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Molecular monitoring of microbial diversity in expanded granular sludge bed (EGSB) reactors treating oleic acid

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Abstract

A molecular approach was used to evaluate the microbial diversity of bacteria and archaea in two expanded granular sludge bed (EGSB) reactors fed with increasing oleic acid loading rates up to 8 kg of chemical oxygen demand (COD) m^{-3} day^{-1} as the sole carbon source. One of the reactors was inoculated with granular sludge (RI) and the other with suspended sludge (RII). During operation, the sludge in both reactors was segregated in two layers: a bottom settled one and a top floating one. The composition of the bacterial community, based on 16S rDNA sequence diversity, was affected most during the oleate loading process in the two reactors. The archaeal consortium remained rather stable over operation in RI, whereas in RII the relative abundance of *Methanosaeta*-like organisms became gradually weaker, starting in the bottom layer. In the range of oleate loads evaluated, 6 kg of COD m^{-3} day^{-1} was found as the maximum value that could be applied to the system. A further increase to 8 kg of oleate-COD m^{-3} day^{-1} induced a maximal shift on the microbial structure of the sludges. At this time point, methanogenic acetoclastic activity was not detected and only very low methanogenic activity on H_2/CO_2 was exhibited by the sludges.

Keywords: Anaerobic digestion; oleic acid; 16S rDNA; DGGE

1. Introduction

Oleic acid (C18:1) is, in general, the most abundant LCFA (long-chain fatty acid) present in industrial and domestic wastewater as well as one of the more toxic ones [1,2]. LCFAs result from hydrolysis of lipids and are especially problematic for anaerobic wastewater treatment. Besides their direct toxicity to the two main trophic groups involved in LCFAs degradation, methanogens and acetogens, they can adsorb onto biomass particles causing biomass flotation and washout [3–5]. The study of these phenomena, as well as reactor operation and alternative configurations in the application of the anaerobic digestion technology to effluents with high lipid/LCFA content have been subject of research work [6–12]. However, there is still a lack of knowledge regarding the microbiological aspects of the complex consortia involved in degradation.

Recent developments in molecular ecology have provided new molecular techniques that make it feasible to investigate complex microbial communities, overcoming the problems associated with the traditional cultivation-dependent methods [13]. Especially in anaerobic bioreactors, where stability and performance is strongly dependent on complex microbial interactions, this development can provide an opportunity to establish the connection between the microbial structure and the functional characteristics of the system. The use of 16S rDNA-based methods employing denaturing gradient gel electrophoresis (DGGE) [14], molecular cloning and sequencing [15,16] and fluorescent in situ hybridization [17–19] can provide an accurate estimate of the microbial composition and diversity in a complex community. Furthermore, when combined with other techniques, chemical, biochemical and/or physiological assays, they can provide considerable information and improve our understanding about the role and dynamics of microorganisms [20–22].

In previous work, it was found that after feeding a re-

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actor with oleate as the sole carbon source, the biomass became encapsulated by a whitish matter. When this biomass was washed to remove the residual substrate, and incubated in batch vials, it was able to produce methane by degradation of the adsorbed substrate [11,12]. The maximum potential methane production due to the degradation of the adsorbed substrate exhibited by the sludge of two expanded granular sludge bed (EGSB) reactors fed with increasing loads of oleic acid was studied in batch assays [23]. The behavior of granular and suspended sludge was compared since, although being more resistant to LCFAs toxicity than the suspended or flocculent sludge, granular stability is critical for lipid/LCFAs containing wastewaters [5,24]. The aim of the present work was to evaluate the microbial diversity of bacteria and archaea in the granular and suspended sludge collected during the operation of these EGSB reactors, using a molecular approach.

2. Materials and methods

2.1. Sludge sources

Sludge samples were obtained from two 10l EGSB reactors operated at mesophilic conditions (37°C) as described elsewhere [23]. One reactor (RI) was inoculated with granular sludge whereas another reactor (RII) was inoculated with suspended sludge. Both reactors were operated in parallel with increasing oleate concentrations from 2 to 8 g chemical oxygen demand (COD) l^{-1}, in a total of four periods. The hydraulic retention time was set at 1 day. Table 1 summarizes the operating conditions and performance of RI and RII. During the operation a significant amount of sludge accumulated as a floating top layer in both digesters. At the end of each period, samples from the bottom and top layers were collected from each reactor, washed and centrifuged ($1681 \times g$, 10 min) twice with the same anaerobic basal medium used in the batch experiments.

2.2. Batch experiments

Methanogenic activity tests were performed using the pressure transducer technique [25]. The pressure increase in sealed vials fed with non-gaseous substrates (acetate, propionate, butyrate and ethanol) or the pressure decrease in vials previously pressurized with gaseous substrates (H_2/CO_2) was monitored. The hand-held pressure transducer was capable of measuring a pressure increase or decrease of two bar (0–±202.6 kPa) over a device range of −200 to +200 mV, with a minimum detectable variation of 0.005 bar, corresponding to 0.05 ml biogas in 10 ml headspace. The basal medium used in the batch experiments, made up with demineralized water, was composed of cysteine–HCl (0.5 g l^{-1}) and sodium bicarbonate (3 g l^{-1}), the pH was adjusted to 7.0–7.2 with 8 N NaOH and was prepared under strict anaerobic conditions. No calcium or trace-nutrients were added.

Methanogenic toxicity tests were also performed using the pressure transducer technique, for oleate concentration ranging from 100 to 900 mg l^{-1}. Acetate was added as co-substrate to characterize the toxicity towards acetoclastic methanogens. The oleate concentration that caused a 50% relative methanogenic acetoclastic activity loss was defined as inhibitory concentration at 50% (IC_{50}). All batch tests were performed in triplicate assays. Methane content of the biogas was measured by gas chromatography using a Chrompack Haysep Q (80–100 mesh) column, with N_2 carrier gas at 30 ml min^{-1} and a flame-ionization detector. Temperatures of the injection port, column, and flame-ionization detector were 120, 40, and 130°C, respectively.

2.3. Microbial community analysis

2.3.1. DNA extraction and amplification

Total DNA was extracted from approximately 1 ml of homogenized sludge sample as previously described by Harmsen et al. [26]. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using a *Taq* DNA polymerase kit (Life Technologies, Gaithersburg, MD,

Table 1
Operating conditions and performance of RI-granular sludge and RII-suspended sludge (mean ± 95% confidence intervals) (adapted from Pereira et al., 2002)

Time (days)	Influent COD (g l^{-1})	Influent oleate-COD (g l^{-1})	COD removal efficiency (%)		Effluent VSS (g l^{-1})		Methane production (l CH_4 day^{-1})	
[period]			RI	RII	RI	RII	RI	RII
0–70	3.8	1.9	96.5	85.8	0.38	0.65	10.6	7.9
[1]	(±0.3)	(±0.2)	(±0.6)	(±3.2)	(±0.07)	(±0.04)	(±1.0)	(±1.2)
70–119	3.8	3.8	83.4	74.4	0.85	0.72	2.3	2.6
[2]	(±0.3)	(±0.3)	(±4.8)	(±5.5)	(±0.22)	(±0.15)	(±0.5)	(±0.5)
119–162	6.2	6.2	74.2	74.6	1.96	1.57	1.6	2.0
[3]	(±0.7)	(±0.7)	(±3.8)	(±2.9)	(±0.43)	(±0.17)	(±0.2)	(±0.8)
162–219	8.2	8.2	68.8	69.4	2.71	2.50	1.5	2.2
[4]	(±0.5)	(±0.5)	(±3.4)	(±5.5)	(±0.57)	(±0.58)	(±0.2)	(±0.6)

USA). 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)TACCTTGTTACGACTT-3′) primers [27] with the following thermocycling program: 94°C for 5 min; 25 cycles of 94°C for 30 s, 52°C for 20 s, and 68°C for 40 s; and 68°C for 7 min. The reactions were subsequently cooled to 4°C. For DGGE a specific region of eubacterial 16S rDNA (V6–V8 region) was amplified using 968-GC-f (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′) and 1401-r (5′-CGGTGTGTACAAGACCC-3′) primers [28] with the same thermocycle program, but increased number of cycles to 35 and an annealing temperature of 56°C. For archaea, primers A109-f (5′-AC(G/T)GCTCAGTAACAGTAACACGT-3′) [29] and 1510-r were used for complete 16S rDNA amplification and A109(T)-f (original Grosskopf et al. [29], third nucleotide changed into T only, Hans G.H.J. Heilig personal communication) and 515-GC-r (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGCTGCTGGCAC-3′) [27] for V2–V4 region amplification for DGGE use. Both reactions were performed with the following thermocycle program: 94°C for 5 min; 24 (34 for DGGE use) cycles of 52°C for 40 s, 68°C for 1 min and 94°C for 30 s; 52°C for 40 s and 68°C for 7 min. All primers were purchased from MWG-Biotech (Ebersberg, Germany). The size and amount of PCR products were estimated by 1% agarose gel (w/v) electrophoresis and ethidium bromide staining.

DGGE analysis of the amplicons was done as described by Zoetendal et al. [30]. 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 (Bio-Rad, 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 [31]. 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. [32]. Similarity 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/).

2.3.2. Cell fixation and fluorescent in situ hybridization

After being washed and resuspended in phosphate-buffered saline, sludge samples were fixed overnight according to Amann [33]. Fixed samples were submitted to sonication for 5 min at 150 W, spotted to wells on gelatine-coated slides, dried for 20 min at 45°C and dehydrated [33]. Thereupon, in situ hybridization was performed with the MX825-CY3 probe (5′-TCGCACCGTGGCCGACACCTAGC-3′; target group: *Methanosaeta*; [17]) as detailed by Manz et al. [34]. For detection of all DNA, 4,6-diamidino-2-phenylindole was added to the wash buffer at a final concentration of 100 ng ml^{-1}. After rinsing the slides in water, they were immediately air-dried and mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Digital images of the slides, viewed with a Leica (Wetzlar, Germany) DMR HC epifluorescence microscope, were taken with a Leica DC 250 digital camera. These images were analyzed with Leica QFluoro image analysis software at a Leica Q550 FW computer. Phase contrast microscopy was performed with a Zeiss (Oberkochen, Germany) Axioscope microscope.

3. Results and discussion

3.1. Specific methanogenic activity

Both granular and suspended sludge inocula were characterized in batch experiments in terms of specific methanogenic activity and oleic acid toxicity toward acetoclastic methanogens (Table 2). The granular sludge exhibited significantly higher activities with acetate, propionate, ethanol and H_2/CO_2. However, methanogenic activity with butyrate was not detected in this sludge whereas a

Table 2
Methanogenic activity for both seed sludge and at the end of reactors operation

	Seed sludge		End of operation	
	granular	suspended	RI-granular	RII-suspended
Methanogenic activity in presence of:	ml $CH_{4(STP)}$ (g^{-1} VSS day^{-1})			
Acetate	327 ± 11	107 ± 6	(n.d.)	(n.d.)
Propionate	160 ± 10	48 ± 14	(n.d.)	(n.d.)
Butyrate	(n.d.)	52 ± 3	(n.d.)	(n.d.)
Ethanol	514 ± 94	106 ± 2	(n.d.)	(n.d.)
H_2/CO_2	597 ± 16	487 ± 31	27.8 ± 1.1	12.4 ± 2.2
Oleic acid toxicity limit (IC50) (mg l^{-1})	345 ± 26	133 ± 16		

Oleic acid toxicity for granular and suspended seed sludge (mean ± 95% confidence interval). (n.d.) – Not detected. STP – Standard temperature and pressure conditions.

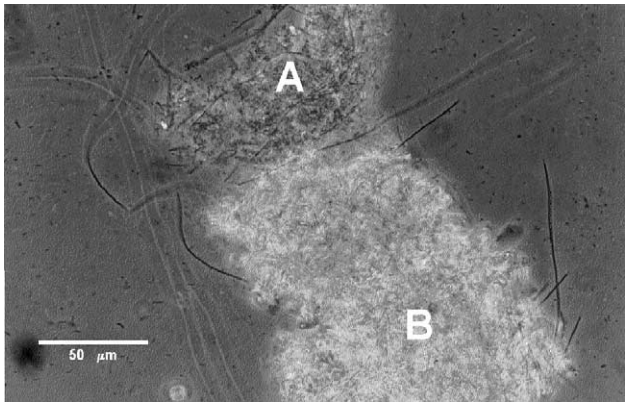

Fig. 1. Microscopic aspect of the encapsulated sludge: A – biomass, B – whitish matter.

value of 52 ml $CH_{4(STP)}$ (g^{-1} volatile suspended solids (VSS) day^{-1}) was detected in the suspended sludge. The toxicity limit (IC_{50}) of oleic acid towards acetoclastic methanogens was higher for the granular than for the suspended sludge, indicating the higher resistance of the granular inoculum to the toxicant studied. This result is consistent with the higher resistance to LCFA toxicity of granular sludge when compared with suspended or flocculent sludge previously reported by Hwu et al. [35].

At the end of operation, the biomass from both reactors was also characterized in terms of methanogenic activity with acetate, propionate, butyrate, ethanol and H_2/CO_2. Both sludges exhibited low activity with H_2/CO_2 and no activity with the other substrates (Table 2). The granular sludge (from RI) exhibited higher methanogenic activity for hydrogenotrophic methanogens, an important group that acts syntrophically with proton reducing acetogenic bacteria such as LCFA-degraders. Furthermore, in this sludge methane production from H_2/CO_2 proceeded without delay, whereas in the suspended sludge (from RII) a lag-phase of 520 h preceding the initial methane production was found.

When considering digestors performance (Table 1), a clear methane production decrease of 20–30% of the initial production, could be observed during the operation. In the last operation period (organic loading rate at 8 kg oleate-COD m^{-3} day^{-1}) the effectively to methane converted fraction of COD, was 27 l CH_4 (kg $COD_{removed})^{-1}$ in RI and 39 l CH_4 (kg $COD_{removed})^{-1}$ in RII. Only 7.7–11% of the removed oleate-COD was used for methane production. Phenomena such as precipitation with divalent cations and adsorption onto the biomass can also be responsible for LCFAs removal [5,36]. In Fig. 1, the encapsulated sludge is shown and clear whitish zones, that represent the absorbed substrate, can be observed surrounding the biomass. This accumulation of non-degraded substrate onto the biomass due to adsorption can hinder the transfer of substrate and products, inducing a delay on initial methane production as well as a reduction of the methane production rate. The potential maximum methane production from the adsorbed substrate exhibited by both sludges during the four operation periods was also evaluated [23]. It was found that the suspended sludge had a higher capacity of LCFAs adsorption, which can explain the lower methane production rates and higher delays on initial methane production found for this sludge in the activity tests.

3.2. Population dynamics

DNA extractions from the granular samples (I) and suspended sludge samples (II), collected from the bottom (b) and the floating top layer (t) of both reactors (RI and RII, respectively) at the end of each operation period (1, 2, 3 and 4), were used as template for amplification of the V6–V8 bacterial regions and the V2–V4 archaeal regions. These amplicons were separated by DGGE and the obtained band patterns of each lane, which corresponded to each sample, were compared. Figs. 2 and 3 present the obtained results.

For the bacterial domain (Fig. 2), comparison of DGGE band-patterns revealed a clear shift in the community structure with a decrease in the similarity indices between the bottom and top of RI-granular sludge from 86.8, in period 1, to 56.7 in period 4. At the end of the operation, the similarity index between bottom sludge and the inoculum was 42.8, and between top sludge and the inoculum 17.3, suggesting that a higher shift in the community structure occurred in the top than in the bottom sludge. This shift was maximal when the oleate loading rate was increased from 6 to 8 kg COD m^{-3} day^{-1} (the similarity index between the top sludge at the end of periods 3 and 4 was of only 14.9). In RII-suspended sludge, the similarity index between bottom and top sludge attained a minimum value of 29.4 at the end of period 3 and increased to 83.1 at the end of the operation, indicating that at the end of period 3 the microbial structure from the top sludge was significantly different from the bottom sludge. This was not the case at the end of the operation, when both layers exhibit also low similarity indices to the inoculum (28.7 between bottom and inoculum and 15.2 between top and inoculum). Furthermore, the suspended top sludge characterized at the end of period 3 revealed the highest methane production capacity from the adsorbed substrate (1145 ± 307 ml $CH_{4(STP)}$ g^{-1} VSS) [23], suggesting that the microbial structure of this sludge includes groups of microorganisms particularly important for the degradation of LCFA to methane. The lowest similarity index among the community patterns of the top layer sludge was found between period 3 and 4, i.e. 27.3. This maximal shift induced in the predominant bacterial composition may include the loss of microorganisms important for the degradation of LCFA to methane, and thus explain the sharp decrease on the methanization capacity of the adsorbed substrate exhibited by this sludge at the end of period 4 (111 ± 24 ml $CH_{4(STP)}$ g^{-1} VSS) [23].

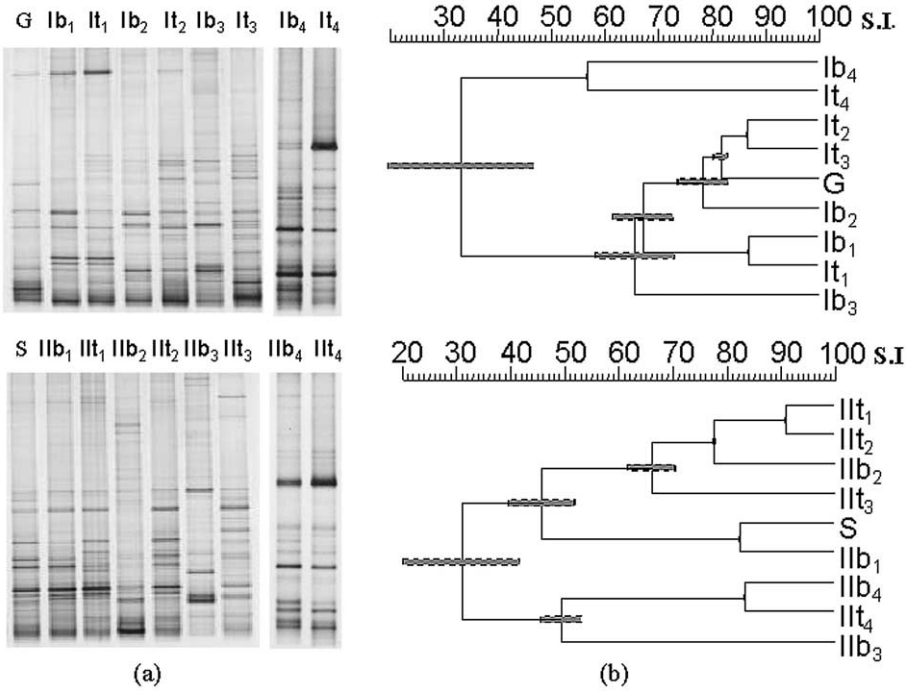

Fig. 2. DGGE profiles of bacterial amplicons from sludge samples (a) and correspondent similarity index (S.I.) dendogram (UPGMA clustering) (b). I – granular sludge, II – suspended sludge, b – bottom layer, t – top layer, 1,2,3 and 4 – operation periods, G – granular inoculum, S – suspended inoculum.

In the archaeal consortium (Fig. 3) no significant shift in RI-granular sludge community patterns was detected indicating that the dominant microbial composition remained rather stable over operation, whereas in RII-suspended sludge the diversity decreased, starting in the bottom layer.

The differences observed between the two sludges can be understood based on their different morphological and physiological structure. In fact, contrary to the weak and random microorganisms aggregation of suspended and flocculent sludge, granules form dense aggregates with a layered microbial organization, in which the internal core

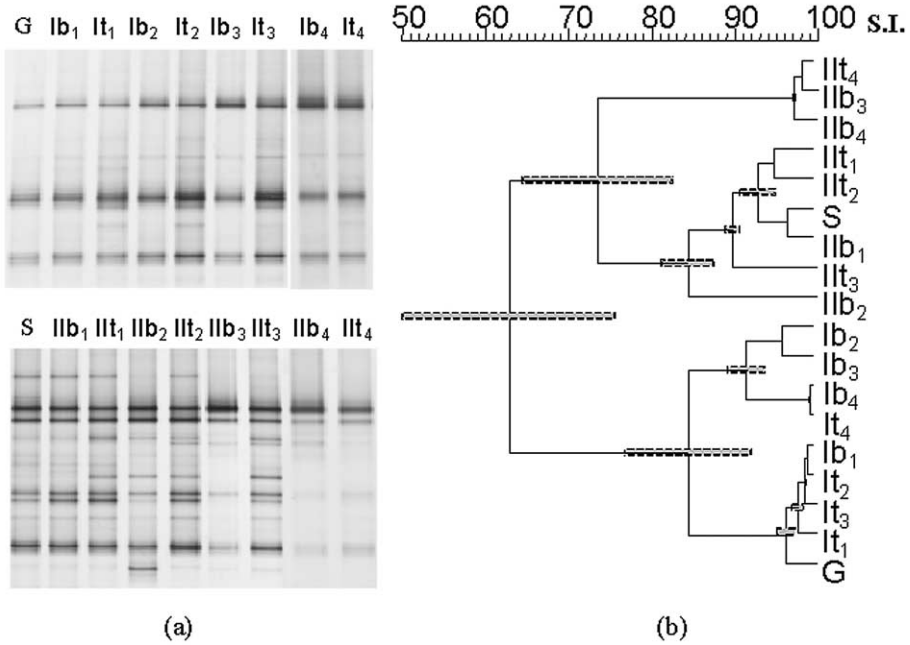

Fig. 3. DGGE profiles of archaeal amplicons from sludge samples (a) and correspondent similarity index (S.I.) dendogram (UPGMA clustering) (b). I – granular sludge, II – suspended sludge, b – bottom layer, t – top layer, 1,2,3 and 4 – operation periods, G – granular inoculum, S – suspended inoculum.

consists mostly of acetoclastic methanogens, surrounded by a second layer of acetogenic and hydrogenotrophic bacteria, with a peripheral layer comprising acidogenic, sulfate-reducing and hydrogenotrophic bacteria [37–40]. This layered structure was observed in granules fed with various substrates including brewery wastes, as was the case of the granular inoculum used in RI. Thus, being located in the inner core of the granules, methanogenic organisms (archaeal domain) can be more protected from the toxic effect and from the hydraulic shear stress, which can explain the insignificant shift in archaeal population observed in this sludge as opposed to the case of suspended sludge. The lower protection offered to the archaeal consortium in the case of the suspended sludge did not prevent the toxic effect, mainly on the community present at the feed inlet, i.e. the bottom layer.

Besides microbial organization, good settling properties are also pointed as an advantage of biomass aggregation [37]. However, for lipid/LCFA containing wastewaters, granule stability is very problematic and disintegration is often observed [6,24,41]. In the thermodynamic respect, the disintegration of granules is predictable, when in contact with this compound, because at neutral pH, LCFAs act as surfactants, lowering the surface tension, compromising the aggregation of hydrophobic bacteria, like most acetogens (LCFA-degraders) [42]. Thus, the decrease in the similarity indices between the bacterial consortium (located in the outer layer) from bottom and top layers on RI-granular sludge with the increase in the toxicant fed to the reactors can be a result of disintegration. The granules suffering from disintegration by contact with oleate would become lighter and accumulated, together with smaller fragments from disintegration, in a floating top layer. Results obtained in a previous work by applying image analysis to characterize the morphology of the granules during the operation of this reactor (RI) confirmed the occurrence of disintegration [24].

3.3. DGGE bands identification

To identify the prominent bands in the DGGE patterns of sludge samples from the last operation period, bacterial and archaeal 16S rRNA genes from samples Ib_4, It_4, IIb_4 and IIt_4 were amplified, cloned and sequenced. The DGGE mobility of the obtained amplicons from the clones were compared to the DGGE profiles of the sludge samples in order to determine to which fragments they corresponded. Due to the considerable shift induced on the bacterial structure during the last operation period and, to retrieve more information, the same procedure was applied to sample IIt_3. This sample, taken from the top of RII-suspended sludge at the end of period 3, when oleate was fed at 6 g COD l^{-1}, was chosen since it exhibited the highest methane production capacity from the adsorbed substrate [23], and thus, would include groups of microorganisms particularly important for the degradation of LCFAs to methane. Table 3 summarizes the sequencing results and Fig. 4 illustrates their corresponding position in each DGGE profile.

Sequencing and BLAST searching of the bacterial clones resulted mainly in matches with unknown and uncultured microorganisms assigned to the Gram-positive group (clones B1, B3, B6, B9), *Proteobacteria* (clone B2) and *Spirochaetales* (clone B7). Close relatives to *Desulfovi-*

Table 3
Sequencing results of the sludge clones

Clone	GenBank accession number	Sequence length (bp)	Closest relatives (% sequence similarity)
B1	AF455055	811	Uncultured eubacterium WCHB1-71 (94%), *Syntrophomonas sapovorans* (94%)
B2	AF455056	813	Uncultured bacterium clone C (97%), *Buchnera aphidicola* (91%)
B3	AF455057	800	Uncultured bacterium mle l-42 (97%), *Aminomonas paucivorans* (88%)
B4	AF455058	903	*D. mexicoense* (98%)
B5	AF455059	736	*Pseudomonas stutzeri* (96%)
B6	AF455060	904	Gram-positive bacterium MOL361 (87%), *Erysipelothrix rhusiopathiae* (88%)
B7	AF455061	657	Unidentified eubacterium clone VadinBA43 (93%), *Spirochaeta africana* (88%)
B8	AF455062	906	*Syntrophomonas sapovorans* (97%)
B9	AF455063	902	Uncultured bacterium SJA-88 (90%), *Clostridium cellobioparum* (87%)
B10	AF455064	804	*Syntrophomonas* sp. MGB-C1 (97%)
B11	AF455065	812	*T. thiogenes* (97%)
A1	AF455066	441	*Methanobacterium formicicum* (97%)
A2	AF455067	815	*Methanosaeta concilli* (97%)
A3	AF455068	775	*Methanobacterium* sp. DSM 11106 (95%)
A4	AF455069	884	*Methanosaeta concilli* (98%)
A5	AF455070	827	Uncultured archaeon TA05 (98%), *Methanosaeta concilli* (98%)
A6	AF455071	852	*Methanobacterium formicicum* strain FCam (98%)
A7	AF455072	774	*Methanobacterium* sp. DSM 11106 (96%)

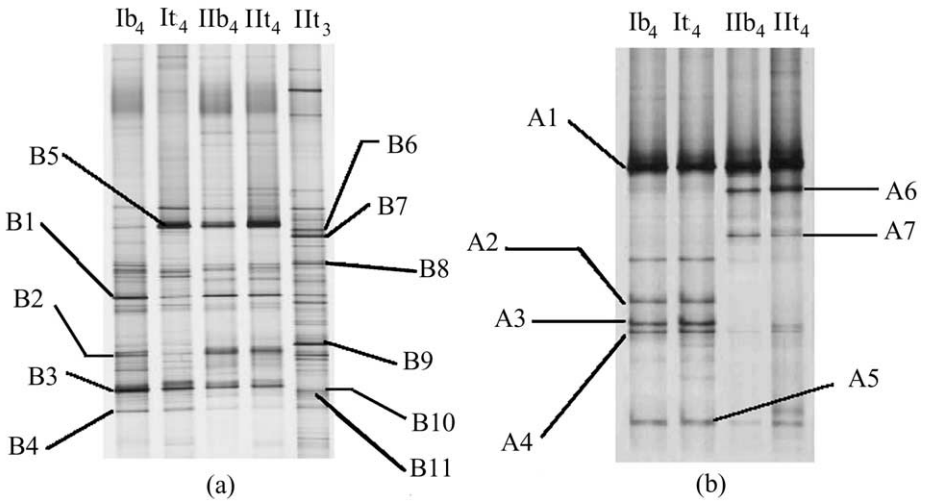

Fig. 4. Correspondent position of each bacterial (a) and archaeal (b) sludge clone in the total DGGE profiles of the analyzed samples. I – granular sludge, II – suspended sludge, b – bottom layer, t – top layer, 3,4 – operation periods.

brio mexicoense (clone B4) and *Trichlorobacter thiogenes* (clone B11) belonging to the delta subdivision of *Protebacteria* were also found, although corresponding to very diffuse fragments on the DGGE profiles. Clones B8 and B10 were closely related to the *Syntrophomonas* genus. The presence of saturated fatty acid-β-oxidizing syntrophic bacteria is desirable to maximize the consumption of butyrate and higher fatty acids resulting from LCFA conversion into acetate and hydrogen through β-oxidation mechanism by the proton reducing acetogenic bacteria [43]. *Syntrophomonas*-like organisms were found in sample IIt_3 but corresponded to very diffuse fragments in the DGGE profiles from the other samples (end of operation), suggesting that the presence of these microorganisms became weaker by increasing the oleate concentration from 6 to 8 g COD l^{-1}. *Pseudomonas*-like organisms (clone B5) corresponded to strong DGGE-bands in the suspended sludge at the end of digesters operation, whereas in the granular sludge they were found only in the top layer.

For the archaeal domain the clone sequences were affiliated with the two main groups, the acetoclastic *Methanosaeta* and the hydrogenotrophic *Methanobacterium*. In the last operation period *Methanosaeta*-like organisms (clones A2, A4 and A5), known to be sensitive to LCFAs [11,44] were clearly present in the granular biomass, but corresponded to very faint bands in the suspended sludge profiles (Fig. 4b). Along the operation, these DGGE bands exhibited stable relative intensities in the RI-granular sludge profiles, whereas in RII-suspended sludge they gradually faded, indicating that the relative abundance of this group became weaker in this sludge during the operation but remained quite constant in the granular one (Fig. 3a). This fact can be related to the layered granular organization referred to before, where *Methanosaeta*-like organisms, being located in the internal granule core, take advantage of a more protected environment against the toxic effect and hydraulic shear stress. This protective concept is also sustained by the higher toxicity limit (IC_{50}) of oleic acid towards acetoclastic bacteria exhibited by the granular inoculum when compared with the suspended sludge (Table 2). In a previous work, Zheng and Raskin [45] used a genus-specific probe to evaluate the levels of *Methanosaeta* sp. in a number of bioreactor samples and found that they were more abundant in granular sludge

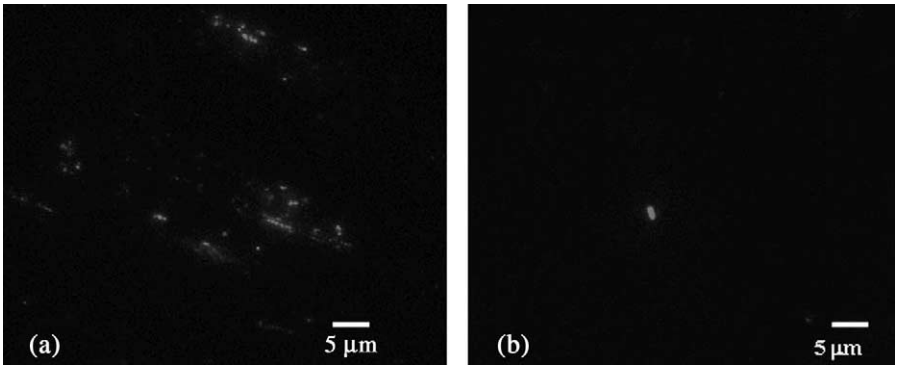

Fig. 5. Epifluorescence photographs showing in situ hybridization with probe MX825-CY3 of (a) granular and (b) suspended sludge present in the bottom layer of reactors, at the end of operation.

than in flocculent sludge. In this work, the *Methanosaeta* genus specific probe MX825 was also used to evaluate the levels of this group of microorganisms in both reactors at the end of operation (samples Ib_4 and IIb_4). The results obtained by fluorescent in situ hybridization were consistent with previous data found by DGGE/sequencing. In the suspended sludge only a weak fluorescent signal was observed whereas in the granular sludge small chains of *Methanosaeta*-like organisms were still detected (Fig. 5). However, in both sludges no acetoclastic activity was detected at the end of operation (Table 2).

In this work, it is shown that the combination of physiological and molecular-based sludge characterization can be a powerful approach to study and optimize the anaerobic digestion process for treating lipids/LCFA-based effluents. Although in general, the methanogenic sludge population can be characterized relatively well, for the group of microorganisms involved in β-oxidation, the presence of many unidentified species in the sludge underlines the need for future research on classical isolation and characterization studies. This need is of utmost importance as it was observed that the bacterial domain was more affected by the contact with increasing oleic acid concentrations. Furthermore, the increase in the organic loading rate to 8 kg oleate-COD m^{-3} day^{-1} induced a maximal shift on the microbial community structure of the sludges. Also at this time point, methanogenic acetoclastic activity was not detected and only a very low methanogenic activity on H_2/CO_2 was still exhibited by the sludges. These results, together with the higher methane production capacity from the adsorbed substrate exhibited by the sludges in the previous organic loading rate evaluated, i.e. 6 kg oleate-COD m^{-3} day^{-1} [23], suggest that this should be the maximum organic loading rate applied to these reactors when treating oleic acid-based effluents.

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References

[1] Komatsu, T., Hanaki, K. and Matsuo, T. (1991) Prevention of lipids inhibition in anaerobic processes by introducing a two-phase system. Water Sci. Technol. 23, 1189–1200.

[2] Galbraith, H., Miller, T.B., Paton, A.M. and Thompson, J.K. (1971) Antibacterial activity of long chain fatty acids and reversal with calcium, magnesium, ergocalciferol and cholesterol. J. Appl. Bacteriol. 34, 803–813.

[3] Koster, I.W. and Cramer, A. (1987) Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. Appl. Environ. Microbiol. 53, 403–409.

[4] Rinzema, A., Boone, M., VanKnippenberg, K. and Lettinga, G. (1994) Bactericidal effect of long chain fatty acids in anaerobic digestion. Water Environ. Res. 66, 40–49.

[5] Hwu, C.-S., Tseng, S.-K., Yuan, C.-Y., Kulik, Z. and Lettinga, G. (1998) Biosorption of long-chain fatty acids in UASB treatment process. Water Res. 32, 1571–1579.

[6] Sam-Soon, P., Loewenthal, R.E., Wentzel, M.C. and Marais, G.V.R. (1991) A long-chain fatty acid, oleate, as sole substrate in upflow anaerobic sludge bed (UASB) reactor systems. Water SA. 17, 31–36.

[7] Rinzema, A. (1988) Anaerobic Treatment of Wastewater With High Concentration of Lipids or Sulfate. Ph.D. Thesis. Wageningen Agricultural University, Wageningen.

[8] Hwu, C.-S., Molenaar, G., Garthoff, J., van Lier, J.B. and Lettinga, G. (1997) Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids: impact of reactor hydrodynamics. Biotchnol. Lett. 19, 447–451.

[9] Hwu, C.-S., van Lier, J.B. and Lettinga, G. (1997) Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids: effect of washed out biomass recirculation. Biotechnol. Lett. 19, 453–456.

[10] Alves, M.M., Mota Vieira, J.A., Álvares Pereira, R.M., Pereira, M.A., Novais, J.M. and Mota, M. (2001) Effects of lipids and oleic acid on biomass development in anaerobic fixed bed reactors. Part I: Biofilm growth and activity. Water Res. 35, 255–263.

[11] Alves, M.M., Mota Vieira, J.A., Álvares Pereira, R.M., Pereira, M.A., Novais, J.M. and Mota, M. (2001) Effects of lipids and oleic acid on biomass development in anaerobic fixed bed reactors. Part II: Oleic acid toxicity and biodegradability. Water Res. 35, 264–270.

[12] Pereira, M.A., Mota, M. and Alves, M.M. (2001) Degradation of oleic acid in anaerobic filters: effect of inoculum acclimatization and biomass recirculation. Water Environ. Res. 73, 612–621.

[13] Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

[14] Muyzer, G. and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel eletrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73, 127–141.

[15] Godon, J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. 63, 2802–2813.

[16] Wu, J.-H., Liu, W.-T., Tseng, I.-C. and Cheng, S.-S. (2001) Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. Microbiology 147, 373–382.

[17] Raskin, L., Stomley, J.M., Rittmannand, B.E. and Stahl, D.A. (1994) Group-specific 16S rRNA hybridization probes to descrive natural communities of methanogens. Appl. Environ. Microbiol. 60, 1232–1240.

[18] Harmsen, H.J.M., Kengen, H.M.P., Akkermans, A.D.L., Stams, A.J.M. and de Vos, W.M. (1996) Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. Appl. Environ. Microbiol. 62, 1656–1663.

[19] Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A. and Harada, H. (1999) Fluorescence in situ hybridization using 16S rRNA-target oligonucleotides reveals localization of Methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol. 65, 1280–1288.

[20] Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E. and Stahl, D.A. (1994) Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Appl. Environ. Microbiol. 60, 1241–1248.

[21] Verstraete, W., De Beer, D., Pena, M., Lettinga, G. and Lens, P.

(1996) Anaerobic bioprocessing of waste. World J. Microbiol. Biotechnol. 12, 221–238.
[22] Oude Elferink, S.J.W.H., van Lis, R., Heilig, H.G.H.J., Akkermans, A.D.L. and Stams, A.J.M. (1998) Detection and quantification of microorganisms in anaerobic bioreactors. Biodegradation 9, 169–177.
[23] Pereira, M.A., Pires, O.C., Mota, M. and Alves, M.M. (2002) Anaerobic degradation of oleic acid by suspended and granular sludge: identification of palmitic acid as a key intermediate. Water Sci. Technol. (in press).
[24] Amaral, A.L., Pereira, M.A., Neves, L., da Mota, M., Pons, M.-N., Vivier, H., Mota, M., Alves, M.M. and Ferreira, E.C. (2001) Characterisation of anaerobic sludge from two EGSB reactors treating oleic acid: automatic detection of granules desintegration by image analysis. In: Proc. 9th World Congress on Anaerobic Digestion 2001 (van Velsen, A.F.M. and Verstraete, W.H., Eds.), Part 1, pp. 89–94. Technologisch Instituut, Antwerpen.
[25] Colleran, E., Concannon, F., Goldem, T., Geoghegan, F., Crumlish, B., Killilea, E., Henry, M. and Coates, J. (1992) Use of methanogenic activity tests to characterize anaerobic sludges, screen for anaerobic biodegradability and determine toxicity thresholds against individual anaerobic trophic groups and species. Water Sci. Technol. 25, 31–40.
[26] Harmsen, H.J.M., Kengen, H.M.P., Akkermans, A.D.L. and Stams, A.J.M. (1995) Phylogenetic analysis of two propionate-oxidizing bacteria in enrichments cultures. Syst. Appl. Microbiol. 18, 67–73.
[27] Lane, D.J. (1991) 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E.R. and Goodfellow, M., Eds.), pp. 115–175. John Wiley and Sons Ltd., Chichester.
[28] Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W. and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.
[29] Grosskopf, R., Janssen, P.H. and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. 64, 960–969.
[30] Zoetendal, E.G., Akkermans, A.D.L., Akkermans-van Vliet, W.M., de Visser, J.A.G.M. and de Voss, W.M. (2001) The host genotype affects the bacterial community in the human gastrointestinal tract. Microb. Ecol. Health Dis. 13, 129–134.
[31] Häne, B.G., Jäger, K. and Drexler, H. (1993) The Pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis 14, 967–972.
[32] Heilig, H.G.H.J., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D.L. and de Voss, W.M. (2002) Molecular diversity of *Lactocobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. 68, 114–123.
[33] Amann, R.I. (1995) In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Molecular Microbial Ecology Manual (Akkermans, A.D.L., van Elsas, J.D. and de Bruigin, F.J., Eds.), Vol. 3.3.6, pp. 1–15. Kluwer Academic Publishers, Dordrecht.
[34] Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: Problems and solutions. Syst. Appl. Microbiol. 15, 593–600.
[35] Hwu, C.-S., Donlon, B. and Lettinga, G. (1996) Comparative toxicity of long-chain fatty acid to anaerobic sludges from various origins. Water Sci. Technol. 34, 351–358.
[36] Roy, F., Albagnac, G. and Samain, E. (1985) Influence of calcium addition on growth of highly purified syntrophic cultures degrading long chain fatty acids. Appl. Environ. Microbiol. 49, 702–705.
[37] MacLeod, F.A., Guiot, S.R. and Costerton, J.W. (1990) Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Appl. Environ. Microbiol. 56, 1598–1607.
[38] Guiot, S.R., Pauss, A. and Costerton, J.W. (1992) A structured model of the anaerobic granule consortium. Water Sci. Technol. 25, 1–10.
[39] Fang, H.H.P., Chui, H.K. and Li, Y.Y. (1994) Microbial structure and activity of UASB granules treating different wastewaters. Water Sci. Technol. 30, 87–96.
[40] Quarmby, J. and Forster, C.F. (1995) A comparative study of the internal architecture of anaerobic granular sludges. J. Chem. Technol. Biotechnol. 63, 60–68.
[41] Hawkes, F.R., Donnely, T. and Anderson, G.K. (1995) Comparative performance of anaerobic digesters operating on ice-cream wastewater. Water Res. 29, 525–533.
[42] Daffonchio, D., Thaveesri, J. and Verstraete, W. (1995) Contact angle measurement and cell hydrophobicity of granular sludge from upflow anaerobic sludge bed reactors. Appl. Environ. Microbiol. 61, 3676–3680.
[43] Weng, C.N. and Jeris, J.S. (1976) Biochemical mechanisms in the methane fermentation of glutamic and oleic acids. Water Res. 10, 9–18.
[44] Hanaki, K., Matsuo, T. and Nagase, M. (1981) Mechanisms of inhibition caused by long chain fatty acids in anaerobic digestion process. Biotechnol. Bioeng. 23, 1560–1591.
[45] Zheng, D. and Raskin, L. (2000) Quantification of *Methanosaeta* species in anaerobic bioreactors using genus- and species-specific hybridization probes. Microb. Ecol. 39, 246–262.