Anaerobic degradation of oleic acid by suspended and granular sludge: identification of palmitic acid as a key intermediate

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Abstract The aim of the present work was to study the maximum potential methane production in batch assays of sludge samples taken along the operation of two EGSB reactors (RI inoculated with granular sludge and RII inoculated with suspended sludge) fed with increasing oleic acid concentrations between 2 and 8 gCOD/l (HRT = 1 day). After removing the residual substrate, the sludge was incubated in batch vials without any added carbon source. A maximum methane production rate of 152 ± 21 $mlCH_{4(STP)}$/gVS.day was obtained for the suspended sludge taken on day 70, when oleate at a concentration of 2 g COD/l was fed with a co-substrate (50% COD). The maximum plateau achieved in the methane production curve was 1145 ± 307 $mlCH_{4(STP)}$/gVS, obtained for the suspended sludge taken on day 162, when oleate was fed as the sole carbon source at 6 g COD/l. The methanization rate of the adsorbed substrate was enhanced under stirring conditions and was inhibited by adding oleic acid. Extraction and GC analysis confirmed that the main adsorbed substrate was palmitate, and not oleate. Accumulated palmitate adsorbed onto the sludge and further β-oxidation was inhibited when in the presence of oleic acid. If oleic acid was removed from the medium β-oxidation proceeded with methane production. Suspended sludge was more efficient than granular sludge.
Keywords EGSB; granular sludge; LCFA; oleic acid; palmitic acid; suspended sludge

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Introduction

Lipids are one of the major components of organic matter in wastewater. Along with slaughterhouses and edible oil and fat refineries, dairy product industries are important contributors for the total lipid emission (Rinzema, 1988). Lipids are easily hydrolysed to Long Chain Fatty Acids (LCFA), which are further converted to acetate and hydrogen through β-oxidation mechanism by the proton reducing acetogenic bacteria (Weng and Jeris, 1976). LCFA are especially problematic compounds for anaerobic wastewater treatment. Tentative application of granule-based digesters to lipid-containing wastewaters revealed that, although granular sludge was more resistant to LCFA toxicity than suspended or flocculent sludge, physical stability of granules is critical for lipid containing wastewaters. This problem arises because these compounds adsorb onto the biomass and, besides the acute toxic effect, induce granular sludge flotation, which occurs for concentrations far below the toxicity limit (Hwu *et al*., 1998). Results from batch assays suggested that LCFA exert a bactericidal effect on methanogenic bacteria and no adaptation was observed. The recovery after a lag phase usually observed in batch assays was attributed to the growth of few survivors (Rinzema, 1998). However, results from continuous experiments with a gradual replacement of a co-substrate by oleic acid ($C_{18:1}$), revealed that acetoclastic bacteria increased the tolerance to oleic acid toxicity (Alves *et al*., 2001). Moreover, a digester inoculated with an acclimated sludge exhibited higher methane yields than a digester inoculated with a non-acclimated sludge (Pereira *et al*., 2001).

In a continuous digester fed with oleate at concentrations as high as 12 gCOD/L anaerobic sludge became encapsulated by a whitish matter, which was *a priori* supposed to be oleic acid. When this encapsulated sludge was incubated in batch vials, after two consecutive washings for removing the residual substrate, methane was produced at a rate of 99 ml $CH_{4(STP)}$/gVSS.day, achieving a plateau of 736 ± 20 ml $CH_{4(STP)}$/gVSS, without any added carbon source. This methane production rate seemed to be delayed when oleic acid was added to the vials (Alves *et al*., 2001). The aim of the present work was to characterize the potential maximum methane production from the adsorbed substrate exhibited by the sludge of two EGSB reactors fed with increasing loads of oleic acid. Behaviour of granular and suspended sludge was compared. Sodium oleate was used as a LCFA model since it is, in general, the most abundant of all LCFA present in wastewater (Komatsu *et al*., 1991) as well as one of the more toxic (Galbraith *et al*., 1971).

Methods

Experimental set-up and operation mode

Two 10 l EGSB reactors (RI and RII) were operated in parallel with increasing oleate concentrations between 2 and 8 g COD/l. Hydraulic retention time was set at 1 day. RI was inoculated with granular sludge and had an internal settler. RII was inoculated with suspended sludge and was equipped with an external settler. The amount of adsorbed substrate and the corresponding potential maximum degradation rate were evaluated for a range of oleate loading rate between 2 and 8 kg COD/m^3.day. In both digesters, a significant amount of sludge accumulated in a floating layer.

Batch experiments

For each applied oleate loading rate, samples from the bottom and top layers were collected, washed and centrifuged twice with anaerobic basal medium and incubated in batch vials of 25 ml at 37°C, 150 rpm under strict anaerobic conditions, without any added substrate. The methane production was followed by measuring the pressure developed in each vial, using a hand-held pressure transducer capable of measuring a pressure variation of two bar (0 to ± 202.6 kPa) over an output range of –200 to +200 mV (Colleran *et al*., 1992). The basal medium used in all the batch experiments, made up with demineralised 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. No calcium or trace-nutrients were added. The initial maximum methane production rate and the maximum plateau achieved were determined for each vial. Values were corrected for Standard Temperature and Pressure (STP) conditions. Methanogenic activity, toxicity and biodegradability tests were also performed using this technique. All the batch experiments were performed in triplicate assays.

Substrate

In the first 70 days the substrate was made of skim milk (50% COD) and oleic acid (50% COD). From day 70, the carbon source was exclusively oleic acid. Macro- and micro-nutrients were added according to the composition described elsewhere (Alves *et al*., 2001).

Seed sludge

The granular sludge was obtained from an UASB treating a brewery effluent located in Oporto, Portugal. Suspended sludge was collected from a laboratory digester treating an oleic acid synthetic effluent. Before inoculation this sludge was incubated in batch during about 15 days, in order to degrade the adsorbed substrate. 1.6 l of granular sludge (20.2 g VSS/l) and 2 l of suspended sludge (18.0 g VSS/l) were added to the digesters RI and RII, respectively. Both inocula were characterized in terms of specific methanogenic activity, oleic acid toxicity towards acetoclastic bacteria and oleic acid biodegradability. Table 1 summarizes the corresponding results.

Granular sludge exhibited activities significantly higher than suspended sludge for acetoclastic, hydrogenophilic and syntrophic propionate and ethanol degrading bacteria. Only methanogenic activity with butyrate as substrate was non-detectable in this sludge whereas a value of 52 ml $CH_{4(STP)}$/gVSS.day was detected in the suspended sludge. The toxicity limit of oleic acid towards acetoclastic bacteria was higher for the granular than for the suspended sludge, which agrees with the work of Hwu *et al.* (1996). The biodegradation rate was slightly, but not significantly higher in the granular than in the suspended sludge, for the range of concentrations studied.

Extraction and GC analysis of LCFA adsorbed onto the sludge

Samples from the reactors were collected washed and centrifuged (4,000 rpm, 10 min) twice with the anaerobic basal medium. An aliquot of each sample was dried at 105°C, weighted and placed into separating funnels. A solution of internal standards (C7 and C15) was added to the sample, and, after acidification to pH 2, a multiple extraction with 5 × 1 ml of petroleum ether was applied. The ether phase was transferred to glass vials, immediately capped, and stored at –4°C. LCFAs concentration was determined by a gas chromatograph (CP-9001 Chrompack) equipped with a flame ionization detector (FID) and a split/splitless injector. LCFAs were separated on a FFAP-CB 25m × 0.32mm × 0.3 μm column (Chrompack), using nitrogen (N_2) as carrier gas at 35 KPa, 30:1 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

Table 2 summarises the operating conditions and performance of RI and RII. During the first 70 days skim milk was introduced as a co-substrate, representing 50% of the total COD fed. For the first two operating conditions, RI exhibited higher removal efficiencies and higher methane production than RII, but in the last two operation periods, higher methane productions and lower VSS levels were obtained in RI. From day 70 onwards, oleate was the sole carbon source fed to both digesters, and the methane production decreased to 20–30% of the initial value. Along the trial period the methane yield decreased from 289 and 242 lCH_4/kg $COD_{removed}$ to 27 and 39 l CH_4/kg $COD_{removed}$ in RI and RII, respectively. Figure 1 represents the results from the batch experiments for the sludge taken from RI and

Table 1 Methanogenic activity, oleic acid toxicity and biodegradability for the granular and suspended seed sludge (± 95% confidence interval)

		Granular	Suspended
Methanogenic activity in presence of:		(ml$CH_{4(STP)}$/gVSS·day)	
	Acetate	327 ± 11	107 ± 6
	Propionate	160 ± 10	48 ± 14
	Butyrate	(n.d.)	52 ± 3
	Ethanol	514 ± 94	106 ± 2
	H_2/CO_2	597 ± 16	487 ± 31
Oleic acid toxicity limit (IC_{50}) (mg/l)		345 ± 26	133 ± 16
Biodegradability for oleate concentrations:		(ml$CH_{4(STP)}$/gVSS·day)	
	100 mg/l	(n.d.)	6 ± 1
	300 mg/l	14 ± 1	7 ± 2
	500 mg/l	10 ± 1	9 ± 1
	700 mg/l	8 ± 1	8 ± 2
	900 mg/l	10 ± 2	7 ± 2

STP – Standard temperature and pressure conditions; n.d. – non-detectable

Table 2 Operating conditions and performance of RI and RII (± 95% confidence intervals)

Period	Time (days)	HRT (± 0.01) (days)	Influent COD (g/l)	Influent oleate COD (g/l)	COD removal efficiency (%)		Effluent VSS (g/l)		Methane production (lCH_4/l.day)	
					RI	RII	RI	RII	RI	RII
I	0–70	1.01	3.8 (± 0.3)	1.9 (± 0.2)	96.5 (± 0.6)	85.8 (± 3.2)	0.38 (± 0.07)	0.65 (± 0.04)	1.06 (± 0.1)	0.79 (± 0.1)
II	70–119	1.01	3.8 (± 0.3)	3.8 (± 0.3)	83.4 (± 4.8)	74.4 (± 5.5)	0.85 (± 0.22)	0.72 (± 0.15)	0.23 (± 0.05)	0.26 (± 0.05)
III	119–162	1.01	6.2 (± 0.7)	6.2 (± 0.7)	74.2 (± 3.8)	74.6 (± 2.9)	1.96 (± 0.43)	1.57 (± 0.17)	0.16 (± 0.02)	0.20 (± 0.08)
IV	162–219	1.01	8.2 (± 0.5)	8.2 (± 0.5)	68.8 (± 3.4)	69.4 (± 5.5)	2.71 (± 0.57)	2.50 (± 0.58)	0.15 (± 0.02)	0.22 (± 0.06)

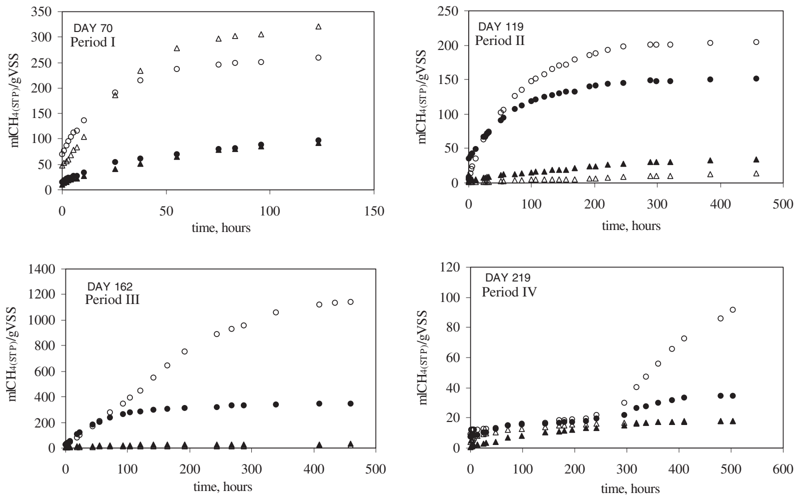

Figure 1 Specific methane production in the batch assays. (●) RI top, (○) RII top, (▲) RI bottom, (△) RII bottom

RII in the bottom and top layers at the end of operating periods I, II, III, and IV. Table 3 summarises the results obtained.

On day 162, when oleate was fed at 6 kg COD/m^3.day, the highest plateau in the methane production curve was obtained for the RII-top sludge (1145 ± 307 ml $CH_{4(STP)}$/gVSS) which was more than threefold that obtained in the RI-top sludge. For the bottom sludges, no significant differences were obtained (Table 3).

For the oleate organic load of 8 kg COD/m^3.day (period IV), a clear inhibition of the adsorbed substrate degradation was observed. A lag-phase of 300 hours preceded the initial methane production and a clear decrease in the methane production rates and plateaux were observed. When comparing the methane production rates in continuous operation and in batch mode for the oleate loading rate of 6 kg COD/m^3.d, in RII, it is concluded that more methane would be produced per day if the feed was suppressed, provided that optimal conditions were assured. In fact, considering the amount of VSS present in the RII top layer (30 g), 2.6 l CH_4 would be produced per day, whereas in continuous mode only 2 l CH_4 were produced in the whole reactor (Table 2). This suggests adsorbed matter can be degraded efficiently, if no oleate is present. This was confirmed in batch assays (Figure 2a). When oleate was added to a sample of washed-encapsulated sludge, the methane production rate was significantly lower than that exhibited by the sludge without any added substrate. A decrease from 41 ml $CH_{4(STP)}$/gVSS.day to 8.1 ml $CH_{4(STP)}$/gVSS.day, was detected when

Table 3 Maximum plateaux (ml $CH_{4(STP)}$/gVSS) and methane production rate (ml $CH_{4(STP)}$/gVSS.day) obtained in the batch experiments (± 95% confidence intervals)

		RI top	RII top	RI bottom	RII bottom
DAY 70	Maximum "plateau"	97 ± 13	260 ± 28	92 ± 6	321 ± 42
Period I	Methane production rate	40 ± 4	152 ± 21	35 ± 6	133 ± 17
DAY 119	Maximum "plateau"	152 ± 6	204 ± 41	34 ± 12	14 ± 2
Period II	Methane production rate	30 ± 3	50 ± 7	4 ± 1	1 ± 1
DAY 162	Maximum "plateau"	349 ± 38	1145 ± 307	34 ± 2	19 ± 3
Period III	Methane production rate	70 ± 2	85 ± 3	5 ± 1	3 ± 1
DAY 219	Maximum "plateau"	35 ± 4	111 ± 24	18 ± 1	18 ± 3
Period IV	Methane production rate	4 ± 1	7 ± 1	3 ± 1	2 ± 1

100 mg/l oleic acid were added. This suggests that adsorbed matter is no longer oleic acid, but probably an intermediate of its degradation, such as stearate or palmitate (Weng and Jeris, 1976).

Experiments made under static and stirring conditions led to the conclusion that methane production rate is significantly enhanced by stirring as can be observed in Figure 2b. A lag phase of about 300 hours was observed preceding the initial methane production under static conditions. As the substrate is already in intimate contact with the biomass, substrate diffusion limitations are not expected to limit the degradation rate. However, product diffusion limitation, e.g. biogas release was observed to be difficult under static conditions, which may justify the different methane production patterns.

Figure 3 represents a phase contrast microphotograph from the encapsulated sludge where a very clear whitish zone is detected. The extraction and GC analysis of this adsorbed matter confirmed the suspicion that it was not composed mainly of oleic acid. Only traces of this LCFA were detected and palmitate was the main compound detected (Figure 4).

This is evidence that accumulated palmitic acid adsorbed onto the sludge and further β-oxidation was inhibited in the presence of oleic acid. In batch assays, when oleic acid was removed from the medium, β-oxidation proceeded with methane production. The maximum accumulated palmitate and the rate of methane production were highly dependent on biomass structure, suspended sludge being more efficient than granular sludge. From these results it can be concluded that for treating effluents with high lipid content, it should be advantageous to sequence cycles of adsorption and degradation in order to enhance methane production.

Conclusions

Operation of two EGSB reactors (RI inoculated with granular sludge and RII inoculated with suspended sludge) fed with oleic acid as the sole carbon source revealed that methane production decreased to 20–30% of the value exhibited when a co-substrate was fed as 50%

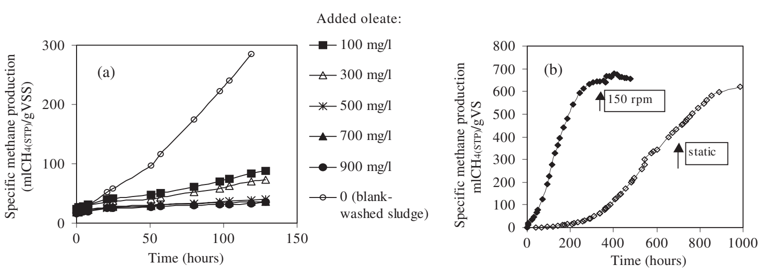

Figure 2 Effect of adding oleate; (a) effect of stirring and (b) in specific methane production in batch assays.

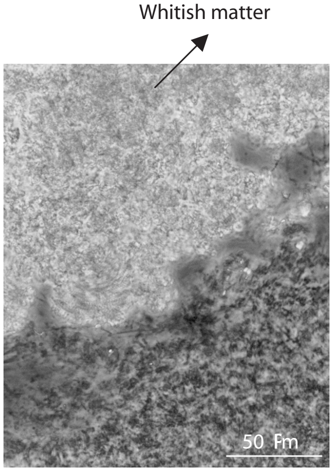

Figure 3 Microscopic examination of encapsulated sludge

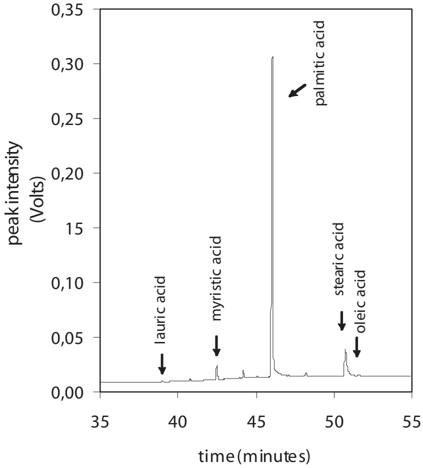

Figure 4 Example of a chromatogram obtained by GC analysis after extraction of the adsorbed matter

COD. Methane yields as low as 25 lCH_4/kg COD removed, were obtained. Maximum rate of methane production due to degradation of adsorbed substrate in batch vials was 152 ± 21 ml$CH_{4(STP)}$/gVS.day obtained for the suspended sludge taken on day 70, when oleate at a concentration of 2 g COD/l was fed with a co-substrate (50% COD). The maximum plateau achieved in the methane production curve was 1145 ± 307 ml$CH_{4(STP)}$/gVS, obtained for the RII-suspended sludge taken on day 162, when oleate was fed as the sole carbon source at 6 g COD/l.

The degradation of the adsorbed substrate was inhibited by adding oleic acid and was enhanced by stirring conditions. Extractions and gas cromatography (GC) analysis of the adsorbed matter revealed that it was mainly composed of palmitic acid. Accumulated palmitic acid adsorbed onto the sludge and further β-oxidation was inhibited in the presence of oleic acid. When oleic acid was removed from the medium (batch experiments), β-oxidation proceeded with methane production. When treating effluents with high lipid content, it should be advantageous to run sequencing cycles of adsorption and degradation in order to enhance methane production. Provided that it can be retained in the reactor, suspended sludge is advantageous over granular sludge due to its higher capacity of LCFA adsorption and degradation.

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