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## Anaerobic biodegradation of oleate by a highly loaded biomass before and after degrading the associated substrate

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**Abstract** Oleic Acid (C18:1) is an unsaturated Long Chain Fatty Acid (LCFA), described as especially problematic in anaerobic digestion processes. In this work, a sludge heavily loaded with 5985 mgCOD.gVS<sup>-1</sup> was studied in terms of its capability to biodegrade oleic acid in batch assays, in concentrations ranging from 100 to 1500 mg.l<sup>-1</sup>. Lag phases before the onset of methane production increased with the oleic acid concentration, up to 17 days for 1500 mg.l<sup>-1</sup> and methane production did not exceed 50% recovery for the highest concentrations tested (1000 and 1500 mg.l<sup>-1</sup>). After the mineralization of the biomass-associated substrate, and for the same concentration range, maximum lag phases of three days and methane recoveries up to 80% were obtained, evidencing an improving capacity of the sludge to biodegrade oleic acid. Also the specific methanogenic activity with acetate and H<sub>2</sub>/CO<sub>2</sub> as individual substrates increased 3.6 and 2 times respectively, after the mineralization of the biomass associated substrate, confirming the reversibility of the inhibitory effect of LCFA, even when the sludge was heavily loaded with more than 5 g COD.gVS<sup>-1</sup>.

**Keywords** Biodegradation; inhibitory effect; LCFA; oleic acid

## INTRODUCTION

Many problems are encountered during the anaerobic treatment of lipid-containing wastewaters such as inhibition and sludge flotation and washout, in part due to the adsorption of LCFA to the microbial cell walls. Besides the potential inhibition effect, LCFA accumulation onto the sludge can create a physical barrier and hinder the transfer of substrates and products (Pereira *et al.*, 2005).

Accumulation of LCFA on anaerobic sludge was studied before in several works, during bioreactors operation, and the general conclusion pointed forward that sequencing accumulation and degradation steps was a strategic procedure to achieve an efficient rate of methane production as well as a stoichiometric methane yield. One of the most interesting results reported by Pereira *et al.* (2002), demonstrated that when feeding continuously oleic acid to an upflow anaerobic sludge bed (UASB) reactor there was an accumulation of palmitic acid onto the sludge which was only biodegraded when oleic acid was not present in the medium. This clearly suggested that batch sequential operation would be the optimal strategy to treat LCFA rich wastewater. Recent results proved that the sequential operation is an essential step during start-up but, after few cycles, the sludge is able to efficiently convert continuously organic loading rates up to 8 kg COD.m<sup>-3</sup>.day<sup>-1</sup> (50% COD as oleate), with methane conversion efficiencies higher than 80% (Cavaleiro *et al.*, 2007).

Some studies reported biodegradability and activity measurements of anaerobic sludge encapsulated with different amounts of LCFA (Pereira *et al.*, 2002, 2004). Also the kinetics of biodegradation of biomass associated LCFA was already established in a previous work (Pereira *et al.*, 2004). However, the behavior of the sludge is highly dependent on the degree of acclimation to LCFA. A sludge acclimated to discontinuous or pulsed feeding of fat is probably the best inoculum to use for LCFA-rich wastewater treatment, according to previous results. Therefore, it was considered important to study a sludge previously acclimated to LCFA in those conditions. In this work, a

biomass highly loaded with LCFA, and acclimated as further explained, was studied before and after the degradation of the biomass-associated LCFA in terms of: (i) biodegradability of oleic acid in concentrations ranging from 100 to 1500 mg.l<sup>-1</sup>, and (ii) specific methanogenic activity in acetate and H<sub>2</sub>/CO<sub>2</sub>. Sodium oleate was used as a model for long chain fatty acids because it is, in general, the most abundant of all LCFA present in wastewaters, has a good solubility and is considered one of the more toxic LCFA (Alves *et al.*, 2001).

## METHODS

### Biomass source

The suspended biomass used in this study was collected from a lab scale reactor fed with a synthetic dairy wastewater (made by dilution of skim milk with tap water) containing sodium oleate. The reactor was operated in continuous during 67 days, with an organic loading rate of 4.0 kg COD.m<sup>-3</sup>.day<sup>-1</sup>, at a constant temperature of 37 ± 1°C during the trial period. Skim milk and oleate contributed each for 50 % of total COD fed. This substrate was supplemented with macro and micronutrients and 5 g NaHCO<sub>3</sub> were added per litre of feeding (Zehnder *et al.*, 1980). At the end of the operation the biomass was encapsulated by a whitish matter.

### Biomass characterization

#### *Specific methanogenic activity tests*

Specific methanogenic activity, in the presence of acetate and H<sub>2</sub>/CO<sub>2</sub>, was determined before and after the degradation of the biomass associated LCFA. Specific methanogenic activity tests were performed using a pressure transducer technique as previously described by Colleran *et al.* (1992) and Coates *et al.* (1996). The methane content of the biogas was determined by gas chromatography using a Porapak Q (100–180 mesh) column, with He as carrier gas at 30 ml.min<sup>-1</sup> and a thermal conductivity detector. Temperatures of the injection port, column and detector were 110, 35 and 110°C, respectively. Specific methanogenic activity values were converted to mg COD-CH<sub>4</sub>.gVS<sup>-1</sup>.day<sup>-1</sup>) and background methane production due to the residual substrate (blank controls) was subtracted.

#### *Extraction and GC analysis of LCFA accumulated onto the biomass*

The extraction and derivatization of LCFA was performed in duplicate as described elsewhere (Neves *et al.*, 2007) and esterified LCFA were further separated by gas chromatography (Chrompack CP 9001), with a TR-WAX (eq.CP-Sil 52 CB) 30 m × 0.32 mm × 0.25 µm capillary column connected to a flame ionization detector and to a split/splitless injector (split ratio 1:10). Helium was the carrier gas at a flow rate of 1 ml.min<sup>-1</sup>, H<sub>2</sub> (30 ml.min<sup>-1</sup>) was the combustion gas and N<sub>2</sub> (30 ml.min<sup>-1</sup>) was the make-up gas. The column, injector and detector temperatures were maintained at 50 to 225°C (10°C/min), 220°C and 250°C respectively. LCFA were analysed in the solid and in the liquid phase.

### Oleate biodegradability before and after degradation of biomass associated LCFA

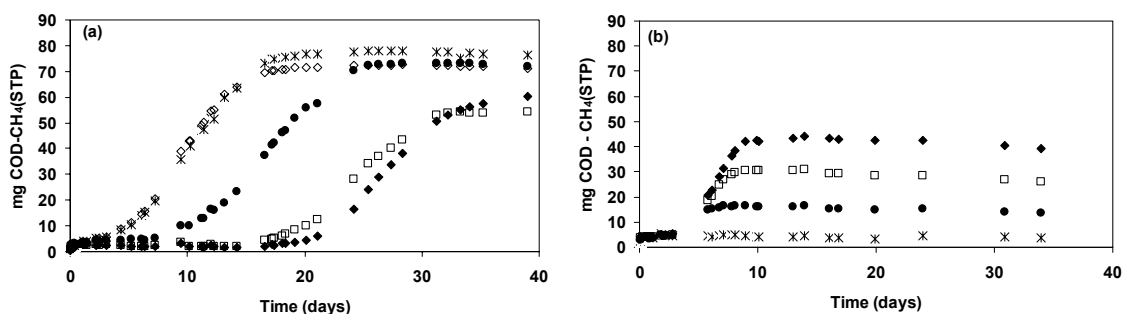
Oleate biodegradability capacity was studied before and after degradation of the biomass associated LCFA. A set of 20 vials was prepared as described elsewhere (Pereira *et al.*, 2004). After an overnight incubation (37°C, 150 rpm), a volume of 1.25 ml of oleate stock solutions, 10 fold concentrated, was added to 8 of these vials, in order to obtain the following concentrations inside the vials: 100, 500, 1000 and 1500 mg.l<sup>-1</sup> (duplicate assays). Other 8 vials, received the same amount of substrate, but it was added only after the stabilization of the biogas production due to the degradation of the biomass associated LCFA. Before oleate addition, the vials were vented and the headspace washed with N<sub>2</sub>/CO<sub>2</sub> (80:20). A blank control assay (without oleate addition) was

performed in parallel, corresponding to the remaining 4 vials. From the moment of substrate addition, the methane production was determined by measuring periodically pressure and methane content of the biogas accumulated in the headspace, until stabilization.

## RESULTS AND DISCUSSION

The sludge analyzed in this study was sampled from a lab-scale reactor, operated as described above. During the reactor operation, methane production was significantly reduced and the biomass became surrounded by a whitish matter, which was expected to be palmitic acid as it was observed previously (Pereira *et al.*, 2002). Extraction and GC analysis of individual LCFA between C12:0 and C18:2, confirmed that palmitate was present in a concentration of  $268 \pm 9$  mg palmitate.gTS<sup>-1</sup>, which represented 91% of the total LCFA accumulated onto the sludge, and also traces of myristate ( $14 \pm 0.4$  mg myristate.gTS<sup>-1</sup>) and oleate ( $12 \pm 0.4$  mg oleate.gTS<sup>-1</sup>) were detected.

Figure 1 (a) and (b) presents the cumulative methane production obtained in the biodegradability assays performed before and after the degradation of the biomass-associated substrate, respectively, and Table 1 summarizes the percentage of methane-COD recovered in both experiments. When the encapsulated biomass was placed in batch vials, without any added substrate, it was capable of degrading the adsorbed substrate (Figure 1 (a) -  $\diamond$  - no oleate added). The onset of methane production was observed after three days of incubation, suggesting some difficulties on substrate degradation or in the methane formation/release to the headspace. The addition of increasing oleate concentration resulted in a proportional increase of the lag phase preceding the initial methane production, except when 100 mg.l<sup>-1</sup> of oleate were added. In this case, methane production presented the same behavior of the blank assay (in which methane production resulted from the degradation of the accumulated substrate) during the first 16 days. After this period, methane production in the blank assay stopped and in the 100 mg.l<sup>-1</sup> vials continued, reaching a plateau of 78 mg COD-CH<sub>4</sub> instead of the 73 mg COD-CH<sub>4</sub> obtained in the blank assay (Table 1).



**Figure 1.** Anaerobic biodegradation of oleate (a) before and (b) after the degradation of the biomass-associated substrate.  $\diamond$  - no oleate added;  $*$  - 100 mg.l<sup>-1</sup> oleate;  $\bullet$  - 500 mg.l<sup>-1</sup> oleate;  $\square$  - 1000 mg.l<sup>-1</sup> oleate;  $\blacklozenge$  - 1500 mg.l<sup>-1</sup> oleate.

When 500 mg.l<sup>-1</sup> were added, the same plateau of the blank assay was reached after a lag phase of 7 days, but when higher concentrations were present a lower plateau was observed in the time course of the experiment. In all cases, except for the vials where 100 mg.l<sup>-1</sup> oleate were added, the theoretical methane production from the degradation of the oleate added, in addition to the accumulated substrate was never achieved (Table 1). In the presence of 1000 and 1500 mg.l<sup>-1</sup> the sludge was able to convert to methane only about 50% of the total substrate present in the vials, and a lag phase of 17 days before methane production started was observed. These results show that oleate addition influenced the delay observed before methane production started and also the maximum plateau reached in each vial. These effects may be due to mass transfer limitations (Pereira *et al.*, 2005), inhibition or a combination of both phenomena. In this work, no effect was

caused by a concentration as low as 100 mg.l<sup>-1</sup>, but a moderate effect was observed with 500 mg.l<sup>-1</sup> of oleate and a more evident one was caused by the highest concentrations (1000 and 1500 mg.l<sup>-1</sup>). Other authors reported that an oleate concentration of 100 mg.l<sup>-1</sup> was already inhibitory, either in terms of methane production rate or in terms of plateau obtained (Lalman & Bagley, 2002; Perle *et al.*, 1995; Pereira *et al.*, 2002).

**Table 1.** Percentage of COD-CH<sub>4</sub> recovered before and after the degradation of biomass associated LCFA, in the presence of different concentrations of oleate.

Test Concentration (mg.l <sup>-1</sup> )	Before degrading the biomass associated LCFA			After degrading the biomass associated LCFA		
	Initial COD (mg COD)	Methane produced (mg COD)	% COD – CH <sub>4</sub> recovered	Initial COD (mg COD)	Methane produced (mg COD)	% COD – CH <sub>4</sub> recovered
0	73 ± 3 <sup>(*)</sup>	73 ± 3		2.64 ± 0.04 <sup>(*)</sup>	2.64 ± 0.04	
100	76	78 ± 4	102.5 ± 5.8	7	5.1 ± 0.2	81.0 ± 3.6
500	91	73 ± 5	80.9 ± 6.1	22	16.9 ± 1.1	81.7 ± 5.4
1000	109	55 ± 0.1	50.9 ± 1.2	40	30.8 ± 0.1	79.4 ± 0.2
1500	127	62 ± 6	48.6 ± 4.9	58	44.0 ± 2.6	77.5 ± 4.6

(\*) indirectly estimated by the plateau in the cumulative methane production curve (“blank assay”)

After degrading the associated LCFA, oleate biodegradation capacity was enhanced, since the conversion of oleate to methane was in the range of 78 and 82%, for all the concentrations tested (Table 1). Lag phases (during approximately 3 days) were considerably smaller than the ones observed previously.

On the assay performed without any added oleate (blank assay), the amount of biomass-associated substrate was indirectly estimated from the plateau achieved in the cumulative methane production curve (5985 ± 600 mg COD-CH<sub>4</sub>.gVS<sup>-1</sup>). This value is higher than the maximum one reported in the literature by Pereira *et al.* (2005), who described the accumulation of 4.6 g COD-LCFA.gVS<sup>-1</sup> when feeding pure oleic acid at an organic loading rate of 6 kg COD.m<sup>-3</sup>.day<sup>-1</sup>. In that case, the accumulated substrate was mainly composed of LCFA, 83% as palmitic acid. In the present experiment, the sludge was loaded with 4 kg COD.m<sup>-3</sup>.day<sup>-1</sup> of a complex substrate containing oleate, which is a more realistic approach than the strategy followed in some previous tests where oleate was the sole organic carbon source fed to the reactors.

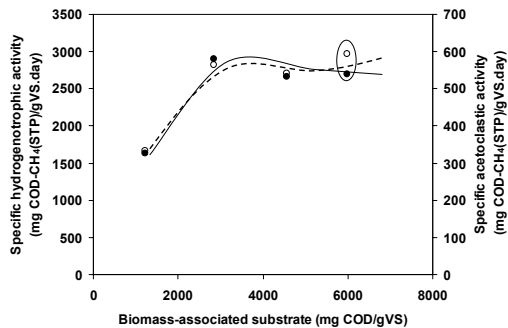
Table 2 presents the specific methanogenic activity in the presence of acetate and H<sub>2</sub>/CO<sub>2</sub>, measured before and after the degradation of the accumulated substrate. The extremely high value of substrate associated to the sludge by mechanisms of adsorption, precipitation and/or entrapment did not inhibit completely the specific methanogenic acetoclastic activity, as was previously observed for sludges loaded with amount of substrate in the range of 2.8 to 4.5 g COD.gVS<sup>-1</sup> (Pereira *et al.*, 2004). After degrading the associated LCFA the biomass SMA increased about 2 times in the presence of H<sub>2</sub>/CO<sub>2</sub> and approximately 4 times in the presence of acetate (Table 2).

**Table 2.** Specific methanogenic activities exhibited by the biomass, before and after the degradation of the accumulated substrate (mean ± SD).

SMA in the presence of:	(mg COD-CH <sub>4</sub> .g VS <sup>-1</sup> .day <sup>-1</sup> )	
	Before substrate degradation	After substrate degradation
Acetate	148 ± 39	539 ± 55
H <sub>2</sub> /CO <sub>2</sub>	1416 ± 90	2970 ± 327

In a previous work made by Pereira *et al.* (2004) three sludges with different loads of biomass-associated substrate were characterized in terms of SMA before and after its degradation. Figure 2 represents the influence of the amount of substrate accumulated onto the sludge on the specific methanogenic activity measured after its degradation, and was produced with experimental data

obtained in this work and also by Pereira *et al.* (2004). A trend towards a maximum specific acetoclastic activity of about 550 mg COD-CH<sub>4</sub>.gVS<sup>-1</sup>.day<sup>-1</sup> and a maximum specific hydrogenotrophic activity of about 2800 mg COD-CH<sub>4</sub>.gVS<sup>-1</sup>.day<sup>-1</sup> is evident, suggesting that heavy accumulation, followed by degradation of complex substrates with high lipid content, is beneficial to develop an active methanogenic consortium. Furthermore, it is relevant to note that, despite the very high amount of accumulated substrate that was in contact with the sludges, both trophic groups, hydrogen and acetate consumers, showed a similar pattern of SMA increasing, which is particularly interesting since the acetoclastic group is usually reported as more sensitive to LCFA toxicity than hydrogenotrophic group (Hanaki *et al.*, 1981). The hypothesis already proposed by Pereira *et al.* (2005) about the important role of mass transfer limitations in LCFA degradation processes, is likely the reason for the reported “high toxicity” of LCFA towards acetoclastic bacteria. Since acetate is a larger molecule, when comparing with H<sub>2</sub>, is likely more difficult to be transported through a layer of LCFA surrounding the cells in an encapsulated sludge.



**Figure 2.** Influence of the amount of substrate accumulated onto the biomass, in the specific methanogenic activity in (●) acetate and (○) H<sub>2</sub>/CO<sub>2</sub> after its degradation. The marked points represent the values obtained in the present work. The other three points were obtained by Pereira *et al.* (2004).

The results obtained in this work confirm the idea presented before by Pereira *et al.* (2003, 2004, 2005), that the inhibitory effect caused by oleic acid is reversible, even when concentrations in the order of 1500 mg.l<sup>-1</sup> are applied and when the biomass was severely loaded with LCFA (this work).

Because LCFA degradation depends on the interaction of different metabolic groups, which act in syntrophy, the loss of activity of a group, even if it is temporary, influences negatively the overall process, causing a delay on the degradation of LCFA and consequently on methane production. Therefore, besides SMA, it is also important to study the capacity of this sludge to biodegrade the accumulated substrate and compare it with previously studied sludges. In this work, an initial methane production rate of 146 ± 10 mg COD-CH<sub>4</sub>.gVS<sup>-1</sup>.day<sup>-1</sup> was obtained from the consumption of the accumulated substrate. Comparing this value with others presented by Pereira *et al.* (2004) it is possible to suggest that sludge adaptation has a major role on the methane production rate from the accumulated substrate. Even when high amounts of substrate are present (5985 mgCOD.gVS<sup>-1</sup>) relatively high methane production rates are achieved (in this work, 146 ± 10 mg COD-CH<sub>4</sub>.gVS<sup>-1</sup>.day<sup>-1</sup>).

## CONCLUSIONS

A biomass highly loaded with LCFA, exhibiting low methanogenic activity was severely inhibited by the addition of oleate concentrations in the range of 500 to 1500 mg.l<sup>-1</sup>. However, after degrading the adsorbed substrate, the biomass performance improved considerably since the methanogenic activity increased 2 and 3.6 times in H<sub>2</sub>/CO<sub>2</sub> and acetate as individual substrates, respectively. Also, the ability to degrade oleate, in the same concentration range, was enhanced, since lag phases preceding the initial production of methane decreased considerably and methane recovery increased from 50 to 80%. The recovery of the methanogenic activity after the degradation of the accumulated substrate (LCFA) was more evident when higher amounts of biomass associated substrate (3000 to 6000 mg COD.g VS<sup>-1</sup>) were present.

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