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 Methanoseta-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 H₂/CO₂ 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]. LCFAAs 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 LCFA 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-
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 10 l 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 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 (H₂/CO₂) 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% (IC₅₀). 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 N₂ 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

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Influent COD (g l⁻¹)</th>
<th>Influent oleate-COD (g l⁻¹)</th>
<th>COD removal efficiency (%)</th>
<th>Effluent VSS (g l⁻¹)</th>
<th>Methane production (l CH₄ day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[period]</td>
<td>RI</td>
<td>RII</td>
<td>RI</td>
<td>RII</td>
<td>RI</td>
</tr>
<tr>
<td>0–70</td>
<td>3.8</td>
<td>(± 0.3)</td>
<td>96.5</td>
<td>85.8</td>
<td>0.38</td>
</tr>
<tr>
<td>[1]</td>
<td>(± 0.2)</td>
<td>(± 0.6)</td>
<td>(± 3.2)</td>
<td>(± 0.07)</td>
<td>(± 0.04)</td>
</tr>
<tr>
<td>70–119</td>
<td>3.8</td>
<td>(± 0.3)</td>
<td>83.4</td>
<td>74.4</td>
<td>0.85</td>
</tr>
<tr>
<td>[2]</td>
<td>(± 0.3)</td>
<td>(± 0.3)</td>
<td>(± 4.8)</td>
<td>(± 5.5)</td>
<td>(± 0.22)</td>
</tr>
</tbody>
</table>

Complete bacterial 16S rDNA was selectively amplified for cloning and sequencing using 7-f (5'-AGAGTTTGATC(T/A)GGGCTGAG-3') and 1510-r (5'-ACGGTGTGTTAACGACTTT-3') primers [27] with the same 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'-CGCCGGGGCGGGCGGGGCGGGCGG-GGGGGGCGACGGGGAACGAGAACCTT-3') and 1401-r (5'-CGGTGTGTTAACAGGACC-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(AG/G)TGCAGTAAACGTAAACGT-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'-CGCCGGGGCGGGCGGGCGGGGACGGGCGTGCGTGGC-AC-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'-GATT- TAGGTGACATATAG-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'-TCGCCACCTGTCGCC-GACACCTAGC-3'; target group: Methanoseta; [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 QFluor image analysis software at a Leica Q550 FW computer. Phase contrast microscopy was performed with a Zeiss (Oberkochen, Germany) Axioskop 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 H₂/CO₂. However, methanogenic activity with butyrate was not detected in this sludge whereas a

<table>
<thead>
<tr>
<th>Table 2</th>
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<td>Methanogenic activity for both seed sludge and at the end of reactors operation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Seed sludge</th>
<th>End of operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RI-granular</td>
</tr>
<tr>
<td>Methanogenic activity in presence of:</td>
<td>ml CH₄ (STP) (g⁻¹ VSS day⁻¹)</td>
</tr>
<tr>
<td>Acetate</td>
<td>327 ± 11</td>
</tr>
<tr>
<td>Propionate</td>
<td>160 ± 10</td>
</tr>
<tr>
<td>Butyrate</td>
<td>(n.d.)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>514 ± 94</td>
</tr>
<tr>
<td>H₂/CO₂</td>
<td>597 ± 16</td>
</tr>
<tr>
<td>Oleic acid toxicity limit (IC50) (mg l⁻¹)</td>
<td>345 ± 26</td>
</tr>
</tbody>
</table>

Oleic acid toxicity for granular and suspended seed sludge (mean ± 95% confidence interval). (n.d.) – Not detected. STP – Standard temperature and pressure conditions.
value of 52 ml CH4(STP) (g−1 volatile suspended solids (VSS) day−1) was detected in the suspended sludge. The toxicity limit (IC50) 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 H2/CO2. Both sludges exhibited low activity with H2/CO2 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 H2/CO2 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 CH4 (kg CODremoved)−1 in RI and 39 l CH4 (kg CODremoved)−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 LCFA 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 LCFA 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 CH4(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 CH4(STP) g−1 VSS) [23].

Fig. 1. Microscopic aspect of the encapsulated sludge: A – biomass, B – whitish matter.
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
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 pop-
ulation 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 incon-
tact with this compound, because at neutral pH, LCFAs
act as surfactants, lowering the surface tension, comprom-
isng the aggregation of hydrophobic bacteria, like most
acetogens (LCFA-degraders) [42]. Thus, the decrease in
the similarity indices between the bacterial consortium (lo-
cated 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. Re-

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 I b 4, I t 4, II b 4
and II t 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 I t 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 exhib-
ted the highest methane production capacity from the
adsorbed substrate [23], and thus, would include groups
of microorganisms particularly important for the degrada-
tion of LCFAs to methane. Table 3 summarizes the se-
quencing results and Fig. 4 illustrates their corresponding
position in each DGGE profile.

Sequence and BLAST searching of the bacterial
clones resulted mainly in matches with unknown and un-
cultured microorganisms assigned to the Gram-positive

group (clones B 1, B 3, B 6, B 9), Proteobacteria (clone B 2)
and Spirochaetales (clone B 7). Close relatives to Desulfovi-

<table>
<thead>
<tr>
<th>Clone</th>
<th>GenBank accession number</th>
<th>Sequence length (bp)</th>
<th>Closest relatives (% sequence similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>AF455055</td>
<td>811</td>
<td>Uncultured eubacterium WCHB1-71 (94%), Syntrophomonas sapovorans (94%)</td>
</tr>
<tr>
<td>B2</td>
<td>AF455056</td>
<td>813</td>
<td>Uncultured bacterium clone C (97%), Buchnera aphidicola (91%)</td>
</tr>
<tr>
<td>B3</td>
<td>AF455057</td>
<td>800</td>
<td>Uncultured bacterium mle 1-42 (97%), Aminomonas paucivorans (88%)</td>
</tr>
<tr>
<td>B4</td>
<td>AF455058</td>
<td>903</td>
<td>D. mexicorum (98%)</td>
</tr>
<tr>
<td>B5</td>
<td>AF455059</td>
<td>736</td>
<td>Pseudomonas stutzeri (96%)</td>
</tr>
<tr>
<td>B6</td>
<td>AF455060</td>
<td>904</td>
<td>Gram-positive bacterium MOL361 (87%), Erysipelothrix rhusiopathiae (88%)</td>
</tr>
<tr>
<td>B7</td>
<td>AF455061</td>
<td>657</td>
<td>Unidentified eubacterium clone VadinBA43 (93%), Spirochaeta africana (88%)</td>
</tr>
<tr>
<td>B8</td>
<td>AF455062</td>
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<td>Syntrophomonas sapovorans (97%)</td>
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<tr>
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<td>902</td>
<td>Uncultured bacterium SJA-88 (90%), Clostridium cellubiovarum (87%)</td>
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<tr>
<td>B10</td>
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<td>804</td>
<td>Syntrophomonas sp. MGB-C1 (97%)</td>
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<tr>
<td>B11</td>
<td>AF455065</td>
<td>812</td>
<td>T. thiogenes (97%)</td>
</tr>
<tr>
<td>A1</td>
<td>AF455066</td>
<td>441</td>
<td>Methanobacterium formicicum (97%)</td>
</tr>
<tr>
<td>A2</td>
<td>AF455067</td>
<td>815</td>
<td>Methanoseta concilii (97%)</td>
</tr>
<tr>
<td>A3</td>
<td>AF455068</td>
<td>775</td>
<td>Methanobacterium sp. DSM 11106 (95%)</td>
</tr>
<tr>
<td>A4</td>
<td>AF455069</td>
<td>884</td>
<td>Methanoseta concilii (98%)</td>
</tr>
<tr>
<td>A5</td>
<td>AF455070</td>
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<td>Uncultured archaeon TA05 (98%), Methanoseta concilii (98%)</td>
</tr>
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<td>A6</td>
<td>AF455071</td>
<td>852</td>
<td>Methanobacterium formicicum strain FCam (98%)</td>
</tr>
<tr>
<td>A7</td>
<td>AF455072</td>
<td>774</td>
<td>Methanobacterium sp. DSM 11106 (96%)</td>
</tr>
</tbody>
</table>
brio mexicóense (clone B4) and Trichlorobacter thiogenes (clone B11) belonging to the delta subdivision of Proteobacteria 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 II\textsubscript{t3} 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\textsuperscript{-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 LCFA\textsubscript{s} [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\textsubscript{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. 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.](image)

![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.](image)
than in flocculent sludge. In this work, the *Methanoseta* 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 Ib4 and IIb4). 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 *Methanoseta*-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 less 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 H₂/CO₂ 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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