Image analysis, methanogenic activity 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 give insight into the mechanisms of sludge disintegration, flotation and washout The molecular characterization of microbial community indicated that the bacterial domain was affected during sludge disintegration, flotation and washout. The archaeal domain was less affected although no acetoclastic and only a residual hydrogenotrophic activity were detected at the end of the operation.

Keywords Long Chain Fatty Acids; EGSB reactor; granular sludge; image analysis; molecular techniques; methanogenic activity

Introduction

Oleic acid is an abundant long chain fatty acids (LCFA) present in wastewater and it is one of the more toxic ones. In addition to the acute toxicity towards the acetogenic and methanogenic bacteria, LCFA 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. Consequently the aggregation of hydrophobic bacteria, like most of acetogens (LCFA -degraders), is unfavorable (Hwu et al. 1997). The aim of the present work was to monitor granular sludge from an EGSB reactor fed with increasing loads of oleic acid, using an integrated approach combining image analysis, methanogenic activity and molecular ecology techniques to quantify changes in the granular sludge at morphological, physiological and microbial levels.

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 a 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. During the first 70 days the substrate was made of skim milk (50% COD) and oleic acid (50% COD). From day 70 onwards, the carbon source was exclusively composed by oleic acid. The total operation lasted 219 days. Macro and micronutrients with a composition described elsewhere (Alves *et al.*, 2001) were added. For each applied oleate loading rate, 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 same biomass samples were 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 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 $\pm$ 202.6 kPa) over an output range of -200 to +200 mv (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. Methanogenic activity tests were also performed using this technique. All the batch experiments were performed in triplicate assays.

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 100x magnification. Images for the quantification of morphological parameters in the aggregates 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. 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 is enhanced by means of a Mexican-hat filter (Russ, 1995), followed by a background homogenisation, Wiener filtering and histogram equalization. Subsequently, a defined threshold binarises the image. In order to identify the filaments skeletonisation and a 10 pixels length end-point removal is performed.

Floc program. This program was applied only to the granular sludge. The background from the original image is subtracted and then a previously defined threshold binarises the image. 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 (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 from Life Technologies (Gaithersburg, Md.). Complete bacterial 16S rDNA was selectively amplified for cloning and sequencing using 7-f and 1510-r primers. For DGGE a specific region of eubacteria 16S rDNA (V6-V8 region) was amplified using 968-GC-f and 1401-r primers. For archaea, primers A109-f and 1510-r were used for complete 16S rDNA amplification and A109(T)-f and 515-GC-r for V2-V4 region amplification for DGGE use.

DGGE analysis of the amplicons was performed on 8% polyacrylamide gels containing a urea plus formamide gradients from 35 to 50% for bacterial amplicons separation and from 30 to 45% for the archaeal ones. A 100% denaturant corresponds to 7M urea and 40% (vol/vol) formamide. Electrophoresis was performed in 0.5 x Tris-acetic acid-EDTA buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA pH 8) at 85V and 60ºC for 16 hours using a Dcode System apparatus (BioRad, Hercules, CA). DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Molecular Analyst 1.12 software package (BioRad). 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. The amplified total 16S rDNA products were cloned in E. coli JM109 by using the pGEM®-T Easy vector system (Promega, The Netherlands) according to the manufacture's manual. The inserts were screened by Restriction Fragment Length Polymorphism (RFLP) analysis and by mobility comparison on DGGE. Sequencing analysis of the selected transformants was carried out with the Sequenase sequencing kit (Amersham, United Kingdom) using the sequencing primer Sp6. The sequences were automatically analysed on a LI-COR (Lincoln, USA) DNA sequencer 4000L and corrected manually. Homology search of the partial 16S rDNA sequences derived from the sludge clones was performed using the NCBI sequence search service available in the internet (http.//www.ncbi.nlm.nih.gov/blast/).

Results and Discussion

Figure 1 summarises the results from the batch experiments. The granular sludge present in the top layer exhibited higher amounts of absorbed substrate attaining a maximum value of 349±38 mlCH$_4$/g.VSS on day 162, when oleate was fed at 6 kgCOD/m^3.day.
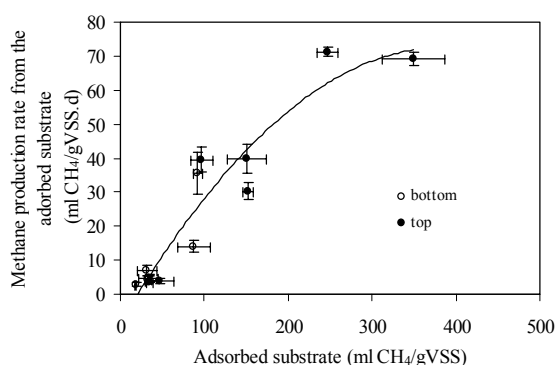

Figure 1.Effect of the amount of adsorbed substrate on the methane production rate.

Methane production rate from the absorbed substrate was also maximal in these conditions (70 mlCH$_4$/gVSS.day). For the oleate organic load of 8 kgCOD/m^3.day, a clear inhibition of the adsorbed

substrate degradation was found as a lag-phase of 300 hours preceding the methane production (Pereira *et al.*, 2001 – results not shown). A clear decrease was observed in both the amount of adsorbed substrate effectively degraded and in the corresponding methane formation rate in batch assays. The excessive accumulation of non-degraded substrate adsorbed onto the biomass can hinder the transfer of gas products, inducing a delay on 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 where only a residual hydrogenotrophic activity was detected (Table 1).

Table 1. Methanogenic activity for the granular sludge (±95% confidence interval).

		Granular Inoculum	Final sludge (end of operation)
Methanogenic activity in presence of:		$(mlCH_{4(STP)}/gVSS^.d)$	
	Acetate	327±11	(n.d.)
	Propionate	160±10	(n.d.)
	Butyrate	(n.d.)	(n.d.)
	Ethanol	514±94	(n.d.)
	H_2/CO_2	597±16	27.8±1.1

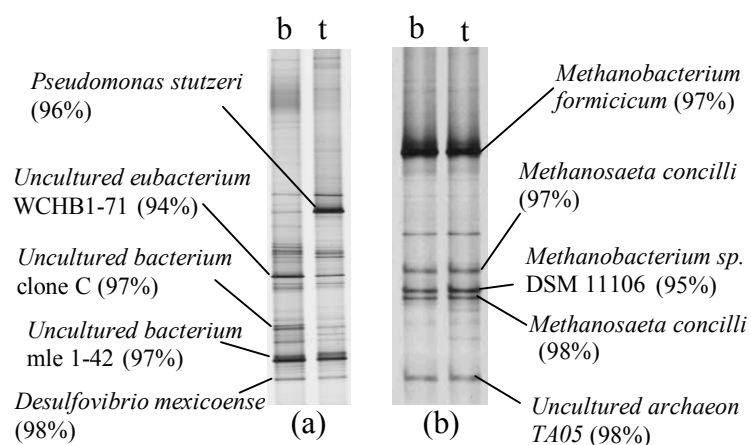STP–Standard temperature and pressure conditions; n.d.- non-detectable; (n.d.)-Non-detected

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

Image analysis provided information about the morphology and aggregation status of the granular sludge.

Granule disintegration occurred along the trial period, as the fraction of fine particles (with a minimal Feret diameter smaller than 1 mm) increased steadily from 27% to 76%, in terms of projected area. In the bottom sludge, the size of the non-fine aggregates increased with the amount of adsorbed substrate to 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 disintegration (Amaral *et al.*, 2001). The number of free filaments (per unit of VSS concentration) increased also with the amount of absorbed LCFA, suggesting that granular disintegration was simultaneous with a release of filamentous organisms to the medium. When the size of fine aggregates increased in the range of 0.2-0.3 mm the degradation rate of the adsorbed LCFA decreased sharply, possibly due to internal diffusion limitations of intermediates such as acetate and products such as methane.

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, suggesting the 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 (0-70 days), to 56.7 in the last operation period (oleate load of 8 kg COD/m^3.day). At the end of the operation, 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.

For the archaeal consortium no significant shift in the granular community patterns was detected, which can be 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 4 illustrates the molecular characterisation of the samples at the end of operation.

Sequencing of the bacterial clones resulted mainly in matches with unknown and uncultured microorganisms. For the archaeal domain the cloned organisms were affiliated with the two main genera, the acetoclastic *Methanosaeta* and the hydrogenotrophic *Methanobacterium*. However, although exhibiting some hydrogenotrophic activity, no acetogenic activity was detected in this sludge (Table 1). Fluorescent in situ hybridisation with the *Methanosaeta* genus specific probe MX825 was used to evaluate the levels of this group of microorganisms. Only small chains of *Methanosae*ta-like organisms exhibiting a weak fluorescent signal were detected.

Conclusions

Image analysis was a powerful tool that allowed the quantification of morphological changes in anaerobic granular sludge fed with oleic acid. The combination of this technique with data on the accumulation of adsorbed long chain fatty acid allowed to understand the mechanisms of sludge disintegration, flotation and washout. The molecular characterization of microbial community indicated that bacterial domain was the most affected during the process of sludge disintegration, flotation and washout. Archaeal consortium remained less affected although no acetoclastic and only a residual hydrogenotrophicactivity were detected at the end of the operation. In the range of oleate loads evaluated, the value of 6 kg of oleate-COD/m^3.day was found the practical maximum oleate loading rate applicable. In these conditions adsorbed substrate can be efficiently methanised at a rate of 70 ml CH_4/gVSS.day. A further increase to 8 kg COD/m^3.day caused a clear inhibition in the degradation of the adsorbed substrate and a clear decrease in the microbial diversity especially in the bacterial domain.

Acknowledgements

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