	98.9 \pm 12.0	1.67 \pm 0.20
90.9 \pm 34	1.48 \pm 0.04	133.7 \pm 47.4	1.70 \pm 0.17
93.7 \pm 10.9	1.53 \pm 0.07	276.0 \pm 37.4	1.63 \pm 0.04
96.6 \pm 33.1	1.51 \pm 0.05	430.0 \pm 67.4	1.58 \pm 0.05
97.1 \pm 5.4	1.46 \pm 0.08	433.7 \pm 17.1	1.53 \pm 0.03
250.6 \pm 53.4	1.79 \pm 0.07	704.9 \pm 34.6	1.64 \pm 0.04
262.9 \pm 16.6	1.65 \pm 0.03	997.7 \pm 107.1	1.47 \pm 0.04

Table 4 Similarity matrix calculated from: (I) bacterial DGGE profiles and (II) archaeal DGGE profiles of each sludge sample

(I)	G	b ₁	b ₂	b ₃	b ₄	t ₁	t ₂	t ₃	t ₄
G	100								
b₁	61.3	100							
b₂	75.8	77.1	100						
b₃	56.1	67.2	77.4	100					
b₄	42.8	37.5	45.0	62.7	100				
t₁	68.0	<u>86.8</u>	66.2	60.7	43.2	100			
t₂	82.9	71.4	<u>84.9</u>	72.0	37.9	71.1	100		
t₃	80.3	59.1	74.2	<u>60.3</u>	35.1	62.6	86.6	100	
t₄	17.3	18.4	22.3	44.6	<u>56.7</u>	19.9	23.3	14.9	100

(II)	G	B ₁	b ₂	b ₃	b ₄	t ₁	t ₂	t ₃	t ₄
G	100								
b₁	97.6	100							
b₂	92.4	97.0	100						
b₃	79.5	87.6	95.7	100					
b₄	72.0	78.7	88.7	93.5	100				
t₁	95.9	<u>97.7</u>	93.3	83.5	74.4	100			
t₂	95.8	98.7	<u>94.8</u>	84.5	76.4	98.4	100		
t₃	94.7	98.3	97.2	<u>90.5</u>	84.2	96.6	98.5	100	
t₄	75.2	81.4	89.8	93.6	<u>98.9</u>	77.9	79.6	86.7	100

G – granular inoculum; b – bottom sludge; t – top sludge; 1,2,3 and 4-operation periods

Similarity indices between the bottom and top sludge along toxicant feed increase are underlined

related to the more protected environment against the toxic effect and hydraulic shear stress offered to the methanogenic organisms (archaea domain) normally located in the inner core of the granules.

Figure 2 illustrates the molecular characterisation of the samples at the end of operation. The sequencing of the bacterial clones resulted mainly in matches with unknown and uncultured microorganisms. For the archaeal domain the cloned sequences were affiliated with the two main groups, the acetoclastic *Methanosaeta* and the hydrogenotrophic *Methanobacterium*. However, although exhibiting some hydrogenotrophic activity, no acetoclastic activity was detected in this sludge (Table 1). This suggests that the presence of this group of microorganisms became weaker during the oleate load operation. Phenomena such as the acute toxicity of oleate to this group of microorganisms (Alves *et al.*, 2001) and substrate unavailability either due to diffusion limitations through the LCFA accumulation on the aggregates or due to the selective washout of acetogenic bacteria (responsible for the acetate production) could be responsible for this.

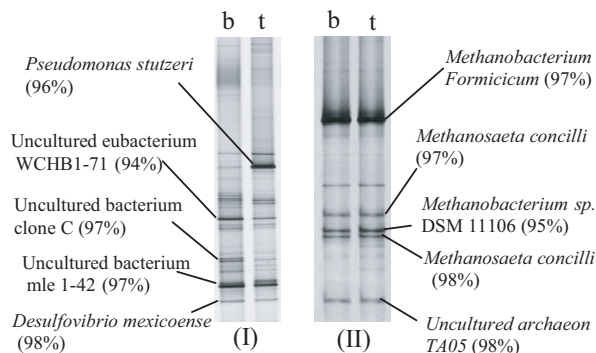


Figure 2 Molecular characterization of the granular sludge at end of operation: (I) bacterial domain, (II) archaeal domain (b bottom, t top)

Conclusions

Monitoring of granular sludge at microbial, morphological and physiological levels allowed the understanding of the mechanisms of sludge disintegration, flotation and washout in an EGSB reactor fed with increasing loads of oleic acid. Image analysis was a powerful tool that provided the quantification of morphological changes in the anaerobic granular sludge, evidencing the occurrence of sludge disintegration. The morphology of the aggregates could be related to the amount of adsorbed substrate and to the degradation rate of this substrate, in batch assays. When the amount of adsorbed LCFA increased, a migration of aggregates towards a top floating layer was observed with a simultaneous decrease in size. The molecular characterization of the microbial community indicated that the bacterial domain was most affected during the process of sludge disintegration, flotation and washout. The archaeal consortium remained less affected although no acetoclastic and only a residual hydrogenotrophic activity was detected at the end of the operation.

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