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ANAEROBIC DEGRADATION AND INHIBITORY EFFECTS OF LINOLEIC ACID

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Abstract—The anaerobic degradability and inhibitory effects of linoleic acid, an 18 carbon acid with two double bonds (C18:2) commonly found in vegetable oils, were examined at 21°C. Linoleic acid was anaerobically degraded to unsaturated and saturated products. Unsaturated oleic (C18:1) and palmitoleic (C16:1) acids were transiently formed while saturated palmitic (C16:0) and myristic (C14:0) acids were stoichiometrically produced and may have inhibited their own subsequent degradation. Ultimately, all detected byproducts, including lauric (C12:0) and hexanoic (C6:0) acids, were degraded with the exception of acetic acid which accumulated. Linoleic acid at concentrations of 30 mg l^{-1} or more completely inhibited aceticlastic methanogenesis. Although hydrogenotrophic methanogenesis was slightly inhibited by 30 mg l^{-1} of linoleic acid or more, hydrogen consumption remained robust. Anaerobic systems to treat wastewaters with linoleic acid-containing vegetable oils may require two separate stages to minimize the inhibition of aceticlastic methanogens by linoleic acid.

Key words—anaerobic, linoleic (C18:2) acid, biodegradation, inhibition, acetate, hydrogen

INTRODUCTION

Wastewaters from many food-processing facilities may contain emulsified and dissolved vegetable oils (glycerol triesters). Under anaerobic conditions, the vegetable oils hydrolyze to long-chain fatty acids (LCFAs) and glycerol (Hanaki *et al.*, 1981). The glycerol then degrades to 1,3-propanediol (Qatibi *et al.*, 1991) and subsequently to acetate (Dubourguier *et al.*, 1986). In contrast, while slowly degradable under anaerobic conditions, the LCFAs may also inhibit anaerobic microbial activity (Koster and Cramer, 1987; Hanaki *et al.*, 1981). The design of anaerobic systems to treat wastewaters containing vegetable oils must account for both the slow degradation of and potential inhibition by LCFAs.

Linoleic acid (an 18 carbon acid with two double bonds, designated as C18:2), oleic acid (C18:1) and stearic acid (C18:0) are among the most common long chain fatty acid (LCFAs) present in vegetable oils. For example, safflower oil contains between 73 and 79% linoleic acid, 13–21% oleic acid and 1–4% stearic acid (all percentages by weight) while peanut oil contains 13–27% linoleic acid, 53–71% oleic acid and 3–6% stearic acid (Sonntag, 1979). Because of the differing amounts of saturation, these C18 LCFAs may be expected to degrade at different rates and provide different levels of inhibition. Therefore, the response of anaerobic systems for each LCFA must be determined.

The β-oxidation half-reaction for an LCFA with $n + 2$ carbons under anaerobic conditions is indicated in equation (1) (Weng and Jeris, 1976; Jeris and McCarty, 1965). For each LCFA molecule degraded, one carboxylic acid molecule containing two fewer carbons, one molecule of acetic acid and four electrons are produced.

$$CH_3(CH_2)_nCOOH + 2H_2O \rightarrow CH_3(CH_2)_{n-2}COOH + CH_3COOH + 4e^- + 4H^+ \quad (1)$$

The electrons (carried in the cell as FADH and NADH) must be deposited on an appropriate electron acceptor to allow the β-oxidation half-reaction to continue to completion. One potential electron acceptor is H^+, forming H_2 (equation (2)).

$$4e^- + 4H^+ \rightarrow 2H_2 \quad (2)$$

The hydrogen formed is then available to be consumed by hydrogenotrophic methanogenic organisms to form methane, provided that these organisms are not inhibited by the presence of LCFAs. In the absence of other electron acceptors

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(such as sulfate), the acetic acid formed in equation (1) would be consumed by aceticlastic methanogenic organisms, again providing that the organisms are not inhibited by the LCFAs.

The β-oxidation mechanism sequentially converts saturated C18 LCFAs to palmitic (C16:0), myristic (C14:0), lauric (C12:0), capric (C10:0) acids, etc., until complete conversion to acetic acid is achieved. In practice, palmitic and myristic acids have been often observed (Novak and Carlson, 1970). Trace amounts of lauric, capric, caprylic (C8:0) and butyric (C4:0) acids have also been detected as byproducts from the degradation of a mixture of linoleic, oleic, stearic, palmitic and myristic acids (Novak and Carlson, 1970), but not hexanoic (C6:0) acid. However, caprylic, hexanoic and butyric acids were detected in degradation studies conducted with capric acid by Rinzema *et al.* (1994). The inability to detect β-oxidation intermediates in the bulk solution is not unexpected, however, as the reactions occur in the cell. To the contrary, the detection of intermediates may indicate either a saturation of the organisms' capability to process the intermediates or the requirement of different organisms to degrade different intermediates.

Although degradable, LCFAs are inhibitory to anaerobic systems. Hydrogen consumption was inhibited in systems at 37°C receiving whole milk that contained a mixture of LCFAs including linoleic, oleic, and stearic acids as well as both saturated and unsaturated C16, C14, C12, and C10 acids (Hanaki *et al.*, 1981). Koster and Cramer (1987) showed that aceticlastic methanogens were inhibited by between 300 and 900 mg l^{-1} of oleic, myristic, lauric, capric and caprylic acids at 30°C. Later studies reported that between 100 and 200 mg l^{-1} oleic and 500 mg l^{-1} stearic acid inhibited the degradation of acetate, propionate and butyrate added to anaerobic systems at 55°C (Angelidaki and Ahring, 1992).

Although LCFAs in general have been studied in anaerobic systems, linoleic acid has been studied either only in mixtures or not at all, even though it is a major constituent of many vegetable oils. Additionally, the responses of anaerobic systems to LCFAs, in particular linoleic acid, at temperatures lower than 30°C have not been examined. Understanding LCFA degradation and inhibition in anaerobic systems operating at temperatures lower than 30°C may be especially important for reactors operating in cold climates. Finally, although the inhibition of aceticlastic methanogenic activity by LCFAs in general has been studied, little is known about the impact of LCFAs, especially linoleic acid, on hydrogenotrophic methanogenesis. Inhibition of hydrogen consumption may indirectly inhibit the degradation of linoleic acid and other LCFAs as per equations (1) and (2).

One objective of this work was to determine the anaerobic degradability of linoleic acid at 21°C. In particular, degradation byproducts are identified. The other objective of this work was to determine the extent that linoleic acid inhibits aceticlastic and hydrogenotrophic methanogenesis at 21°C.

EXPERIMENTAL METHODS

Inoculum source

Experiments were conducted using a 1:6 mixture of digester sludge and granulated anaerobic biomass from the Toronto Main Treatment Plant and a food processing plant in Cornwall, Ontario, respectively. The mixture contained approximately 20,000 mg l^{-1} volatile suspended solids (VSS) and was maintained with 1000 mg l^{-1} glucose (BDH, Canada) in a 4-l semi-continuous reactor (designated reactor A) at 21°C. Inoculum from Reactor A was diluted with basal medium into a second 4-l semi-continuous reactor (Reactor B) to achieve 1500 mg l^{-1} VSS. Reactor B was maintained with 1000 mg l^{-1} glucose every 5–6 days (time when acetate and gas production measurements indicated that all glucose and byproducts were consumed) and served as the biomass source for all experiments. The basal medium used for dilution (adapted from Weigant and Lettinga, 1985) had a pH of 8.0–8.2 and contained the following constituents (milligrams per liter of distilled water): $NaHCO_3$, 6000; NH_4HCO_3, 70; KCl, 25; K_2HPO_4, 14; $(NH_4)_2SO_4$, 10; yeast extract, 10; $MgCl_2{\cdot}4H_2O$, 9; $FeCl_2{\cdot}4H_2O$, 2; resazurin, 1; EDTA, 1; $MnCl_2{\cdot}4H_2O$, 0.5; $CoCl_2{\cdot}6H_2O$, 0.15; Na_2SeO_3, 0.1; $(NH_4)_6MoO_7{\cdot}4H_2O$, 0.09; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $NiCl_2{\cdot}6H_2O$, 0.05; and $CuCl_2{\cdot}2H_2O$, 0.03.

Linoleic acid studies

Linoleic acid degradation and inhibition studies were conducted in 160-ml serum bottles prepared under a 70% N_2/30% CO_2 atmosphere with 100 ml of liquid in each bottle. The bottles were inoculated with 96, 98 or 100 ml of culture from Reactor B, depending on the condition examined, and sealed with Teflon®-lined silicone rubber septa and aluminum crimp caps. Bottles were covered in aluminum foil to inhibit photosynthetic activity and photodegradation, and agitated in an orbital shaker (Lab Line Instruments) at 200 rpm and 21°C $\pm$ 1°C for the duration of each study. To avoid the formation of a negative pressure in the headspace during sampling, culture bottles received 20 ml overpressure of 70% N_2/30% CO_2 immediately after inoculation. Liquid and headspace samples were periodically withdrawn to measure selected parameters. At the completion of each experiment, culture bottles were sacrificed to measure pH, alkalinity (as $CaCO_3$), total suspended solids (TSS) and VSS.

When added to an aqueous system at concentrations in excess of its approximate solubility of 3 mg l^{-1} (Ralston and Hoerr, 1942), pure linoleic acid does not disperse well and adheres to the surfaces of the reactor system. One approach to resolve this problem is to prepare LCFA emulsions in NaOH and to then add the emulsion to batch microbial cultures or continuous flow reactors (Hwu, 1997; Angelidaki and Ahring, 1992; Novak and Carlson, 1970). However, the emulsion itself is also very surface active and the precision with which small quantities of the emulsion can be added to 160 ml serum bottles is poor due to surface adherence. Increasing the volume of emulsion added increases precision but also culture pH. Therefore, for this work linoleic acid (Aldrich Canada) was prepared in a diethyl ether (Aldrich Canada) stock solution at a concentration of 5000 mg l^{-1}.

Differing amounts of linoleic acid stock solution up to 2 ml were added to culture bottles to provide initial linoleic acid concentrations of 0, 10, 30, 50 and 100 mg l^{-1}. Bottles receiving less than 2 ml of linoleic acid stock sol-

ution received additional diethyl ether solution so that the total volume of linoleic acid stock solution and diethyl ether solution added was always 2 ml and the concentration of diethyl ether in any culture was always 14.2 g l^{-1}. There was no evidence of diethyl ether degradation during the experiments. Initial studies were also conducted with bottles receiving pure linoleic acid which was gravimetrically added to bottles prior to adding the inoculum.

For the inhibition studies, either acetic acid or hydrogen was added to culture bottles in addition to linoleic acid. Sufficient (2 ml) acetic acid stock solution (5000 mg l^{-1} in deionized water) was added to provide an initial acetic acid concentration of 100 mg l^{-1}. Sufficient hydrogen (6 ml of gas) was added to the headspace to provide a partial pressure of 10.13 kPa.

After adding the diethyl ether and acetic acid stock solutions, additional $FeCl_2$ and Na_2S were added to ensure anaerobic conditions in the culture bottles throughout the course of the experiment (indicated by the resazurin dye remaining colorless). All conditions receiving linoleic acid were conducted in triplicate. Controls (with and without diethyl ether, acetic acid and/or hydrogen) receiving no linoleic acid were conducted in duplicate.

Analytical methods

Periodically, 2-ml mixed liquor samples were withdrawn from the serum bottles and split into two 1-ml aliquots, one for the measurement of volatile fatty acids (VFAs) and the other for measurement of LCFAs. The VFA aliquots were diluted with 2 ml deionized water and centrifuged at 1750 *g* for 5 min. Centrate was removed, filtered through H-cartridges (Dionex Canada) and diluted with deionized water to provide a detector response within the range of the calibration curves. Samples were analyzed using a Dionex Ion Chromatograph equipped with a 25 μl sample loop, a conductivity detector (CD 20), a 24-cm × 4-mm diameter AS11 column, an AMMSII micromembrane supressor and a IonPac® ATC-1 cartridge (all from Dionex). The three eluents used were deionized water (eluent A), 5 mM NaOH sodium hydroxide (eluent B) and 50 mM NaOH sodium hydroxide (eluent C). The eluent flows (as a percent of the total flow of 2 ml min^{-1}) were as follows: 0–2 mins., 93% A, 7% B; 2–6 mins., A ramped from 93 to 0%, B from 7 to 100%; 6–9 mins., B ramped from 100 to 50%, C ramped from 0 to 50% and then held until 9.99 mins; and from 10–17 mins., 93% A, 7% B. This method provided detection of lactic acid, acetic acid, propionic acid, iso- and n-butyric acids, iso- and n-valeric acid and n-hexanoic acid. The effective detection limits (incorporating dilution) were 0.2 mg l^{-1} for propionic, i -butyric, n-valeric and i -valeric acid; 0.3 mg l^{-1} for acetic and n-butyric acid and 0.4 mg l^{-1} for hexanoic acid.

The LCFA aliquots were placed into a 5 ml vial and received 0.05 g NaCl, two drops of 50% H_2SO_4 and 2 ml of a 50:50 hexane:methyl tertiary butyl ether (MTBE) mixture. The vials were capped with Teflon® lined septa, shaken at 200 rpm using an orbital shaker (Lab Line Instruments Inc. Model No. 3520) for 15 min and centrifuged at 1750 *g* for 5 min. The organic layer was removed and 1-μl was analyzed by gas chromatography using a Hewlett Packard 5890 chromatograph equipped with a flame ionization detector (FID) at 250°C, injector at 250°C and a 30-m × 0.53-mm diameter Nukol (Supelco) column. The helium carrier gas flow was 5 ml min^{-1} and the oven temperature program was 90°C for 0.5 min, with a 20°C min^{-1} ramp to 180°C and a final hold at 180°C for 9 min. This method quantified C8 to C18 saturated and unsaturated LCFAs with a minimum 85–90% extraction efficiency. The effective detection limits ranged from 1 mg l^{-1} (in the bottle) for C8 to C16 acids to 2 mg l^{-1} for stearic, oleic and linoleic acids.

Head space gas samples (20 μl) were removed and analyzed isothermally at 100°C for hydrogen and methane using a Hewlett Packard 5890 chromatograph equipped with a thermal conductivity detector (TCD) at 250°C, injector at 250°C and a 30-m × 0.53-mm diameter Carboxen® (Supelco) plot column. A nitrogen carrier gas set at 5 ml min^{-1} was used. Hydrogen and methane detection limits were 0.0628 and 0.0486 kPa, respectively. Volatile suspended solids (VSS), alkalinity and pH were determined according to *Standard Methods* (APHA, AWWA and WEF, 1992). The pH for all cultures was 7.4–7.6 with an alkalinity of approximately 4800 mg l^{-1} (as $CaCO_3$).

RESULTS

Linoleic acid partitioning in serum bottles

The partitioning of linoleic acid to the aqueous phase, the microbial solids and the serum bottle surfaces was examined by comparing two sets of triplicate bottles each receiving 100 mg l^{-1} linoleic acid (0.07 mg linoleic acid per mg microbial solids). One set of bottles received pure linoleic acid and the other set received 2 ml of linoleic acid stock solution (in diethyl ether). Three distinct phases were visually observed in bottles receiving pure linoleic acid: the aqueous phase, the microbial solids phase and a third phase of linoleic acid particles. The linoleic acid phase adhered to the serum bottle surfaces and could not be recovered quantitatively without sacrificing the bottles.

In bottles that received linoleic acid stock solution only two phases were visually observed: the aqueous phase and the microbial solids phase. Therefore, two mixed liquor samples were taken from each bottle. One set of samples was extracted without further processing. The average measured linoleic acid concentration (± standard deviation) was 100 ± 5 mg l^{-1}, indicating that linoleic acid was uniformly distributed throughout the mixed liquor in the bottle and showed little affinity for the surfaces of the serum bottle. The second set of samples was centrifuged at 1750 *g* for 5 min. The centrate

Table 1. Maximum concentration of byproducts formed during linoleic acid degradation

Product formed	Initial linoleic acid concentration (μmol l^{-1})[a]		
	36	179	357
	Byproduct concentration (μmol l^{-1})[b]		
Stearic (C18:0) acid	ND[c]	ND	ND
Oleic (C18:1) acid	13 ± 2	30 ± 1	19 ± 2
Palmitoleic (C16:1) acid	ND	ND	41 ± 18
Palmitic (C16:0) acid	13 ± 0.4	113 ± 12	178 ± 9
Myristic (C14:0) acid	21 ± 3	40 ± 4	127 ± 14
Lauric (C12:0) acid	ND	ND	< 5
Capric (C10:0) acid	ND	ND	ND
Caprylic (C8:0) acid	ND	ND	ND
Hexanoic (C6) acid	ND	ND	322 ± 113
n-Butyric (C4) acid	< 57	< 57	< 57
Acetic (C2) acid	483 ± 32	2567 ± 50	3292 ± 115

[a]36 μmol l^{-1} = 10 mg l^{-1}, 179 μmol l^{-1} = 50 mg l^{-1}, 357 μmol l^{-1} = 100 mg l^{-1}.
[b]Average ± standard deviation for triplicate bottles.
[c]ND = not detected.

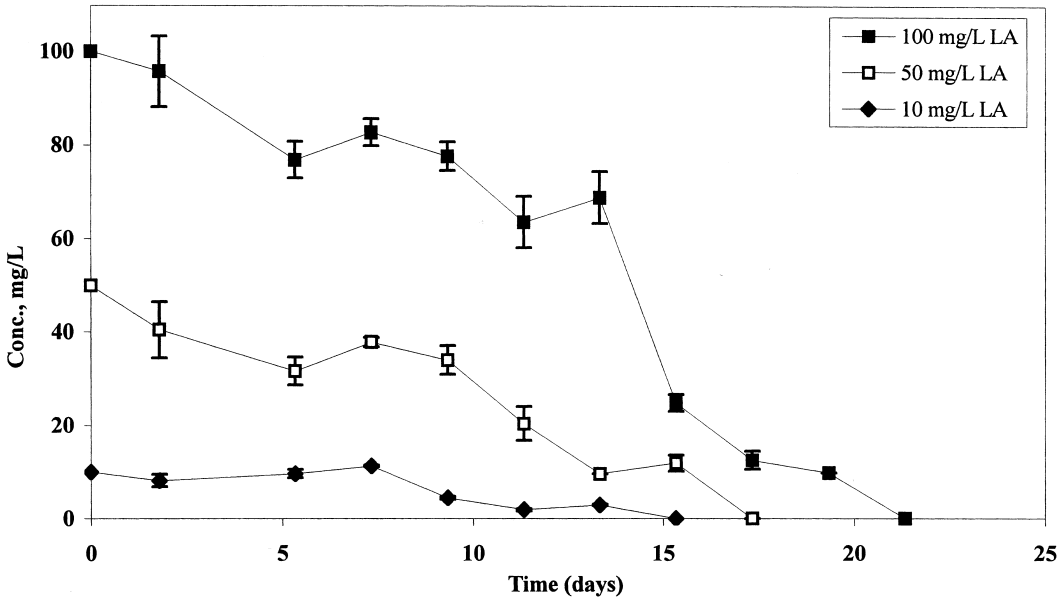

Fig. 1. Linoleic acid removal (average (points) and standard deviation (error bars), LA = linoleic acid).

was removed and extracted. The average linoleic acid concentration measured in the centrate was 5.8 ± 0.6 mg l^{-1}, slightly higher than the expected aqueous solubility of 3 mg l^{-1} (Ralston and Hoerr, 1942), perhaps because of the presence of diethyl ether.

Linoleic acid degradation studies

Linoleic acid added to cultures via the diethyl ether stock solution was degraded under anaerobic conditions at 21°C (Fig. 1) and within 25 days it was not detected. Saturated β-oxidized LCFA byproducts, palmitic, myristic and trace amounts of lauric (data not shown) acids, were observed together with hexanoic and acetic acids (Fig. 2) in cultures receiving 100 mg l^{-1} linoleic acid. Additionally, the unsaturated LCFAs oleic and palmitoleic acids were observed (Table 1). Butyric acid was detected at concentrations below 5 mg l^{-1} (Table 1). However, butyric acid appeared shortly

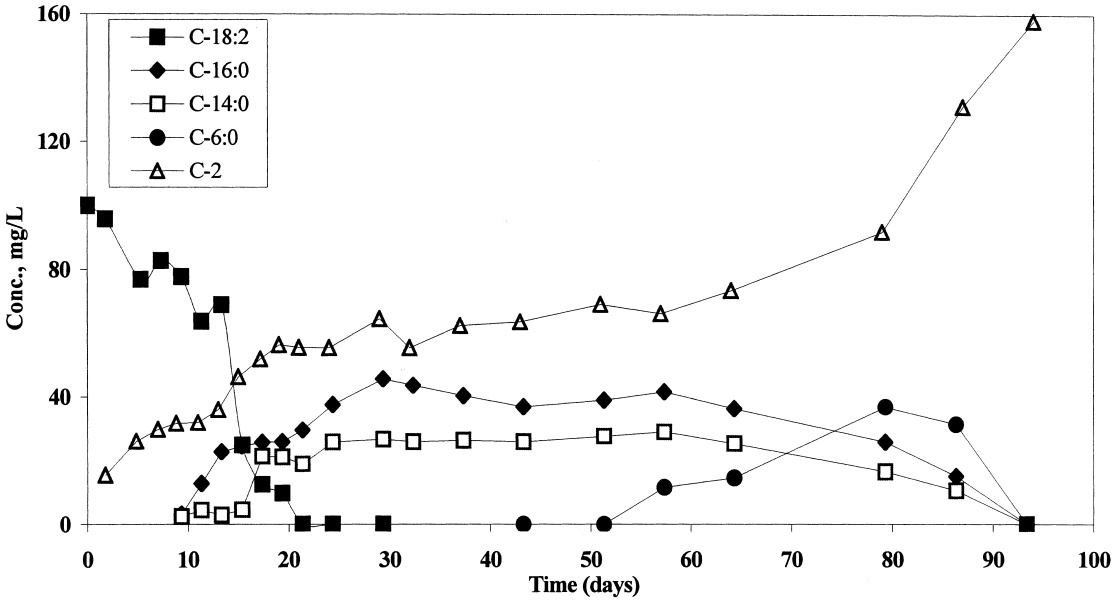

Fig. 2. Linoleic acid byproducts. Initial linoleic acid concentration = 100 mg l^{-1}.

after the studies commenced, long before hexanoic acid was detected, and its concentration remained essentially constant throughout the study. The degradation profiles for cultures receiving 10 and 50 mg l^{-1} linoleic acid were similar (data not shown) with the exception that neither palmitoleic nor hexanoic acids were observed. Methane production was inhibited in cultures receiving 30 mg l^{-1} linoleic acid or greater (data not shown).

Carbon mass balances for cultures receiving 10, 50 and 100 mg l^{-1} linoleic acid, based on converting the byproducts to a linoleic acid basis, are shown in Fig. 3. Although there were fluctuations, in particular for cultures receiving 100 mg l^{-1} linoleic acid, the mass balances indicate that the products of linoleic acid degradation were detected and measured.

The maximum concentrations (μmol l^{-1}) of byproducts detected as a result of linoleic acid degradation are shown in Table 1. As expected for a sequential degradation process, the maximum concentrations of individual byproducts did not necessarily occur at the same time. For example, the palmitic and myristic acid concentrations both peaked in the cultures receiving 100 mg l^{-1} (357 μmol l^{-1}) linoleic acid at 30 days, shortly after all linoleic acid was removed (Fig. 2). Oleic acid and palmitoleic acid concentrations (not shown in Fig. 2), however, peaked at 11 and 15 days while linoleic acid was still present and well before the palmitic and myristic acid peaks. The hexanoic acid concentration peaked at 80 days (Fig. 2), after both palmitic and myristic acids had begun to degrade, and acetic acid concentration was highest after all other intermediates were consumed.

The distribution of byproducts formed, as indicated by the maximum concentration detected, varied with initial linoleic acid concentration. At the lowest linoleic acid concentration (36 μmol l^{-1}), at least 36% (molar basis) of the linoleic acid was converted to oleic acid as an intermediate during degradation. The fractions decreased to 17 and 5% as the initial linoleic acid concentrations increased from 179 to 357 μmol l^{-1}, respectively. The fraction of shorter chain acids also shifted as the initial linoleic acid concentration increased. The maximum detected palmitic and myristic acid concentrations were 35 and 58%, respectively, of the initial linoleic acid concentration of 36 μmol l^{-1}, 63 and 22%, respectively, of the initial linoleic acid concentration of 179 μmol l^{-1}, and 50 and 36%, respectively, of the initial linoleic acid concentration of 357 μmol l^{-1}. The shift for hexanoic acid was the greatest, ranging from 0% detected at the two lowest initial linoleic acid concentrations to 90% for the highest initial linoleic acid concentration.

Acetate inhibition studies

Acetate degradation in the presence and absence of diethyl ether is shown in Fig. 4 (average of duplicates). Cultures containing neither linoleic acid nor diethyl ether consumed the added 100 mg l^{-1} acetic acid within 2 days. In the presence of 2.0 ml diethyl ether (14.2 g l^{-1} in the culture), the acetate degradation rate was retarded and complete consumption was accomplished within approximately 14 days. Stoichiometric amounts of methane were produced

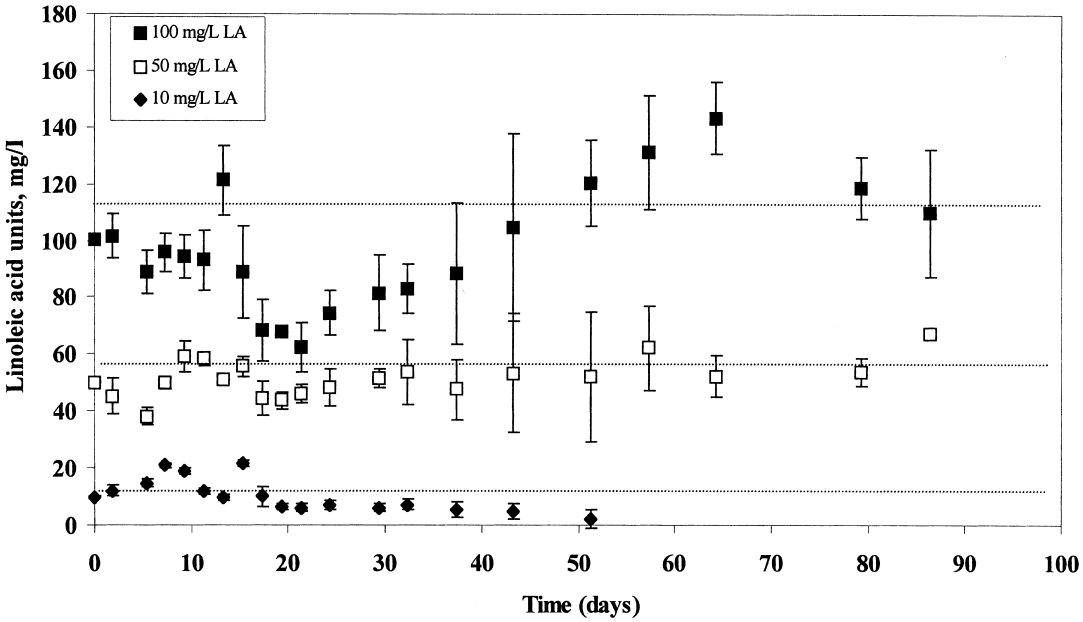

Fig. 3. Carbon mass balance profile for 10, 50 and 100 mg l^{-1} linoleic acid.

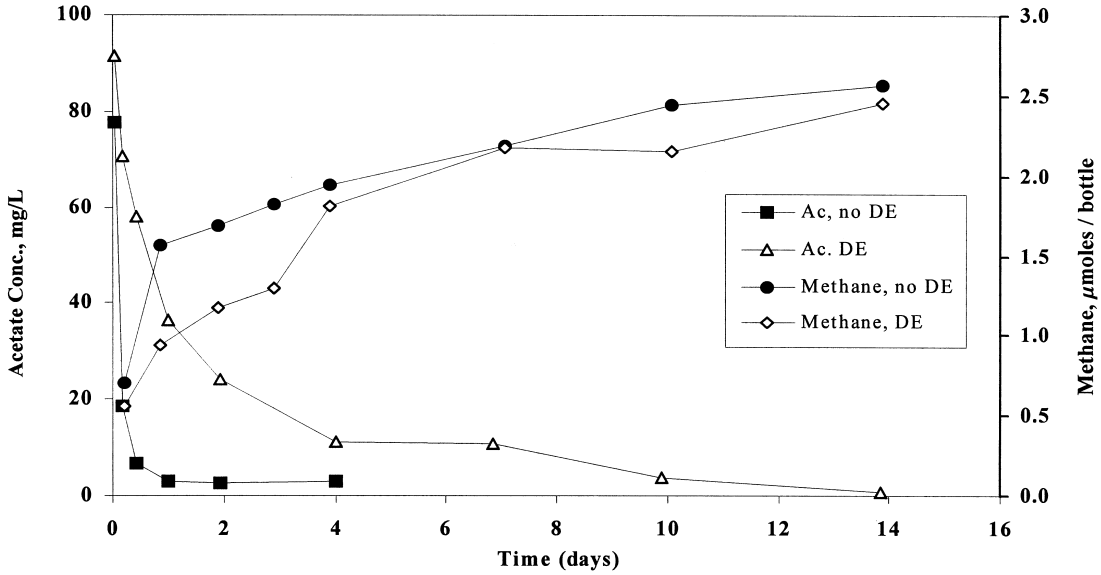

Fig. 4. Acetic acid removal in the absence of linoleic acid (Ac = acetate, DE = diethyl ether).

in cultures fed acetate in the absence and presence of diethyl ether (Fig. 4).

Acetate degradation profiles for cultures fed with linoleic acid are presented in Fig. 5. A set of cultures inoculated at the same time and receiving 30, 50 and 100 mg l^{-1} were examined first (test 1). Other cultures were then subsequently prepared, also from Reactor B, with 10 and 100 mg l^{-1} (test 2). In cultures receiving 10 mg l^{-1} linoleic acid, no inhibition was observed in comparison to cultures receiving only diethyl ether. However, linoleic acid inhibited acetic acid consumption when added at concentrations of 30 mg l^{-1} or greater. The test 2 results indicate that inhibition due to 100 mg l^{-1} linoleic acid was relieved after approximately 15 days, in contrast to the earlier results. The linoleic acid was degraded in both sets of cultures, producing byproduct profiles similar to those in Fig. 2 (data not shown). No other differences between the sets of cultures was noted. Methane was not produced when acetic acid degradation was inhibited.

Hydrogen inhibition studies

Hydrogen consumption was examined in the pre-

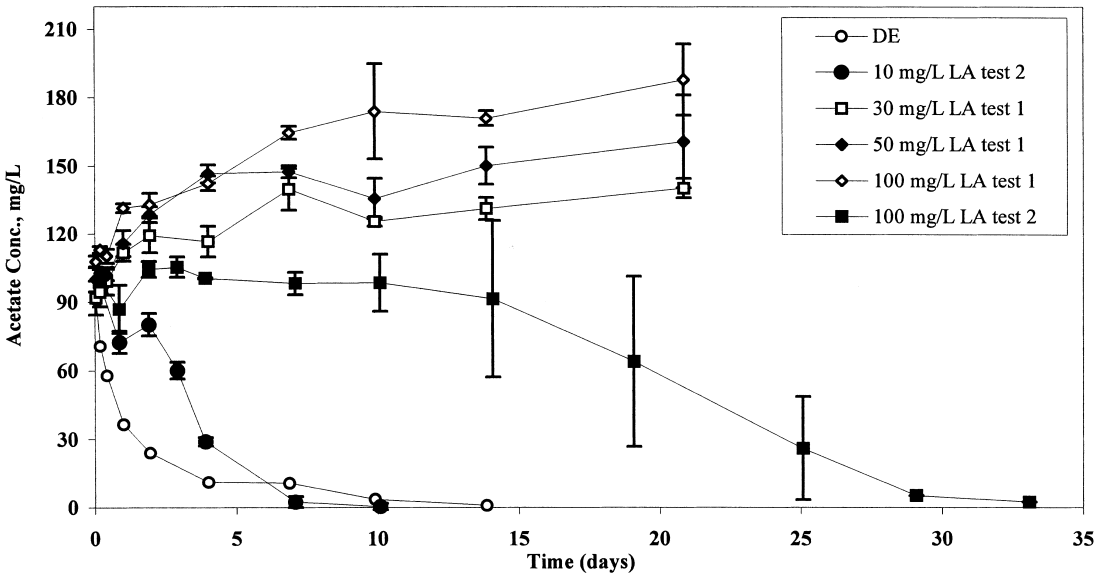

Fig. 5. Acetic acid removal in the presence of linoleic acid.

sence and absence of diethyl ether and in the presence of different concentrations of linoleic acid. All cultures received approximately 250 μmol of hydrogen 1 day after linoleic acid was added (86–95% of the initially added linoleic acid was still present and no byproducts were detected). In all cases, added hydrogen was consumed within 12 h. Methane production was stoichiometric at all conditions examined (data not shown). The hydrogen degradation curves were not linear but followed a first order degradation expression. Therefore, to provide a means to compare degradation rates, the data sets were fit to first order expressions and the first order rate constants were estimated. Although least-squares regression provided $r^2 > 0.97$ for all data sets examined, no implication of a kinetic mechanism should be drawn.

The average hydrogen removal first order rate constants for the conditions examined are shown in Table 2. Based on the Tukey's paired comparison procedure (Box *et al*., 1978) at a 95% confidence level, the rate constants for cultures receiving 0 (without diethyl ether), 0 (with diethyl ether) and 10 mg l^{-1} linoleic acid were not significantly different from each other but were significantly different from those for cultures receiving 30, 50 and 100 mg l^{-1} linoleic acid. The rate constants for cultures receiving 50 mg l^{-1} linoleic acid were not significantly different from those for cultures receiving either 30 mg l^{-1} or 100 mg l^{-1} but the constants for the cultures receiving 30 mg l^{-1} were significantly different from those for cultures receiving 100 mg l^{-1}.

DISCUSSION

Byproducts of linoleic acid degradation

The direct β-oxidation of linoleic acid would have produced a C16:2 acid. While this may have occurred within cells, no C16:2 product was detected. Oleic acid, however, was detected in all cases examined (Table 1), indicating that at least some linoleic acid was hydrogenated prior to β-oxidation. Additional hydrogenation to stearic acid may have also occurred within the cells prior to β-oxidation, although the detection of palmitoleic acid indicates that β-oxidation of an unsaturated C18 acid also occurred. Palmitoleic acid may be formed via two independent pathways. In one pathway, linoleic acid is hydrogenated at carbon 12 to produce oleic acid. Subsequently, oleic acid undergoes β-oxidation and a double bond isomerization to form palmitoleic acid. In the other possible pathway linoleic acid is hydrogenated at carbon number 9 and the resulting C18:1 acid is β-oxidized to form palmitoleic acid. The production of palmitoleic acid from oleic acid has been observed previously (Canovas-Diaz *et al*., 1991) and supports the hypothesis that the palmitoleic acid detected in the present study formed as a β-oxidation product of oleic acid that was formed from the added linoleic acid.

Table 2. Hydrogen removal first order rate constants

Linoleic acid concentration mg l^{-1}	First order rate constant[a] h^{-1}
0 (without DE)	0.387 ± 0.001
0 (with DE)	0.379 ± 0.006
10	0.374 ± 0.006
30	0.325 ± 0.02
50	0.307 ± 0.004
100	0.301 ± 0.005

[a]Average $\pm$ standard deviation for triplicate bottles.

In all cases both palmitic and myristic acids accumulated essentially stoichiometrically (the sum of both acids equalled from 85 to 93% (molar) of the added linoleic acid). These acids remained recalcitrant for a lengthy period (approximately 60 days in Fig. 2) prior to being degraded. The accumulation of these intermediates suggests that either linoleic acid or the intermediates themselves inhibited further β-oxidation. Alternatively, organisms capable of degrading myristic acid may have initially been present in very small quantities. Canovas-Diaz *et al*. (1991) observed that myristic acid added at much higher concentrations, 1000–3000 mg l^{-1}, required 80 days to be degraded and that myristic acid formed as a byproduct of oleic acid degradation was removed within approximately 70 days. Finally, the lower temperature examined in the present study (21°C) would have been expected to slow LCFA degradation rates. Hwu (1997) reported three-fold lower degradation rates for oleic acid when the temperature was lowered from 55 to 30°C.

The implication of these results is that wastewaters containing vegetable oils with a significant fraction of linoleic acid may be challenging to treat not only because of linoleic acid, but also because of palmitic and myristic acids produced. Additional work examining the potential of palmitic and myristic acids to inhibit their own degradation as well as inhibiting other anaerobic microbial processes is required.

Linoleic acid–methanogenic inhibition studies

The addition of 30 mg l^{-1} of linoleic acid or more (> 0.02 mg linoleic acid per mg VSS) led to complete, although not necessarily permanent, inhibition of aceticlastic methanogenesis (Fig. 5). The presence of the cosolvent diethyl ether at concentrations more than 140 times those of the added linoleic acid retarded but did not completely inhibit aceticlastic methanogenesis in the absence of linoleic acid (Fig. 4). The inhibitory effects of linoleic acid may have been enhanced by the presence of diethyl ether. Such enhancement may have been related to allowing linoleic acid to be uniformly contacted with the microbial solids.

The duration of the acetic acid experiments was sufficient to allow linoleic acid to degrade (as is indicated by the increase of acetic acid concentration for most inhibited cultures in Fig. 5). Therefore, inhibition later in the experiments may have been due to byproducts instead of linoleic acid. The complete inhibition of acetic acid consumption immediately after the addition of linoleic acid, however, must be considered to be due to linoleic acid (as possibly enhanced by diethyl ether). The results of test 2 at 100 mg l^{-1} suggest that the linoleic acid byproducts may be less inhibitory than linoleic acid as acetic acid consumption was observed to begin at the time linoleic acid concentrations began to decrease (data not shown). The reduced temperature of the present study (21°C) would be expected to have increased the inhibitory effects of linoleic acid and its byproducts. Hwu (1997) reported a six-fold increase in inhibition by oleic acid when the temperature was reduced from 55 to 30°C. The reasons why test 2 culture was more resilient to linoleic acid inhibition were not determined. The only experimentally observed difference between test 1 and test 2 cultures was that test 2 cultures were prepared at a later date.

The addition of 30 mg l^{-1} of linoleic acid or more slightly inhibited hydrogenotrophic methanogenesis but diethyl ether did not (Table 2). As linoleic acid concentrations were increased a clear trend of decreasing first order rate constants was observed. Nevertheless, in all cases the added hydrogen was consumed in less than 12 h, during which time no detectable linoleic acid degradation occurred. Previous work with linoleic acid and hydrogen (Demeyer and Henderickx, 1967) indicated that linoleic acid at concentrations greater than 70 mg l^{-1} was inhibitory. From a practical standpoint, the inhibition of aceticlastic methanogenesis in a system treating linoleic acid-containing vegetable oils would be expected to more significantly affect treatment than the inhibition of hydrogenotrophic methanogenesis.

The biomass used for these experiments was enriched using glucose in the absence of linoleic acid. In the continuous presence of linoleic acid or other LCFAs, acclimation may occur, reducing the inhibitory effects. If so, the relevant concern becomes determining the maximum LCFA concentration that can be withstood by the system, a concern that must be answered experimentally on a case by case basis.

CONCLUSIONS

Although linoleic acid is a major constituent of vegetable oils, neither its anaerobic degradability nor its inhibitory properties have been well examined. The conclusions from this study are:

1. Linoleic acid is anaerobically degraded at 21°C.
2. Unsaturated C18 and C16 byproducts form but do not accumulate significantly while saturated C16 and C14 byproducts accumulate and may inhibit their own subsequent degradation.
3. Linoleic acid inhibits aceticlastic methanogenesis at 21°C although the inhibition may have been enhanced by the presence of the cosolvent diethyl ether.
4. Linoleic acid slightly inhibits hydrogenotrophic methanogenesis at 21°C.

Anaerobic systems to treat wastewaters with linoleic acid-containing vegetable oils may require two separate stages to minimize the exposure of aceticlastic methanogens to linoleic acid. Hydrogenotrophic methanogens which may be needed to facilitate β-oxidation by consuming produced hydrogen would be expected to survive in the linoleic acid degradation stage.

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