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The important role of mass transfer limitations caused by Long Chain Fatty Acids accumulation onto the anaerobic sludge

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Abstract In this work, the role of transport limitations caused by LCFA accumulation onto anaerobic sludge was evaluated. Two sludges were compared in terms of the specific methanogenic activity in the presence of acetate, propionate, butyrate and H_2/CO_2, before and after the mineralization of LCFA accumulated onto the biomass. In both cases the main LCFA accumulated was palmitic acid, but in one of the sludges (referred as encapsulated sludge) it was mainly adsorbed, surrounding the cells, and in the other (non-encapsulated sludge) it was mainly precipitated in white spots in between the sludge. The LCFA loaded, but non-encapsulated sludge exhibited a considerable initial methanogenic activity on all the tested substrates, with the single exception of butyrate. However, for the encapsulated sludge only methane production from ethanol and H_2/CO_2 was detected, after a lag phase of about 50 hours. The obtained results evidenced that, besides the potential toxic effect, LCFA accumulation onto the sludge can create a physical barrier and hinder the transfer of substrates and products, inducing a delay on the initial methane production.
Keywords Long chain fatty acids (LCFA), methanogenic activity; transport limitations

Introduction

When an oleic acid based effluent is continuously fed to an anaerobic reactor, a significant accumulation of LCFA occurs, associated to the sludge, palmitic acid representing more than 80% of the most expected β-oxidation intermediates (C10:0 to C18:0) (Pereira *et al*., 2002). Adsorption, precipitation and entrapment are the mechanisms responsible for the LCFA accumulation. Adsorption is widely reported in the literature and, besides problems of sludge flotation, this phenomenon was considered to be the main factor of LCFA toxicity due to physical interactions with the cell wall, affecting its transport and protective functions (Galbraith and Miller, 1973). A permanent toxic effect and a bactericidal toxic effect of LCFA were reported (Rinzema *et al*, 1994, Angelidaki and Ahring, 1992) and accepted for years. However, studies conducted in our research group clearly demonstrated that LCFA toxicity is not permanent and that LCFA do not exert a bactericidal effect towards methanogens. It was found that LCFA, provided they are associated to the biomass, could be efficiently mineralized and that after the mineralization, the specific methanogenic activity was significantly enhanced. This finding was observed for sludges with specific LCFA contents in the range from 1 to 5 g COD/gVSS (Pereira *et al*., 2004). A reasonable hypothesis to explain this behavior is that LCFA exert reversible inhibitory effect. However, transport limitation effects imposed by the LCFA that accumulates onto the anaerobic sludge may also occur and, therefore, the aim of this work was to add insights on the role of mass transport limitations imposed by the biomass-associated LCFA when acetate, propionate, butyrate, ethanol and H_2/CO_2 were mineralized.

Materials and Methods

Experimental set-up and operation mode

Two 1 l expanded granular sludge bed (EGSB) reactors (R1, R2), inoculated with suspended sludge, were continuously fed with oleic (R1) or palmitic (R2) acid at a constant influent concentration of 4 g chemical oxygen demand (COD)/l (HRT≅1 day), under mesophylic conditions

(37±1 ºC). The reactors were equipped with an external settler (vol = 200 ml) from where biomass was recycled at a rate of 4 l/day. During the first 28 days, start-up period, the substrate consisted of skim milk (50% COD) and the corresponding individual LCFA (50% COD) diluted with tap water. Macro and micro-nutrients were added according to the composition described elsewhere (Alves *et al*., 2001). To give suitable alkalinity 5 g $NaHCO_3$/l was added to the feed. From the day 28 on, the carbon source was exclusively composed by sodium oleate in R1 and palmitic acid in R2. During this time, a nitrogen supplement was added to the macronutrients solution in order to maintain a ratio COD/N/P of 200:5:1.1. Due to the low solubility of palmitic acid in aqueous media (7.2 mg/l in water at 20ºC) this feed stock was homogenized in a Euroturrax T20 (IKA Labortechnik, Germany) to form a fine emulsion. To overcome problems of solid sedimentation in the feed tank and to promote a homogeneous emulsion, the feed stock was continuously stirred and the monitoring of the influent COD revealed that the desired value was assured. The influent pH was kept near 8.

Routine analysis. COD and VSS were determined according to Standard Methods (1989). VFA were determined by high-performance liquid chromatography (HPLC) using a Chrompack column (300×6.5 mm) and a mobile phase of 5 mM sulphuric acid (H_2SO_4) at 0.7 ml/min. The column was set at 40°C and the detection was spectrophotometric at 220 nm. Methane content of the biogas was measured by gas chromatography (GC) using a Chrompack Haysep Q (80 to 100 mesh) column, with N_2 carrier gas at 30 ml/min and a flame-ionization detector. Temperatures of the injection port, column, and flame-ionization detector were 120, 40, and 130ºC, respectively.

Batch experiments

Specific methanogenic activity (SMA) tests: These tests were performed using a pressure transducer technique, as described by Colleran *et al*., 1992. The pressure increase developed in the batch vials fed with non gaseous substrates (30 mM acetate, 30 mM propionate, 15 mM butyrate and 30mM ethanol) or pressure decrease in vials previously pressurized (1 bar) with gaseous substrates (H_2/CO_2 - 80:20 vol/vol. The basal medium used in all the batch experiments was made up with demineralized water, was composed of cysteine-HCL (0.5 g/l) and sodium bicarbonate (3 g/l), the pH was adjusted to 7.0-7.2 with NaOH 8N and was prepared under strict anaerobic conditions. Methane content of the biogas was measured by gas chromatography as previously described. The values of methane production were corrected for the standard temperature and pressure conditions (STP). Blank controls were used for liquid substrates (no added substrate) and for gaseous substrates (pressurized with N_2/CO_2 - 80:20 vol/vol at 1 Bar). The batch experiments were performed in triplicate assays. SMA values were determined dividing the initial slope of the methane production curve by the VSS content of each vial at the end of the experiment and were expressed in ml CH_4/(gVSS.day), which were then converted to the equivalent COD. Background methane production due to the residual substrate was discounted.

Assessment of SMA before and after allowing the mineralization of biomass-associated LCFA: The SMA of the sludge before and after degrading the biomass-associated LCFA accumulated during the continuous load was compared using parallel assays. After washed and centrifuged (4000 rpm, 10 min) twice with anaerobic basal medium, the sludge was incubated in two sets of vials at 37 °C, 150 rpm under strict anaerobic conditions. In the first set the SMA against acetate, propionate, butyrate, ethanol and H_2/CO_2 were determined in duplicate as described before. In the other set of vials, no substrate was added but the degradation of the biomass-associated LCFA was followed until stabilization. After this stabilization the vials were depressurized, vented with N_2/CO_2 (80:20 vol/vol) and the SMA against acetate, propionate, butyrate, ethanol and H_2/CO_2 measured as described for the first set of vials.

The cumulative methane production obtained at the end of the blank control assays, was considered an indirect measurement of the amount of biomass-associated LCFA. The specific LCFA content

was then determined by dividing the maximum plateau achieved in the methane production curve by the VSS content of each vial at the end of the experiment and was expressed as mg COD/gVSS.

Toxicity and biodegradability tests. Toxicity tests were performed using the same pressure transducer technique previously described for the methanogenic activity measurements. Oleic and palmitic acids were the potential individual toxicants at concentrations in the range of 100 to 900 mg/l. Acetate (30 mM) was added, in order to evaluate the influence of each LCFA concentration on the aceticlastic activity. Due to the lower toxicity of palmitic acid towards the trophic group under study, concentrations up to 2500 mg/l were tested. In the biodegradability experiments, oleic or palmitic acids were the sole organic carbon source added to the vials at concentrations ranging from 100 to 900 mg/l.

Extraction and GC analysis of LCFA accumulated onto the sludge

After two consecutive washings and centrifugations (4000 rpm, 10 min) with anaerobic basal medium, an aliquot of sludge sample was dried at 105ºC, weighed and placed into separating funnels. A solution of internal standard (pentadecanoic-C15) was added to the sample, and, after acidification to pH 2, a multiple extraction with 5x1 ml of petroleum ether was applied. The ether phase was transferred to glass vials, immediately capped, and stored at –20ºC. LCFA (C10 to C18) concentration was determined by a gas chromatograph (CP-9001 Chrompack) equipped with a flame ionization detector (FID) and a split/splitless injector. LCFA were separated on a FFAP-CB 25m x 0,32mm x 0,3μm column (Chrompack), using nitrogen (N_2) as carrier gas at 35KPa, 31:1 split rate. Oven temperature was 40ºC for 2 min, with a 5ºC/min ramp to 250ºC, and a final hold at 250ºC for 15 min.

Results and Discussion

Prior to inoculation the suspended sludge was characterized in terms of specific methanogenic activity, oleic or palmitic acid toxicity towards the aceticlastic trophic group, as well as in terms of biodegradation capacity of the two individual LCFA. The sludge exhibited no detectable activity against propionate and a SMA against acetate, butyrate, ethanol and H_2/CO_2 of 146±15, 80±18, 72±4 and 581±33 mg COD-CH_4/gVSS, respectively. In terms of toxicity limit to the LCFA under study, a sixteen-fold higher IC_{50} value was found for palmitic (1100±50 mg/l), than for oleic acid (70±10 mg/l) revealing a higher tolerance of aceticlastic methanogens to this LCFA than to oleic acid. In terms of biodegradation capacity (Table 1), and for the range of concentrations studied, the sludge exhibited slightly higher biodegradation rates for oleic than for palmitic acid, but during oleic acid biodegradation, significantly higher lag phases preceding the maximum methane production were detected.

During the continuous operation, R2 exhibited a better performance in terms of removal efficiency and VFA and VSS effluent levels (Table 2). However, in R1 a methane production 25% higher than that achieved in R2 was obtained. Nevertheless, in both reactors a considerable low mineralization of the fed LCFA was achieved, with methane yields as low as 33 and 29 l CH_4/kg $COD_{removed}$, in R1 and R2 respectively, revealing a considerable accumulation of non-mineralized substrate. Acetate was the only VFA detected in the effluent of both reactors representing 39 and 29% of the total soluble effluent COD, in R1 and R2, respectively.

At the end of the continuous operation (day 75), the sludge fed with oleate (sludge 1) exhibited the typical whitish greasy aspect (Figure 1 (a)) referred in the literature as related to LCFA adsorption onto the sludge (Hwu, 1997; Pereira *et al.*, 2002). However, the sludge fed with palmitic acid (sludge 2) kept its original dark aspect, where whitish spots were eye perceptible (Figure 1 (b)), suggesting the accumulation of LCFA mainly as individualized precipitates rather than adsorbing onto the sludge.

Table 1 Oleic and palmitic acid biodegradation capacity exhibited by the seed sludge (Mean±SD).

Maximum biodegradation rate (mg COD-CH_4/(gVSS.day))			Lag-phase (hours)
Oleic acid concentrations:			
	100 mg/l	17±7	0
	300 mg/l	25±1	0
	500 mg/l	56±5	70±5
	700 mg/l	62±2	90±2
	900 mg/l	62±8	110±10
Palmitic acid concentrations:			
	100 mg/l	3±2	0
	300 mg/l	15±8	0
	500 mg/l	12±7	0
	700 mg/l	23±4	50±2
	900 mg/l	40±4	60±3

Table 2 Operating conditions and performance of R1 and R2 (Mean±SD).

Time (days)	HRT (±0.01) (days)	Influent COD (g/l)	COD Removal efficiency (%)	Effluent VFA-COD (g/l)	Effluent VSS (g/l)	CH_4 (%)	Biogas (l/(l.day))
R1							
0-28	2.7-1.18	3.9 (±0.3)	65.4-93.1	(*)	(*)	48.4-69.4	(*)-0.28
28-75	1.18	3.9 (±0.3)	80.3 [a] (±5.4)	0.32 [a] (±0.09)	0.88 [a] (±0.24)	54.9 [a] (±8.3)	0.16 [a] (±0.05)
R2							
0-28	2.7-1.14	3.7 (±0.3)	61.6-90.1	(*)	(*)	47.2-62.4	(*)-0.25
28-75	1.14	3.7 (±0.3)	92.8 [a] (±2.5)	0.08 [a] (±0.03)	0.76 [a] (±0.46)	51.1 [a] (±6.4)	0.13 [a] (±0.07)

(*) not determined, [a] pseudo steady-state values.

Therefore, sludge 1 appeared to be "encapsulated" by the accumulated LCFA and, thus, to have a higher degree of intimate association with the accumulated LCFA than sludge 2. Extraction and GC analysis of the accumulated matter present in both sludges revealed that palmitic acid (C16:0) was the main LCFA present, representing 83% of the total LCFA present in the sludge 1 and the totality of the LCFA present in the sludge 2. In terms of specific LCFA content, a value of 4570±257 and of 5200±9 mg COD/gVSS were present in sludge 1 and 2, respectively.

During the methanogenic activity measurements (Figure 2), sludge 2 exhibited a considerable initial methanogenic activity on all the tested substrates, with the single exception of butyrate. Also the blank control tests, where only the biomass-associated palmitic acid was present, exhibited a significant initial methane production rate. However, for the sludge 1 (encapsulated) only methane production from ethanol and H_2/CO_2 was detected, after a lag phase of about 50 hours. This sludge had a high amount of LCFA in intimate contact with the biomass, and thus, besides the potential toxic effect, it is reasonable to expect that the accumulated LCFA can create a physical barrier and hinder the transfer of substrates and products (e.g. biogas release), inducing a delay on initial methane production. This hypothesis is reinforced by the fact that H_2, the smallest substrate, was

rapidly transformed into methane. Methane production from ethanol was also observed, likely because it dissolved the accumulated LCFA, overcoming, to some extent, diffusion limitations. Sludge 2 had a higher specific LCFA content, but in a loosely association with the cells, and thus mass transfer limitations are not expected to occur.

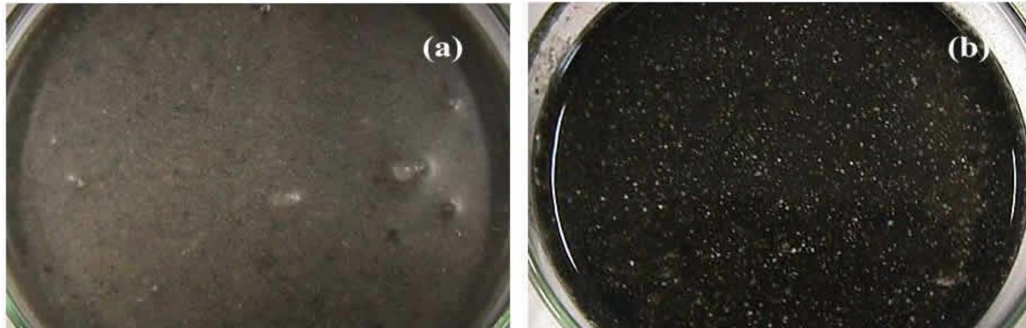

Figure 1 Sludge aspect after the continuous load with oleate (a) and palmitate (b).

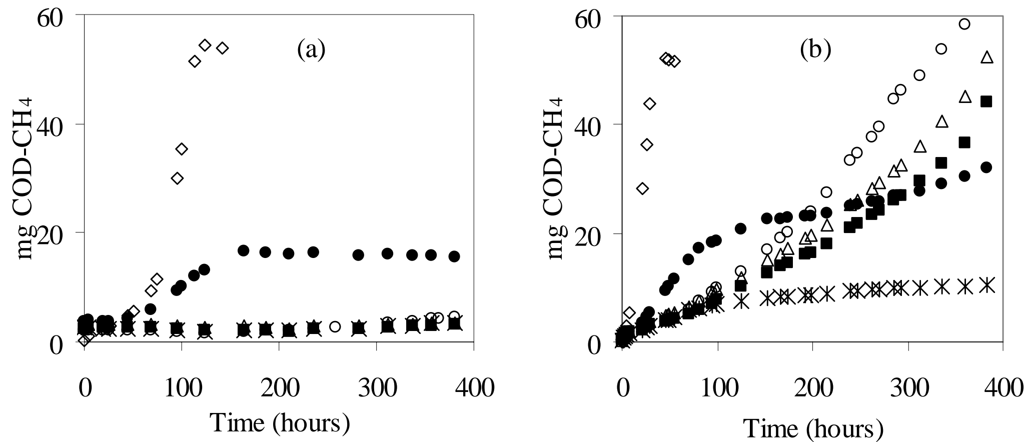

Figure 2 Cumulative methane production curves obtained for (a) sludge 1 and (b) sludge 2, during the methanogenic activity measurement with (△) acetate, (■) propionate, (x) butyrate, (●) ethanol and (◊) H_2/CO_2 as substrates, and (o) in the "blank" control (mineralization of the biomass-associated LCFA).

Notwithstanding the importance of transport limitations, a possible reversible metabolic inhibition should not be disregarded. In fact, in the sludge 2 the presence of acetate, propionate and butyrate induced a delay on the degradation of the biomass-associated LCFA ("blank" control). Since, as described in the Methods, the specific methanogenic activity values are, by definition, obtained after discounting the background methane production obtained in the blank controls (mineralization of the biomass-associated LCFA), this delay will, thus, result in null activity values against those substrates (Table 3).

After depletion of the biomass-associated LCFA, both sludges exhibited activities of similar order of magnitude and significantly higher than before (Table 3). One may therefore hypothesize that both sludges had also similar intrinsic activities before degrading the accumulated LCFA and that the different pattern on the methane production curves, before degrading the biomass-associated LCFA (Figure 2), resulted mostly from the different way/degree of LCFA accumulation onto the biomass. Since sludge 1 resulted from the continuous load with oleic acid, apparently more toxic than palmitic acid, the possibility of being submitted to higher toxic load, which may have induced a lower activity, could therefore be speculated. However, the transformation of oleic acid to

palmitic acid was found to be a fast and non-limiting step in oleic acid degradation (Pereira *et al*, 2002), and thus, palmitic acid was, in fact, the main LCFA that contacted for a long term with both sludge. Hence, the hypothesis that they may have a similar intrinsic activity, while LCFA-loaded, seems to be reasonable.

Table 3 Specific methanogenic activities exhibited by sludge 1 and sludge 2, before and after degrading the biomass-associated LCFA (Mean±SD).

Specific methanogenic activity (mg COD-CH_4/(gVSS.day))				
In the presence of:	**Sludge from R1**		**Sludge from R2**	
	Before	After	Before	After
Acetate	0	533±95	0	454±5
Propionate	0	16±4	0	41±3
Butyrate	0	224±71	0	110±9
Ethanol	24±6	67±1	155±8	138±11
H_2/CO_2	437±12	2709±38	2351±33	3224±182

CONCLUSIONS

The results obtained in this work add some insights to the role of physical transport limitations imposed by the LCFA in the anaerobic digestion process. Besides the potential toxic effect, LCFA accumulation onto the sludge can create a physical barrier and hinder the transfer of substrates and products (e.g. biogas release), inducing a delay on the initial methane production. By allowing the mineralization of the biomass-associated LCFA, the inhibitory/transport limitation effects are eliminated.

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