EFFECT OF STEARIC, OLEIC AND LINOLEIC ACIDS ON THE ANAEROBIC FERMENTATION OF GLUCOSE AND LACTOSE

By

IRINA V. KOMJAROVA

Engineer

Mendeleyev University of Chemical Technology of Russia

Moscow, Russia

1997

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2002

EFFECT OF STEARIC, OLEIC AND LINOLEIC ACIDS

ON THE ANAEROBIC FERMENTATION OF

GLUCOSE AND LACTOSE

Thesis Approved:

Jerald Lalman

Thesis Adviser

Daniel E. Bellmer

John M. Clarkson

Timothy J. Pettitone

Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to express my gratitude to my major advisor, Dr. Jerald Lalman for his supervision and guidance and my committee members Drs. William Clarkson and Danielle Bellmer for serving in my committee. I wish to thank Hector Cumba for providing support assistance during this work.

I would like to give my special appreciation to my parents and my husband, Ivan, for love, support and encouragement at this difficult time. I would not make it without you.

Finally, I would like to thank the Department of Biosystems and Agricultural Engineering for financial support.

TABLE OF CONTENTS

Chapter **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

1.0 INTRODUCTION

1.1 Context

Anaerobic technology is widely used to treat industrial wastewaters with a high organic load. However, treating waste with high concentration of lipids (fats, oils and greases) has been problematic due to methanogenic inhibition and sludge flotation. Methanogenic inhibition is caused by toxic effects of long chain fatty acids (LCFAs). LCFAs are slowly biodegradable. For example, Lalman *et.al* (submitted) observed stearic acid degradation rates between 0.31 to 3.11 μg LCFA·mg $VSS^{-1}\cdot d^{-1}$ and linoleic acid degradation rates between 2.32 to 12.16 μg LCFA·mg $VSS^{-1}\cdot d^{-1}$. Sludge flotation followed by washout is another problem caused by the adsorption of lipids around the biomass particles. Although the majority of lipid-containing effluents receive some form of preliminary physico-chemical treatment before entering the biological stage, such as trapping, flotation or intercepting, the remaining colloidal and emulsified lipid particles are still able to inhibit the microorganisms (Foster, 1992). Several researchers have examined the influence of LCFAs on anaerobic treatment. Factors such as toxicity and biodegradability, prior adaptation, sludge origin, physical parameters and waste composition have been investigated. Several researchers have used individual LCFAs or their mixtures as the only carbon source (Koster and Cramer, 1987; Hwu *et al.,* 1996; Hanaki *et al.,* 1981; Angelidaki and Ahring, 1992). However, industrial wastewaters are complex and contain carbohydrates and/or proteins in addition to lipids. Consequently, fermentation of carbohydrates might be affected by LCFAs. Some evidence describing the effect of LCFAs on carbohydrate fermentation has been documented. Laman and

Bagley (2002) and Hanaki *et.al.*(1981) have reported glucose fermentation in the presence of LCFA at 21°C and 37°C, respectively. However, the degradation of mono- and disaccharides in the presence of LCFA at mesophilic conditions has not been examined. The objective of the present work is to investigate the effect of different C18 LCFAs on carbohydrate degradation and to determine kinetic parameters of glucose degradation at 37°C.

1.2 General Principles of Anaerobic Digestion

Anaerobic digestion is a biological process, where organic compounds are degraded by a population of microorganisms in the absence of oxygen. Final products of this process are methane, carbon dioxide and water. Organisms mediating the process include hydrolytic acidogens, acetogens and methanogens.

Organic polymers are first hydrolyzed into monomers. For example, lipids are hydrolyzed to glycerol and LCFA, proteins are degraded to a variety of amino acids and polysaccharides are converted to sugar monomers. In the next step, acidogens convert monomeric substrates into volatile fatty acids (VFAs), alcohols, carbon dioxide and hydrogen gas. In the third step called acetogenesis, the organic fermentation products (higher fatty acids) are oxidized to acetic acid, carbon dioxide and hydrogen by acetogenic bacteria. Hydrogen ions or carbon dioxide serve as electron acceptors. The resulting products serve as substrates for hydrogenotrophic methanogens. Finally, acetoclastic methanogens degrade acetate into methane and carbon dioxide.

The organisms involved in the hydrolysis and acidogenesis grow relatively rapidly. The fermentation processes give greater energy yield compared to the methane

formation reaction (McCarty, 1964). For this reason, methanogens are more slowly growing organisms. Low cell yield makes methanogens to be rate-limiting organisms in the system. In addition, they are pH sensitive with a working range between 6.5 and 7.6. Another important requirement for the growth of anaerobic microorganisms is sufficient amount of macronutrients, such as nitrogen (5-15 $mg \cdot g^{-1}$ COD, desired excess 50 $mg \cdot g^{-1}$ COD), phosphorus (0.8-2.5 $mg \cdot g^{-1}$ COD, desired excess 10 $mg \cdot g^{-1}$ COD), and sulfur (1-3 $mg \cdot g^{-1}$ COD). Trace amounts of heavy metals are needed for enzyme activation. For example, iron, cobalt and nickel are key enzymes for methane producing microorganisms. Other important trace metals are zinc, copper, manganese, molybdenum, and selenium.

1.3 Anaerobic Degradation of Lipids

Agricultural and food industries, such as dairy, oil and fat refining, margarine, palm oil processing and wool scouring are producers of high-strength (more than 100 $mg \cdot l^{-1}$) lipid-containing wastewater (Hwu *et al.*, 1998). In contrast, domestic sewage also contains lipids, but in lower concentration between 40 to100 $mg \cdot l^{-1}$. Depending on their physical properties, lipids behave differently during anaerobic treatment. Therefore, it is important to review the structure and physical properties of lipids.

1.3.1. *Structure and Physical Properties of Lipids*

Lipids are a diverse group of biological compounds characterized by their insolubility in water and high degree of solubility in non-polar organic solvents. Dietary lipids are mostly fatty acid esters of glycerol, called triglycerides (Figure.1.1). The

majority of naturally accruing lipids contain unbranched saturated or unsaturated long chain fatty acids with even number of carbon atoms, typically between 12 to 24.

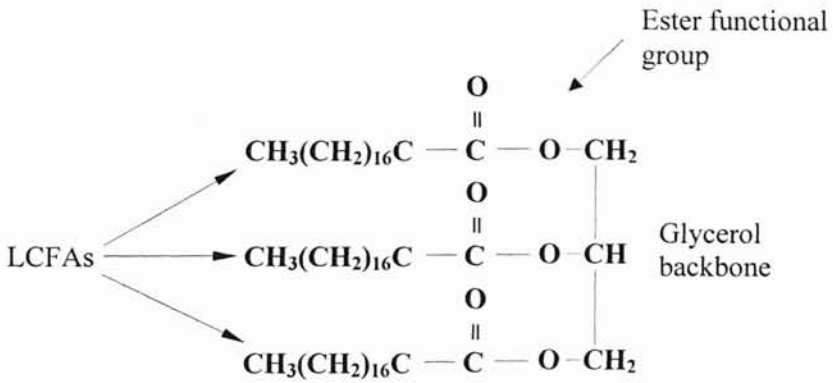

Figure 1.1: Structure of a triacylglycerol (Lehninger *et al.*, 1999)

Under anaerobic conditions, triglycerides are hydrolyzed into glycerol plus three free fatty acids. Physical properties of LCFA depend on the length of carbon chain and the degree of saturation. Melting point decreases with shorter carbon chain and higher number of double bonds. Animal fats are primarily composed of saturated fatty acids, which are solid at room temperature. Vegetable oils, on the contrary, are unsaturated fatty acids, so they are liquid at room temperature. Table 1.1 shows typical fatty acid composition of several dietary lipids (Altman and Ditmer, 1964)

1.3.2. *Anaerobic Degradation of Lipids*

Under anaerobic conditions, fats and oils are hydrolyzed to glycerol and LCFA (saturated fatty acids with 12-16 carbon atoms and unsaturated fatty acids with 18 carbon atoms). Previous work by Hanaki *et.al.*(1981) has shown that hydrolysis is not a limiting

step during anaerobic degradation. The rate-limiting step is the degradation of LCFAs to acetic acid and hydrogen gas by β-oxidation mechanism (Pavlostathis, 1991).

Table 1.1: Fatty acid composition of selected fats and oils (mole %)

LCFA	Animal Fats		Vegetable Oils				
	Lard	Beef tallow	Olive	Peanut	Corn	Soybean	Linseed
Myristic acid C_{14}	1-2	2-5	0-1		1-2	1-2	
Palmitic acid C_{16}	25-30	24-34	5-15	7-12	7-11	6-10	4-7
Stearic acid $C_{18:0}$	12-18	15-30	1-4	2-6	3-4	2-4	2-4
Oleic acid $C_{18:1}$	48-60	35-45	67-84	30-60	25-35	20-30	14-30
Linoleic acid $C_{18:2}$	6-12	1-3	8-12	20-38	50-60	50-58	14-25
Linolenic acid $C_{18:3}$	0-1	0-1				5-10	45-60

The mechanism of LCFA degradation consists of the following steps: transport and activation, formation of acyl-CoA intermediate and β-oxidation (Batstone, 1999). Adsorption of LCFA onto the surface of microorganisms precedes their bioconversion. Oxidation of fatty acids occurs in the mitochondria. In order to be degraded, first, LCFAs must be activated in the cytoplasm. This process requires an energy source in form of ATP and is performed only once per fatty acid degraded. After the activation, fatty acid is transported into the mitochondria, where β-oxidation occurs. The β-carbon is oxidized

from CH_2 to C=O by three reactions and oxidized β group becomes susceptible for the attack. The fatty acid is cleaved into *acetyl-CoA* plus a shorter chain fatty acid with two carbon atoms removed. The cycle repeats until the LCFA is completely converted to acetic acid. Oxidation of fatty acids with an odd number of carbon atoms will eventually yield acetyl-CoA and propionyl-CoA. Finally, methanogenic microorganisms use acetate, carbon dioxide and hydrogen to produce methane. To illustrate the β-oxidation mechanism, the conversion of stearic acid to methane and carbon dioxide is presented as follows:

$$CH_3 - (CH_2)_{14} - {}^{\beta}\mathbf{CH_2} - CH_2 - COOH \quad (1)$$

$$\downarrow$$

$$CH_3 - (CH_2)_{14} - {}^{\beta}\mathbf{CH_2} - CH_2 - COO\text{-}S\text{-}CoA$$

$$\downarrow$$

$$CH_3 - (CH_2)_{14} - {}^{\beta}\mathbf{CO} - CH_2\text{-}COO\text{-}S\text{-}CoA$$

$$\downarrow$$

$$CH_3 - (CH_2)_{14} - {}^{\beta}\mathbf{COO}\text{-}S\text{-}CoA + CH_3 - COO\text{-}S\text{-}CoA$$

$$\vdots \quad \textit{Cycle repeats}$$

$$CH_3 - COO\text{-}S\text{-}CoA + CH_3 - COO\text{-}S\text{-}CoA$$

Overall: $C_{18}H_{36}O_2 + 16\ H_2O \rightarrow 9\ CH_3COOH + 16\ H_2$

$9\ CH_3COOH \rightarrow 9\ CH_4 + 9\ CO_2$

$4\ CO_2 + 16\ H_2 \rightarrow 4\ CH_4 + 8\ H_2O$

Finally: $C_{18}H_{36}O_2 + 8\ H_2O \rightarrow 13\ CH_4 + 5\ CO_2$ (1)

β-oxidation of unsaturated fatty acids follows the same pathway with prior hydrogenation of the double bond; stearic acid was detected as a byproduct of oleic acid degradation (Komatsu and Hanaki, 1991; Lalman and Bagley, 2001).

The oxidation of fatty acids yields significantly more energy per carbon atom than does the oxidation of carbohydrates. For example, oxidation of 1 mole of oleic acid (18 carbon atoms) results in the generation of 146 moles of ATP (2 moles are used during the activation process), while oxidation of 3 moles of glucose (18 carbon atoms total) yields 114 moles of ATP in aerobic pathway and 6 moles of ATP in anaerobic fermentation (Solomons, 1992).

1.4 Toxicity of Long-Chain Fatty Acids

1.4.1 *LCFA Structure and Concentration*

LCFAs have been reported to inhibit several Gram-positive microorganisms and methanogenic bacteria, which have the cell wall structure similar to Gram-positive organisms, at concentrations between 0.02 to 2 mM (Galbraith and Miller, 1971). Koster (1987) reported LCFA inhibition is a rapid process with 50% of the methanogenic activity lost after 7.5 minutes. Recovery of an anaerobic system took place after a few months and was attributed to the growth of surviving organisms. Inhibitory effects of LCFAs have been reported to have physico-chemical properties (Hwu *et al.,* 1998). Adsorption of LCFAs onto cell surfaces causes alteration of protective and transporting functions of cells.

LCFA inhibitory effects have been reported to increase with the number of double bonds and type of isomers (Rinzema, 1988). To explain this phenomenon, consider the

1.4.2 *Influence of Sludge Origin on the Anaerobic Biodegradability of LCFAs*

Sorption of LCFAs onto cell wall and cell membrane is the main mechanism of inhibition. Disruption of the cell membrane function limits the transport of substrates and products in and out of the cell (Galbraith and Miller, 1973). Sorption of fatty matter is a relatively rapid process. Approximately 80% of lipids can be adsorbed by an activated sludge within 20 minutes (Hwu *et al.*, 1998). Hanaki *et al.*, (1981) observed LCFAs disappeared from the aqueous phase and accumulated onto a solid phase within 24 hours of incubation. Petruy and Lettinga (1997) reported 70% of the LCFAs they added were adsorbed within 24 hours.

Hwu *et al.* (1998) reported adsorption of an LCFA mixture containing 35% palmetic, 15% stearic and 50% oleic acids took place within 3 hours and 90% of LCFAs were removed from solution. Based on these observations, Hwu *et al.* (1998) suggested that the sorption process could be described by a multilayer adsorption theory. They also proposed a hypothesis explaining the relationship between biosorption and biodegradation of LCFAs. According to the proposed theory, after introduction into a bioreactor, LCFAs rapidly adsorb onto granular biomass surfaces forming multilayers. No significant methane production and no biodegradation occur at this stage. LCFA adsorption is concentration dependent, therefore, at high LCFA concentrations large amounts are adsorbed with time. This causes long lag periods prior to methanation. LCFA desorption from the biomass surface could occur next with a subsequent disappearance of LCFA from the aqueous phase. The process is accompanied by methane production. Finally, biodegradation of all adsorbed LCFA into methane takes place.

A logical conclusion from the adsorption theory is that granulated biomass might be less affected by LCFAs when compared to suspended or flocculated biomass. LCFAs adsorb onto the surface of a sludge granule and subsequently affect the microorganisms residing inside the granule. Hwu *et al.* (1996) reported IC_{50} values of sodium oleate (the concentration at which 50 % of microorganisms are inhibited) for 5 different granular sludges were between 1.75 to 3.34 mM (494 to 942 $mg \cdot l^{-1}$). For flocculent and suspended cultures, IC_{50} values were between 0.53 and 0.26 mM (150 and 73 $mg \cdot l^{-1}$respectively). Hanaki *et al.* (1981) and Alves *et al.* (2001) reported IC_{50} values of sodium oleate for suspended cultures between 10 to 295 $mg \cdot l^{-1}$. In comparison, Koster and Cramer (1987) also reported a conflicting value of 1322 $mg \cdot l^{-1}$ for granular sludge. However, data reported by Koster and Cramer (1987) may be attributed to the high calcium levels added to the medium. In the presence of calcium, LCFAs form insoluble salts. Thus, the concentration of free LCFA was less than assumed.

1.4.3 *Influence of pH on LCFA toxicity*

The activity of LCFAs is highly dependent on the pH of the medium. Galbraith and Miller (1973) examined the influence of pH on the inhibitory effect of LCFAs toward Gram-positive aerobic bacteria. Since the structure of the cell wall of methanogens resembles that of Gram-positive organisms, conclusions derived from these studies could be extrapolated to anaerobic organisms. A molecule of fatty acid consists of hydrophilic and hydrophobic moieties. The hydrophilic part of a molecule is responsible for the solubility of LCFA and the hydrophobic properties are important for uptake into bacterial cells. According to Galbraith and Miller (1971), the balance between hydrophilic and

hydrophobic functionality of a LCFA molecule is pH dependent. The solubility of LCFA increases with higher pH values. Experimental data reported by Galbraith and Miller (1971) showed that at pH 6 lauric acid (C_{12}) was more toxic to methanogens when compared to myristic (C_{14}), palmitic (C_{16}) or pentadecanoic (C_{15}) acids, but at pH 8 lauric acid was less active. Thus, with increasing pH, the toxicity of longer chain LCFAs is increasing because of their greater solubility and greater degree of ionization at higher pH than at lower pH.

A few studies have reported LCFA degradation under different pH conditions. Beccari *et al.*(1996) examined the anaerobic degradation of oil mill effluent and found that pH 8.5 was the most optimum condition with an initial lipid concentration 0.96 $g \cdot l^{-1}$. Decreasing the pH to 6.0 caused a dramatic decrease in VFA production. Similar results were reported by Komatsu and Hanaki (1991), who noticed greater inhibitory effect of oleic, linoleic and myristic acids at neutral pH values than at pH 8.3.

1.4.4 *Influence of prior sludge acclimatization on the biodegradability of LCFAs*

Culture adaptation to LCFAs may increase its tolerance to elevated levels of LCFAs. Rinzema *et al.* (1994) suggest that the recovery after a lag phase observed in batch assays is attributed to the growth of a few surviving organisms. In another study, Hwu *et al.* (1997) observed a long lag period of methane production for non-acclimated granular sludge fed with oleate compared to acclimated biomass. In continuous flow experiments, a reactor inoculated with the non-acclimated sludge failed at influent concentrations of 500 mg COD LCFA$\cdot l^{-1}$, while a reactor with pre-exposed sludge was successfully able to treat an influent containing 4000 mg COD LCFA$\cdot l^{-1}$. In work

reported by Alves *et al.*(2001), the performance of a control reactor fed with non-fat substrate was compared to the performance of the reactor gradually shifted from the original composition to oleate as a sole carbon source. They showed that gradual increase of the oleate concentration in the influent from 0 $mg \cdot l^{-1}$ to 1968 $mg \cdot l^{-1}$COD oleate produced a biomass more tolerant to oleic acid (IC_{50}=300 mg COD oleate$\cdot l^{-1}$), compared to sludge in a control digester (IC_{50}=140 mg oleate COD$\cdot l^{-1}$). An increasing oleic acid biodegradation capacity was observed only for the acclimated sludge. Maximum methane production rates were reported between 33 and 46 ml $CH_4 \cdot gVS^{-1} \cdot day^{-1}$. Maximum percentages of methanization (between 85 and 98%) were obtained for oleate concentration between 500 and 900 $mg \cdot l^{-1}$

In contrast, Canovas-Diaz *et al.* (1992) showed that the inoculum acclimated with LCFAs over a 40 day period and then reused for additional batch experiments, was less effective in degrading LCFAs when compared to unacclimated inoculum. It was suggested that the sludge lost valuable enzymes contained in fresh sludge during the inoculation process.

1.4.5 *Temperature Effect*

The rate of metabolic reactions increase with increasing temperatures. Hence, increasing the temperature of a digester may improve LCFA degradation rates. On the other hand, higher temperatures increase the detergent/surfactant toxicity of LCFAs and their salts, which results in a higher degree of cell lysis. However, the surfactant effect of LCFAs on methanogenic organisms can be less pronounced due to the structure of their cell wall. To prove this assumption, Hwu and Lettinga (1997) performed a toxicity study

using oleic acid on acetate-utilizing methanogens with four different anaerobic sludges (three granular and one flocculent). The temperatures investigated were 30°C, 40°C and 55°C. They reported the toxic effect of oleic acid increases with increasing temperature. Under thermophilic conditions, IC_{50} values they reported for granular biomasses were between 0.35 to 0.79 mM, and 2.35 to 04.30 at 30°C. The flocculent sludge showed the same trend, yet the toxic effect of oleate was 12 times higher. In addition, Hwu and Lettinga (1997) reported there is no relationship between the methanogenic activity and oleate toxicity.

Becarri *et al.* (1996) also investigated the influence of temperature on the anaerobic digestion of olive mill effluent. At 35°C biodegradation of lipids in olive mill effluent was higher than at 25°C. In contrast, studies conducted by Broughton *et al.* (1998) reported a negative influence of thermophilic conditions on the degradation of LCFAs. They found sheep tallow, composed of mostly oleic and stearic acids, was rapidly degraded at 35°C, but the fermentation process was refractory at 50°C. Angelidaki and Ahring (1995) were able to enrich a stable anaerobic stearate ($C_{18:1}$) degrading culture, which appeared to be thermophilic (optimum growth temperature was 55°C). Propionate, acetate and methane production were monitored to determine if LCFA biodegradation had occurred. The culture degraded oleic($C_{18:1}$) palmitic (C_{16}) , capric (C_{10}) and other LCFAs with a lower number of carbon atoms.

1.4.6 *Addition of Cosubstrates*

Thermodynamically, degradation of LCFA is an unfavorable process as illustrated in reaction 2.

$$C_{18}H_{36}O_2 + 2\,H_2O \rightarrow C_{16}H_{32}O_2 + CH_3COO^- + H^+ + 2H_2 \quad \Delta G' = +48.1 \text{ kJ/mol} \qquad (2)$$

Consequently, presence of easily biodegradable cosubstrate, such as glucose, would supply energy for both cell growth and biodegradation of LCFA. In addition, essential enzymes would be provided to mediate the degradation process. Experimental data show the biodegradation of LCFA was more efficient in the presence of cosubstrates. Alves *et al.* (2001) used skim and whole milk as cosubstrates, and they were able to acclimate the inoculum to LCFAs over a long time period. Vidal *et al.*(2000) used a synthetic feeding mixture consisting of fats, proteins and carbohydrates in COD proportion 1.7 : 0.57 : 1 with total COD ranging from 1 to 20 $g \cdot l^{-1}$. They observed approximately 99 % oleic acid removal with initial concentration between 0.9 $g \cdot l^{-1}$ to 1.5 $g \cdot l^{-1}$.

Beccari *et al.* (1996) compared degradation rates of oleic versus a mixture of glucose plus oleic acid at concentrations of 6$g \cdot l^{-1}$ and 3$g \cdot l^{-1}$ respectively. The presence of glucose activated the degradation of oleic acid, yielding a decrease of oleic acid in the solution from 1.19 $g \cdot l^{-1}$ to 0.13 $mg \cdot l^{-1}$ during a 66-day period.

1.5 Carbohydrate Fermentation

Anaerobic fermentation of carbohydrates is a complex process which involves acidogenic, acetogenic and methanogenic organisms. Under anaerobic conditions, complex carbohydrate polymers are first hydrolyzed to simple sugars. Then, sugar monomers are converted into ethanol, formic, acetic, propionic, butyric and lactic acids by acidogenic organisms with subsequent conversion to methane and carbon dioxide (Zoetemeyer *et al.*, 1982). In the presence of hydrogen utilizing microorganisms, a

reduction in ethanol and an increase in acetate production is observed (Pavlostathis, 1991).

Glucose fermentation is carried out by a variety of microorganisms and each population mediates the conversion of glucose to a different product. Kalyuzhnyi and Davlyatshina (1997) examined the stoichiometry of glucose fermentation at 37°C and initial pH 7. The following equation was adapted from Kalyuzhnyi and Davlyatshina (1997):

$$C_6H_{12}O_6 + 0.12\ H_2O \rightarrow 0.92\ H_2 + 1.14\ CO_2 + 0.34\ C_2H_5OH +$$
$$1.31\ CH_3COOH + 0.24\ C_3H_7COOH + 0.2\ C_2H_5COOH \qquad (3)$$

During anaerobic digestion of formed intermediates, ethanol, propionate and butyrate are transformed into acetate and hydrogen gas. However, relative amounts of glucose fermentation products are pH dependent. Zoetemeyer *et al.* (1982) reported butyric acid as a major product of glucose fermentation at pH 5.7. However, at pH 6.4, the concentration of butyric acid decreased, while the concentrations of lactic and formic acids increased. Changes in pH values did not affect acetate and ethanol production.

Kisaalita *et al.* (1989) studied metabolic pathways of lactose fermentation and proposed a model of lactose degradation. According to Kisaalita *et al.* (1989), during anaerobic fermentation, lactose is first broken down to glucose and galactose, which are subsequently converted into pyruvate. Pyruvate is then transformed into butyrate and lactate. In the presence of hydrogen reducing methanogens, lactate is rapidly into acetate. The process is accompanied by a low propionate production. Butyrate is also transformed into acetate. Finally, acetate, hydrogen and carbon dioxide are converted into methane.

1.6 Objectives

Data on the effects of individual LCFAs on the fermentation of monosaccharides and disaccharides at 37°C is limited. Therefore, the objectives of this study are:

1) To investigate fermentation of glucose at 37°C in the presence of 50 to1000 $mg \cdot l^{-1}$ C18 LCFAs
2) To investigate lactose fermentation at 37°C in the presence of 50 to1000 $mg \cdot l^{-1}$ C18 LCFAs.

2.0 MATERIALS AND METHODS

2.1 Experimental plan

Experiments were designed to achieve the objectives and a design matrix is presented in Table 2.1 Each condition examined was conducted in triplicate sets. Parameters monitored included glucose, acetate, propionate, butyrate, methane, and LCFAs concentrations.

Table 2.1: Experimental outline

LCFA	LCFA Concentration, $mg \cdot l^{-1}$						
	0	50	100	300	500	700	1000
SA	G	G	G	G	G	G	G
OA	G	G	G	G	G	G	G
LA	G	G	G	G	G	G	G
SA	L	L	L	L	L	L	L
OA	L	L	L	L	L	L	L
LA	L	L	L	L	L	L	L

Note: Glucose and lactose concentrations were 2000 $mg \cdot l^{-1}$
G = glucose, L = lactose, SA = stearic acid, OA = oleic acid, LA = linoleic acid

2.2 Reagents

During each experimental condition examined, D-(+)glucose anhydrous (reagent grade, Spectrum Quality Products, Inc., Gardena, CA) and LCFA (Lancaster Synthesis, Pelham, NH) were used as substrates. The gas chromatograph (GC) (Agilent Technologies 6890) was equipped with flame ionization and thermal conductivity detectors. The GC was calibrated with linoleic ($C_{18:2}$) (99%), oleic ($C_{18:1}$) (99%, Aldrich Chem. Co., Milwaukee, WI), stearic ($C_{18:2}$) (99%), palmitic ($C_{16:0}$) (95%), myristic ($C_{14:0}$)

(98%), lauric ($C_{12:0}$) (98%), capric ($C_{10:0}$) (99%) and caprilic ($C_{8:0}$) (98%) acids (Lancaster Synthesis, Pelham, NH) dissolved in n-hexane (HPLC grade, Pharmco Products Inc., Brookfield, CT). For LCFA extraction a 50%:50% mixture of n-hexane and methyl-tertiary-butyl ether (MTBE) (HPLC grade, Spectrum Quality Products, Inc., Gardena, CA) was used. Methane (100%, Matheson Tri-Gas, Twinburg, OH) was used to calibrate GC for the head space analysis. Carrier gases were nitrogen (99.999%) and helium (99.999%) (UHP grade, Stillwater Steel and Supply, Stillwater, OK).

The Dionex DX-600 Ion Chromatograph (IC) was equipped with an AS 40 autosampler, a gradient pump (GP40), conductivity detector (ED50) and integrated amperometry detector (ED50). The IC was calibrated with acetic and propionic acids (reagent grade, Spectrum Quality Products, Inc., Gardena, CA), butyric acid (99+%, Lancaster Synthesis, Pelham, NH) and lactic acid (85%, Spectrum Quality Products, Inc., Gardena, CA). Calibrations were also conducted with glucose, lactose and galactose (all 99+%, Lancaster Synthesis, Pelham, NH).

2.3 Batch Reactors

Anaerobic cultures were obtained from the Stillwater Wastewater Treatment plant. Cultures were diluted with basal medium (1:4) and placed in the main semi-continuous 4-L reactor (reactor A1). Total suspended solids (TSS) and volatile suspended solids (VSS) were 30,000 and 25,000 $mg \cdot l^{-1}$respectively. Biomass from the main reactor (A1) was diluted with basal medium to 2000 $mg \cdot l^{-1}$ VSS into a second 4-L semi-continuous reactor designated as A2. Anaerobic conditions and an operating temperature of 37°C were maintained in both reactors. Serum bottles (160 ml) for individual batch

experiments were prepared using the inoculum from reactor A2. Preparation of the basal medium composition was adapted from Lalman and Bagley (2002) and had a pH between 7.8 to 8.0

Table 2.2: Basal medium composition

Compound	Concentration, $mg \cdot l^{-1}$
$NaHCO_3$	6000
$(NH_4)SO_4$	4.125
Heavy metal stock	0.5 ml
Resazurin	1.0
Yeast extract	100
K_2HPO_4	14.032[a)]
NH_4HCO_3	81.82[a)]
$FeCl_2 \cdot 4H_2O$	156
$Na_2S \cdot 9H_2O$	307

Notes: a) -amounts of NH_4HCO_3 and K_2HPO_4 were calculated based on the ratio of COD : N : P = 100 : 1.35 : 0.25

Table 2.3: Heavy metal stock solution composition

Compound	Concentration, $mg \cdot l^{-1}$
$MgCl_2 \cdot 4H_2O$	9
KCl	25
H_3BO_3	0.05
$FeCl_2 \cdot 4H_2O$	2
$ZnCl_2$	0.05
$MnCl_2 \cdot 4H_2O$	0.5
$CuCl_2 \cdot 2H_2O$	0.03
$(NH_4)_6MoO_7 \cdot 4H_2O$	0.09
$CoCl_2 \cdot 6H_2O$	0.15
$NiCl_2 \cdot 6H_2O$	0.05
Na_2SeO_3	0.1
EDTA	1.0

2.4 Inoculum Reactors Operation

Reactors A1 and A2 were operated in batch mode. Reactor A1 was fed with 7 $g \cdot l^{-1}$ glucose and 100 ml of basal medium every other day (time when VFA and gas measurements indicated all glucose and byproducts were consumed). Reactor A2 was fed with 2.0 $g \cdot l^{-1}$ glucose and 100 ml of basal medium once every 4 days. Both reactors were monitored for pH, alkalinity (as $CaCO_3$), TSS, VSS and VFA.

2.5 Serum Bottles Preparation

Degradation and inhibition studies were conducted in 160 ml serum bottles at 37°C with a total liquid volume of 100 ml. Serum bottles inoculations were conducted inside a Coy® anaerobic chamber (Coy Laboratory Products, Inc.) under an 80% N_2 and 20% CO_2 atmosphere. Varying biomass aliquots were added to the serum bottles to provide a total volume of 100 ml after addition of LCFAs and glucose stock solutions. Volume of glucose solution added was constant (4 ml of a 50,000 $mg \cdot l^{-1}$ solution) and amount of LCFAs added varied. For example, to provide 50,100, 300, 500, 700 and 1000 $mg \cdot l^{-1}$ of individual LCFA in 100 ml of solution using 10,000 $mg \cdot l^{-1}$ of a stock solution, 0.5, 1, 3, 5, 7 and 10 ml of stock were added. Control bottles contained 96 ml of the biomass. All conditions were prepared in triplicate bottles. After inoculation, the bottles were sealed with Teflon®-lined silicon rubber septa and aluminum crimp caps and pressurized with 20 ml of gas mixture (80% N_2 and 20% CO_2) to avoid the formation of a negative pressure in the headspace during sampling.

2.6 Gas Measurements

Analysis of a serum bottle headspace for methane (20 µl samples) was conducted using a GC (Agilent 6890) equipped with thermal conductivity detector (TCD). The column was a capillary CARBOXEN™ 1006 PLOT (Supelco) 30 m × 0.53 mm column with a 0.01 mm film thickness. Inlet and detector temperature conditions were 200°C and 250°C respectively. Nitrogen was used as a carrier gas at a flow rate of 10 ml·min^{-1}.

Gas calibration standards were prepared in 160 ml serum bottles. After purging with nitrogen (99.99%) for 5 min, the bottles were sealed with Teflon® lined septa and capped with aluminum crimp seals. Then, 0.25, 0.5, 1, 3, 5, 10, 15 and 20 ml of methane were injected into the bottles. Triplicate measurements (20 µl) were taken from each bottle and a calibration curve was plotted in terms of response versus µg of carbon·ml^{-1}. Prior to the headspace analysis, three methane standards were prepared and analyzed to ensure calibration.

2.7 Volatile Fatty Acid Measurements

Volatile fatty acid (VFA) concentration was determined by ion chromatography. The Dionex DX-600 IC was equipped with a conductivity detector (ED50), AS11-HC IonPac® column, ASRS suppressor, ATC-1 trap column and 25 µl loop. Eluents used were deionized water (A), 5mM NaOH (B) and 50mM NaOH (C). Total flow was 2.0 ml·mim^{-1}. During each sample run, eluents were mixed in the following proportions: from 0 to 2 minutes - 93% A, 7% B; from 2 to 6 minutes -100% B; from 6 to 9 minutes - 50% B , 50% C; from 9 to 18.99 minutes - 50% B , 50% C and from 19 to 26 minutes 93%A, 7%B.

Acetic, propionic and butyric acids were used to prepare VFA standards. Triplicate standards (1, 2, 3, 4, 5, 10, 15, and 20 $mg \cdot l^{-1}$) were prepared in diluted 1:10 basal medium from a 5000 $mg \cdot l^{-1}$ stock solution. Standards were filtered through a cartridge containing 0.8 g of Chelex®100 resin (biotechnology grade, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA) and analyzed by IC.

To analyze for VFA concentration in the serum bottles, 1ml samples were periodically withdrawn using a 2.5 ml syringe. The aliquots were placed in test tubes containing 8 ml of de-ionized water and centrifuged at 1750 g for 5 minutes. The centrate was removed, filtered through a Chelex®100 resin cartridge and analyzed. To avoid carry over, vials with de-ionized water were placed in between every 15 to 20 samples. The retention times were 6.9, 6.5, 6.3 and 5.9 minutes for butyric, propionic, acetic and lactic acids respectively. The detection limit was 0.5 $mg \cdot l^{-1}$ for each acid.

2.8 Carbohydrate Measurements

Glucose, lactose and galactose analyses were performed using the Dionex IC equipped with a 25 cm × 4 mm CarboPac™ MA1 column. A sodium hydroxide solution (612 mM) was used as an eluent with a 0.4 $ml \cdot min^{-1}$ flow rate. The analysis time was 26 minutes and the detection times were 18.5 minutes for glucose, 21 minutes for galactose and 22 minutes for lactose. The detection limit was 1 $mg \cdot l^{-1}$.

Sample preparation was the same as for the VFA analysis, except the aliquots were filtered through a Millipore glass fiber filter paper. Triplicate standards were prepared in the same manner as for VFA analysis. Standards and vials with de-ionized water samples were placed in between every 15 to 20 samples.

2.9 LCFA Delivery Method

Long chain fatty acids are water insoluble. However, their sodium salts are soluble in aqueous solutions. Based on this property of LCFA salts, a LCFA delivery method developed by Angelidaki and Ahring (1992) was used in this study. LCFAs were combined with a known amount of 50% w/w NaOH and heated until all the LCFAs dissolved.

2.10 LCFA Measurement Method

The LCFA analysis procedure was adapted from Lalman and Bagley (2000). Since LCFAs are water insoluble, an extraction method was developed. The extraction efficiency was lowest for caprylic acid (from 80 to 85%), however, for other LCFAs the extraction efficiencies were between 88 to 94%. The extraction procedure is described below as follows: remove 1- ml aliquot from a serum bottle and add 0.05 g NaCl and 2 drops of 50% H_2SO_4. Next, add 2 ml of 50:50 hexane:MTBE mixture and shake using an orbital shaker (Lab Line Instruments, Inc. model No. 3520) for 20 min at 200 rpm, then centrifuge for 5 min at 1750 g and remove the organic phase. Analyze 1 μL of the organic phase using a GC (Agilent 6890) equipped with a flame ionization detector (FID) and 30×0.53 mm analytical column (Nukol, Supelco). Detector and injector temperatures were set at 250°C. The helium carrier gas flow was set at 13 $ml \cdot min^{-1}$ flow rate with a split ratio of 7:1. The oven temperature program was 100°C for 1 min, followed by $20°C \cdot min^{-1}$ temperature ramp until a final temperature 200°C was achieved for 5 minutes.

3.0 BATCH REACTOR OPERATION

3.1 Experimental Results

3.1.1 Carbohydrates Consumption

Glucose, lactose and galactose degradation profiles for reactor a diluted reactor A2 are shown in Figure 3.1. Complete glucose and lactose degradation was achieved within 1.5 to 2.5 hours and the degradation rates for both substrates were similar. Since reactor was not acclimated to galactose, a lag-phase was observed and no galactose was detected after nine hours (Figure 3.1)

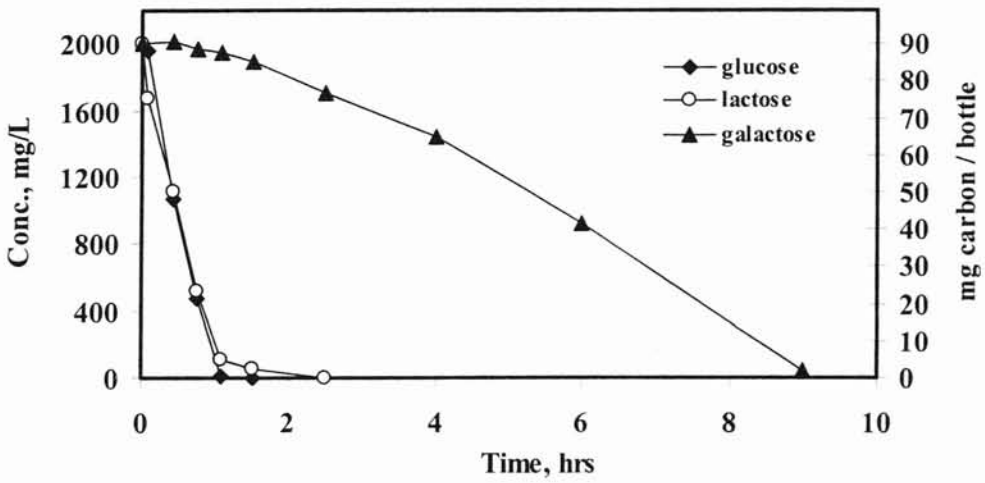

Figure 3.1: Carbohydrates degradation profiles for diluted reactors

Glucose degradation rates were within the range 21.5 to 24.6 μg substrate·VSS^{-1}·min^{-1}. Using glucose degradation rates at 25 °C reported by Lalman *et al.* (in press) and correcting for temperature using equation 4: (Rittmann and McCarty, 2001), the calculated glucose degradation rate is 24 μg substrate·VSS^{-1}·min^{-1}:

$$r_t = r_{20} * \theta^{(t-20)}, \text{ where} \qquad (4)$$

r_t – substrate removal rate at given temperature, μg substrate·VSS^{-1}·min^{-1}

r_{20}- substrate removal rate at 20°C, μg substrate·VSS^{-1}·min^{-1}

θ – temperature coefficient. For activated sludge process it is 1.04

t - temperature, °C

In the studies where lactose was used as a carbon source, two diluted reactors were used. The degree of acclimatization of these reactors to lactose was different from study to study, which significantly affected the time of complete lactose consumption by the control cultures (Figure 3.2). Initially, the biomass from Stillwater Water Treatment Plant was acclimated to lactose for one month in the main reactor A1. Then, this biomass was used to prepare a diluted reactor A2 with a VSS concentration equal to 2000 mg·l^{-1}. Reactor A2 was fed twice with lactose and then the culture was used for serum bottles inoculation. Experiments were conducted with SA and OA on 09/03/02 and 09/10/02, respectively. Immediately after preparing the serum bottles, a new diluted reactor A2 was set up to be used for conducting experiments with LA. While the SA and OA experiments were conducted, reactor A1 was fed continuously with lactose. This resulted in a longer acclimatization time of the biomass to lactose. As a result, lactose degradation time for the control cultures in the LA study conducted on 09/24/02 was less when compared to the OA and SA studies.

Because of the significant differences in the lactose controls, a decision was made to repeat the previous experiments with SA and OA. Since all biomass from the main reactor was depleted, a new diluted reactor A2 was prepared using an inoculum acclimated to glucose. The biomass was acclimated to lactose by gradually increasing the

amount of substrate to 2000 $mg \cdot l^{-1}$ before the conducting the experiment. The entire acclimation process took one and one-half weeks. Since the biomass was previously acclimated to glucose, a product of lactose hydrolysis, the removal time in the OA and SA experiments conducted on 11/12/02 and 11/13/02, respectively was approximately 6 hours (Figure 3.2). However, compared to LA study performed on 09/24/02, the removal time was longer.

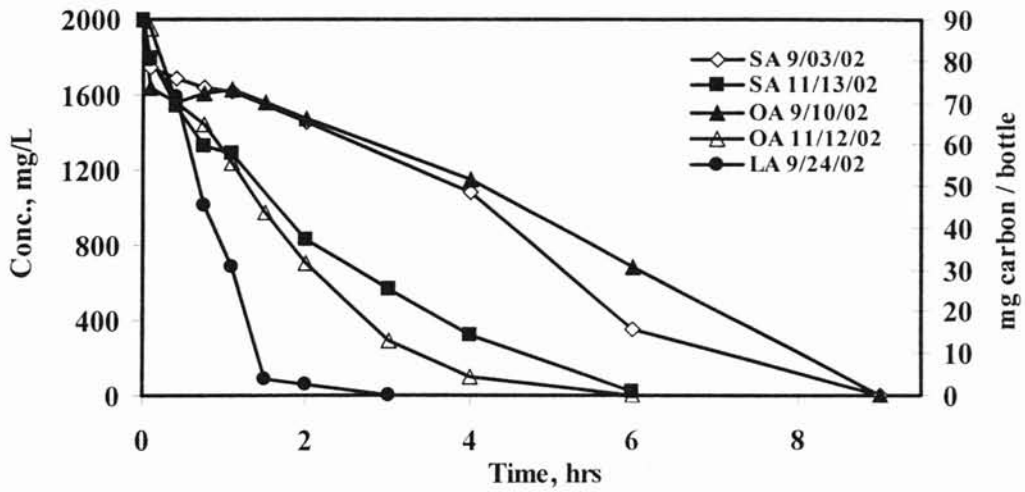

Figure 3.2: Lactose degradation profiles for control cultures

3.1.2. VFAs Production

VFA profiles for cultures fed with glucose and lactose are shown in Figures 3.3 and 3.4. Degradation of glucose resulted in the production of propionic and acetic acids. The maximum VFA concentration was observed at approximately 12 hours and within 72 hours, complete VFA degradation was observed. In contrast, lactic and acetic acids were produced during lactose fermentation with a maximum acid concentration detected at

approximately 10 hours. Complete removal of acetate, propionate and lactate was observed within 72 hours.

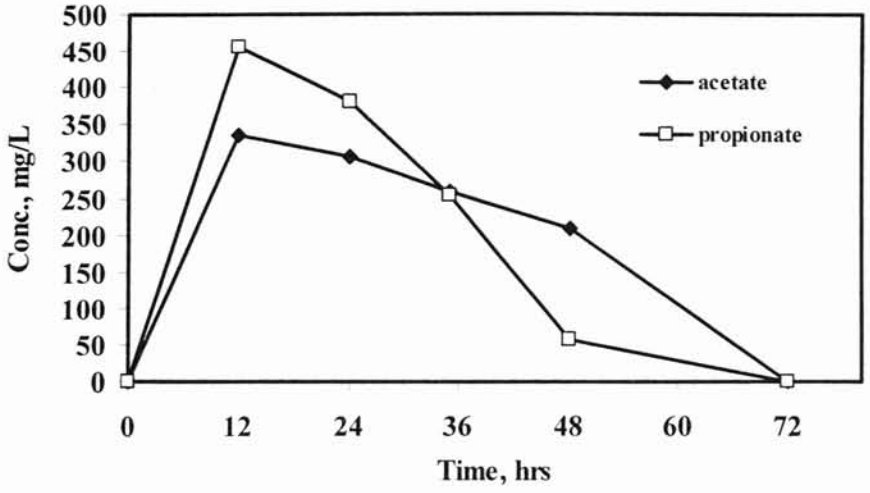

Figure 3.3: VFA profiles for the reactor fed with glucose

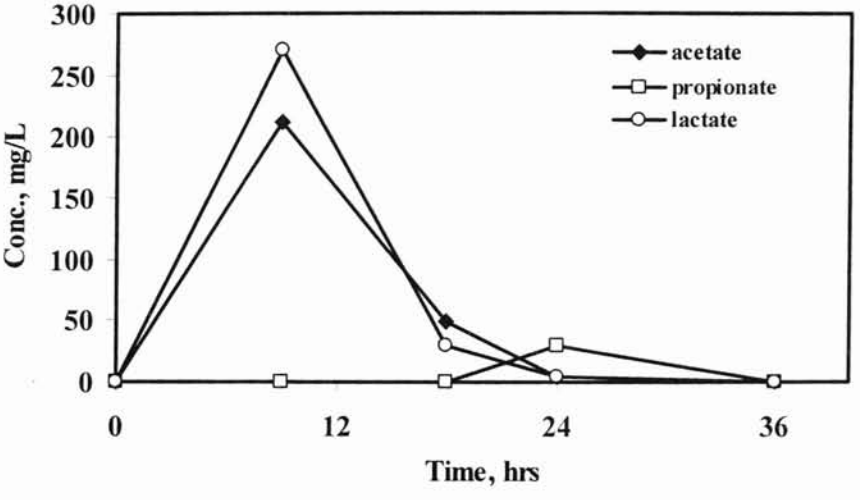

Figure 3.4: VFA profiles for the reactor fed with lactose

3.1.3 Gas Production

Total gas production for the diluted reactor fed with glucose substrate is shown in Figure 3.5. The theoretical gas production was calculated based on the assumption that all the glucose was converted to methane and carbon dioxide in a 1:1 molar ratio. The maximum gas production was achieved within four days, the time when all VFAs were consumed.

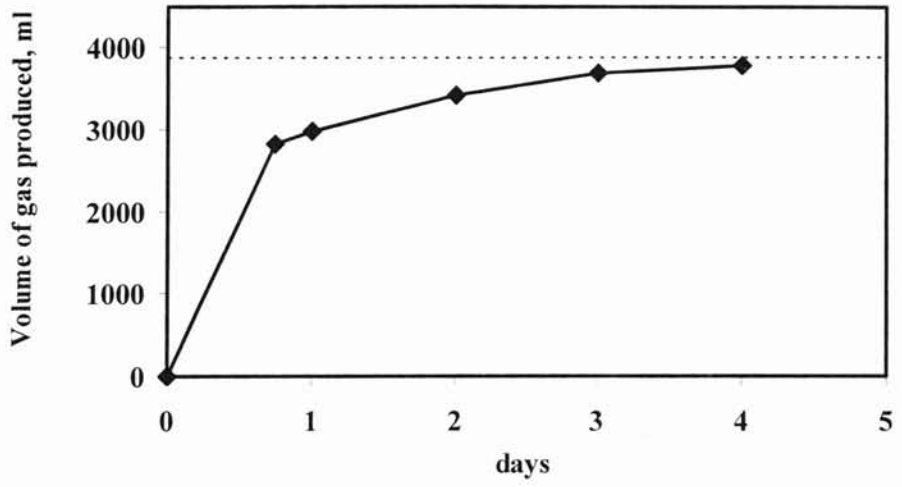

Figure 3.5: Gas production profile for the diluted reactor fed with glucose
-------- represents theoretical gas production

4.0 EFFECT OF STEARIC, OLEIC AND LINOLEIC ACIDS ON GLUCOSE DEGRADATION

4.1 Experimental Results

The first objective of this research project was to examine the effect of individual LCFAs on glucose fermentation. To fulfill this objective, experiments were designed to examine the effect of C18 LCFAs on glucose fermentation. Glucose, volatile fatty acids, LCFAs and methane were monitored over the duration of the study.

4.1.1 Glucose Degradation

Glucose degradation profiles for cultures receiving stearic, oleic and linoleic acids in various concentrations are shown in Figures 4.1 to 4.3. In all experiments, the initial glucose concentration was maintained constant at 2000 $mg \cdot l^{-1}$. Within 1.5 hours, more than 95% of the glucose was consumed in the control cultures and no traces were detected after 2.5 hours. No statistical difference has been found between the initial degradation rates for all controls (Table 4.1).

When compared to cultures fed with oleic and linoleic acids, stearic acid (SA) had the least effect on glucose consumption (Figure 4.1). No detectable levels of glucose were observed after 2.5 hours for cultures fed with SA. Similar glucose removal rates were observed for the control cultures. Initial glucose degradation rates for cultures receiving SA were statistically compared using the Tukey's paired comparison procedure (Steel, et al., 1987). Initial glucose degradation rates for cultures receiving 0 to 700 $mg \cdot l^{-1}$stearic acid were statistically the same. However, initial degradation rates for cultures receiving 1000 $mg \cdot l^{-1}$ were statistically different.

Table 4.1 Initial glucose degradation rates for cultures receiving stearic, oleic and linoleic acids

	LCFA Concentration						
	0 mg/L	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
	Glucose Degradation Rate (μg glucose·VSS^{-1}·min^{-1})						
LA	24.61 ± 3.25^{a}	20.04± 0.59^{b}	18.80± 0.49^{b}	14.28± 0.16^{c}	6.39±0.04^{d}	0.82 ±0.06^{e}	0.75±0.04^{e}
OA	24.26 ± 0.99^{a}	25.11 ± 3.79^{a}	22.41 ± 0.88^{a}	20.49 ± 0.23^{a}	18.22±1.30^{b}	14.66 ±0.77^{b}	7.95 ±0.63^{c}
SA	21.57 ± 0.11^{a}	21.60 ± 0.77^{a}	22.40 ± 0.58^{a}	21.59 ± 0.93^{a}	20.08 ±0.55^{a}	20.37 ±0.58^{a}	18.9 ±0.78^{b}

SA=stearic acid, OA=oleic acid, LA=linoleic acid. Averages and standard deviations for triplicate samples are shown. Means followed by the same letter are not statistically different within the rows.

Oleic acid (OA) had a more pronounced inhibitory effect on glucose degradation, with the degree of inhibition being concentration dependent. Degradation trends presented in Figure 4.2 were linear from 0 to 300 mg·l^{-1} OA and exponential from 500 to1000 mg·l^{-1}. Between 500 and 700 mg·l^{-1} of OA, a concentration threshold is observed. In cultures receiving 0 to 500 mg·l^{-1} of OA, glucose removal took place within 2.5 hours, while for cultures fed with 700 and 1000 mg·l^{-1} OA, the removal times were 4 and 8 hours, respectively.

The Tukey's pairwise comparison procedure was used to compare initial glucose degradation rates for cultures receiving OA. Degradation rates for cultures receiving 0 to 300 mg·l^{-1} OA were statistically the same. However, in comparison to the controls, cultures fed with 500 to 1000 mg·l^{-1} OA were statistically different. No statistical difference was found between initial degradation rates for cultures fed with 500 and 700 mg·l^{-1} OA.

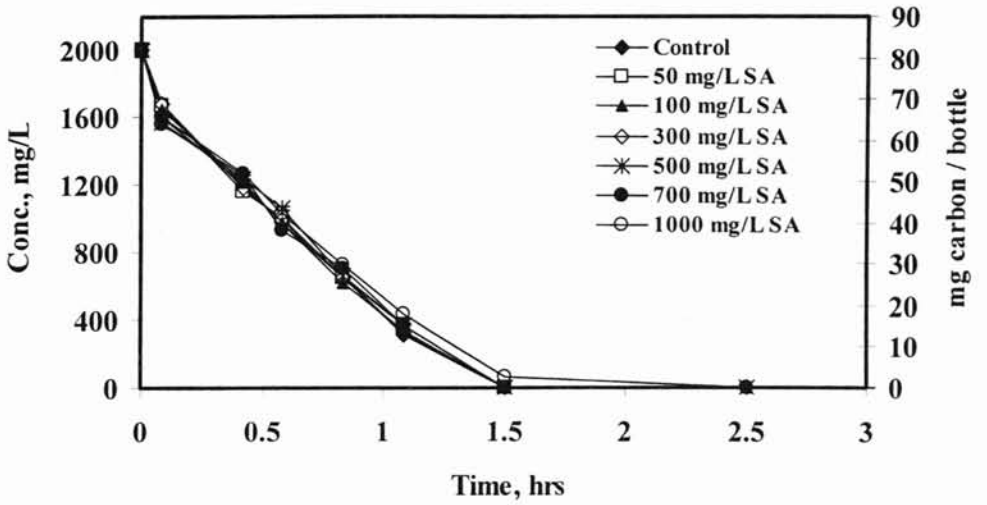

Figure 4.1: Glucose degradation profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ glucose (SA = stearic acid, averages for triplicate samples)

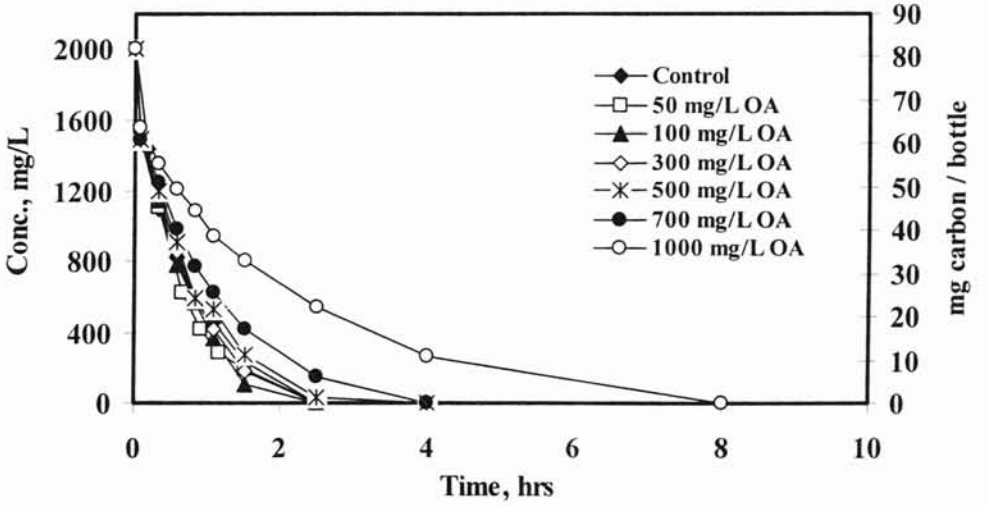

Figure 4.2: Glucose degradation profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ glucose (OA = oleic acid, averages for triplicate samples)

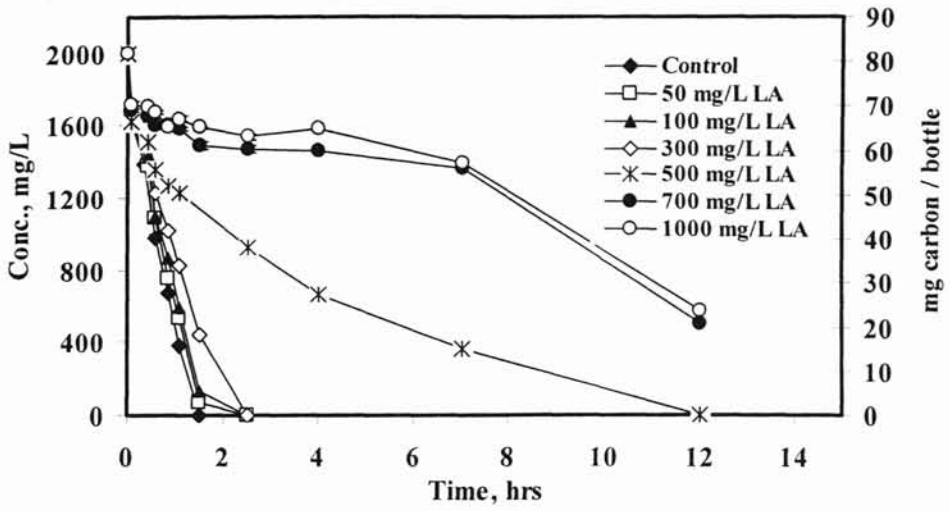

Figure 4.3: Glucose degradation profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ glucose (LA = linoleic acid, averages for triplicate samples)

Linoleic acid was the most inhibitory toward glucose consumption among the LCFAs examined. A concentration threshold was observed between 300 to 500 $mg \cdot l^{-1}$ LA. At 1.5 hours, cultures fed with 0 to 100 $mg \cdot l^{-1}$ LA had residual glucose concentrations below 10%, while about 25% glucose still remained in solution at the same time in cultures receiving 300 $mg \cdot l^{-1}$ LA. Nevertheless, at 2.5 hours no glucose was detected in cultures receiving up to 300 $mg \cdot l^{-1}$LA. However, a different trend was observed for cultures receiving greater than 300 $mg \cdot l^{-1}$LA. The degradation trend gradually changed from linear (0 to 300 $mg \cdot l^{-1}$LA) to exponential (500 $mg \cdot l^{-1}$ of LA) and then an extended lag-phase (up to 7 hours) was observed for cultures fed with 700 and 1000 $mg \cdot l^{-1}$ LA. At 12 hours, complete glucose uptake was achieved for cultures receiving 500 $mg \cdot l^{-1}$ LA, however, cultures inoculated with 700 and 1000 $mg \cdot l^{-1}$ LA had

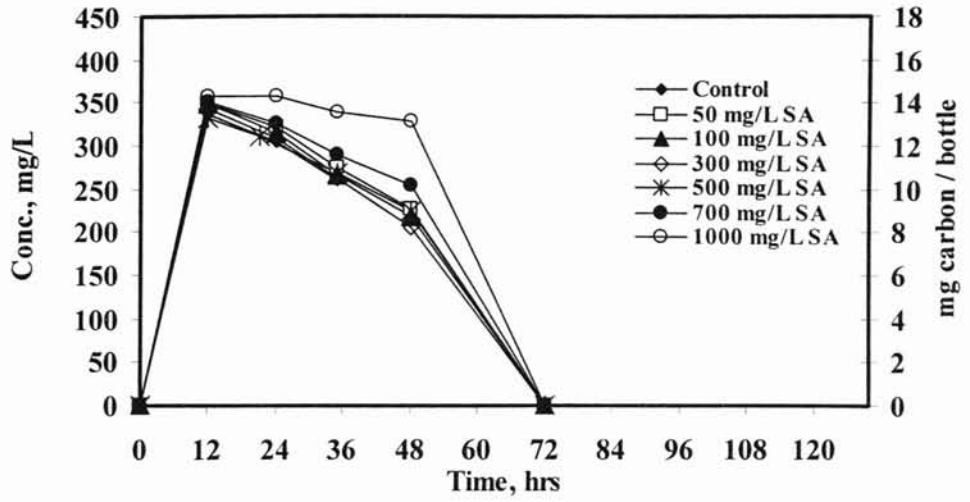

Figure 4.4: Acetic acid production profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ glucose (SA = stearic acid , averages for triplicate samples)

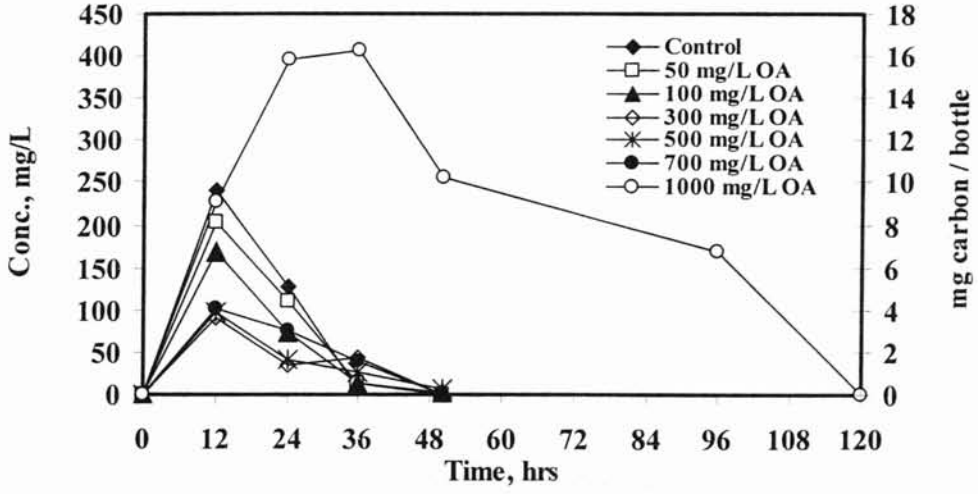

Figure 4.5: Acetic acid production profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ glucose (OA = oleic acid, averages for triplicate samples)

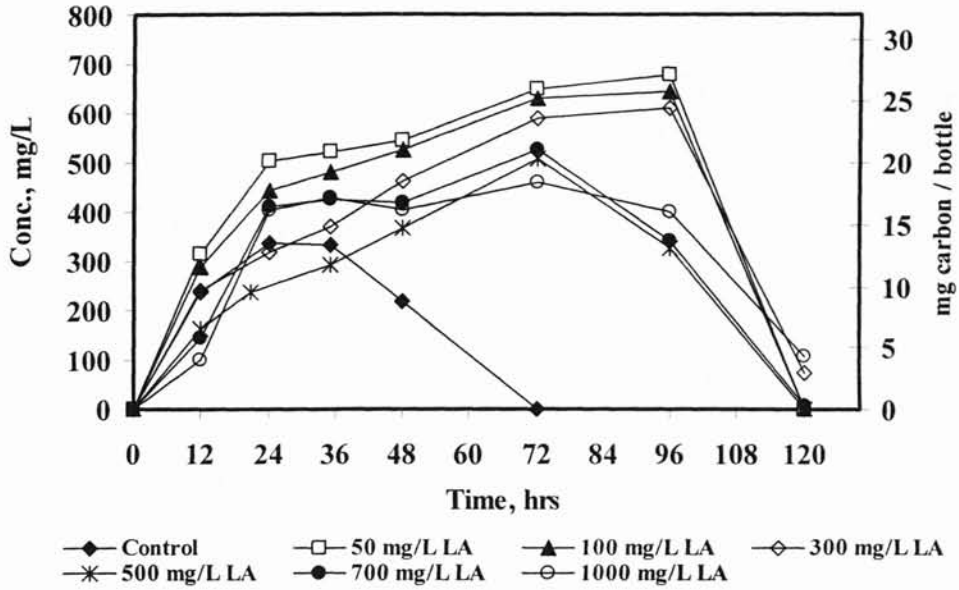

Figure 4.6: Acetic acid production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ glucose (LA = linoleic acid, averages for triplicate samples)

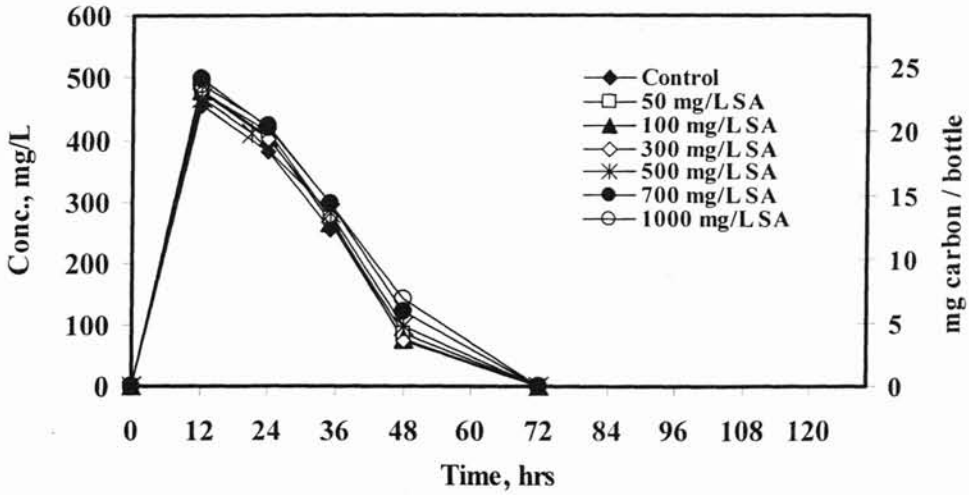

Figure 4.7: Propionic acid production profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ glucose (SA = stearic acid, averages for triplicate samples)

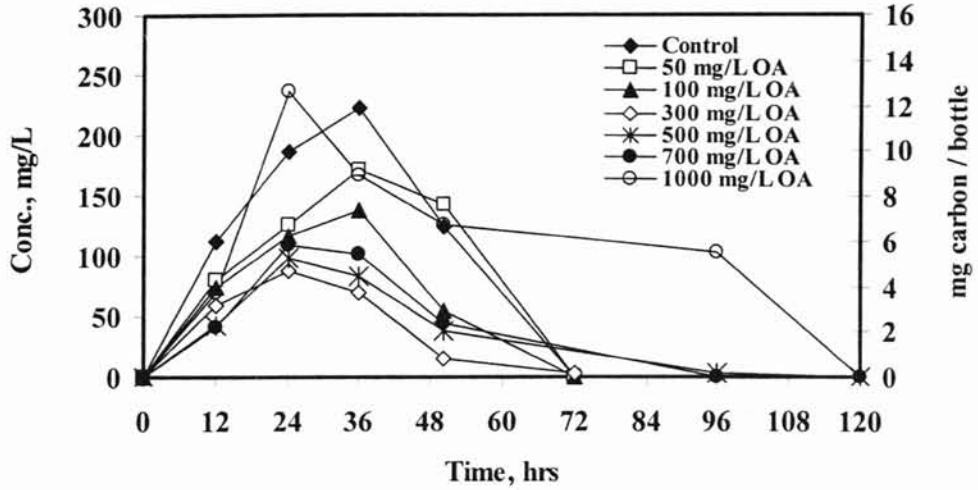

Figure 4.8: Propionic acid production profiles for cultures receiving oleic acid and 2000 mg·l^{-1} glucose (OA = oleic acid, averages for triplicate samples)

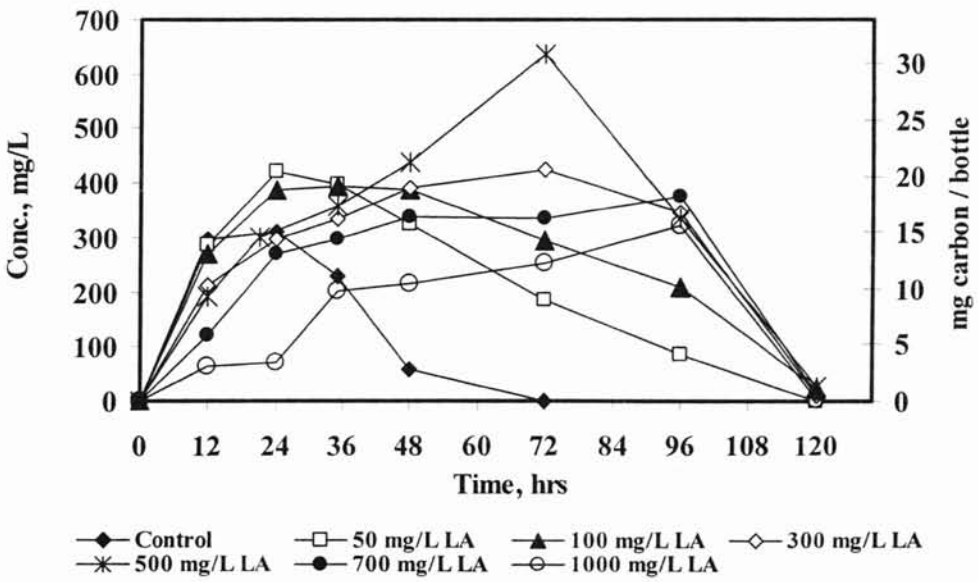

Figure 4.9: Propionic acid production profiles for cultures receiving linoleic acid and 2000 mg·l^{-1} glucose (LA = linoleic acid, averages for triplicate samples)

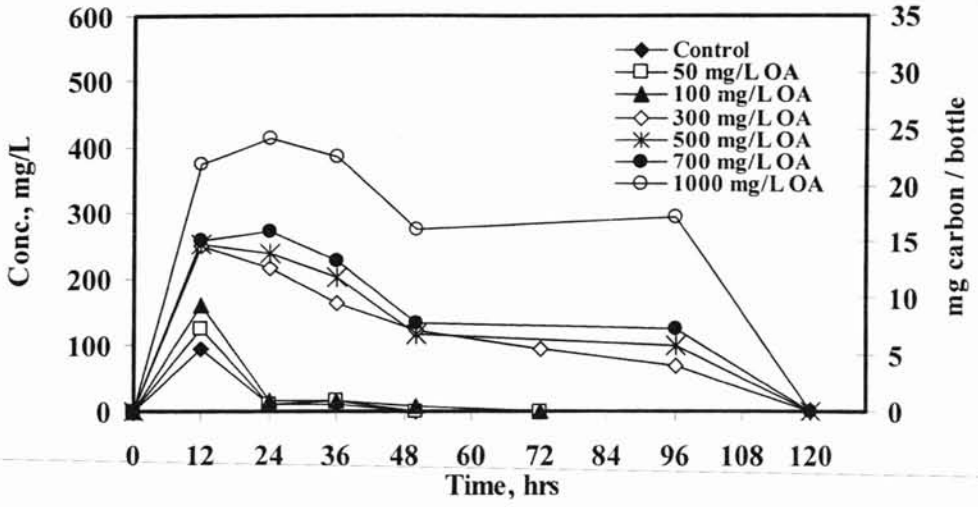

Figure 4.10: Butyric acid production profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ glucose (OA = oleic acid, averages for triplicate samples)

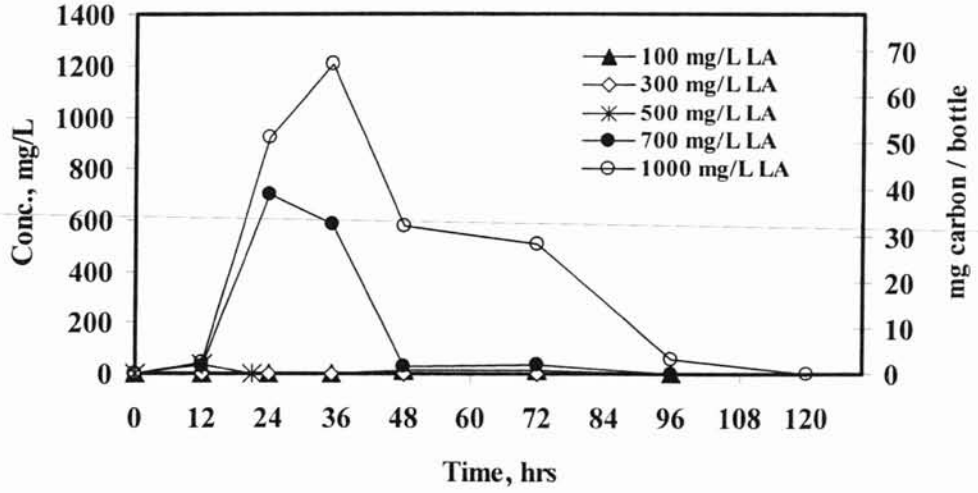

Figure 4.11: Butyric acid production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ glucose (LA=linoleic acid, averages for triplicate samples)

LA inhibited propionate degradation at all conditions examined. In cultures receiving 50 and 100 $mg \cdot l^{-1}$ LA, the maximum propionate concentration was observed after 24 hours of incubation and larger quantities of propionate were observed compared with the controls. The maximum propionate concentration (more than twice higher than in the control) was observed for cultures inoculated with 500 $mg \cdot l^{-1}$ LA after approximately 72 hours of incubation. Cultures receiving 700 and 1000 $mg \cdot l^{-1}$ LA showed a maximum propionate accumulation of approximately 350 to 400 $mg \cdot l^{-1}$ after 96 hours of incubation. In all conditions examined, propionate was removed within 120 hours.

Larger quantities of butyrate, between 700 and 1200 $mg \cdot l^{-1}$, were observed in cultures receiving 700 and 1000 $mg \cdot l^{-1}$LA. Cultures fed with 0 to 100 $mg \cdot l^{-1}$OA produced approximately 100 $mg \cdot l^{-1}$ of butyrate after 12 hours of incubation and complete removal was achieved within 24 hours. Cultures fed with 300 to 700 $mg \cdot l^{-1}$ OA show similar concentration profiles with a maximum butyrate production at approximately 300 $mg \cdot l^{-1}$. All detectable levels of butyrate were slowly degraded within 120 hours. In cultures inoculated with 1000 $mg \cdot l^{-1}$ OA, the butyrate reached a maximum of approximately 400 $mg \cdot l^{-1}$ and was completely removed within 120 hours.

4.1.3. Gas Production

The methane production profiles for each LCFA condition are presented in Figures 4.12 to 4.14. In control cultures all the added glucose was converted to gaseous products in 3 days. The presence of SA seems to have no inhibitory effect on methane

production. Although at day 2, methane production for cultures fed with 1000 $mg \cdot l^{-1}$ SA was less than in the controls and at day 3 more than 95% of the methane was produced.

Lower methane production was observed in cultures fed with greater than 100 $mg \cdot l^{-1}$ OA or LA. In the presence of 300 to 700 $mg \cdot l^{-1}$ OA, methane production was less than in the control cultures. The presence of LA at all concentrations and at 1000 $mg \cdot l^{-1}$ OA, low methane production was observed in comparison to the controls with less than 50% of the methane produced at day 6.

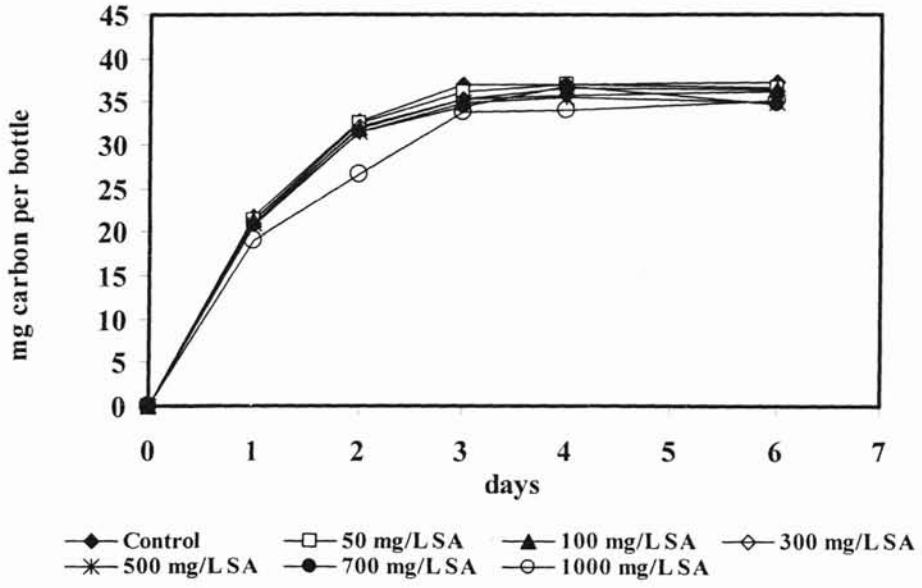

Figure 4.12: Methane production profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ glucose (SA = stearic acid, averaged for triplicate samples)

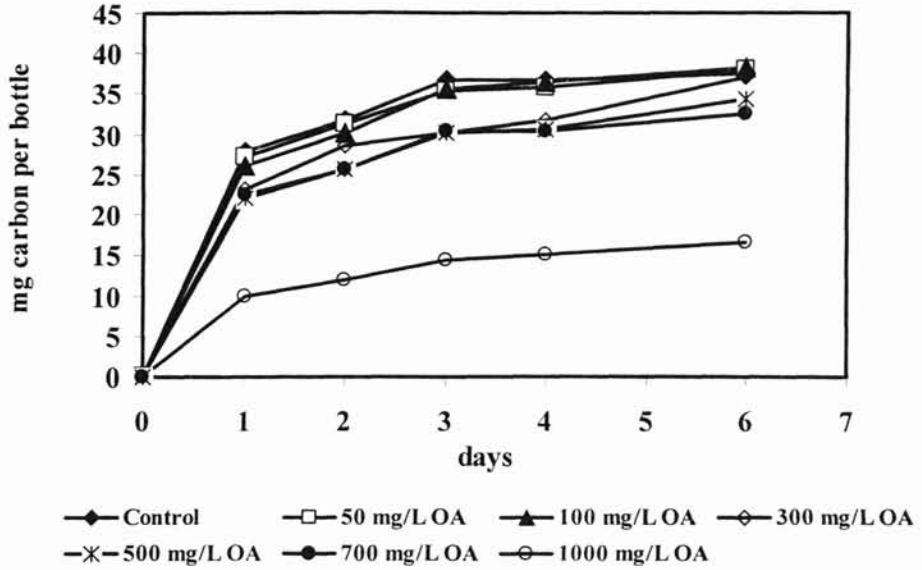

Figure 4.13: Methane production profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ glucose (OA = oleic acid, averaged for triplicate samples)

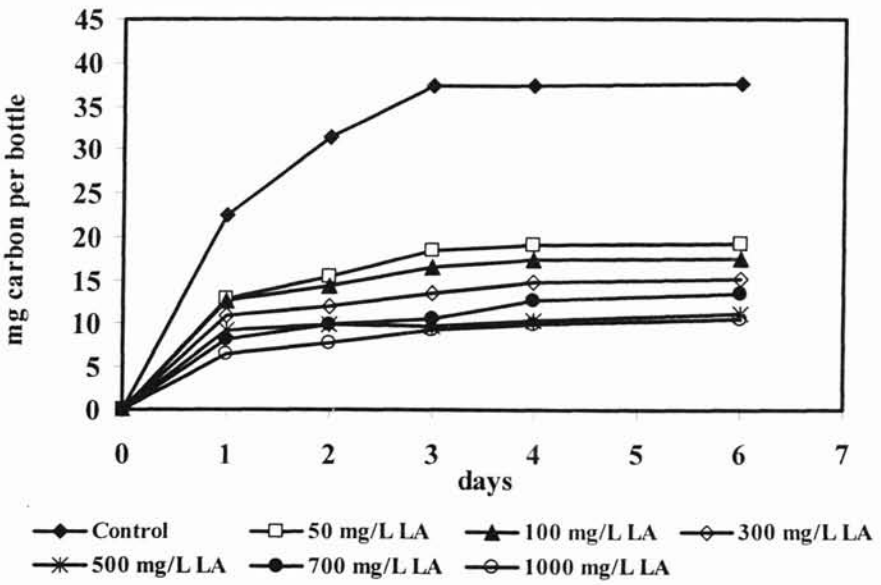

Figure 4.14: Methane production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ glucose (LA = linoleic acid, averaged for triplicate samples)

4.2 Discussion

The inhibitory effects of LCFAs on anaerobic microorganisms should be taken into account during bioreactor design. Under anaerobic conditions lipids are hydrolyzed to LCFA plus glycerol. Glycerol is not toxic toward anaerobic organisms. However, LCFAs can affect several anaerobic populations including acidogens, acetogens and methanogens. Stearic, oleic and linoleic acids were used in this study to investigate the effect of the degree of carbon bond saturation on glucose fermentation at 37°C.

Among three acids examined, LA, which has two double bonds, exhibited the strongest inhibitory effect. Initial glucose degradation rates were lower compared to controls at all LA concentrations examined. Furthermore, the time for complete substrate removal increased between 6 to 12 times when comparing cultures fed with LA to those incubated with OA or SA. In contrast, SA did not inhibit glucose degradation and OA possessed inhibition properties only at concentrations greater than 300 $mg \cdot l^{-1}$. Moreover, the inhibitory effects of LCFAs increase in the following order: SA<OA<LA, with each addition of double bond increasing inhibition of glucose fermentation.

Similarly, Lalman and Bagley (2002) reported that at 21°C, SA and OA at a concentration of 100 $mg \cdot l^{-1}$ did not inhibit glucose degradation in contrast to LA. Although Lalman and Bagley (2002) observed greater glucose inhibition in cultures fed with 300 $mg \cdot l^{-1}$LCFA (100 $mg \cdot l^{-1}$ LA plus 100 $mg \cdot l^{-1}$ OA plus 100 $mg \cdot l^{-1}$ SA), they did not examine individual LCFAs concentrations greater than 100 $mg \cdot l^{-1}$. Research by Alosta (2002) has shown that at 21°C, SA and OA at concentrations greater than 100 $mg \cdot l^{-1}$ and LA at greater than 50 $mg \cdot l^{-1}$ significantly decreased glucose degradation rates. In addition, inhibition of glucose fermentation by LA was reported to be greater

compared to OA and SA. Initial glucose degradation rates were reported from 15.2 μg glucose·VSS^{-1}·min^{-1} for the control cultures to 6.87μg glucose·VSS^{-1}·min^{-1} for cultures fed with 1000 mg·l^{-1} LA and 8.5 to 8.8μg glucose·VSS^{-1}·min^{-1} for 1000 mg·l^{-1} SA and OA (Alosta, 2002). Initial glucose degradation rates obtained in the present study for controls were between 24.61 to 21.57 μg glucose·VSS^{-1}·min^{-1}. Cultures inoculated with 1000 mg·l^{-1} SA, OA and LA showed degradation rates equal to 18.9, 7.95 and 0.75 μg glucose·VSS^{-1}·min^{-1} respectively. Comparing data between this work and those reported by Alosta (2002) clearly shows a decrease in glucose degradation rates for cultures inoculated with 700 and 1000 mg·l^{-1} LA.

Differences in glucose degradation date between this study and those reported by Alosta (2002) can be explained by different operating conditions. Temperature conditions reported by Alosta (2002) were 25°C, while these studies were conducted at 37°C. The wider range of the initial rates suggests that two competitive factors could affect glucose degradation. Elevation of the temperature increases both the reaction rate and inhibitory properties of LCFA (Hwu and Lettinga, 1997; Becarri *et al.*, 1996). Lower glucose degradation rates for cultures fed with 1000 mg·l^{-1} OA and LA compared to cultures inoculated with 1000 mg·l^{-1} SA indicate that higher temperatures increase the inhibitory effect of OA and especially LA. In comparison, the inhibitory properties of SA remained unaffected by an increase in temperature. This observation is in agreement with differences in the molecular structure of LCFAs. Each addition of a double bond decreases the melting point of the substance. Thus, at 37°C LA will be in a liquid state and because of higher solubilities, increased LA uptake by microorganisms is likely. In contrast, at 37°C SA will be solid and as a result, less will be available for uptake.

Consequently, due to increase in the reaction rate and no changes in the SA inhibitory properties, at 37°C higher initial glucose degradation rates for cultures fed with SA should be expected, than compared to those observed at 25°C.

In contrast to data obtained from these studies, Hanaki *et al.(*1981) indicated that LCFA did not affect glucose degradation. Hanaki *et al.(*1981) assumed complete glucose degradation based on increasing acetate concentration. However, this work and previous studies by Alosta (2002) have shown glucose accumulation in cultures fed with LA at 21°C and 37°C.

Accumulation of VFAs is a major indicator of biomass inhibition. Several studies have shown VFA inhibition in the presence of LCFAs (Lalman and Bagley, 2001; Vidal *et al.,* 2000; Canovas – Diaz *et al.,* 1992). In this work, propionate degrading organisms were less affected by LCFAs. Although no acetate accumulation was observed at all SA concentrations examined, acetate accumulation was observed in cultures fed with OA and LA. Inhibition of acetoclastic methanogens, organisms degrading acetate to methane plus carbon dioxide, has been reported (Lalman and Bagley, 2001; Koster and Cramer, 1987). Also, no propionate and butyrate production was observed in the presence of SA. Butyrate accumulation was detected in the presence of more than 100 $mg \cdot l^{-1}$ OA or 700 to 1000 $mg \cdot l^{-1}$ LA. This suggests that under these conditions, the carbon flow was shifted form the methane to butyrate production. A significant reduction in methane production and large butyrate accumulations were observed in the presence of OA and LA. It is possible that other more reduced compounds, such as butanol and ethanol, were also produced, however, they were not monitored in this study.

Canova-Diaz *et al.* (1992) also observed accumulation of acetic acid (up to 2.5 $g \cdot l^{-1}$) and butyric acid (up to 2 $g \cdot l^{-1}$) in the presence of 2000 $mg \cdot l^{-1}$ of oleic or myristic acids at 35^0C. The concentration of propionic acid was much lower (up to 500 $mg \cdot l^{-1}$). Vidal *et al.* (2000) also reported accumulation of VFAs in the cultures treating a synthetic fat and carbohydrate waste at 36^oC. Although the effect of individual LCFAs was not investigated, acetate, propionate and butyrate were observed.

LCFAs also inhibit methane production by aceticlastic methanogens (Koster and Cramer, 1987; Angelidaki and Ahring, 1992; Demeyer and Henderickx, 1967). Demeyer and Hendericks (1967) reported that methane production from pyruvate decreased in the presence of LCFA. In particular, inhibition caused by C_{18} LCFAs increased in the following order: SA<OA<LA. Lalman and Bagley (2001) reported oleic acid inhibited aceticlastic methanogens at concentration above 30 $mg \cdot l^{-1}$, while SA at concentrations up to 100 $mg \cdot l^{-1}$ did not have any effect.

SA did not affect methane production; however, 1000 $mg \cdot l^{-1}$ OA and LA at all concentrations examined decreased methane production by more than 50%. Similar results were reported by Alosta (2002), however the threshold levels for LA was at 300 $mg \cdot l^{-1}$ and a lag-phase was observed for cultures fed with more than 500 $mg \cdot l^{-1}$ OA. The major difference between these results and data reported by Alosta (2002) is due to lower operating temperature.

5.0 EFFECT OF STEARIC, OLEIC AND LINOLEIC ACIDS ON LACTOSE DEGRADATION

5.1 Experimental Results

In this chapter the results of the experiments that investigated the effect of C18 LCFAs on lactose degradation are presented. Experiments were designed to determine the impact of LCFAs on the hydrolysis rate of lactose. Two sets of experiments were conducted with OA and SA. The first series of experiments examined lactose fermentation in the presence of SA, OA and LA were conducted on 09/03/02. Because of the differences in the control cultures, additional experiments were repeated with OA and SA were performed on 11/12/02.

5.1.1 Lactose Degradation

Lactose degradation profiles are presented in Figures 5.1 to 5.3 and initial lactose degradation rates are shown in Table 5.1. In the first series of experiments which were performed (Figures 5.1 A and 5.2 A), lactose uptake profiles for SA and OA were similar and followed a degradation pattern close to the control cultures. A one-hour lag-phase was observed after an initial rapid uptake. Subsequently, the lactose decreased to undetectable levels within 9 hours for cultures receiving SA and OA. In contrast, lactose consumption profiles for cultures receiving LA were different from those receiving SA and OA. Lactose degradation in the control cultures for experiments conducted with LA occurred within 2.5 hours compared to 9 hours for cultures fed with SA and OA. The large difference in the lactose degradation times in the control cultures compared to controls in studies conducted with OA and SA indicate a possible microbial population

shift. Consequently, the effect of SA and OA on lactose degradation was not clearly observed. Therefore, experiments for cultures fed with OA and SA were repeated.

The lactose degradation profiles for studies conducted in the second set of experiments with OA and SA are presented in Figures 5.1 B and 5.2 B. In the control cultures, complete lactose degradation was achieved within 6 hours of incubation. No effect of SA on lactose degradation was observed under all conditions examined. Tukey's procedure used for the statistical comparison showed no statistical difference between the initial degradation rates of cultures inoculated with 0 to 1000 $mg \cdot l^{-1}$ SA.

The inhibiting effects of OA and LA were concentration dependent (Figures 5.2 B and 5.3). At 50 $mg \cdot l^{-1}$ LA and 100 $mg \cdot l^{-1}$ OA, lactose uptake was less compared to the control. As the OA and LA concentration increased, the lactose consumption decreased and achieved undetectable levels for cultures receiving 700 and 1000 $mg \cdot l^{-1}$LA. Nevertheless, complete lactose degradation was observed within 9 hours for all cultures fed with LA and cultures inoculated with 500 $mg \cdot l^{-1}$OA had residual lactose concentration below 10% after 6 hours on incubation. Under selected LA concentration between 500 to 1000 $mg \cdot l^{-1}$, glucose production was observed (Figure 5.4).

The Tukey's procedure showed that initial lactose degradation rates for cultures receiving 100 to 1000 $mg \cdot l^{-1}$OA and 50 to 1000 $mg \cdot l^{-1}$LA were statistically lower than in the controls. No statistical differences between initial lactose degradation rates were found for cultures inoculated with 300 to 500 $mg \cdot l^{-1}$OA and degradation rates for cultures receiving 700 to 1000 $mg \cdot l^{-1}$OA were statistically the same. Similarly, no statistical differences between initial lactose degradation rates were found for cultures inoculated

with 100 to 300 mg·l^{-1}LA and degradation rates for cultures receiving 700 to 1000 mg·l^{-1}OA were statistically the same.

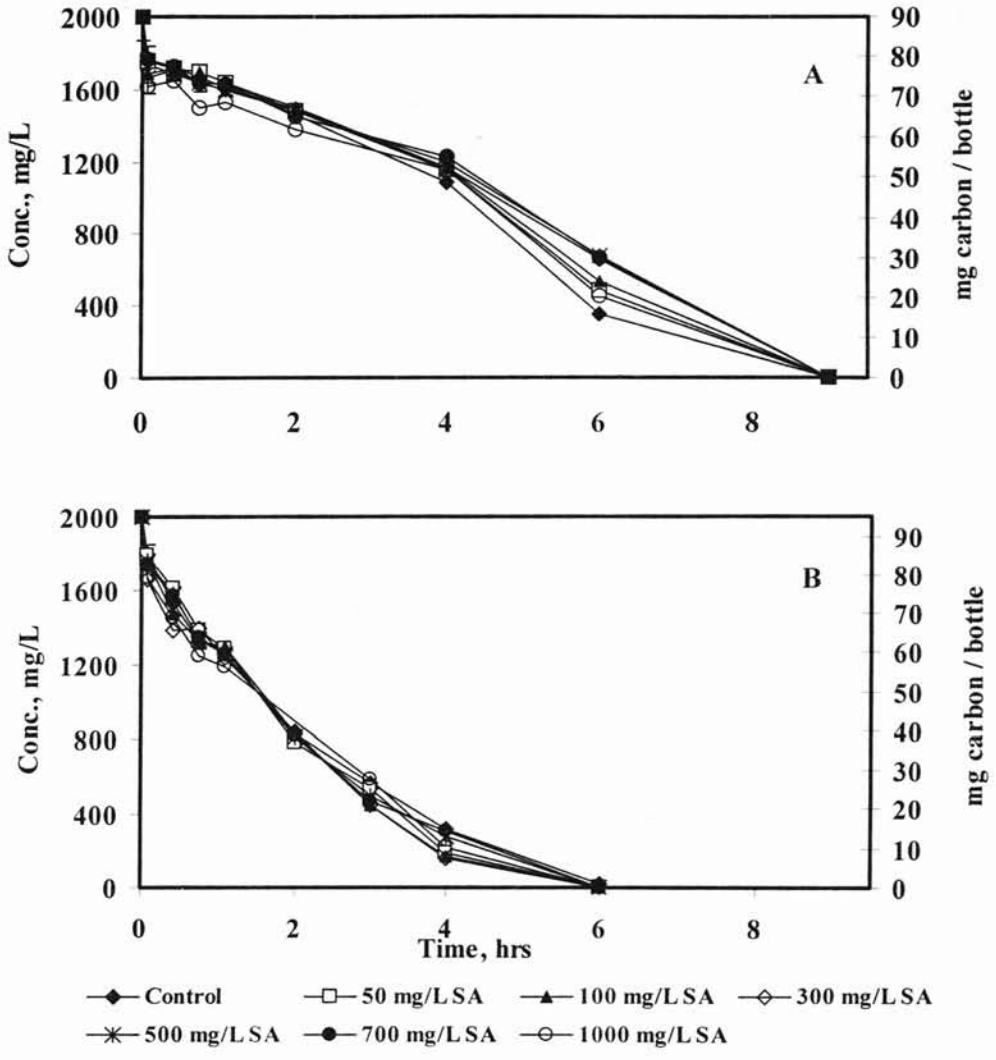

Figure 5.1: Lactose degradation profiles for cultures receiving stearic acid and 2000 mg·l^{-1} lactose for experiments performed on 09/03/02 (A) and 11/13/02 (B) (SA = stearic acid, averages for triplicate samples)

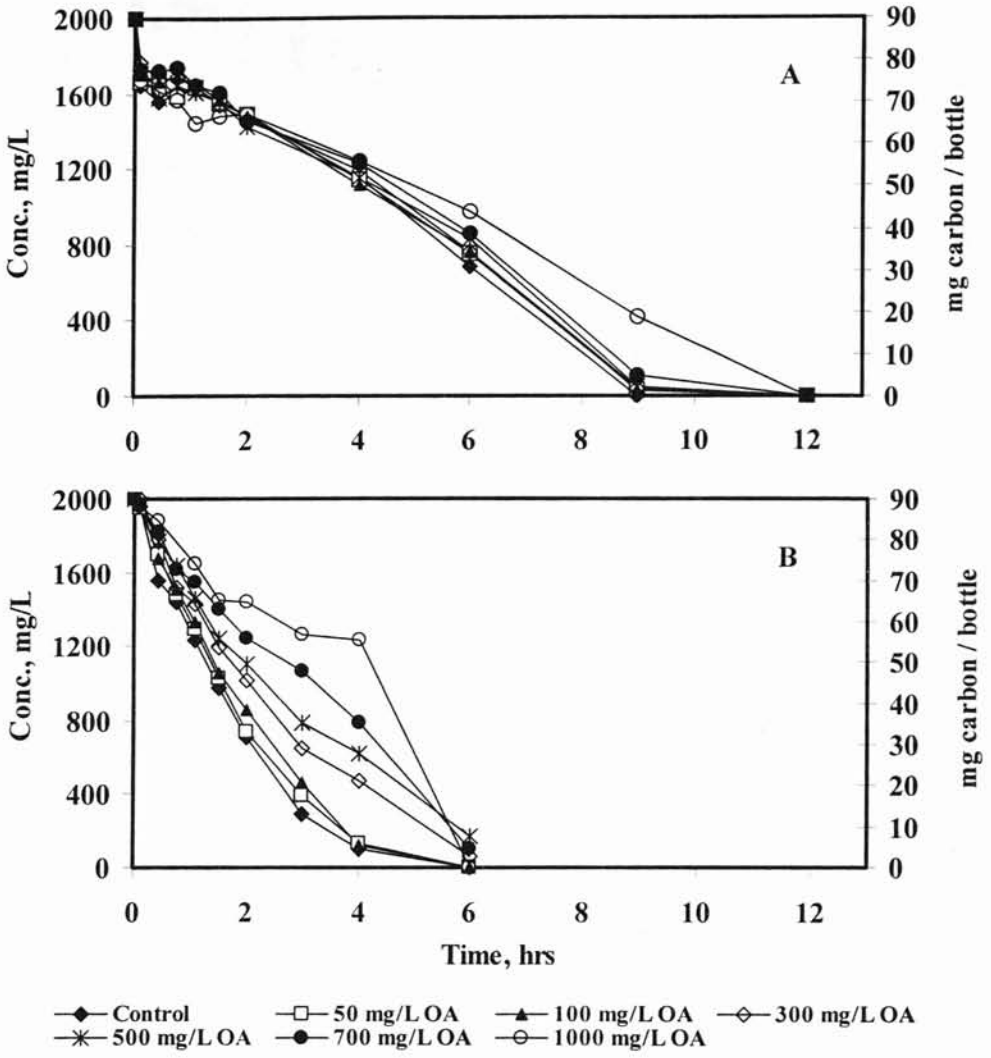

Figure 5.2: Lactose degradation profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ lactose for experiments performed on 09/10/02 (A) and 11/12/02 (B) (OA = oleic acid, averages for triplicate samples)

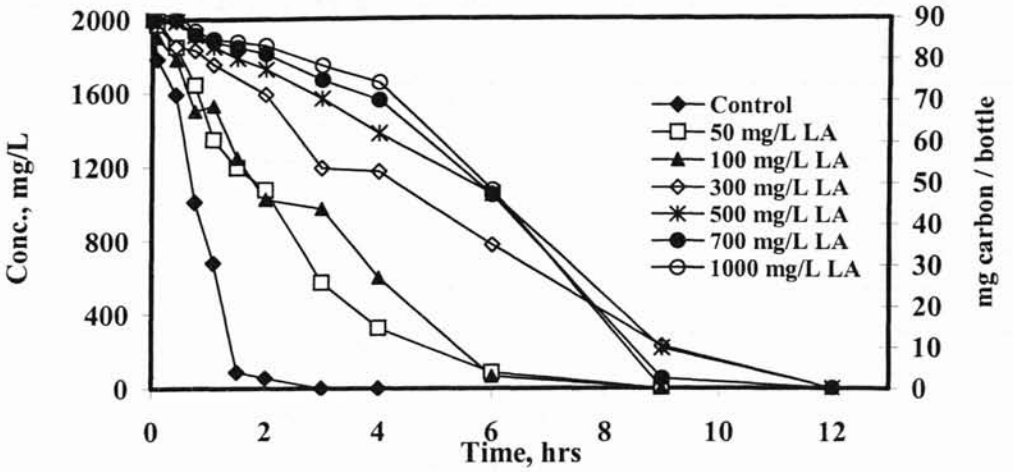

Figure 5.3: Lactose degradation profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ lactose (LA = linoleic acid, averages for triplicate samples)

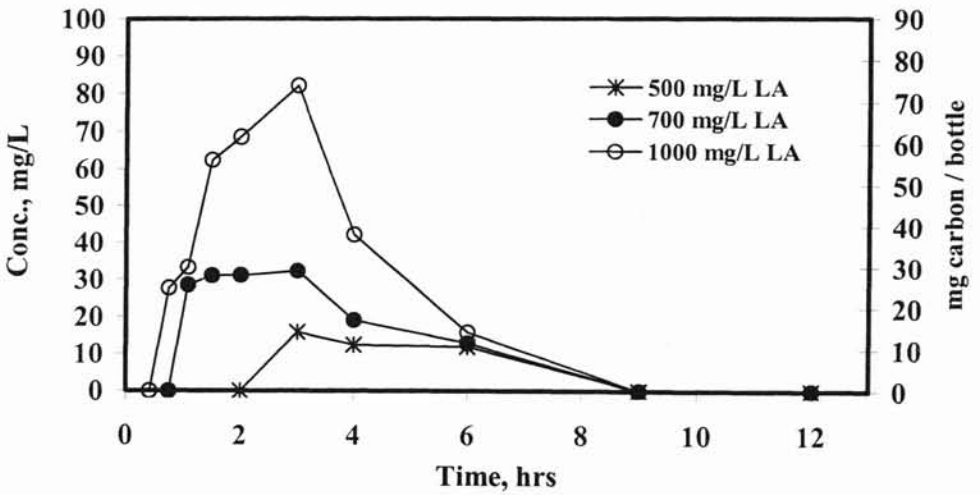

Figure 5.4: Glucose production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ lactose (LA = linoleic acid, averages for triplicate samples)

Table 5.1: Initial lactose degradation rates for cultures receiving stearic, oleic and linoleic acids

	Initial LCFA Concentration, $mg \cdot l^{-1}$						
	0	50	100	300	500	700	1000
	Lactose Degradation Rate (μg glucose$\cdot VSS^{-1} \cdot min^{-1}$) for experiments conducted in September						
LA	16.47 ± 0.38^{a}	6.12 ± 0.48^{b}	1.98 ± 0.03^{c}	1.72 ± 0.09^{c}	1.31 ± 0.20^{e}	1.01 ± 0.02^{f}	0.83 ± 0.05^{f}
OA	1.61 ± 0.03^{b}	1.38 ± 0.02^{e}	1.40 ± 0.02^{e}	1.41 ± 0.01^{e}	$1.36 \pm .05^{e}$	1.35 ± 06^{e}	1.19 ± 0.01^{g}
SA	1.78 ± 0.03^{b}	1.76 ± 0.01^{b}	1.72 ± 0.06^{b}	1.68 ± 0.02^{c}	1.64 ± 0.02^{c}	1.67 ± 0.01^{c}	1.65 ± 0.03^{c}
	Lactose Degradation Rate (μg glucose$\cdot VSS^{-1} \cdot min^{-1}$) for experiments conducted in November						
OA	8.24 ± 0.37^{b}	7.87 ± 0.32^{b}	6.99 ± 0.09^{c}	5.40 ± 0.42^{d}	4.64 ± 0.27^{d}	3.58 ± 0.53^{e}	3.79 ± 0.16^{e}
SA	6.01 ± 0.37^{g}	6.5 ± 0.11^{g}	6.48 ± 0.24^{g}	6.13 ± 0.68^{g}	6.38 ± 0.12^{g}	6.31 ± 0.13^{g}	6.69 ± 0.60^{g}

SA=stearic acid, OA=oleic acid, LA=linoleic acid. Averages and standard deviations for triplicate samples are shown. Means followed by the same letter are not statistically different within the rows.

5.1.2. VFAs Production

VFAs production profiles are shown in Figures 5.5 to 5.15. In the contrast to experiments conducted with glucose, lactic, acetic, propionic and butyric acids were observed with longer removal times. Control cultures had the maximum lactic acid production (289 to 600 $mg \cdot l^{-1}$) between 12 to 24 hours. Complete degradation of lactate was observed within 48 to 96 hours. For cultures inoculated in during the first series of experiments (09/03/02) (Figures 5.5 A, 5.6 A and 5.7), in the presence of 50 to 300 $mg \cdot l^{-1}$ SA or LA, lactic acid profiles were similar to the controls. SA and LA between 500 to 1000 $mg \cdot l^{-1}$ inhibited lactate degradation. At 500 $mg \cdot l^{-1}$ SA lactate was degraded to undetectable levels within 144 hours, but at higher SA concentrations, only 50% of the lactate was degraded within 9 days. LA was more inhibitory and no lactate degradation was observed within 9 days for cultures receiving 500 to 1000 $mg \cdot l^{-1}$ LA. In contrast, for

cultures inoculated during the second series of experiments (11/12/02) (Figures 5.5 B and 5.6 B), complete lactate degradation was observed within 48 hours of incubation for cultures inoculated with 50 to 500 $mg \cdot l^{-1}$ OA and at all SA conditions examined.

Acetate production was significantly lower compared to lactate. In the first series of experiments (Figures 5.8, 5.9A and 5.10A), acetate production and degradation patterns were similar to lactate. However, the maximum acetate production was approximately one-quarter of the lactate concentration for cultures receiving SA and about one-half for cultures fed with OA and LA. Lower acetate degradation was observed for cultures fed with 300 to1000 $mg \cdot l^{-1}$SA and 50 to 300 $mg \cdot l^{-1}$ OA or LA. No acetate degradation was observed in cultures receiving 500 to 1000 $mg \cdot l^{-1}$ of OA or LA. In the second series of experiments (Figures 5.9B and 5.10B), shorter acetate degradation times were observed for cultures inoculated with SA or OA and lower acetate degradation rates were observed in the presence of 700 to 1000 $mg \cdot l^{-1}$ OA.

Propionate and butyrate productions were very low between 40 to 100 $mg \cdot l^{-1}$. After 12 hours of incubation, the propionate concentrations peaked between 700 to 800 $mg \cdot l^{-1}$ for cultures fed with 700 to 1000 $mg \cdot l^{-1}$ LA or 500 to 700 $mg \cdot l^{-1}$OA (Figures 5.12 and 5.13 A). Cultures fed with SA showed variable removal times for propionate and butyrate. No propionate and butyrate degradation occurred in the presence of 300 to 1000 $mg \cdot l^{-1}$ OA and 300 to 1000 $mg \cdot l^{-1}$ LA in the cultures inoculated during the first series of experiments. However, when the study was repeated, lower butyrate concentrations and no propionate were detected in the cultures receiving SA. Lower butyrate degradation rates with subsequent propionate accumulations were observed in cultures fed with 700 to 1000 $mg \cdot l^{-1}$ OA (Figures 5.13 B and 5.15 B).

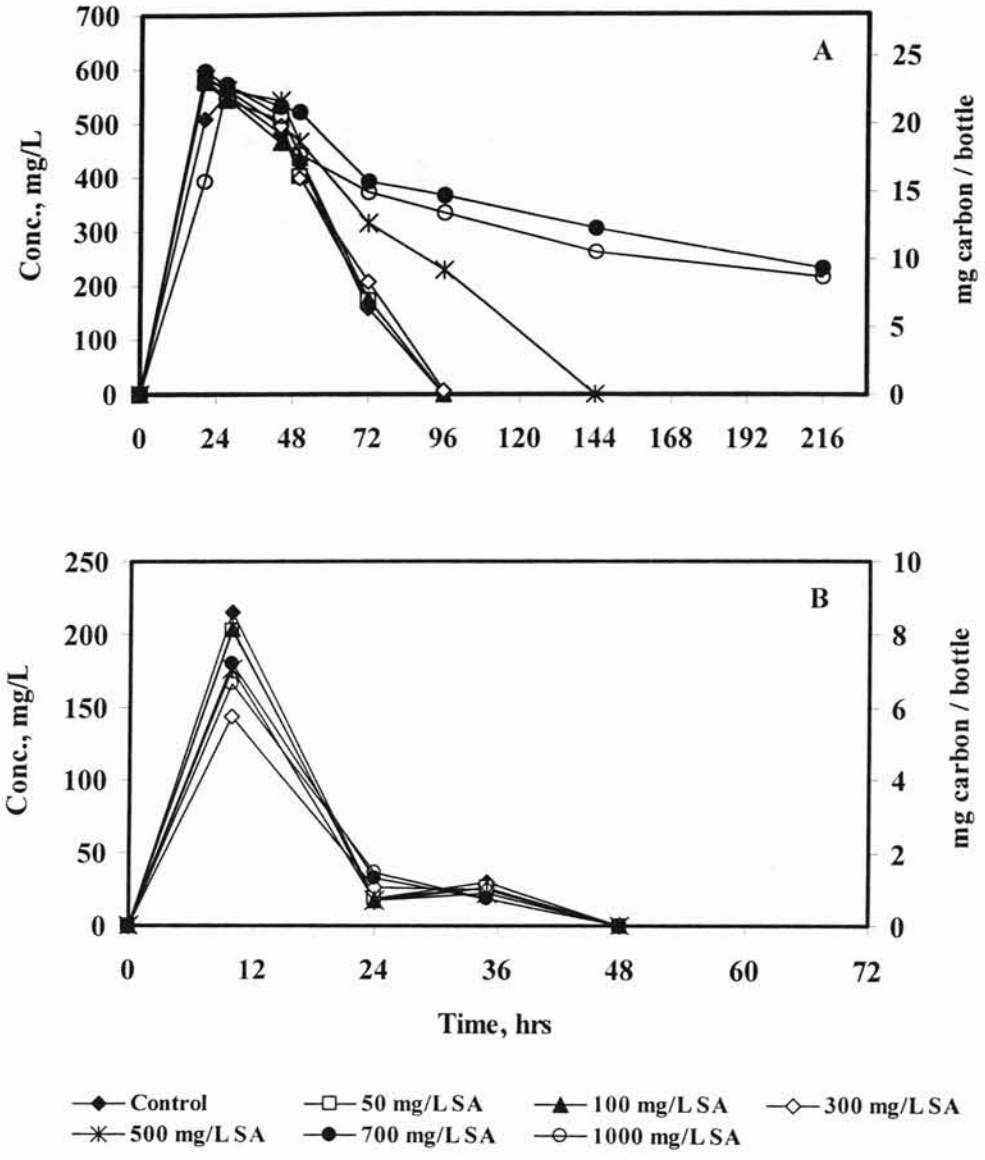

Figure 5.5: Lactic acid production profiles for cultures receiving stearic acid and 2000 mg·l^{-1} lactose for experiments performed on 09/03/02 (A) and 11/13/02 (B) (SA = stearic acid , averages for triplicate samples)

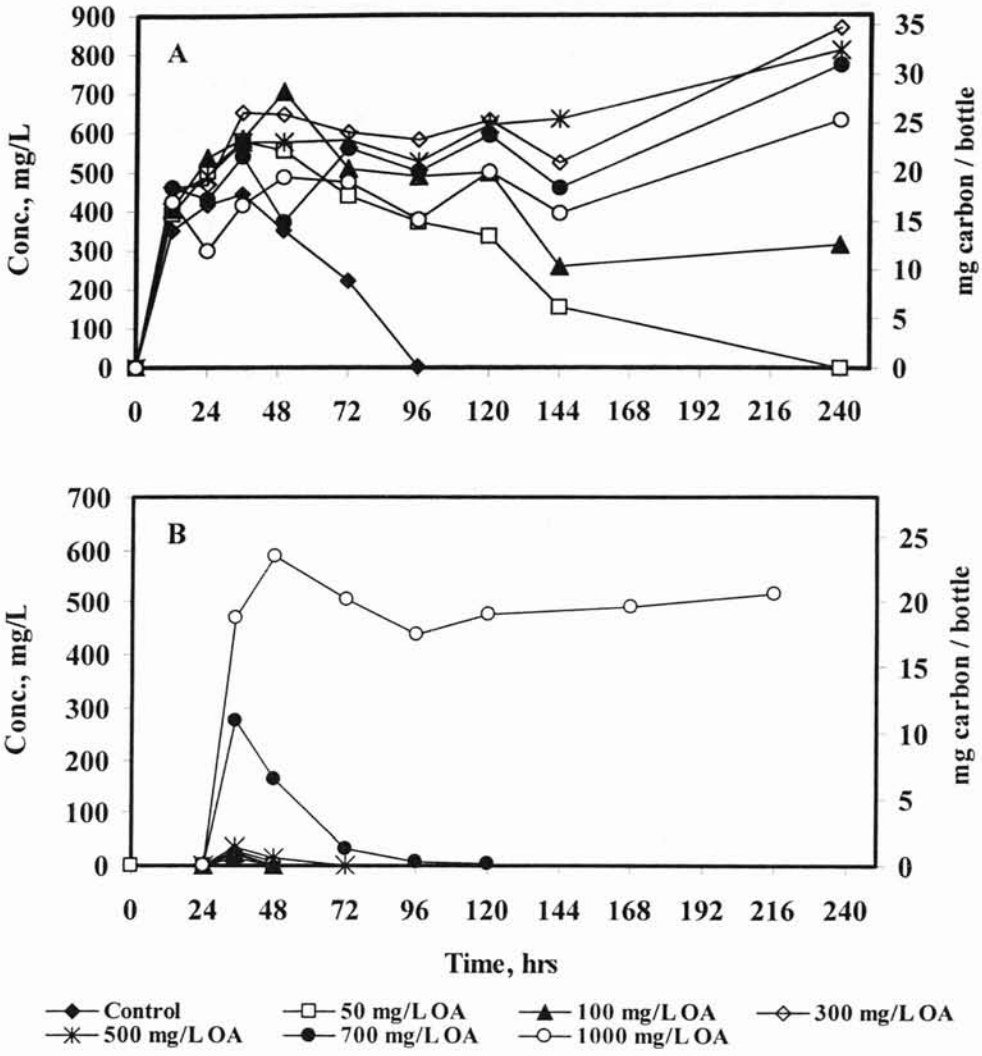

Figure 5.6: Lactic acid production profiles for cultures receiving oleic acid and 2000 mg·l^{-1} lactose for experiments performed on 09/10/02 (A) and 11/12/02 (B) (OA = oleic acid, averages for triplicate samples)

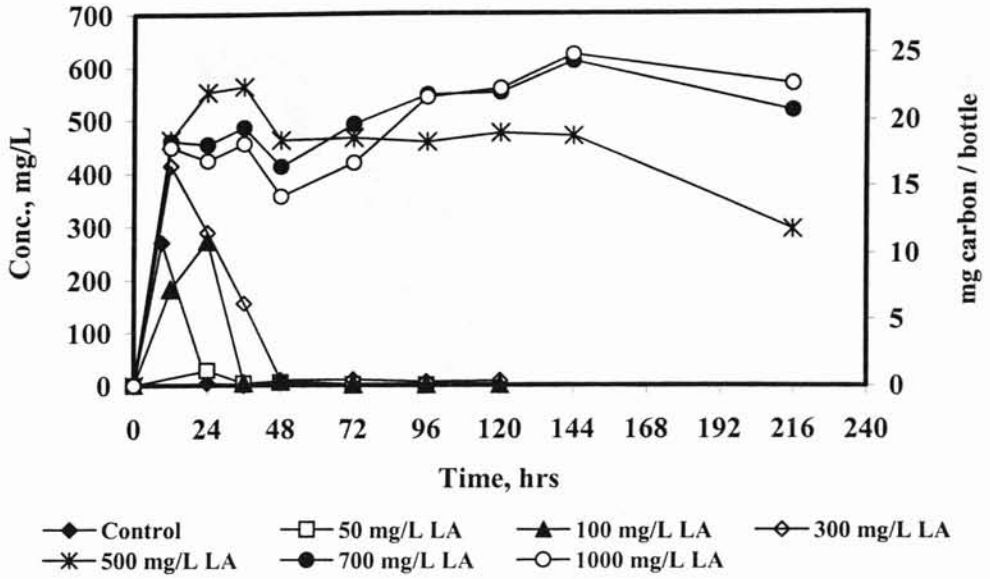

Figure 5.7: Lactic acid production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ lactose (LA = linoleic acid , averages for triplicate samples)

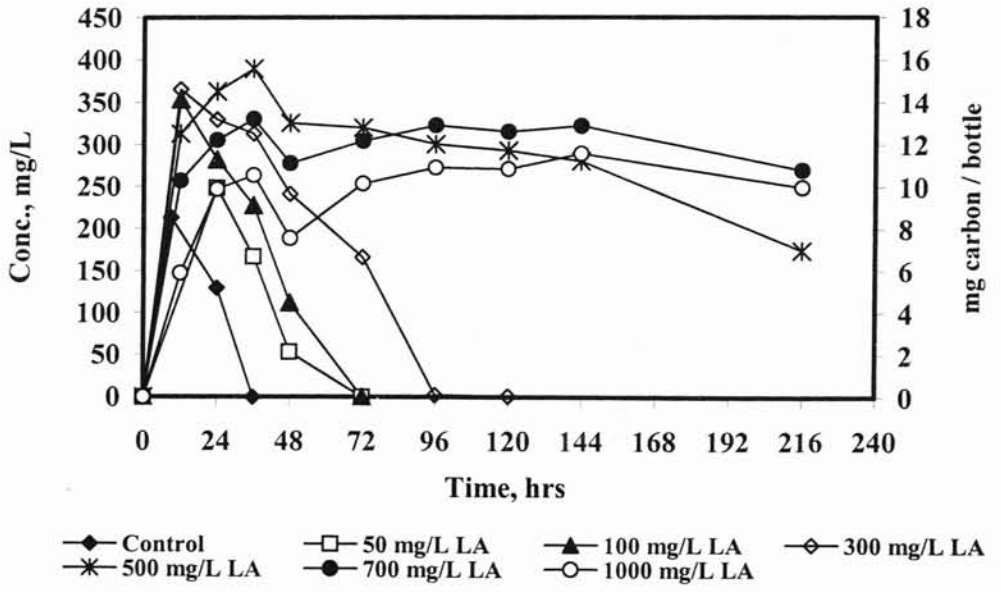

Figure 5.8: Acetic acid production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ lactose (LA = linoleic acid, averages for triplicate samples)

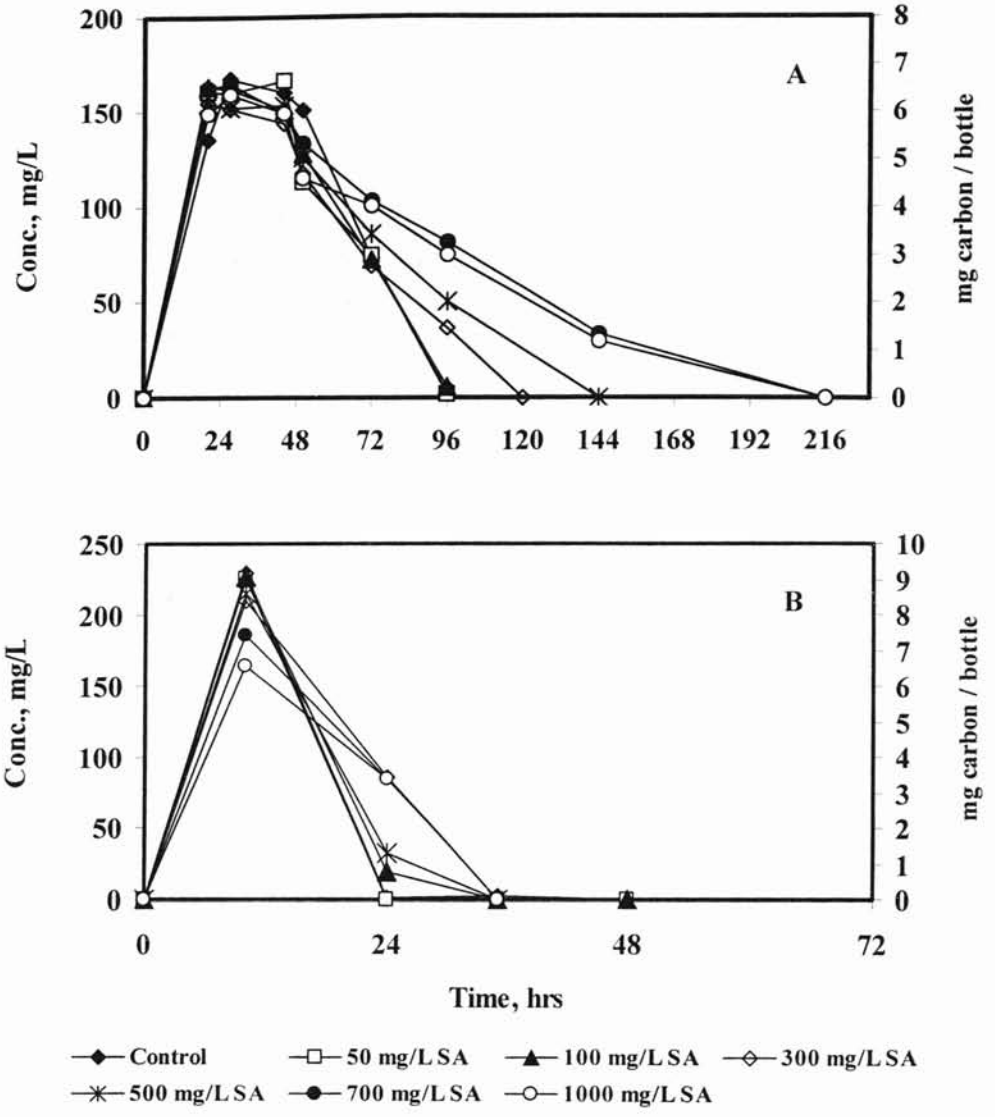

Figure 5.9: Acetic acid production profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ lactose for experiments performed on 09/03/02 (A) and 11/13/02 (B) (SA = stearic acid, averages for triplicate samples)

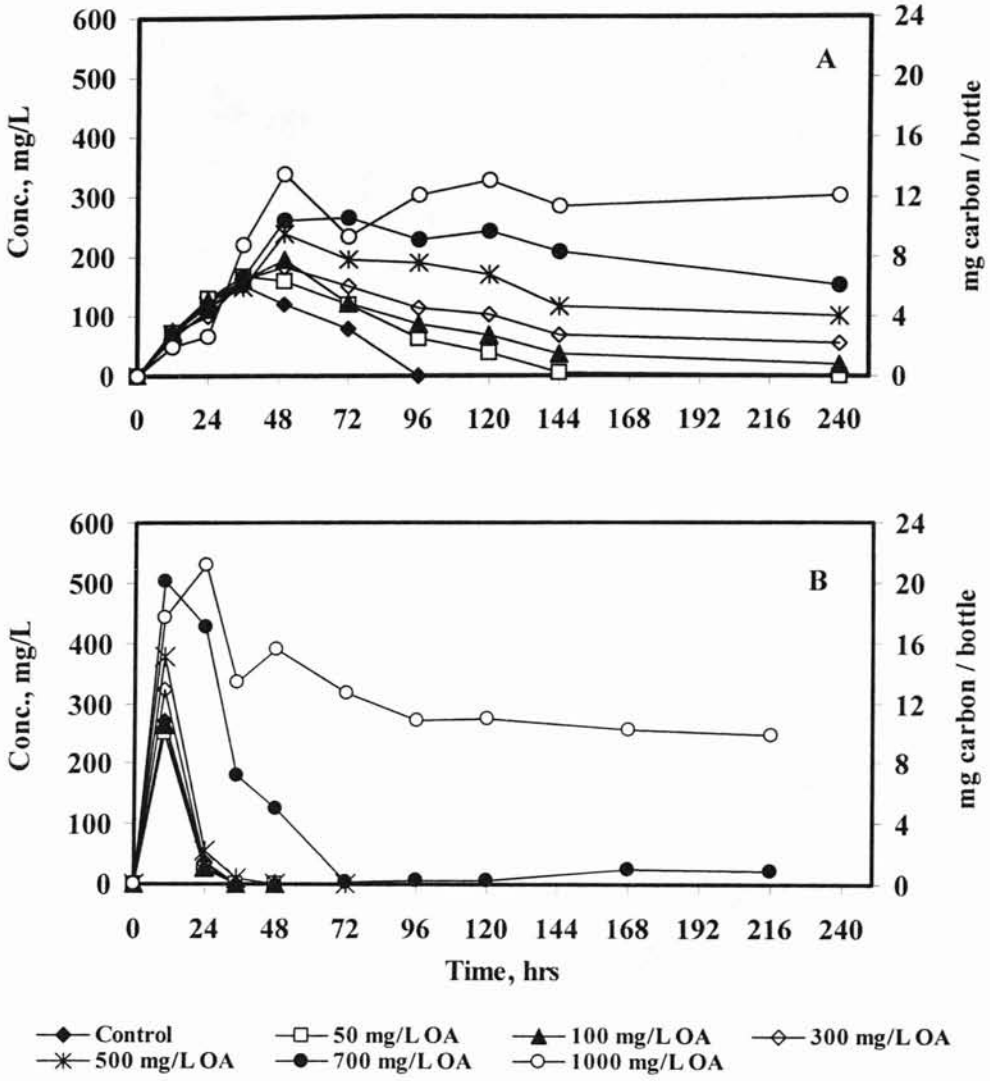

Figure 5.10: Acetic acid production profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ lactose for experiments performed on 09/10/02 (A) and 11/12/02 (B) (OA = oleic acid , averages for triplicate samples)

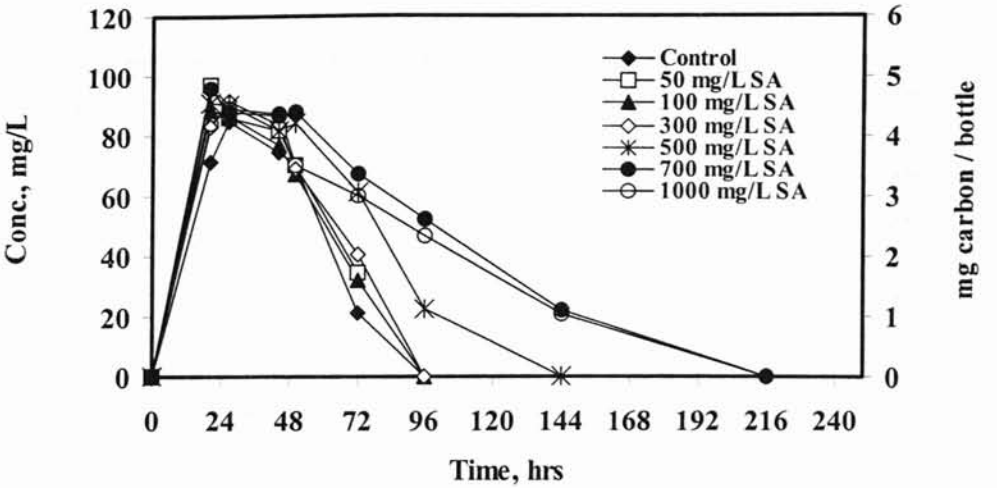

Figure 5.11: Propionic acid production profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ lactose for experiments performed on 09/03/02 (SA = stearic acid, averages for triplicate samples)

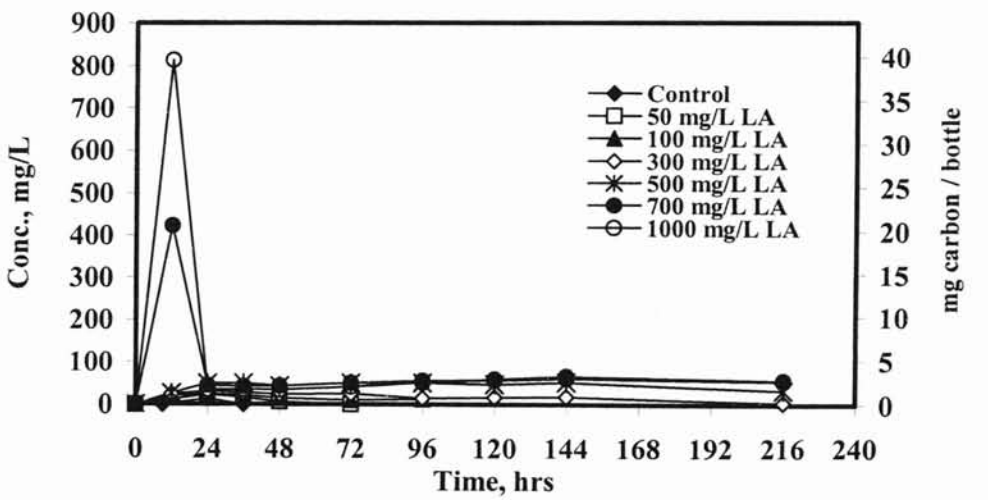

Figure 5.12: Propionic acid production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ lactose (LA= linoleic acid , averages for triplicate samples)

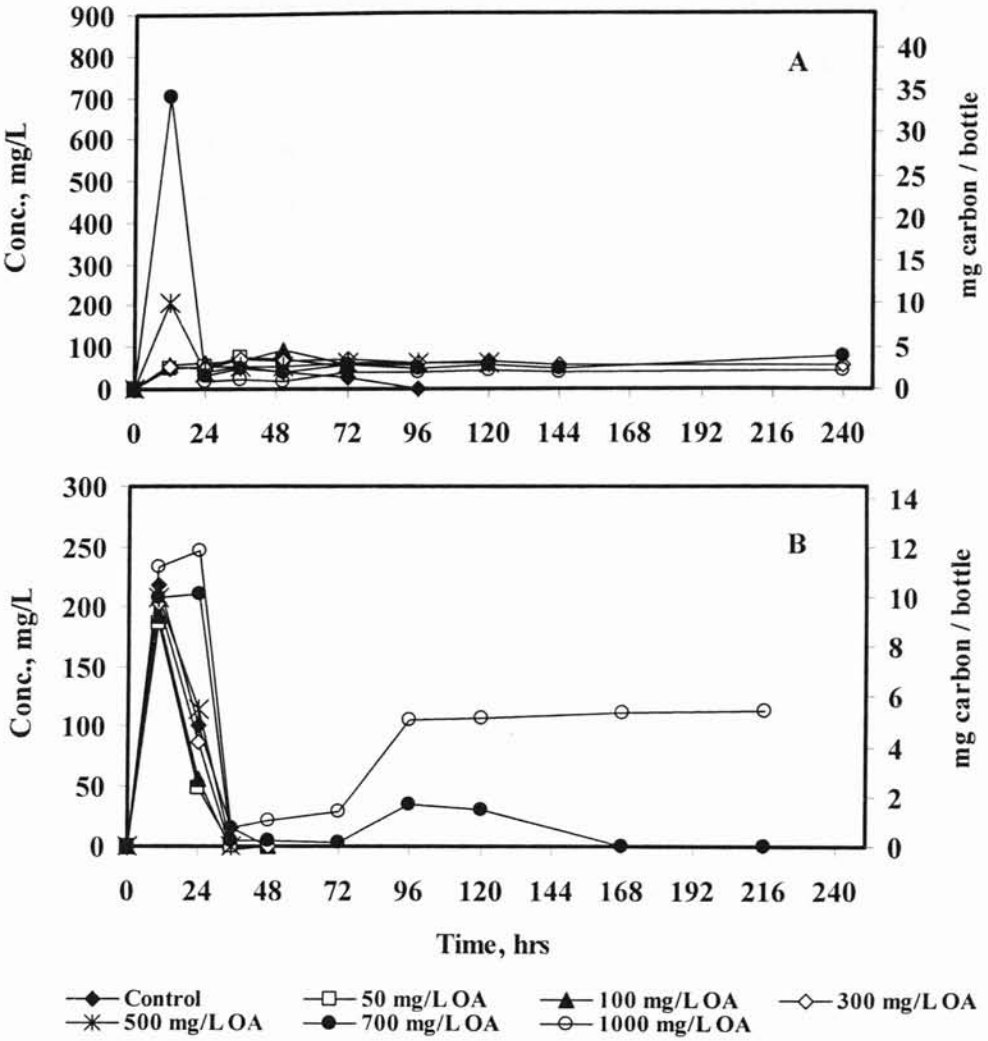

Figure 5.13: Propionic acid production profiles for cultures receiving oleic acid and 2000 $mg \cdot l^{-1}$ lactose for experiments performed on 09/10/02 (A) and 11/12/02 (B) (OA = oleic acid , averages for triplicate samples)

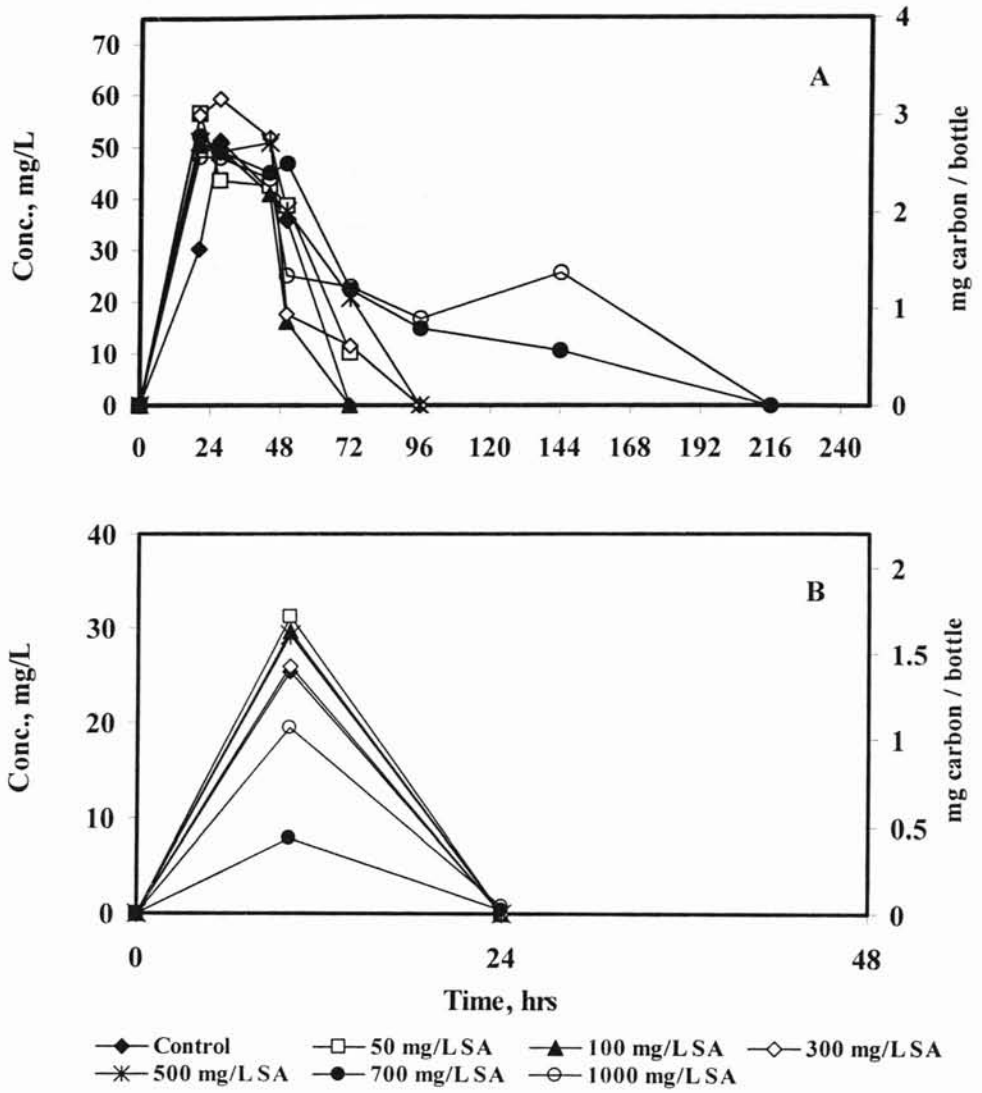

Figure5.14: Butyric acid production profiles for cultures receiving stearic acid and 2000 $mg \cdot l^{-1}$ lactose for experiments performed on 09/03/02 (A) and 11/13/02 (B) (SA = stearic acid , averages for triplicate samples)

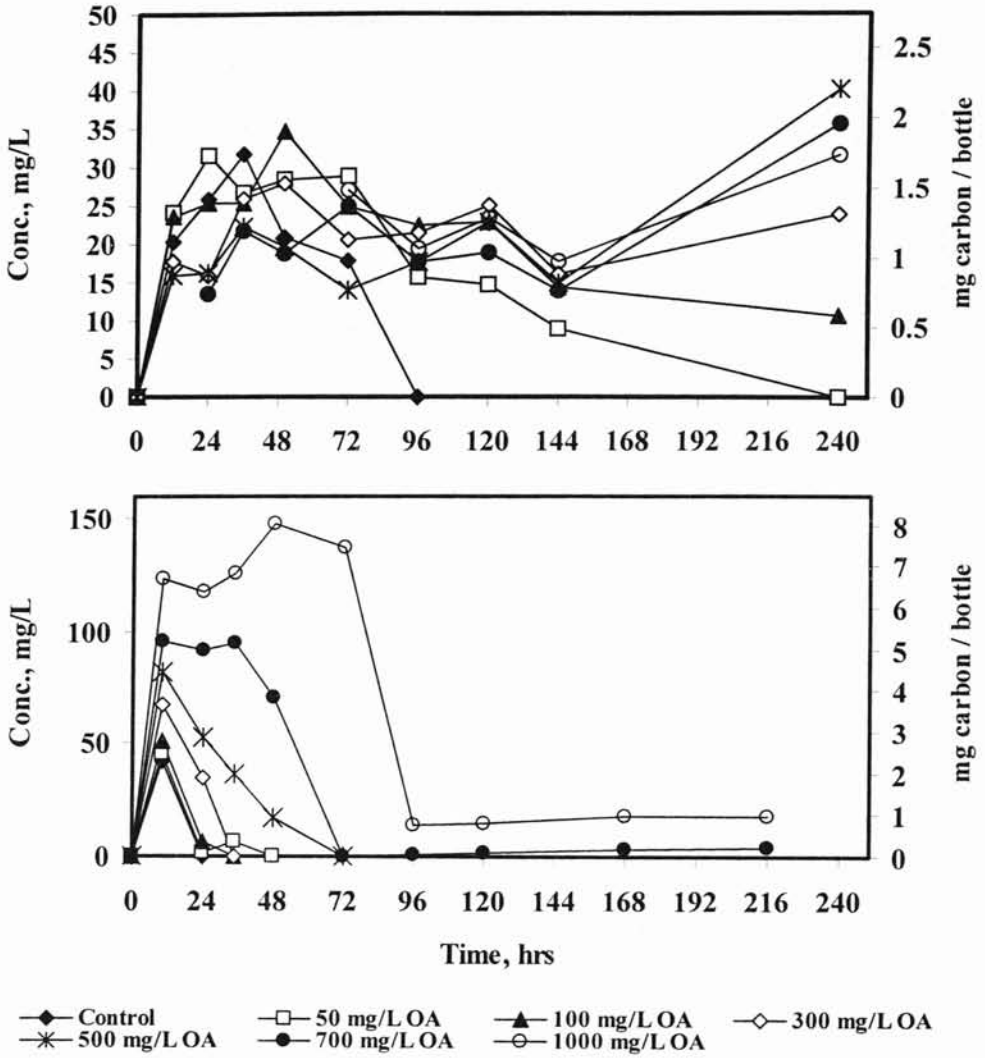

Figure 5.15: Butyric acid production profiles for cultures receiving oleic acid and 2000 mg·l^{-1} lactose for experiments performed on 09/10/02 (A) and 11/12/02 (B) (OA = oleic acid, averages for triplicate samples)

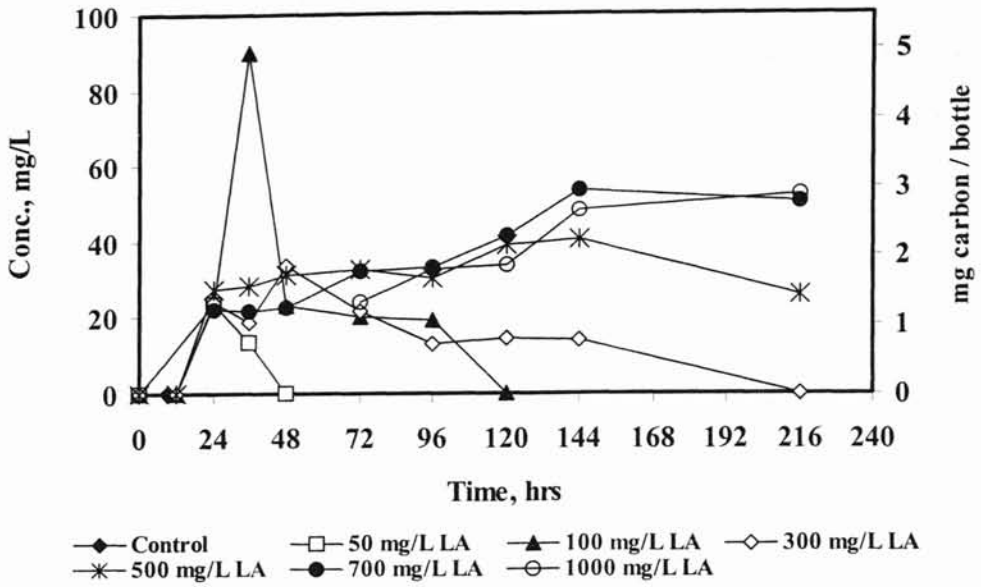

Figure 5.16: Butyric acid production profiles for cultures receiving linoleic acid and 2000 $mg \cdot l^{-1}$ lactose (LA = linoleic acid , averages for triplicate samples)

5.2 Discussion

Anaerobic treatment of dairy effluents, which contain large quantities of lactose, fats and proteins, is difficult due to the inhibitory effect of LCFAs (Clark, 1988; Perle *et al.*, 1995; Vidal *et al.*, 2000). Anaerobic degradation of lactose has been reported by several researchers (Kisaalita *et al.*, 1989; Yang and Guo, 1990; Fu and Mathews, 1999). However, data on the effect of LCFAs on lactose degradation has not been reported.

In general, the initial lactose degradation rates were less compared to initial glucose degradation rates. Lactose, a disaccharide, is hydrolyzed to glucose and galactose prior to metabolism of the sugar monomers. As a result, the degradation of lactose should be expected to take longer compared to glucose. The presence of a lag-phase in the control cultures for the SA and OA experiments and shorter consumption time in the LA experiments and repeated SA and OA studies indicated that biomass adaptation to lactose

takes longer compared to glucose. This could likely be due to slow growth of hydrolytic microorganisms in the mixed culture. This assumption is supported by Yang and Guo (1990), who reported complete degradation of 1500 $mg \cdot l^{-1}$ lactose in 24 hours in the packed bed immobilized cell reactor with total cell mass of 5000 to 10,000 $mg \cdot l^{-1}$. However, after adaptation of the reactor to whey permeate, which contained 0.6 to 2% lactose at 30-37°C for about a year, lactose degradation rate increased. Yang and Guo (1990) reported between 4500 to 5500 $mg \cdot l^{-1}$ of lactose was consumed in 2 to 5 hours and the degradation rates were between 2.1 to 4.8 $g \cdot l^{-1} \cdot h^{-1}$. In comparison, degradation rates from these studies were between 1.92 to 2.01 $g \cdot l^{-1} \cdot h^{-1}$ for cultures receiving LA.

The effects of SA on lactose degradation were similar to studies conducted with glucose. Although SA at concentrations greater than 100 $mg \cdot l^{-1}$ decreased the initial lactose degradation rates in the cultures less acclimated to lactose, the carbohydrate levels were undetectable within the same time period as in the controls. Moreover, the degradation rates for cultures fed with 300 to 1000 $mg \cdot l^{-1}$ SA were statistically the same. In the repeated studies, no effect of SA on lactose degradation was observed. The inhibitory effect of OA on the microorganisms depended on the degree of the biomass acclimatization to lactose. However, in both cases the substrate removal times in cultures fed with OA were close to the controls. LA was the most inhibitory with an extended lag-phase observed in cultures inoculated with 700 to 1000 $mg \cdot l^{-1}$ LA. In cultures receiving between 500 to 1000 $mg \cdot l^{-1}$LA, less inhibition was observed in biomass inoculated with lactose compared to those fed with glucose. For example, the initial glucose and lactose degradation rates for culture inoculated with 700 $mg \cdot l^{-1}$ LA were 0.82 and 1.01 respectively. Because of this difference in degradation rates, glucose

accumulation was observed for cultures inoculated with 500 to 1000 $mg \cdot l^{-1}$ LA (Figure 5.4)

Acidogenic and acetogenic organisms also were affected by LCFAs. In contrast to glucose fermentation, production of lactic acid was observed in each condition examined. This observation has been previously described in work by Fu and Mathews (1999), Peeva and Peev (1997), and Kisaalita *et al.* (1989). Acetic, propionic and butyric acids were also produced, but in lower concentrations. Among these three acids, contribution of acetate was the largest and production of butyrate was the lowest. The presence of 500 to 700 $mg \cdot l^{-1}$ OA and 700 to 1000 $mg \cdot l^{-1}$ LA caused a sudden increase in propionate production after 12 hours of incubation. The presence of threshold LA levels (greater than 300 $mg \cdot l^{-1}$ LA) inhibited lactate and acetate degradation causing VFA accumulation. Similar degradation patterns were observed for cultures fed with OA and SA thus suggesting inhibition of lactate degrading organisms at a threshold concentration. It should be mentioned that the inhibitory effects of OA and SA were more pronounced in cultures less acclimated to lactose, which resulted in lactate and acetate accumulations at lower LCFAs concentrations than compared with the biomass which was acclimated to the substrate over a longer time period. In those cultures, SA had the least effect on acidogens. However, OA at concentrations 700 to 1000 $mg \cdot l^{-1}$ inhibited propionate degrading organisms, which caused accumulation of propionate formed form acetate and butyrate.

The VFAs distribution profiles observed in the lactose fermentation experiments were different from VFA profiles for glucose fermentation. Results from this work support research by Kisaalita *et al.*(1989), who proposed a mechanism of lactose

fermentation using radioactive markers. They reported that in the initial phase lactose is degraded to pyruvate, which is later converted to lactate and butyrate. Lactate serves as precursor for propionate production, and butyrate is converted to acetate. Only a small amount of butyrate was detected in all studies. In the presence of hydrogenotrophic methanogens, lactate degradation is shifted to acetate instead of propionate. This is likely the main reason why propionate and butyrate concentrations were so low in the present study. Finally, degradation of lactose was more affected by LCFAs compared to glucose.

6.0 CONCLUSION

Wastewaters from food and dairy industries often contain mixtures of fats and carbohydrates. LCFA, a hydrolysis product of fats, causes inhibition carbohydrate fermentation. In this work, the effects stearic, oleic and linoleic acids on glucose and lactose fermentation were examined at 37°C. Lactose fermentation resulted in production of lactic acid, which was not observed as a byproduct of glucose fermentation. Consequently, the effects of LCFAs on glucose and lactose degradation were substrate-dependent. In addition, the degree of the biomass acclimatization to a specific substrate was an important factor affecting byproduct distribution profiles and the intensity of LCFAs inhibition.

In general, lactose degradation was less compared to glucose degradation. Lactose is first hydrolyzed with subsequent fermentation to glucose and galactose. Consequently, an additional reaction step precedes glucose degradation. The lower glucose degradation rate compared to the rate observed for lactose is the principal reason for glucose accumulation. The inhibitory effect of LCFAs on hydrolytic, acidogenic and aceticlastic methanogenic organisms depended on the LCFAs structure and increased in the following sequence: SA<OA<LA. This is clearly observed in the methane production levels. SA did not decrease methane production, however, cultures fed with 1000 $mg \cdot l^{-1}$ OA and 50 $mg \cdot l^{-1}$ or greater LA produced 50 % less methane than the controls.

LCFAs affected VFA production and degradation. In studies conducted with glucose, the greatest VFA accumulation was observed in cultures fed with LA. Nevertheless, all VFAs were removed within 5 days. In contrast, OA and LA at concentrations greater than 500 $mg \cdot l^{-1}$, inhibited lactate and acetate degradation

microorganisms and caused VFAs accumulation when lactose was used as a substrate. The inhibitory effects of SA and OA on acidogenic organisms were more intensive in the cultures poorly acclimated to lactose. For example, the addition of up to 1000 $mg \cdot l^{-1}$ SA to a well-acclimated biomass did not cause any significant changes in the VFAs metabolism. In contrast, a decrease in acetate and lactate degradation rates was observed in a less acclimated biomass.

LCFAs also affected metabolic pathways of glucose and lactose fermentation. OA and LA at threshold concentrations shifted carbon flow from methane to butyrate production in cultures fed with glucose. A slow conversion of acetate and butyrate to propionate followed by propionate accumulation was observed in cultures fed with 700 to 1000 $mg \cdot l^{-1}$ OA. In contrasts, in the controls all propionate disappeared from the solution within 35 hours, the same time as acetate and butyrate.

Finally, in comparison to previous studies (Lalman *et al.*, 2002), elevating the temperature from 25°C to 36°C increased both the substrate degradation rate and the inhibitory effects of LCFAs. In cultures fed with SA, temperature elevation increased the reaction rate, but did not significantly affect the inhibitory properties of SA. This resulted in higher initial glucose degradation rates, than compared to those at 25°C. Contrary, higher temperature significantly enhanced the inhibitory LA effect, leading to lower initial glucose degradation rates.

7.0 ENGINEERING SIGNIFICANCE

The major intent of the present work was to identify factors affecting the anaerobic treatment of fat and carbohydrate containing waste. Information from this work can be used by process engineers or for the determination of factors affecting a reactor failure. For example, anaerobic treatment of lactose and oleate waste, which is representative of a dairy waste, can cause accumulation of lactic and acetic acids. As a result, the reactor may be inhibited under high loading conditions. Knowing the possible outcome of such treatment, a designer can propose a two stage treatment process. In the first stage, where the hydraulic retention time is approximately 12-24 hours, the carbohydrates are removed. Then, the effluent with high concentrations of VFAs can be directed into a second methanogenic digester for VFAs degradation. This idea was proposed by Hanaki *et al.* 1981, however, it never was applied.

Another application arises from the ability of oleic and linoleic acids to cause VFA accumulation, which may be used for production of acetic or lactic acid from carbohydrate containing wastes. This would add value to the waste and allow economical production of valuable products. For example, applying of 1000 $mg \cdot l^{-1}$ OA or 500 to 1000 $mg \cdot l^{-1}$ LA to lactose containing waste at hydraulic retention time of 5 days would produce lactic acid at concentration between 500 to 600 $mg \cdot l^{-1}$. However, the product will also contain acetic and butyric acids. Consequently, a separation technique, for example ion exchange column, could be used as a final processing step.

As discussed in the literature review, one of the reasons of LCFA toxicity is their adsorption around sludge granules. Hwu *et al.* (1998) observed sludge granules with thick

white coats (0.2-0.3 mm compared to the diameter of the granules, which was 2-3 mm) in the sludge of an expanded granular sludge bed (EGSB) reactor. Consequently, addition of an alternative adsorption surface, for example bentonite granules, with more LCFA affinity in comparison to sludge granules, would exclude LCFA toxicity. Then, the LCFAs desorbed from the surface would be slowly degraded.

The present work was conducted in a laboratory scale batch reactors. The length of each experiment did not exceeded ten days and the LCFAs were applied to the biomass only once. It is not known how LCFAs would affect a long-term performance of a full scale treatment system. Consequently, it is important to perform experiments over extended periods with several LCFAs feedings. In addition, concentrations and the types of carbohydrates in full scale treatment system are different from the substrate used in this work. Thus, investigation of LCFAs effects on anaerobic fermentation of polysaccharides, such as starch, at different concentrations would be the next stage of this work. Moreover, not only VFAs, but also alcohols should be monitored in order to determine the complete spectrum of fermentation byproducts.

REFERENCES

Alosta H. (2002) Effects of linoleic, oleic and stearic acids on the anaerobic fermentation of glucose. *MS thesis*, Biosystems and Agricultural Engineering Dept, Oklahoma State University, Stillwater, USA

Altman P. L., Ditmer D. S. (1964) *Biology data book*. Wachington D.C.: Federation of American societies for experimental biology

Alves M. M., Vieira J. A. M., Pereira R. M. A., Pereira M. A., Mota M. (2001) Effects of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part II: oleic acid toxicity and biodegradability. *Water Res.* 35(1), 264-270

Angelidaki I., Ahring B. K. (1992) Effects of free long-chain fatty acids on thermophilic anaerobic digestion. *App. Microbiol. Biotechnol.* 37(6), 808-812

Angelidaki I., Ahring B. K. (1995) Establishment and characterization of an anaerobic thermophilic (55^0C) enrichment culture degrading long-chain fatty acids. *Appl. Environ. Microbiol.* 6, 2442-2445

Batstone D. J. (1999) High rate anaerobic treatment of complex wastewater. *PhD. Thesis*, Advanced Wastewater Management Center, The University of Queensland, Brisbane, Australia

Beccari M., Bonemazzi F., Majone M., Riccardi C. (1996) Interaction between acidogenesis and methanogenesis in the anaerobic treatment of olive oil mill effluents. *Water Res.* 30(1), 183-189

Broughton M. J., Thiele J. H., Birch E. J., Cohen A. (1998) Anaerobic degradation of sheep tallow. *Water Res.* 35(5), 1423-1428

Canovas-Diaz M., Sanchez-Roig M. J., Iborra J. L. (1992) Myristic and oleic acid degradation by an acclimated anaerobic consortia: synergetic behavior. *Biomass Energy, Ind. Environ., E.C. Conf., 6th*, London. 580-584

Clark J. N. (1988) Anaerobic digestion of whey in pilot scale UASB digester. Poster Paper Book, *5th Int. Symposium on Anaerobic Digestion*, Bolgna. 489-493

Demeyer D. I., Henderickx H. K. (1967) The effects of C18 unsaturated fatty acids on methane production in vitro by mixed rumen bacteria. *Biochem. Biophys. Acta.* 137, 484-497

Foster C. F. (1992) Oils, fats and greases in wastewater treatment. *J. Chem. Technol. Biotechnol.* 55, 402-404

Fu W., Mathews A. P. (1999) Lactic acid production from lactose by lactobacillus plantarum: kinetic model and effect of pH, substrate and oxygen. *Biochem. Eng. J.* 3, 163-170

Galbraith H., Miller T. B., Paton A. M., Thompson J. K. (1971) Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, egrocalciferol and cholesterol. *J. Appl. Bacteriol.* 34(4), 803-813

Galbraith H., Miller T. B.(1973) Phisicochemical effects of long chain fatty acids on bacterial cells and their protoplasts. *J. Appl. Bacteriol.* 36, 647-658

Hanaki K., Matsuo T., Nagase M. (1981) Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. *Biotechnol. Bioengng.* 23, 591-1610

Hwu C. H., Dolnon B., Lettinga G. (1996) Comparative toxicity of long-chain fatty acids to anaerobic sludges from various origins. *Water Sci. Technol.* 34(5-6), 351-358

Hwu C. H., Lettinga G. (1997) Acute toxicity of oleate to acetate-utilizing methanogens in mesophilic and thermophilic anaerobic sludges. *Enzyme Microb. Technol.* 21, 297-301

Hwu C. H., Tseng S. K., Yuan C. Y., Kulik Z., Lettinga G. (1998) Biosorption of long-chain fatty acids in UASB treatment process. *Water Res.* 32(5), 1571-1579

Hwu C. H., van Lier J. B., Lettinga G. (1998) Physicochemical and biological performance of expanded granular sludge bed reactors treating long-chain fatty acids. *Process Biochem.* 33(1), 75-81

Kalyuzhnyi S. V., Davlyatshina M. A. (1997) Batch anaerobic digestion of glucose and its mathematical modeling. Kinetic investigations. *Biores. Technol.* 59, 73-80

Kisaalita W. S., Lo K. V., Pinder K. L. (1989) Influence of dilution rate on the acidogenic phase products distribution during two-phase lactose anaerobiosis. *Biotechnol. Bioengng.* 34, 1235-1250

Komatsu T., Hanaki K. (1991) Prevention of lipid inhibition in anaerobic process by introducing a two-phase system. *Water Sci. Technol.* 23, 1189-1200

Koster I. W. (1987) Abatement of long-chain fatty acid inhibition of methanogenesis by calcium addition. *Biol. Wastes* 22, 295-301

Koster I.W., Cramer A. (1987) Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. *Appl. Environ. Microbiol.* 53(2), 403-409

Lalman J., Alosta, H., Bellmer, D. Glucose fermentation in the presence of linoleic, oleic and stearic acid by mixed culture. Submitted to *Biores. Technol.*

Lalman J., Bagley D. M.(2000) Anaerobic degradation and inhibitory effects of linoleic acid. *Water Res.* 34(17), 4220-4228

Lalman J., Bagley, D. M. (2001) Anaerobic degradation and methanogenic inhibitory effects of oleic and stearic acids. *Water Res.* 35(12), 2975-2983

Lalman J., Bagley, D. M. (2002) Effect of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation . *Water Res.* 35, 3307-3313

Lehninger A. L., Nelson D. L., Cox M. M. (1999) *Principles of Biochemistry*, 2nd ed., New York, NY: W.H. Freeman & Sons

McCarty P. L.(1964) Anaerobic waste treatment fundamentals, part I, chemistry and microbiology. *Public Works* 95(September), 107-112

Pavlostathis, S.G. (1991) Kinetics of anaerobic treatment: a critical review. *Crit. Rev. Environ. Control* 21(5,6), 411-490

Peeva L., Peev G. (1997) A new method for pH stabilization of the lactoacid fermentation. *Enzyme Microb. Technol.* 21, 176-181

Perle M., Kimchie S., Shelef G. (1995) Some biochemical aspects of the anaerobic degradation of dairy wastewater. *Water Res.* 29(6), 1549-1554.

Petruy R., Lettinga G. (1997) Digestion of a milk-fat emulsion. *Biores. Technol.* 61, 141-149

Rinzema A., Boone M., van Knippenberg K., Lettinga G. (1994) Bacterial effect of long-chain fatty acids in anaerobic digestion. *Water Environ. Res.* 66(1), 40-49

Rinzema A. (1988) Anaerobic treatment of wastewater with high concentration of lipids and sulphate. *Ph.D thesis,* Wageningen Agricultural University, Wageningen, The Netherlands.

Rittmann B., McCarty P. L. (2001) *Environmental Biotechnology: Principles and Applications.* New York, NY: McGraw-Hill Publ.

Steel R. G. D., Torrie J. H., Dickey D. A. (1997) *Principles and procedures of statistics. A biometrical approach, 3rd edition.* New York, NY: McGraw-Hill, Inc.

Solomons T. W. (1992) *Organic Chemistry, 5th eddition.* New York, NY: Wiley & Sons Inc.

Vidal G., Carvalho A., Mendez R., Lema J. M. (2000) Influence of the content in fats and proteins on the anaerobic biodegradability of dairy wastewaters. *Biores. Technol.* 74, 231-239

Yang S. T., Guo M. (1990) Kinetics of methanogenesis from whey permeate in packed bed immobilized cells bioreator. *Biotechnol. Bioengng.* 36, 427-436

Zoetemeyer R. J., Heuvel J. C., Cohen A. (1982) pH influence on anaerobic dissimilation of glucose in an anaerobic digester. *Water Res.* 16, 303-311

APPENDICES

APPENDIX A: Tukey's Procedure for Statistical Comparisons

Tukey's procedure is used for pairwise comparisons of the means. The difference between means is compared to a critical value: $w = q_{\alpha}(t, df_w)S_Y$

If that difference is larger than w, then two means are statistically different.

Experimental conditions for each experiment were:

treatment	1	2	3	4	5	6	7
LCFA concentration, $mg \cdot l^{-1}$	0 0 0	50 50 50	100 100 100	300 300 300	500 500 500	700 700 700	1000 1000 1000

For conditions specified above $\alpha = 0.05$, $df = 21$, $q = 4.6$

Example: Compare initial glucose degradation rates for cultures receiving linoleic acid (see Table 4.1)

C(stearic acid), $mg \cdot l^{-1}$	Average rate	Std	Var, S^2
0	24.61	3.25	10.5625
50	20.04	0.59	0.3481
100	18.79	0.49	0.2401
300	14.28	0.16	0.0256
500	6.39	0.04	0.0016
700	0.82	0.06	0.0036
1000	0.75	0.04	0.0016
		avg	1.597585714

w=q*s*sqr(1/2* (1/3+1/3)) = 4.6* $(1.597585714)^{0.5}$ * $(1/3)^{0.5}$ = 3.3568
Now, the difference between average means is compared to w= 3.3568

	control	50 SA	100 SA	300 SA	500 SA	700 SA	1000 SA
averages	24.61	20.04	18.79	14.28	6.39	0.82	0.75
difference	0	4.57	5.82	10.33	18.22	23.79	23.86
		0	1.25	5.76	13.65	19.22	19.29
			0	4.51	12.4	17.97	18.04
				0	7.89	13.46	13.53
					0	5.57	5.64
						0	0.07
							0

The result of comparison is that

1) Degradation rate of the control is statistically larger than degradation rate of any Treatment
2) Degradation rates of cultures receiving 50 and 100 $mg \cdot l^{-1}$ SA are statistically the same and larger than those of 300, 500, 700, and 1000 $mg \cdot l^{-1}$
3) Degradation rates of cultures receiving 700 and 1000 $mg \cdot l^{-1}$ SA are statistically the same and smaller than any others

Finally, the table was constructed:

	LCFA Concentration						
	0 mg/L	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
	Glucose Degradation Rate (μg glucose$\cdot VSS^{-1} \cdot min^{-1}$)						
LA	24.61 ± 3.25^{a}	20.04 ± 0.59^{b}	18.80 ± 0.49^{b}	14.28 ± 0.16^{c}	6.39 ± 0.04^{d}	0.82 ± 0.06^{e}	0.75 ± 0.04^{e}

Means followed by the same letter are not statistically different within the rows.

VITA 2

Irina Victorovna Komjarova

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF STEARIC, OLEIC AND LINOLEIC ACIDS ON THE ANEROBIC FERMENTATION OF GLUCOSE AND LACTOSE

Major Field: Biosystems Engineering

Biographical:

Education: Received Engineer degree in Environmental Engineering for Mendeleyev university of Chemical technology of Russia, Moscow, Russia in February 1997. Completed requirements for the Master of Science degree with major in Biosystems Engineering at Oklahoma State University in December, 2002

Experience: Previously employed as an Engineer for the department of Biosphere Protection Technology at Mendeleyev university of Chemical technology of Russia, currently employed as a Research Assistant for the Department of Biosystems Engineering, Oklahoma State University, 2000 to present