LCFA accumulation and biodegradation during anaerobic discontinuous treatment of an oleate-rich wastewater

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Abstract: The dynamics of medium and long-chain fatty acids (LCFA) accumulation and biodegradation was studied during the anaerobic treatment of an oleate-rich wastewater. This treatment was made in an upflow sludge bed reactor operated in cycles during 213 days. Five cycles were performed, each one with a feeding phase in continuous and a reaction phase in batch. Saturated and unsaturated fatty acids from C6 to C18 were extracted and analyzed by gas chromatography on biomass samples collected at different key moments of the reactor operation. These biomass samples were also incubated in batch assays and methane production from the accumulated substrate was followed. LCFA accumulated onto the sludge during the first two cycles, reaching a maximum value of 1.7 gCOD-LCFA.gVS^{-1}. Palmitate and stearate were the dominant intermediates quantified, approximately in equal quantities. On the subsequent cycles only residual amounts of LCFA were detected. Methane production on batch assays was higher than expected from the LCFA accumulated, suggesting that other substrates could also be entrapped with the sludge. The results show that during the first two cycles a specialized microbial consortium developed, able to treat oleate-rich wastewaters.

Keywords: Anaerobic treatment; discontinuous operation; long-chain fatty acids; oleic acid; palmitic acid; stearic acid.

INTRODUCTION

Anaerobic treatment of complex lipid-rich wastewaters is frequently impaired by several operational problems, such as inhibition and sludge flotation and washout. These problems result mainly from the accumulation of long-chain fatty acids (LCFA) on the microbial aggregates by mechanisms of adsorption, precipitation and entrapment (Hwu, 1997; Tagawa, 2002; Pereira *et al.*, 2005; Jeganathan *et al.*, 2006). LCFA are produced by lipids hydrolysis, which is a fast process under methanogenic conditions (Hanaki *et al.*, 1981). 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). However, LCFA can be efficiently biodegraded under some defined conditions (Pereira *et al.*, 2004), through β–oxidation pathway, after transport into the bacterial cell (Weng and Jeris, 1976).

Pereira *et al.* (2002) reported that when oleic acid was continuously fed to an upflow sludge bed 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 suggests that sequencing accumulation and degradation steps would be a strategic procedure to achieve an efficient rate of methane production from LCFA-rich wastewaters.

Until now, detection and quantification of LCFA degradation intermediates were only performed on batch assays (Lalman and Bagley, 2001; Alosta *et al.*, 2004; Salvador, 2007). However, the study of the dynamics of LCFA accumulation and biodegradation during the operation of bioreactors can bring

new insights on the process of LCFA anaerobic treatment and contribute to the establishment of the more suitable conditions for an efficient conversion of this compound to methane.

The aim of this work was to study the dynamics of LCFA accumulation and biodegradation during the discontinuous anaerobic treatment of an oleate-rich wastewater.

METHODS

Biomass samples

Nine biomass samples were collected at different key moments of the operation of a mesophilic upflow sludge bed reactor operated in cycles during 213 days, as described elsewhere (Cavaleiro *et al.*, 2007). Five cycles were performed, each one with a feeding phase in continuous and a reaction phase in batch. In the first three cycles an organic loading rate of 4.4 ± 0.6 kg COD.m^{-3}.day^{-1} was applied during 17 days (feeding phase). An increase in the feeding time was introduced in the fourth cycle (28 days), although applying the same organic loading rate, and in the fifth cycle the organic loading rate was increased (8.2 kg COD.m^{-3}.day^{-1}) during a similar feeding time (22 days). The reactor was fed with a mixture of skim milk (50% COD) and commercial sodium oleate (50% COD). Biomass sampling times are presented in Table 1.

Table 1. Biomass sampling times during the reactor operation.

Sample number	Sampling time (days)	Key moment of the operation
1	0	Beginning of the feeding phase of the 1st cycle (F1)
2	16	Beginning of the reaction phase of the 1st cycle (R1)
3	44	Beginning of the feeding phase of the 2nd cycle (F2)
4	62	Beginning of the reaction phase of the 2nd cycle (R2)
5	100	Beginning of the feeding phase of the 3rd cycle (F3)
6	118	Beginning of the reaction phase of the 3rd cycle (R3)
7	139	Beginning of the feeding phase of the 4th cycle (F4)
8	182	Beginning of the feeding phase of the 5th cycle (F5)
9	213	End of the operation

Quantification of the biomass-associated medium and long-chain fatty acids

Biomass-associated saturated and unsaturated medium and long-chain fatty acids from C6 to C18 were extracted and quantified according to the method described by Neves *et al.* (2007). Free fatty acids present in the samples were esterified with propanol in acid medium at high temperature (100 ºC) for 3.5 hours, and extracted with dichloromethane. Quantification was made by gas chromatography (CP-9001 Chrompack) with a flame ionization detector. Fatty acids were separated on a eq.CP-Sil 52 CB 30 m x 0.32 mm x 0.25 µm column (Teknokroma, Tr-wax), using helium (He) as carrier gas at 1.0 mL.min^{-1}. Oven temperature was 50ºC for 2 min, with a 10ºC.min^{-1} ramp to 225ºC and with a final isothermal for 10 minutes. Detector and injector temperatures were 250 ºC and 220 ºC, respectively. Free LCFA composition of the feed was also analyzed in the same way.

Batch assays
Biomass samples were incubated in sealed batch vials at 37 ºC, 150 rpm, under strict anaerobic conditions, without any added organic carbon source, calcium, or nutrients. Only anaerobic basal medium was added, as described elsewhere (Pereira *et al.*, 2003), and methane production was followed. Methane content of the biogas was measured by a Pye Unicam GC-TCD gas chromatograph (Cambridge, England), using a Chrompack column Haysep Q (80-100 mesh). Helium was used as carrier gas (30 mL.min^{-1}) and the temperature of the injection port, column and detector was 110, 35 and 110 ºC, respectively. Methane production values were corrected for standard temperature and pressure conditions (STP). The cumulative methane production at the end of the assay was considered an indirect measurement of the amount of accumulated substrate. The specific substrate content was then determined by dividing the maximum plateau achieved in the methane production curve by the volatile solids (VS) content of each vial at the end of the experiment (g COD.gVS^{-1}). Specific methane production rate was obtained by dividing the slope of the methane production curve by the VS content of each vial at the end of the experiment (g COD.gVS^{-1}.day^{-1}). All batch tests were performed in triplicate.

RESULTS AND DISCUSSION

Analysis of the free LCFA present in the mixture of skim milk (50% COD) and commercial sodium oleate (50% COD) fed to the reactor showed that oleate (C18:1) was the most abundant LCFA, constituting 73-76% of total LCFA. Palmitate (C16:0) and stearate (C18:0) represented 10% and 4-6% of total LCFA, respectively, and laureate (C12:0), myristate (C14:0) and palmitoleate (C16:1) were also detected in lower quantities.

Saturated and unsaturated medium and long-chain fatty acids from C6 to C18 were quantified in the nine biomass samples collected at different key moments of the reactor operation and the results obtained are presented in Figure 1.
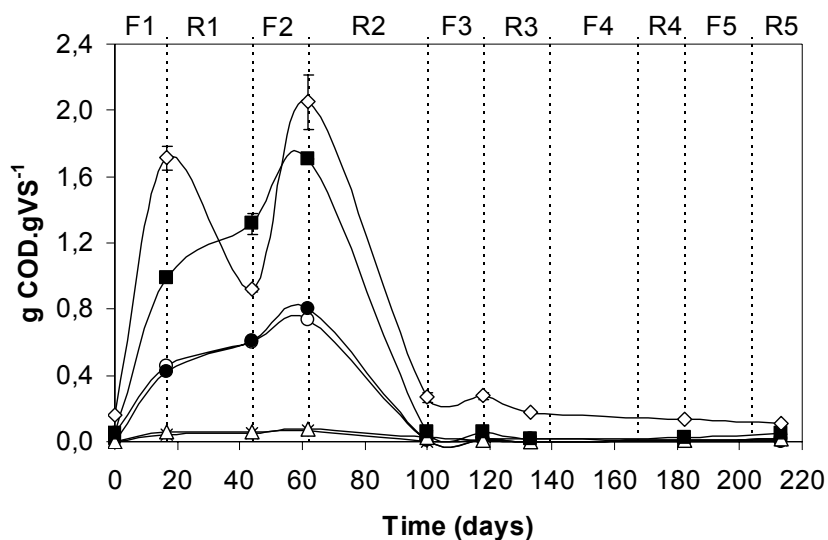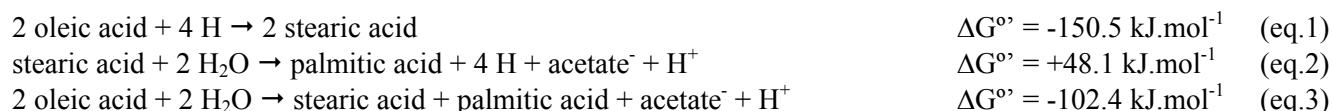

Figure 1. Time course of total LCFA (■), oleate (Δ), stearate (●), palmitate (o) and myristate (✶), as well as the maximum specific cumulative methane production determined in the batch assays (◊), at the beginning of each feed (F) and reaction (R) phase.

LCFA accumulated onto the sludge during the first cycle and in the feeding phase of the second cycle, reaching a maximum value of 1.7 g COD-LCFA.gVS^{-1}. This value is significantly higher than the one suggested by Pereira *et al.* (2004) as the value that would lead to the maximal mineralization rate (1 g COD-LCFA.gVS^{-1}). However, degradation of accumulated LCFA was observed in highly loaded sludge, with substrate accumulated in amounts as high as 6 g COD-LCFA.gVS^{-1} (Salvador, 2007). In the reaction phase of the second cycle biodegradation of accumulated LCFA proceeded efficiently and in the third, fourth and fifth cycles almost no LCFA accumulation occurred (values lower than 0.06 g COD-LCFA.gVS^{-1}).

Palmitate and stearate were the dominant intermediates quantified, approximately in equal quantities and representing around 45-50% of total LCFA each. C6:0, C8:0 and C10:0 were never detected in all the samples analyzed, but C12:0 and C16:1 were found in residual quantities (lower than 0.02 g COD-LCFA.gVS^{-1}) in two of the samples. Other authors have reported palmitic acid as the main product detected from oleic acid degradation (Lalman and Bagley, 2001; Salminen *et al.*, 2001; Pereira *et al.*, 2002). Since β-oxidation reactions occur within the cells, the detection of intermediates may indicate a saturation of the organism's capacity to process the intermediates (Lalman and Bagley, 2001) or a preference for one substrate instead of another. According to Lalman and Bagley (2001), the production of stearic and palmitic acid from oleic acid is energetically favorable ($\Delta G^{0'}$ = -8.6 and -27.8 kJ.mol^{-1}, respectively). However, the production of palmitic acid from stearic acid is not ($\Delta G^{0'}$ = +50.8 kJ.mol^{-1}). If stearic acid is formed from oleic acid reduction, it will be expected to transiently accumulate until environmental conditions change sufficiently to allow its degradation. However, Broughton *et al.* (1998) stated that a fermentation of oleate to palmitate analogous to crotonate fermentation by syntrophic acetogenic bacteria and *Clostridium kluyveri* may explain the formation of stearic and palmitic acids, as shown by the following equations, where reaction 3 is highly exergonic under standard conditions:

2 oleic acid + 4 H → 2 stearic acid	$\Delta G^{o'}$ = -150.5 kJ.mol^{-1}	(eq.1)
stearic acid + 2 H$_2$O → palmitic acid + 4 H + acetate$^-$ + H$^+$	$\Delta G^{o'}$ = +48.1 kJ.mol^{-1}	(eq.2)
2 oleic acid + 2 H$_2$O → stearic acid + palmitic acid + acetate$^-$ + H$^+$	$\Delta G^{o'}$ = -102.4 kJ.mol^{-1}	(eq.3)

When the biomass samples were incubated in batch vials without any added carbon source, calcium or nutrients, methane was produced due to the degradation of the substrate accumulated onto the sludge during the reactor operation. The values of maximum cumulative methane production for each test, considered as an indirect measurement of the amount of accumulated substrate, are also represented in Figure 1. For all the samples studied, except the sample collected in the beginning of the 2nd cycle, methane production on batch assays was higher than expected from the LCFA accumulated, suggesting that other substrates (proteins, sugars or other intermediates) could also be entrapped with the sludge.

Figure 2 presents the specific cumulative methane production in the batch assays performed with all the biomass samples except the inoculum of the reactor (sample 1). The higher plateaux were achieved in the tests with the biomass samples collected during the first and second cycles, corresponding to 2.0, 1.7 and 0.9 gCOD.gVS^{-1} for the samples 4, 2 and 3, respectively. Also in these samples the onset of methane production was preceded by 8-15 days lag phases, probably due to the higher amounts of accumulated substrate present, which can be responsible for mass transfer limitations, inhibition or a combination of both phenomena. The plateaux achieved with the samples from the 3rd cycle until the

end of the operation were lower than 0.3 gCOD.gVS^{-1}, showing relatively low substrate accumulation onto the sludge.
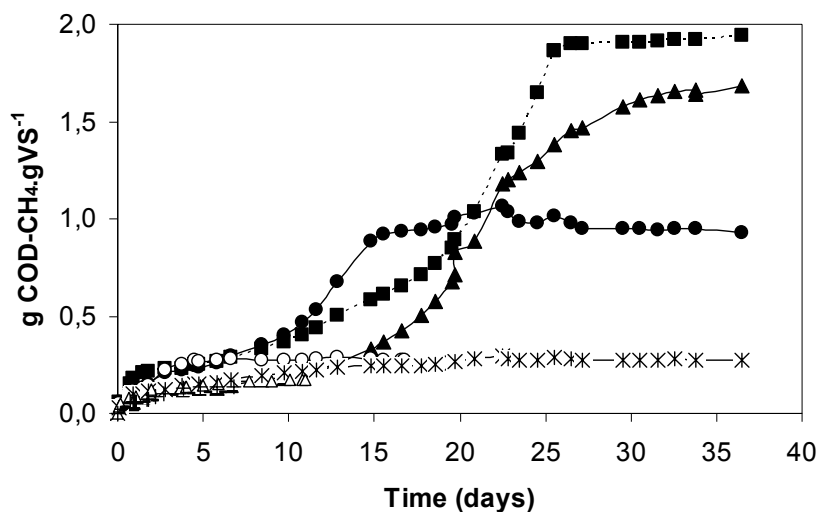

Figure 2. Specific cumulative methane production in the batch assays with the biomass samples collected during the operation of the reactor: sample 2 (▲), sample 3 (•), sample 4 (■), sample 5 (o), sample 6 (✱), sample 7 (Δ), sample 8 (+) and sample 9 (-).

Specific methane production rate was determined for the biomass samples collected during the first two cycles. Similar values were observed for samples 2 and 3 (0.119 and 0.098 gCOD.gVS^{-1}.day^{-1}, respectively), but a slightly higher value was obtained in the assay with sample 4 (0.161 gCOD.gVS^{-1}.day^{-1}), which can be indicative of biomass adaptation.

CONCLUSIONS
LCFA accumulated onto the sludge during the first two cycles of the reactor operation, with palmitate and stearate as the dominant intermediates quantified. On the subsequent cycles only residual amounts of LCFA were detected. Methane production from the accumulated substrate on batch assays was higher than expected from the LCFA accumulated, suggesting that other substrates could also be entrapped with the sludge. The results presented show that during the first two cycles a specialized microbial consortium developed, able to biodegrade oleate-rich wastewaters without LCFA accumulation in the process.

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