# ARTICLES

## Influence of C18 Long Chain Fatty Acids on Hydrogen Metabolism

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During anaerobic treatment, several microorganisms mediate a series of reactions to convert reduced compounds (electron donors) into methane. Inhibitors such as long chain fatty acids (LCFAs) can affect several anaerobic microbial populations and decrease the treatment efficiency. The effects of three C18 LCFAs on hydrogenotrophic methanogens in a flocculated mixed anaerobic culture were assessed in this study. The reaction half-life and the hydrogen versus time profiles were used to characterize the inhibition process. The half-life values and profiles were similar for controls and cultures exposed to LCFAs for 1 h. The hydrogen inhibition was a function of the exposure time and the LCFA concentration except for cultures exposed to stearic acid (SA). A statistical analysis of the reaction half-life for cultures incubated with 1,500 and 2,000 mg $L^{-1}$ LCFAs for 48 h, revealed the following inhibition trend: linoleic acid (LA) > oleic acid (OA) > SA. After 48 h of exposure, no clear inhibition trend was observed for cultures inoculated with LCFA mixtures; however, at levels of 1,500 and 2,000 mg $L^{-1}$, the reaction half-life values were less than that observed for cultures fed with only LA. Based on the reaction half-life data, all of the LCFAs except SA at threshold levels of approximately 1,500 mg $L^{-1}$ inhibited hydrogen metabolism. The greatest inhibition and, hence, the largest amount of accumulated hydrogen was observed in cultures fed with 2,000 mg $L^{-1}$ LA and incubated for 48 h.

### Introduction

Neutral lipids such as fats and oils together with carbohydrates are major constituents in effluents from many food processing frying operations. Fats and oils are composed of a glycerol molecule linked to a variety of long chain fatty acids (LCFAs). Typically, the number of carbons in LCFAs is greater than 14, whereas in medium chain fatty acids the chain length can vary between 8 and 14 carbon atoms. Fatty acids are classified as saturated, monosaturated, or polysaturated. For example, linoleic (C18:2) acid, a common polysaturated LCFA in many edible oils, is present in safflower and tobacco seed oils with a composition of up to 75% w/w, whereas pecan and olive oils contain up to 85% w/w oleic acid, a monosaturated fatty acid (Table 1) (Sonntag, 1979).

Under favorable anaerobic conditions, LCFAs and carbohydrates are degraded to methane, a terminal reaction product, via a series of biochemical reactions. During the fermentation process, reduced carbon compounds are oxidized with a simultaneous liberation of electrons. Hydrogen produced as an intermediate is consumed by hydrogen-consumers in order to sustain favorable thermodynamic conditions. Many bacteria containing hydrogenase enzymes generate hydrogen from electrons derived from the oxidation of substrates such as carbohydrates and LCFAs.

Maintaining low hydrogen levels is important during the operation of anaerobic bioreactors. The inhibition of hydrogen consuming organisms can cause hydrogen to accumulate and eventually adverse conditions are attained. Hydrogen buildup is an indicator of reduced metabolic activity in several microbial populations.

The anaerobic degradation of mixtures consisting of carbohydrates and fats follow different metabolic pathways. Fats and oils are first hydrolyzed into LCFAs and glycerol while carbohydrates are converted into sugar monomers (Gujer and Zehnder, 1983). The next series of biochemical reactions in the pathways involve in the conversion of sugar monomers and intermediates are acidogenesis, acetogenesis and eventually methanogenesis. In contrast, the degradation of LCFAs proceeds by $\beta$-oxidation (reaction 1, $n \geq 2$) to produce a LCFA molecule reduced by two carbon atoms plus acetate and hydrogen (Weng and Jervis, 1976). This cycle is repeated until the LCFA molecule is converted into acetate plus hydrogen.

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

Threshold LCFA levels can impair the metabolic activity of numerous microorganisms and hence, affect the operation of

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10.1021/bp050304v CCC: $33.50 
Published on Web 01/07/2006

**Table 1. Fatty Acid Composition for Selected Vegetable Oils (Sonntag, 1979)**

| long chain fatty acids | | | vegetable oil LCFA composition (w/w %) | | | | |
|---|---|---|---|---|---|---|---|
| common name | systematic name | nomenclature | cotton | sunflower | sunflower (high oleic) | peanut | olive |
| stearic | octadecanoic | C18:0 | 2−3 | 3−7 | 4−5 | 1−7 | 1−3 |
| oleic | *cis*-9-octadecenoic | C18:1 | 13−24 | 14−34 | 74−82 | 35−72 | 65−85 |
| linoleic | *cis,cis*-9,12-octadecadienoic | C18:2 | 45−58 | 55−72 | 9−12 | 13−45 | 4−14 |
| linolenic | *cis,cis,cis*-9,12,15-octadecatrienoic | C18:3 | <0.5 | <0.7 | <0.2 | <1 | 0.5−1.5 |

anaerobic bioreactors. Hydrolytic microorganisms as well as acidogens, acetogens, and methanogens are inhibited by LCFAs (Hanaki et al., 1981; Lalman et al., 2003; Mykhaylovin et al., 2005; Hwu and Lettinga, 1997; Koster and Cramer, 1987). When a particular microbial population is disrupted by LCFAs, a specific substrate begins to accumulate. For example, acetate accumulation is caused by the action of LCFAs on aceticlastic methanogens (Koster and Cramer, 1987).

Hydrogen, produced from acidogenic, acetogenic, and $\beta$-oxidation reactions, is mainly utilized by hydrogenotrophic methanogens to generate methane. Nitrate and sulfate may also function as electron acceptors. In another methanogenic terminal pathway, acetate is converted to methane plus carbon dioxide.

Microorganisms producing methane belong to the archaea domain. Methanogens are classified in five orders: *Methanopyrales, Methanococcales, Methanobacteriales, Methanosarcinales,* and *Methanomicrobiales* (Boone et al., 1993). Methanogens generate methane not only from several substrates such as acetate and carbon dioxide but also from C1 carbon compounds such as methanol and methylamines (Wolfe, 1996; Thauer, 1998). Three hydrogenotrophic methanogens, *Methanocaldococcus jannaschii* (Bult et al., 1996), *Methanothermobacter thermautotrophicus* (Smith et al., 1997), and *Methanopyrus kandleri* (Slesarev et al., 2002), all of which are thermophiles or hyperthermophiles, have been recently characterized using genetic tools. Other hydrogenotrophic methanogens identified include *Methanococcus maripaludis* (Hendrickson et al., 2004) and *Methanobrevibacter acididurans* (Savant and Ranade, 2004). These organisms utilize a series of enzymes to generate methane from carbon dioxide and hydrogen. A large portion of the enzymes in the methane producing pathway have been purified and characterized (Ferry, 2003). Inactivating these enzymes by inhibitors may prevent hydrogen utilization.

Only a few studies have provided evidence describing the effects of individual or LCFAs mixtures on hydrogen metabolism (Hanaki et al., 1981; Lalman and Bagley, 2000; Lalman and Bagley, 2001; Demeyer and Hendrickx, 1967). Evidence provided by Hanaki et al. (1981) has shown a 2,000 mg $L^{-1}$ mixture consisting of C12 to C18 LCFAs at 37 °C inhibited hydrogen metabolism. The cultures readily consumed hydrogen but at a lesser rate when compared to the controls. In similar studies, low LCFA levels did not appear to severely impair hydrogen degradation (Lalman and Bagley, 2002). Other work has shown linolenic (C18:3) acid, a 18 carbon fatty acid with three C═C double bonds, can affect hydrogen-consuming microorganisms (Demeyer and Hendrickx, 1967). In studies conducted by Hanaki et al. (1981), the LCFA concentration ranged from approximately 0 to 2000 mg $L^{-1}$ and the mixture consisted of fatty acid bearing 10−18 carbon atoms. Because of the wide range of LCFAs used by Hanaki et al. (1981), it is unclear which fatty acid inhibited hydrogen utilization. The present work was focused on assessing the inhibitory effects of linoleic acid (LA, C18:2), oleic acid (OA, C18:1), stearic acid (SA, C18:0), and mixtures of these three C18 fatty acids on hydrogen metabolism at 21 °C.

## Experimental Methods

**Inoculum Source.** The anaerobic mixed culture inoculum used to conduct all of the experiments was obtained from an anaerobic wastewater facility treating effluent from an ethanol manufacturing facility (Chatham, ON). The inoculum was maintained in a 4-L semicontinuous reactor (designated Reactor A) at 21 °C and fed with 5,000 mg $L^{-1}$ glucose (Fisher Scientific, ON) every 6−7 days. Reactor A contained approximately 20,000 mg $L^{-1}$ volatile suspended solids (VSS). Inoculum from Reactor A was diluted with basal medium to achieve 2,000 mg $L^{-1}$ VSS in a second 4-L semicontinuous reactor (Reactor B) (Lalman and Bagley, 2000). Volatile fatty acids (VFAs) and the amount of gas produced were used to determine when all of the glucose and byproducts were consumed (every 5−6 days). Reactor B was maintained with 5,000 mg $L^{-1}$ glucose and served as the biomass source for all experiments.

**Experimental Design.** Experiments were designed to investigate the effects of varying quantities of individual and LCFAs mixtures on hydrogen degradation. Cultures were inoculated with LCFAs (99%, TCI America, OR) concentration ranging from 0 to 2,000 mg $L^{-1}$ for 1 and 48 h. After 1 h, 5.0 mL of hydrogen (UHP grade, Praxair, ON) and 1.25 mL of carbon dioxide (UHP grade, Praxair, ON) were injected, and following 48 h of incubation, the same amount of each gaseous substrate was added again. The fatty acids were added in a 1:1 mass ratio for binary mixtures and a 1:1:1 mass ratio for the tertiary mixture.

**Inhibition Studies Using Linoleic, Oleic, and Stearic Acid.** The studies were conducted under similar conditions as previously described by Lalman and Bagley (2000). Serum bottles (160 mL) were prepared under a 70% $N_2$ (UHP grade, Praxair, ON)/30% $CO_2$ atmosphere and received a total liquid volume of 100 mL. Depending on the condition examined, varying amounts of culture (2,000 mg $L^{-1}$ VSS) from Reactor B were added to the serum bottles. Controls prepared for the study consisted of only culture and culture plus hydrogen. The bottles were sealed with 20 mm diameter Teflon-lined silicone rubber septas and aluminum caps. To avoid the formation of a negative pressure in the headspace during sampling, immediately after inoculation, the culture bottles received 20 mL overpressure of 70% $N_2$/30% $CO_2$. An orbital shaker (Lab Line Instruments model 3520, Iowa) was used to agitate the bottles at 200 rpm and 21 ± 1 °C over the duration of the study. Headspace samples were withdrawn periodically to measure the moles of hydrogen remaining in the serum bottle.

Stock solutions (50,000 mg $L^{-1}$) were prepared from LCFAs melted au bain-marie in hot NaOH (Alosta, 2002; Angelidaki and Ahring, 1992). To provide initial LCFA concentrations of 0, 100, 500, 700, 1,000 1,500 and 2,000 mg $L^{-1}$, varying amounts of the 50,000 mg $L^{-1}$ solution were added to the culture. Equilibrium conditions between the gas and liquid phases were assumed to be attained by shaking the bottles at 200 rpm.

For the inhibition studies, 5.0 mL of hydrogen and 1.25 mL of carbon dioxide were added to the cultures at 1 h and again

at 48 h. To ensure that anaerobic conditions were maintained in the bottles (indicated by the resazurin dye remaining colorless), additional $FeCl_2$ and $Na_2S$ (25 mg $L^{-1}$ each) were added. All controls and culture containing LCFAs were prepared in triplicate. A 48-h incubation time was chosen in order to minimize possible interferences by LCFA degradation byproducts. LCFAs degrade relatively very slowly in comparison to substrates such as glucose (Alosta et al., 2004; Shin et al., 2003). Over the 48-h incubation period, approximately 5−10% of the LCFAs are expected to degrade. The byproduct concentration, following 48 h of incubation, will probably not affect the expected hydrogen degradation profile. However, if a larger incubation time was considered, the inhibition could likely include effects due to not only the parent LCFA but also the degradation byproducts (shorter chain LCFAs).

## Analytical Methods

Headspace gas samples (25 $\mu$L) were removed and analyzed via gas chromatography (GC) with a thermal conductivity detector (TCD). The GC (Varian model 3600) was configured with a 2 m × 2 mm i.d. Carbon Shin column (Alltech, Deerfield, IL). The nitrogen carrier gas flow rate was set at 21 mL $min^{-1}$ and the GC injector, detector and oven temperatures were set at 100, 200, and 200 °C, respectively. The detection limit for hydrogen was 0.0036 kPa.

**Hydrogen Degradation Reaction Half-Life and Statistical Analysis.** All results reported in this work are the means of triplicate samples. The hydrogen data set was nonlinearly regressed to an exponential model using Curve Expert. The half-life is the time for the substrate concentration to decrease to one-half of its initial concentration. Intuitively, fast reactions have short half-lives, and hence the half-life can be used as a suitable parameter to determine the LCFA inhibition effect. A potent inhibitor increases the half-life of a substrate by decreasing the enzyme activity.

Statistical differences between the mean values of the reaction half-life values were determined using the Tukey's paired comparison procedure at the 95% confidence level (Box et al., 1978).

## Results

**Hydrogen Consumption in Cultures Receiving Individual LCFAs.** Selected controls consisting of only culture and hydrogen, for experiments conducted with OA, LAOA, and OASA at incubation times of 1 and 48 h are shown in Figure 1. Note that the exponential profiles for the different LCFA conditions and the two incubation times are similar (Figure 1). In controls without any hydrogen added, the methane levels were low in comparison to cultures injected with hydrogen.

The hydrogen removal profiles for cultures receiving an individual LCFA plus hydrogen (LA, OA, or SA) are depicted in Figures 2−4. Approximately 6 h after the first 1 h incubation period, most of the hydrogen was consumed in the controls as well as in the cultures receiving LA (Figure 2A). Forty-eight hours following the first hydrogen injection, 5.0 mL was injected again together with 1.25 mL of carbon dioxide into all of the serum bottles (Figure 2B). The hydrogen removal profile for cultures fed with 0−1,000 mg $L^{-1}$ LA were exponential, whereas in cultures receiving 1,500 and 2,000 mg $L^{-1}$ LA, the degree of curvature was less compared to the controls (Figure 2B). The decrease in curvature is evidence of a reduction in the hydrogen degradation rate at elevated LCFA levels. Under elevated LA levels (1,500 and 2,000 mg $L^{-1}$), 50−60% of the hydrogen remained in the headspace 5.2 h following the second hydrogen injection.

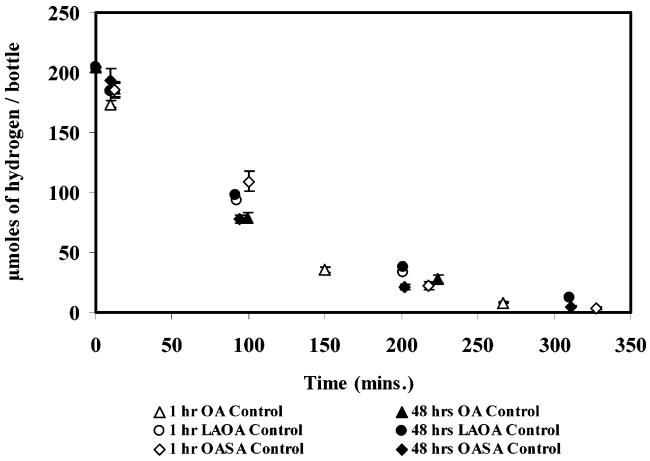


**Figure 1.** Hydrogen degradation profiles for selected control cultures. LA = linoleic acid, OA = oleic acid, SA = stearic acid. Triplicate data set is shown.

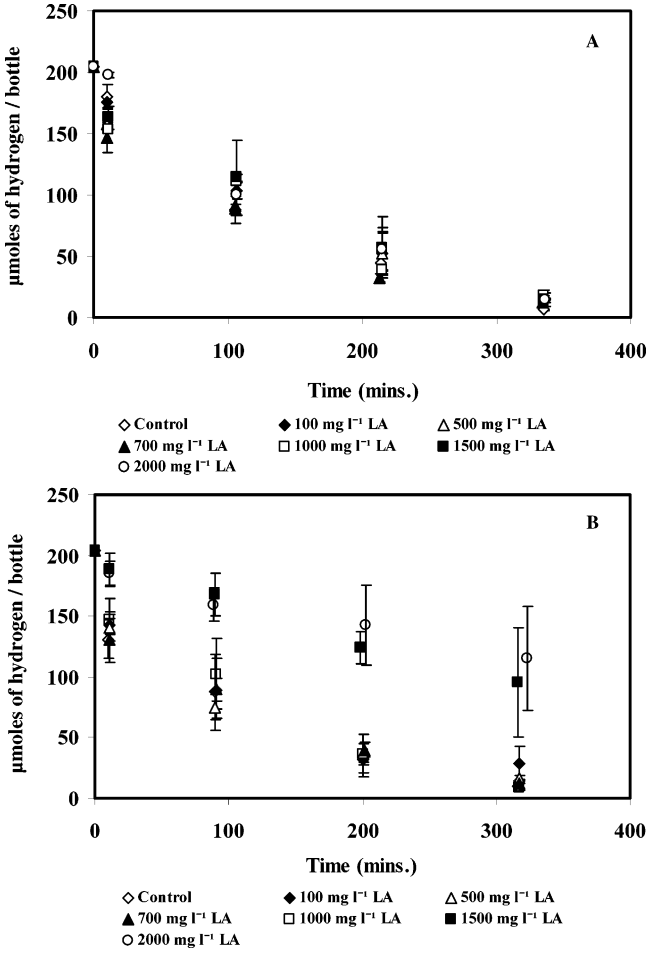


**Figure 2.** Hydrogen degradation profiles for cultures receiving linoleic acid: (A) 1 h incubation; (B) 48 h incubation. LA = linoleic acid. Triplicate data set is shown.

The shape of the hydrogen versus time profile for the controls in studies conducted with LA or OA were similar (Figures 2A and 3A). All of the hydrogen versus time profiles for cultures incubated with OA for 1 h were similar. Following the 48-h incubation period, the hydrogen removal profiles for cultures fed with less than 1,000 mg $L^{-1}$ OA were also similar to the controls (Figure 3B). However, in cultures receiving ≥1,000 mg $L^{-1}$ OA, the hydrogen removal rate was initially low over a 2 h period beginning after the first injection. Subsequently, a greater removal rate was observed between 2 and 4 h (Figure

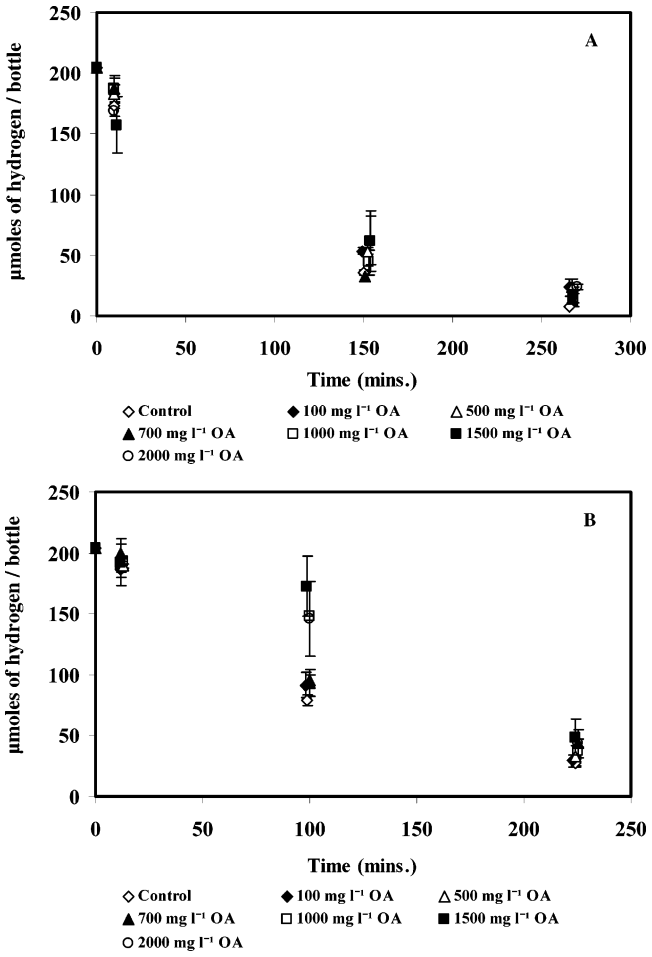


**Figure 3.** Hydrogen degradation profiles for cultures receiving oleic acid: (A) 1 h incubation; (B) 48 h incubation. OA = oleic acid. Triplicate data set is shown.

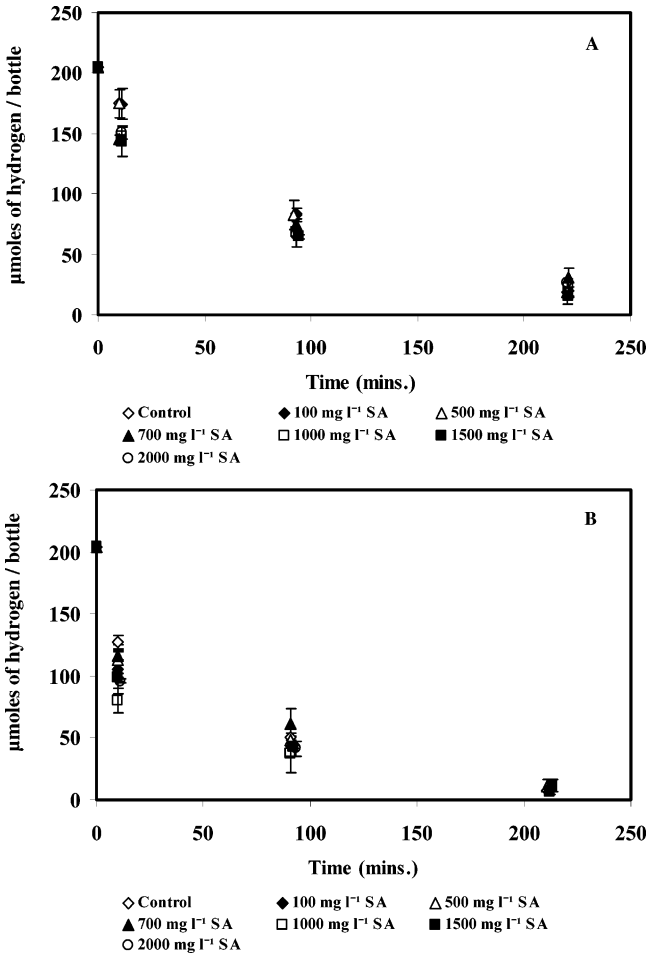


**Figure 4.** Hydrogen degradation profiles for cultures receiving stearic acid: (A) 1 h incubation; (B) 48 h incubation. SA = stearic acid. Triplicate data set is shown.

3B). The amount of hydrogen utilized, after the second injection, in cultures fed with OA was approximately 5% less than in the controls.

The hydrogen profiles for cultures incubated with SA after 1 and 48 h were similar to the controls (Figure 4A and B). The amount of hydrogen (95%) consumed, after 3.5 h, was similar for cultures fed with SA and independent of the incubation time.

**Hydrogen Consumption in Cultures Receiving LCFA Mixtures.** Experiments were also performed to determine the effects of binary and tertiary mixtures containing LA, OA, and SA on hydrogen metabolism. One hour after incubating the culture with LA plus OA, the hydrogen removal profiles were similar for all of the conditions examined (Figure 5A). However, when hydrogen was injected again 48 h following the first injection, the hydrogen profiles remained exponential only for cultures inoculated with 0, 100, 500, and 700 mg $L^{-1}$ of the LCFA mixture (Figure 5B). At increasing LCFA levels from 1,000 to 2,000 mg $L^{-1}$ LA plus OA, the profiles were less concave (Figure 5B) and similar to cultures receiving 1,500 and 2,000 mg $L^{-1}$ LA. In cultures fed with 1,500 and 2,000 mg $L^{-1}$ LA plus OA, 25−30% of the hydrogen added remained approximately 3.2 h after the second injection.

The effect of adding a mixture containing LA plus SA on hydrogen consumption was examined after incubating the culture for 1 and 48 h (Figure 6A and B). Notice the incubation time did not affect the shape of the hydrogen removal profile. At 1 and 48 h, only minor differences in the hydrogen profiles were observed (Figure 6A). The amount of hydrogen remaining, after approximately 3 h, in cultures incubated with LA plus SA for 1 and 48 h were 5% and 10%, respectively.

Hydrogen removal profiles for cultures receiving OA plus SA after a 1 h incubation period were similar to each other (Figure 7A). The amount of hydrogen remaining, after approximately 6 h following the first hydrogen injection, was similar for all of the conditions examined. The profiles for the second hydrogen injection were exponential for cultures inoculated with 0−1000 mg $L^{-1}$ LCFA (Figure 7B). However, profiles at higher OA plus SA levels were linear and also similar to cultures exposed to 1,500 and 2,000 mg $L^{-1}$ LA for 48 h. The fraction of hydrogen remaining in cultures incubated with 1,500 and 2,000 mg $L^{-1}$ OA plus SA at 5.2 h after the second hydrogen injection ranged from 10% to 25%.

Cultures incubated with a mixture consisting of LA, OA, and SA for 1 h had similar exponential hydrogen profiles for all conditions examined (Figure 8A). However, when hydrogen and carbon dioxide were injected again after 48 h, the profiles for cultures receiving 1,500 and 2,000 mg $L^{-1}$ of the three LCFAs were linear (Figure 8B). Under the latter conditions, after 6 h, 50−60% of the hydrogen remained in the headspace, whereas in cultures fed with lower levels of the same LCFA mixture, 10−15% of the hydrogen remained after the same time period (Figure 8B). When compared to cultures fed with a binary LCFA mixture, larger amounts of hydrogen remained in cultures receiving a tertiary mixture consisting of LA, OA, and SA.

**Hydrogen Degradation Reaction Half-Life.** The inhibition process was characterized by the shape of the hydrogen versus

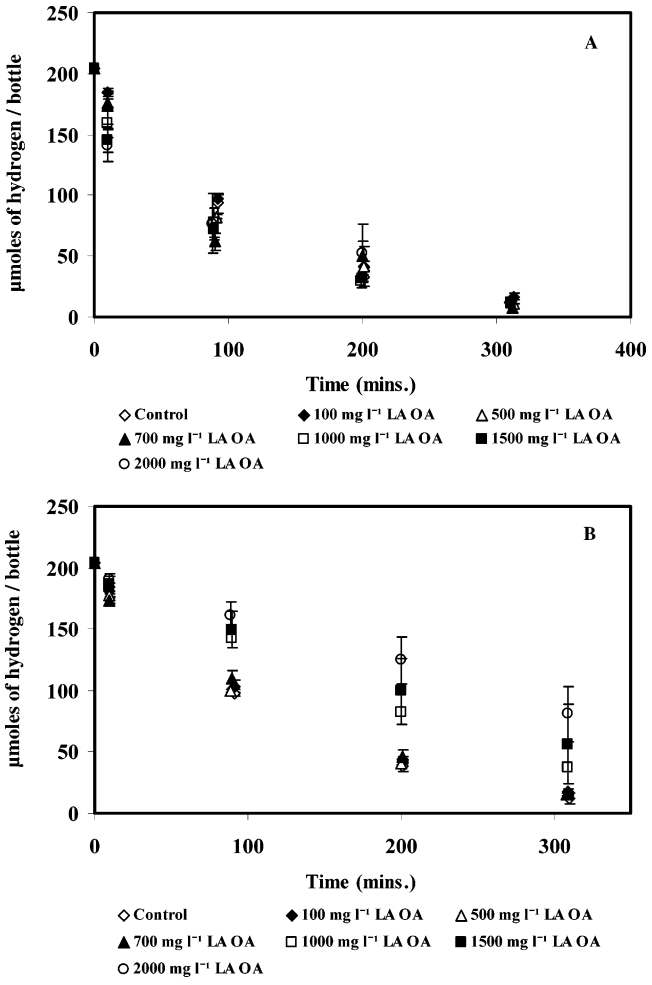


**Figure 5.** Hydrogen degradation profiles for cultures receiving linoleic acid plus oleic acid: (A) 1 h incubation; (B) 48 h incubation. LA = linoleic acid, OA = oleic acid. Triplicate data set is shown. Ratio of LA:OA is 1:1.

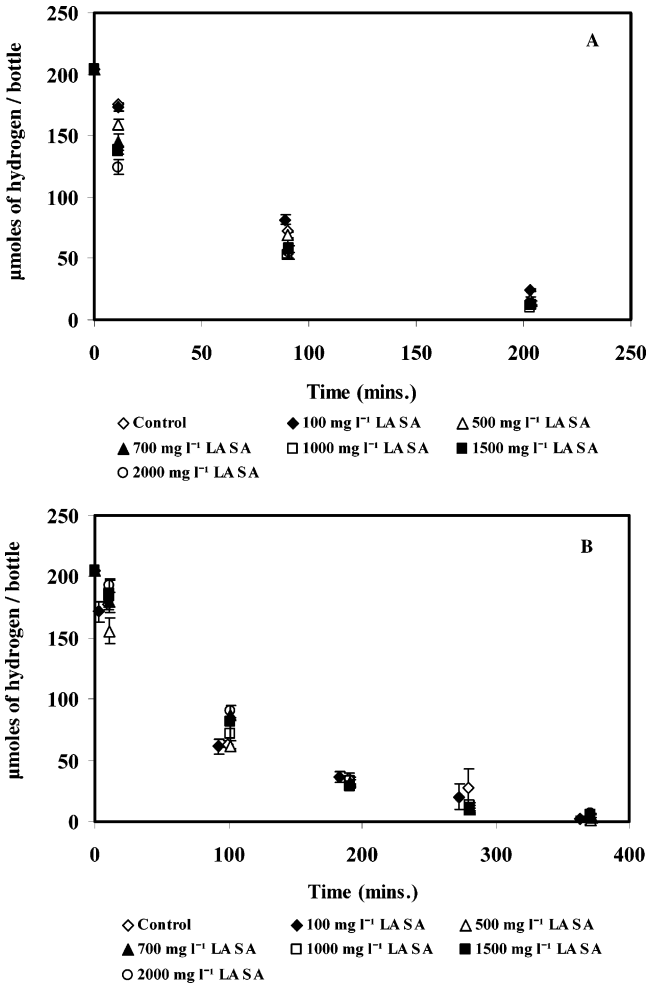


**Figure 6.** Hydrogen degradation profiles for cultures receiving linoleic acid plus stearic acid: (A) 1 h incubation; (B) 48 h incubation. LA = linoleic acid, SA = stearic acid. Triplicate data set is shown. Ratio of LA:SA is 1:1.

time profiles and also by the half-life (Tables 2 and 3) of the substrate reaction. Statistically similar half-reaction values were observed for cultures exposed to LCFA over a 1 h period. However, when the cultures were incubated for a 48 h period, the percent reaction half-life values increased in cultures receiving >1,000 mg $L^{-1}$ LCFA except for cultures fed with SA. Cultures receiving ≤1,000 mg $L^{-1}$ LA or OA had statistically different reaction half-life values compared to those fed with ≥1,500 mg $L^{-1}$ LA or OA. The percent half-life values for cultures receiving SA after 1 and 48 h of incubation were statistically similar based on Tukey's procedure (Tables 2 and 3).

Cultures receiving binary and tertiary LCFAs mixtures behaved similarly to those incubated with LA or OA. For example, after a 1 h incubation period with binary or tertiary LCFA mixtures, all of the hydrogen profiles were similar. However, except for cultures inoculated with SA, a threshold LCFA concentration was observed at 1,500 mg $L^{-1}$ when the cultures were exposed to individual, binary, and tertiary mixtures for 48 h (Table 3).

The half-life values (Table 3) for cultures exposed to individual LCFAs (1,500 and 2,000 mg $L^{-1}$) over 48 h suggest that LA exerted the greatest inhibitory effect followed by OA and then SA. After incubation for 48 h, the percent half-life for cultures inoculated with 1,500 and 2,000 mg $L^{-1}$ of the fatty acid mixture ranged from approximately 120 to 250 min. The inhibition observed for LCFA mixtures did not follow a particular trend; however, the reaction half-life values for cultures fed with 1,500 and 2,000 mg $L^{-1}$ were less than that for cultures fed with only LA (Table 3).

## Discussion

Anaerobic treatment of effluents containing carbohydrates and vegetable oils could be inefficient as a result of the inhibitory effects caused by LCFAs on several microbial populations. Acidogens, acetogens, and aceticlastic methanogens are all affected by threshold LCFAs levels (Mykhaylovin et al., 2005; Lalman et al., 2003; Hwu and Lettinga, 1997; Koster and Cramer, 1987; Hanaki et al., 1981). In a mixed culture treatment system, if a fatty acid such as LA is present at threshold levels, hydrogen metabolism will be likely curtailed. Current evidence from several studies describing the impact of LCFAs on hydrogenotrophic methanogens is inconclusive and insufficient. Lalman and Bagley (2002) concluded the effect of relatively low LCFAs levels (300 mg $L^{-1}$) on hydrogen degradation was negligible, and Hanaki et al. (1981) reported a mixture consisting of C12 to C18 LCFAs was inhibitory. In the former study, the impact of elevated LCFA levels and LCFA mixtures were not examined, and the latter study did not establish the threshold levels of individual LCFAs on hydrogen metabolism. The research in this study addresses these two issues. The microbial production and consumption of hydrogen are essential metabolic steps in establishing a thermodynamically stable environment

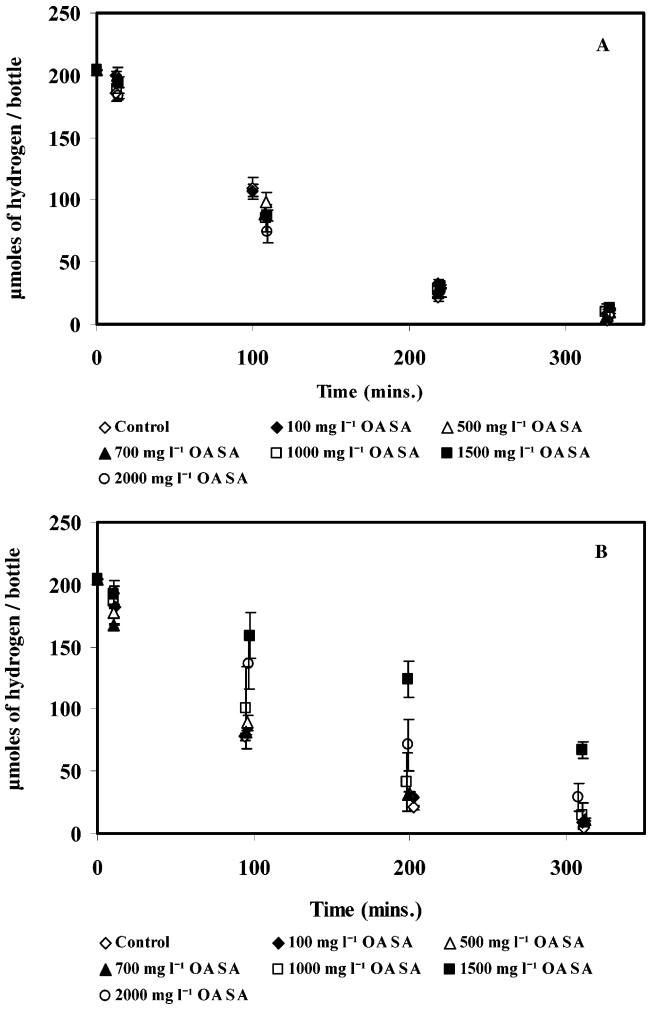


**Figure 7.** Hydrogen degradation profiles for cultures receiving oleic acid plus stearic acid: (A) 1 h incubation; (B) 48 h incubation. OA = oleic acid, SA = stearic acid. Triplicate data set is shown. Ratio of OA:SA is 1:1.

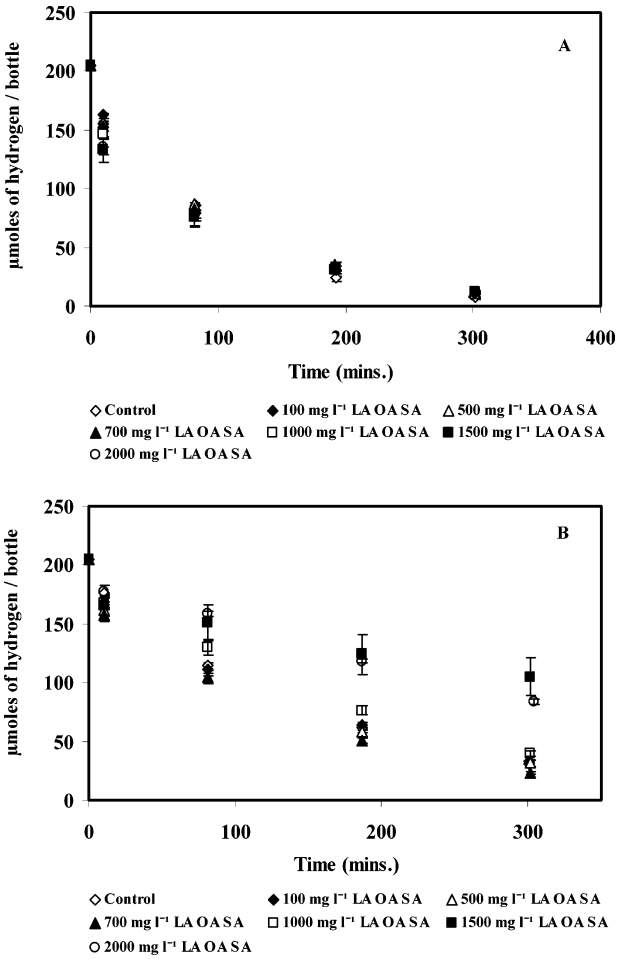


**Figure 8.** Hydrogen degradation profiles for cultures receiving linoleic acid plus oleic acid and stearic acid: (A) 1 h incubation; (B) 48 h incubation. LA = linoleic acid, OA = oleic acid, SA = stearic acid. Triplicate data set is shown. Ratio of LA:OA:SA is 1:1:1.

during anaerobic treatment and the presence of fats and oils could upset this balance.

Hydrogen metabolism is important in acetogenic and methanogenic environments. Efficient hydrogen utilization maintains a low partial pressure, which is required for acetogenic reactions to take place. Within the anaerobic microbial community, there exist a series of syntrophic relationship between different organisms. Hydrogen-producing (reactions 2 and 3, Table 4) and hydrogen-consuming organisms (reactions 4−7) exist syntrophically to maintain low levels of hydrogen (Wolin and Miller, 1982).

Several microorganisms are responsible for utilizing hydrogen in an anaerobic environment. Hydrogenotrophic methanogens consume carbon dioxide and hydrogen, while sulfur-reducing microorganisms synthesize hydrogen sulfide from sulfate and hydrogen. In this study, $CO_2$ is the only available electron acceptor.

Carbon dioxide reduction may follow different routes to form methane, formate, or acetate (Shima et al., 2002; Ljungdahl, 1986). The production of formate and acetate share a common pathway. Carbon dioxide reduction to methane is the predominant pathway based on the large amount of free energy released compared to the formate and acetate route (reactions 4−6). Hence, the hydrogen utilization pathway under consideration in this study is more favorable and mediated by hydrogenotrophic methanogens.

Acetogenic microorganisms in a mixed culture mediating the degradation of butyrate are affected by LA and OA levels ranging from 50 to 1000 mg $L^{-1}$ while SA does not seem to exhibit any inhibitory effects (Mykhaylovin et al., 2005). Similarly, in this work, SA was an ineffective inhibitor but threshold LA or OA levels at approximately 1,500 mg $L^{-1}$ impaired the hydrogen degradation rate. The data presented in Tables 2 and 3 imply the reaction half-life is independent of the concentration and incubation time for cultures inoculated with only SA. The data also show that the inhibition caused by all of the fatty acid mixtures is time dependent based on the half-life data for the 1 and 48 h incubation times.

In an anaerobic reactor, conditions under which LCFAs are inhibitory to volatile fatty acids degrading microorganisms are known to decrease the pH and eventually reduce the process efficiency (Lalman et al., 2003; Koster and Cramer, 1987). Methanogenesis is an important process during the anaerobic degradation of complex substrates. The inhibition of this process will likely cause the formation of reduced organic compounds such as ethanol, propionate, butanol, and butyrate. In methanogenesis, two groups of microorganisms produce methane via different metabolic pathways. Hydrogenotrophic methanogens utilize hydrogen and carbon dioxide to form methane while aceticlastic methanogens metabolize acetate to methane and carbon dioxide. Data from this work provides evidence that hydrogenotrophic methanogenic activity is impaired by greater LCFAs levels compared to aceticlastic methanogens. Acetate degradation is affected by 500 mg $L^{-1}$ OA (Koster and Cramer,

**Table 2. Percent Hydrogen Degradation Half-Life Values (% of Control) for Cultures Incubated with LCFAs for 1 h[a]**

| | LCFA concentration (mg $L^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| LCFA type | 0 | 100 | 500 | 700 | 1000 | 1500 | 2000 |
| LA | 100.0 ± 9.8[a] | 117.2 ± 14.6[a] | 110.1 ± 15.9[a] | 91.6 ± 15.5[a] | 112.3 ± 35.1[a] | 121.0 ± 27.6[a] | 124.1 ± 21.5[a] |
| OA | 100.0 ± 1.7[a] | 139.8 ± 9.5[a] | 124.3 ± 3.3[a] | 107.4 ± 3.1[a] | 120.0 ± 17.4[a] | 127.1 ± 22.5[a] | 179.7 ± 14.6[a] |
| SA | 100.0 ± 6.1[a] | 100.4 ± 10.7[a] | 99.0 ± 17.4[a] | 101.1 ± 10.8[a] | 87.7 ± 12.6[a] | 79.3 ± 8.3[a] | 93.6 ± 15.5[a] |
| LAOA | 100.0 ± 5.6[a] | 111.8 ± 8.9[a] | 97.3 ± 10.6[a] | 84.1 ± 15.0[a] | 84.4 ± 18.6[a] | 77.9 ± 10.6[a] | 90.7 ± 32.6[a] |
| LASA | 100.0 ± 12.3[a] | 117.2 ± 9.8[a] | 96.8 ± 9.0[a] | 83.1 ± 17.2[a] | 125.2 ± 15.7[a] | 78.9 ± 8.1[a] | 74.4 ± 15.5[a] |
| OASA | 100.0 ± 9.2[a] | 120.2 ± 9.2[a] | 119.1 ± 9.9[a] | 104.6 ± 12.5[a] | 106.6 ± 15.3[a] | 115.3 ± 7.9[a] | 98.5 ± 10.6[a] |
| LAOASA | 100.0 ± 16.6[a] | 113.3 ± 12.8[a] | 115.3 ± 10.5[a] | 109.6 ± 14.4[a] | 101.0 ± 24.0[a] | 97.9 ±15.1[a] | 99.5 ± 13.1[a] |

[a] Average and standard deviation for triplicate samples are shown. LA = linoleic acid; OA = oleic acid; SA = stearic acid. The Tukey's comparison is performed with respect to the control. The superscript notations a and b are used to indicate the means that are statistically different within the same row.

**Table 3. Percent Hydrogen Degradation Half-Life Values (% of Control) for Cultures Incubated with LCFAs for 48 h[a]**

| | LCFA concentration (mg $L^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| LCFA type | 0 | 100 | 500 | 700 | 1000 | 1500 | 2000 |
| LA | 100.0 ± 33.1[a] | 113.4 ± 25.3[a] | 98.4 ± 21.3[a] | 103.0 ± 32.1[a] | 110.6 ± 37.6[a] | 474.8 ± 45.1[b] | 524.5 ± 44.7[b] |
| OA | 100.0 ± 8.6[a] | 107.5 ± 8.7[a] | 115.3 ± 11.1[a] | 131.6 ± 13.1[a] | 141.0 ± 8.5[a] | 215.1 ± 18.7[b] | 193.9 ± 8.8[b] |
| SA | 100.0 ± 9.8[a] | 79.1 ± 30.4[a] | 93.9 ± 19.7[a] | 106.1 ± 72.2[a] | 71.4 ± 20.1[a] | 158.1 ± 6.6[b] | 153.7 ± 9.5[b] |
| LAOA | 100.0 ± 10.0[a] | 110.0 ± 10.1[a] | 103.5 ± 7.6[a] | 109.6 ± 12.9[a] | 170.8 ± 14.1[b] | 228.8 ± 49.5[b] | 317.6 ± 36.8[b] |
| LASA | 100.0 ± 19.7[a] | 87.9 ± 18.2[a] | 84.5 ± 17.4[a] | 105.6 ± 22.4[a] | 98.1 ± 19.5[a] | 163.7 ± 12.9[b] | 169.7 ± 10.9[b] |
| OASA | 100.0 ± 4.0[a] | 112.0 ± 5.9[a] | 116.3 ± 4.8[a] | 106.2 ± 3.4[a] | 134.9 ± 36.6[a] | 367.5 ± 9.3[b] | 340.6 ± 17.8[b] |
| LAOASA | 100.0 ± 7.4[a] | 99.6 ± 9.3[a] | 92.1 ± 4.9[a] | 77.2 ± 13.6[a] | 118.1 ± 9.6[a] | 281.9 ± 29.0[b] | 230.7 ± 6.6[b] |

[a] Average and standard deviation for triplicate samples are shown. LA = linoleic acid; OA = oleic acid; SA = stearic acid. The Tukey's comparison is performed with respect to the control. The superscript notations a and b are used to indicate the means that are statistically different within the same row.

**Table 4. Free Energies for Selected Acetogenic and Hydrogen Utilization Reactions**

| reaction | $\Delta G^{\circ\prime}$ (kJ/mol) | reaction |
|---|---|---|
| $CH_3CH_2COO^- + 3H_2O \rightleftharpoons CH_3COO^- + HCO_3^- + H^+ + 3H_2$ | 76.4 | 2 |
| $CH_3CH_2CH_2COO^- + 2H_2O \rightleftharpoons 2CH_3COO^- + 2H_2 + H^+$ | 48.3 | 3 |
| $HCO_3^- + 4H_2 + H^+ \rightleftharpoons CH_4 + 3H_2O$ | −135.6 | 4 |
| $2HCO_3^- + 4H_2 + H^+ \rightleftharpoons CH_3COO^- + 4H_2O$ | −104.5 | 5 |
| $HCO_3^- + H_2 \rightleftharpoons HCOO^- + H_2O$ | −1.4 | 6 |
| $4H_2 + SO_4^{2-} + H^+ \rightleftharpoons HS^- + 4H_2O$ | −176.3 | 7 |

1987), while this study demonstrated hydrogenotrophic methanogens are inhibited by approximately 1,500 mg $L^{-1}$ of the same fatty acid. In general, after a 48-h incubation period, the threshold concentration of LA, OA, or LCFA mixtures affecting hydrogenotrophic methanogens is approximately 1,500 mg $L^{-1}$. The half-life data and the hydrogen versus time profiles indicate that the susceptibility of hydrogenotrophic methanogens in a flocculated culture is dependent on the type of LCFAs, the fatty acid concentration, and exposure time.

Cultures exposed to LCFAs for 1 h were not significantly inhibited compared to those exposed for 48 h. Because LCFAs are not very soluble in an aqueous medium, adsorption onto surfaces is inefficient. With an increase in the incubation time to 48 h, larger amounts of LCFA molecules will likely adhere to cellular surfaces and as a result, an increase in inhibition is observed.

The effect of LCFAs on different microbial populations is dependent on the length of the carbon chain and the number of double bonds in the molecular structure (Demeyer and Hendrickx, 1967). Several studies have reported LA imposes a greater inhibitory effect than OA on many anaerobic microbial populations (Lalman and Bagley, 2002; Lalman and Bagley, 2001; Lalman and Bagley, 2000). The Tukey's analysis of the reaction half-life data provides evidence that LA caused a greater inhibitory effect on hydrogen degradation compared to OA. These findings are substantiated by data from similar studies in which different substrates were utilized. Studies by Lalman and Bagley (2002) have reported that LA exerted a more profound inhibitory effect than OA or SA on microorganisms consuming glucose or butyrate. In this study, a threshold inhibitory LCFA concentration is evident from inspecting the hydrogen versus time profiles and from the data in Tables 2 and 3. The data trend clearly indicates that LA has a larger inhibitory effect on hydrogen metabolism compared to OA and SA.

Mixtures of LA/OA, OA/SA, and LA/OA/SA also demonstrated that a threshold concentration inhibited hydrogen metabolism. In cultures inoculated with threshold levels of the tertiary LCFA mixture, 50−60% of the hydrogen remained in the headspace. In comparison to the control cultures, this confirms the inhibition caused by LCFAs on hydrogen metabolism.

LCFAs inhibit microorganisms via several mechanisms (Lalman, 2000). Before acting on various microbial cellular components, LCFAs must first contact the cell surface and transverse the cellular membrane (Mangroo et al., 1995). LA and OA sodium salts with excellent dispersion characteristics will likely have a greater probability of contacting and entering the cell in comparison to a solution containing SA. Hence, the expected inhibitory trend for the individual LCFAs on hydrogen metabolism is LA > OA > SA. A sodium salt mixture of LA and OA inhibited hydrogen metabolism more compared to mixtures of LA and SA or OA and SA because of the dispersion and inhibitory properties of LA and OA.

Through the association of a number of enzymes, methanogens reduce carbon dioxide to produce methane. The complete LCFA inhibitory mechanism in microorganisms is unknown. However, several possibilities exist for their action on different microbial structures and processes. LCFA could inhibit enzymes in the hydrogen metabolic pathway or they may interfere with cellular surface membrane functions (Mangroo et al., 1995). Before entering the cell, LCFAs may disrupt several surface membrane components and inactivate many energy-linked reactions. For example, they interfere with $H^+$, $K^+$, $Na^+$ regulator proteins and other cell proteins involved with maintaining cell homeostasis (Cheerington et al., 1991).

## Conclusions

The effects of individual and mixtures of LA, OA, and SA on hydrogen degradation by a flocculated culture consisting of a mixed anaerobic population were examined under anaerobic conditions at 21 °C. The conclusions of this study are:

- Under the conditions examined, the hydrogen profiles for cultures exposed to LCFAs for 1 h were similar. However, when the cultures were exposed for 48 h, the hydrogen degradation inhibitory trend was LA > OA > SA.
- The inhibition of hydrogen metabolism was a function of the LCFA concentration, the type of LCFA, and the incubation time for cultures inoculated with LA, OA, LA/OA, LA/SA, OA/SA, and LA/OA/SA over a 48-h period. Exposing the culture to SA for 48 h did not influence the hydrogen removal profile or the reaction half-life.
- A statistical analysis of the half-life values (Table 3) clearly established an inhibition trend and threshold levels for cultures inoculated with individual LCFAs.
- After incubating the culture for 48 h, hydrogen metabolism was inhibited more in the presence of LA and OA mixture compared to cultures inoculated with LA plus SA or OA plus SA mixtures.
- The greatest inhibition and hence, the largest amount of hydrogen accumulation was observed only in cultures inoculated with 2,000 mg $L^{-1}$LA for 48 h.
- The inhibition trend for cultured inoculated with LCFA mixtures for 48 h was unclear, however, the effect caused by 1,500 and 2,000 mg $L^{-1}$ of the fatty acid mixtures on hydrogen degradation was less than that imposed by the same levels of LA.

## Acknowledgment

Financial assistance for this work was provided by the Natural Sciences and Engineering Research Council of Canada.

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Accepted for publication December 6, 2005.

BP050304V