EFFECTS OF LINOLEIC ($C_{18:2}$), OLEIC ($C_{18:1}$), AND STEARIC ($C_{18:0}$) ACIDS ON THE ANAEROBIC FERMENTATION OF GLUCOSE

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Nomenclature

COD	chemical oxygen demand
GC	gas chromatography
IC	ion chromatography
K_I	inhibitor binding affintity
$K_{M\ app}$	substrate apparent binding affinity
LA	linoleic acid
LCFA	long-chain fatty acids
OA	oleic acid
SA	stearic acid
TSS	total suspended solids
VSS	volatile suspended solids
VFA	volatile fatty acids
Vmax	maximum reaction velocity

1.0 INTRODUCTION

1.1 Context

Industrial effluents such as effluents from edible oil (Paredes *et al.,* 1999), egg (Xu *et al.,* 2001), cheese (Harper *et al.,* 1971), and ice-cream processing industries (Borja and Banks, 1995) contain significant amounts of fats, oils, and carbohydrates. The composition of these effluents, however, is variable and depends of the processing industry. These types of effluents are of greater concern in anaerobic treatment systems due to the inhibitory effects of long-chain fatty acids (LCFAs) against microorganisms.

When treated anaerobically, carbohydrates and lipids in wastewater are first hydrolyzed to simple sugars and LCFAs respectively. In turn, sugars and LCFAs are converted to VFAs which is ultimately degraded to acetate and subsequently to methane carbon dioxide. Fats and oils from animals and vegetables are known for their ability to inhibit anaerobic microorganisms. Nieman (1954) reported that the inhibitory effects of unsaturated fatty acids increased as the number of double bonds increased. Similarly, Lalman and Bagely (2000, 2001) reported that LCFA inhibitory effects on acetogenesis increased as follows: linoleic acid (LA) > oleic acid (OA) > stearic acid (SA). Long-chain fatty acids (LCFAs) toxicity has been proposed to be the mechanism of inhibition for suspended or flocculent sludge (Hwu *et al.* 1996). Demeyer and Henderickx (1967) explained that unsaturated LCFAs toxicity is caused by adsorption onto the bacterial cell with subsequent alteration of the cell permeability.

The effects of LCFAs on acetogenesis and methanogenesis have been reported by several researches (Alves *et al.* (2001), Lalman and Bagley (2001), Lalman and Bagley

(2000), Rinzema *et al.* (1994), Angelidaki and Ahring (1992), Koster and Cramer (1987), Hanaki *et al.* (1981)). Lalman and Bagley (in press) and Hanaki *et al.* (1981) have reported some work on the effects of LCFAs on glucose fermentation. However, there exist several unanswered questions on the effects of LCFAs. The objectives of this research is to assist in filling the gap of knowledge which still exist in the literature.

1.2 Objectives

The objective of this work is to examine the degradation of three C_{18} LCFAs and inhibition of LCFAs on the degradation of glucose. Except for the work of Hanaki *et al.* (1981) and Lalman and Bagley (in press), no other work has reported the effects of LCFAs on carbohydrates fermentation.

In the first objective, the effects of three LCFAs on glucose degradation will be investigated. The LCFAs under investigation include linoleic ($C_{18:2}$), oleic ($C_{18:0}$), and stearic ($C_{18:1}$) acids. LCFA initial degradation rates from control cultures will be compared to those from cultures fed with 1000 and 2000 mg/L glucose.

In the second objective, the kinetics of LCFA inhibition on glucose fermentation will be examined. Cultures receiving individual LCFAs will be compared with those receiving LCFA mixtures to determine if any synergistic effects exist. This is particularly important because many food processing wastewater effluents contain LCFA mixtures. LCFA mixtures under consideration include LA/SA, LA/OA, SA/OA, and LA/SA/OA. This part of the study is of particular importance because, to date, no inhibition kinetics data on individual or mixtures of LCFAs are available.

1.3 Literature Review

Long-chain fatty acids (LCFAs) refer to saturated or unsaturated straight-chain even or odd-numbered carbon atom carboxylic acids that are commonly found in lipids. Most commercial fatty acids are obtained through hydrolysis or saponification of animal and vegetable fats and oils. Fats and oils and their fatty acids may be consumed edibly or used in the manufacture of many products. Animal fats are produced at both meatpacking plants and independent rendering plants. Animal fat, an important raw material for LCFA manufacturing, is a primary source of stearic and oleic acids. In contrast, vegetable oils are an important raw material for fatty acid and fatty ester production principally because of their high LCFA content.

Typically, LCFAs are the major constituents of oils and fats. LCFAs can be found in vegetable oils like cottonseed, soybean, and olive oils and animal fats like whale oil, fish liver oil, and lard oil. Shorter-chain acids (C_4-C_{10}) are mostly present in milk fat whereas longer chain acids (C_{12}-C_{24}) are found in seed oil and animal fat (Gunstone, 1967). The percent of LCFAs in these oils, however, is variable. Cottonseed oil, for example, consists of the glycerol esters of linoleic (44%), oleic (32%), palmitic (21%), stearic (2%), and myristic (0.5%) acids. Whereas olive oil consists of oleic (84.6%), palmitic (8%), linoleic (5%), stearic (2%), myristic (0.2%), and arachidic (0.2%) acids (% by weight).

1.4 Long-Chain Fatty Acids Industrial Applications

Fatty acids in the form of esters, amides, and alcohol sulfates are used in soap manufacturing due to their antibacterial properties. They are also used as surface-active

agents and for removing odor-causing substances. Fatty acids represent a very important ingredient in the coating industry such as paint industry, where natural unsaturated oils and heat-polymerized oils are the dominant constituent of exterior paints, and epoxy resin esters industry. In cosmetic manufacturing, fatty acids and their derivatives are widely used with stearic and palmitic acids in many formulations. The rubber industry also represents a major user of fatty acids and their derivatives. They are used as components for emulsifiers and emulsion polymerization. In the textile industry, many synthetic fatty-acid-based detergents are used in textile scouring operations. Also, sulfated oleic acid esters are widely used to assist in dyeing operations. Food processing is also another industry where LCFAs are commonly used. LCFAs are main ingredients for shortenings, dairy products like margarine and ice cream, confections, and starch products like macaroni and potato products. The majority of fatty acids and their derivatives are used as emulsifiers, solubilizers, dispersing agents, or stabilizers in pharmaceuticals. They are also used as a processing aid in antibiotics manufacturing, as protective coatings for medicinal agents, as ointment bases, and most importantly as bactericides.

1.5 Lipids In Wastewater

Lipids are one of the major organic compounds found in domestic and industrial wastewaters (Kramer, 1971). In domestic sewage, lipids concentration range from 40 mg/L to 100 mg/L (Foster, 1992). Since lipids are water insoluble, they exist either as an emulsion or as a separate layer (Kramer, 1971). Industrial wastewater effluents contain much higher concentrations of lipids and are of greater concern due to the lipids' potential toxicity to wastewater treatment microorganisms. Edible oil processing, egg-

processing, slaughterhouses, fishmeal factories, dairy processing, and potato chips and confectionery industries are examples of food processing industries generating effluents containing LCFAs.

Edible oil industries are one of the major contributors to wastewater with high lipids contents. This wastewater arises during fruit extraction and usually contains pulp, mucilage, and oil (Paredes *et al.* 1999). Paredes *et al.* (1999) reported that olive mill wastewater collected from different mills in southern Spain contains fats and carbohydrates in varying amounts. The fat content varied between 0.55 to 11.37 % and carbohydrates ranged from 3.37 to 32.91 % (dry weight). Oil and grease concentration in wastewater effluents, from palm oil industries in Malaysia, is approximately 8,000 mg/L (Ma and Ong, 1986). In terms of biological oxygen demand (BOD), this concentration is 100 times more polluting than domestic sewage. In the Mediterranean region, olive oil mill wastewater is a substantial pollutant of both terrestrial and aquatic ecosystems. This region alone generates 3.0×10^7 m^3/year of wastewater containing high organic loads (D'Annibale *et al.* 1999) with Spain and Italy being the greatest contributors (Paredes *et al.*, 1999).

It is estimated that egg processing in the U.S. generates more that 9.46 billion liters (2.5 Billion gallons) of wastewater annually ranging from 4,000 to 14,000 mg COD (chemical oxygen demand)/L and containing a substantial amount of egg fat (32 to 42% by weight) (Xu *et al.* 2001). Moreover, Xu *et al.* (2001) reported that these organic compounds in wastewater cause serious pollution problems. Several approaches, mainly chemical precipitation or coagulation technologies, have been proposed to remove these

organics. However, the increasing costs for chemical and physical treatment make biological treatment more economically feasible.

Slaughterhouses are also among the major contributors of oil and grease waste. The concentration is variable and ranges from 100 mg/L (Sayed *et al.* 1987) to 897 mg/L (Sachon, 1984). The reason for this variation is reported to be caused by the type of process, water consumption, and animal type and size (Johns, 1995).

Performance results of a pilot–scale anaerobic treatment system treating wastewater from a poultry processing plant have been presented by Harper *et al.* (1990). They reported that fats, oils, and greases in wastewater varied between 169 mg/L to 1316 mg/L. In a survey of the U.S. dairy industry, Harper *et al.* (1971) reported the average amount of wastewater generated for cheese producers was 3.14 m^3 wastewater per ton of milk processed. In contrast, Danalewich *et al.* (1998) reported the amount of wastewater generated was 2 to 3 times less than the data provided by Harper *et al.* (1971). According to Danalewich *et al.* (1998), this reduction is due to increased plant size, automation in product processing, and waste minimization practices by dairy industries. The mean total chemical oxygen demand (COD) of the wastewater was 2,855 mg/L. However, they did not report what percentage of the total COD was derived from fat instead they reported that the whole milk was 4% fat by weight. On the other hand, Hwang and Hansen (1998) estimated that wastewater from a cheese processing industry produce as much as 62,838 mg/L of total COD of which 3.3% (2,060 mg/L) was derived from fat. The reason for the variation in COD concentration reported by Danalewich *et al.* (1998) is likely due to the dilute character of wastewater which included wastewaters from cheese manufacturing as well as utility operations while Hwang and Hansen (1998) reported the total COD for

wastewater arising only from cheese manufacturing. In comparison, Yu and Fang (2001a) reported dairy wastewaters containing 9,870 mg/L carbohydrates and 12,600 mg/L lipids. Yu and Fang (2001b) also reported a dairy wastewater containing 1,240 mg/L carbohydrates and 1,670 mg/L lipids.

Effluents from ice-cream industries also contain significant amounts of carbohydrates and lipids. Borja and Banks (1995) investigated the response of an anaerobic fluidized bed reactor treating ice-cream wastewater. They reported the average amount of carbohydrate, starch, and total fat (saturated, monosaturated, and polyunsaturated) were 0.21%, 0.001%, and 0.063% (of the total weight) respectively.

Tuna processing is also another major industry contributing to wastewater containing LCFAs. Nair (1990) reported that the waste load discharged from tuna canning factories contains BOD levels ranging from 3,000 mg/L to 5,000 mg/L with highly variable oil and grease levels. Guerrero *et al.* (1998) also reported that fishmeal effluent is rich in polyunsaturated LCFAs. The estimated COD range for this effluent was between 30,000 to 120,000 mg/L with a fat and carbohydrate content at approximately 21 and 7% respectively.

El-Gohary (1999) examined the anaerobic digestion of wastewaters from potato chip and confectionery factories. Oil and grease concentration averaged 367 mg/L for confectionery wastewater and 170 mg/L for potato chips wastewater. In addition to oil and grease, wastewater effluents from potato processing industries contain significant quantities of carbohydrates.

In wastewaters from dairy, ice cream, and potato processing industries, the presence of both carbohydrates and LCFAs is of interest because of the inhibition caused

by LCFAs during anaerobic treatment. Carbohydrates and lipids are first hydrolyzed to simple sugars and LCFAs respectively. In turn, sugars and LCFAs are converted to VFAs which is ultimately degraded to methane. Figure 1.1 shows the pathways of both carbohydrates and lipids in wastewater. Several researchers have shown that LCFAs are inhibitory to acidogens and methanogens (Hanaki *et al.*, 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1992; Rinzema *et al.*, 1994; Lalman and Bagley, 2000; and Alves *et al.*, 2001). However, except for the work of Hanaki *et al.* (1981) and Lalman and Bagley (in press) no other work investigated the effects of LCFAs on acidogens and methanogens.

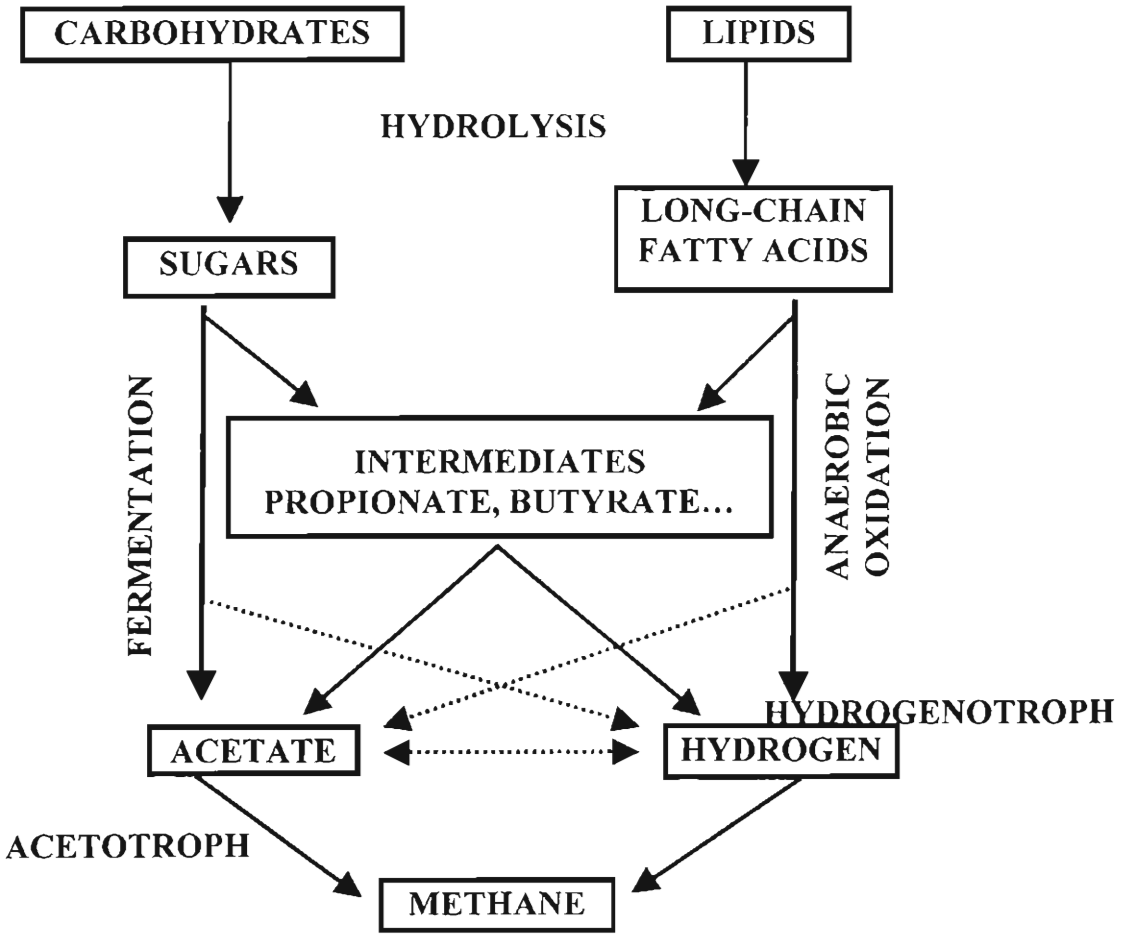


Figure 1.1. Degradation pathways of carbohydrates and lipids in wastewater (adapted from Gujer and Zehnder, 1983)

To reduce pollution caused by wastewaters containing high fat concentrations, some industries, particularly dairy industries, recycle and reuse waste components such as for example, using cheese whey for animal feed (Perle *et al.* 1995). Another method to reduce pollution is to use physical-chemical treatment. However, this type of treatment is costly and provides poor results in removing soluble COD (Vidal *et al.* 2000). This is a major concern since the largest fraction of the fat and grease present in wastewater is present in a nonsettleable form (Kramer, 1971). Typically, anaerobic treatment is suitable and cost-effective for this type of wastewater in spite of the problems associated with treating effluents containing high fat concentrations (Martinez *et al.* 1995; Saxena *et al.* 1986; Sayed *et al.* 1988). Anaerobic treatment has several advantages over aerobic treatment because it can accommodate higher organic loadings, it eliminates the cost associated with aeration, less biomass is produced, and usually the end products are more valuable.

1.6 LCFA β-Oxidation

Several microorganisms are reported to carry out the hydrogenation of unsaturated LCFAs. Selected microorganisms reported to mediate the hydrogenation of linoleic and oleic acids are listed in Table 1.1.

Table 1.1. Selected microorganisms performing LCFAs hydrogenation reactions

LCFA	Product	Species	Reported by
LA	*Trans*-11-$C_{18:1}$ *Cis*-9-$C_{18:1}$ $C_{18:0}$	*Fusocillus T344*	Hazlewood *et al.* (1976) Kemp *et al.* (1975)
OA	10-hydroxystearic acid	*Nocardia paraffinae*	Latrasse *et al.* (1997)

Novak and Carlson (1970) reported that complete LCFA saturation is required before β-oxidation is initiated. In contrast, several researchers have reported shorter carbon chain unsaturated LCFAs by-products from the degradation of linoleic and oleic acids (Canovas-Diaz et al. 1991; Lalman and Bagley 2000). These studies provide evidence that complete saturation is not a complete prerequisite for β-oxidation. Figure 1.2 shows the interaction between LA and the active sites as reported by Harfoot (1978). The substrate is attached to hydrogen bond and hydrogen donor sites, biohydrogenation of LA to trans-11-octadecenoic acid is catalyzed by linoleic acid Δ^{12}-*cis*, Δ^{11}-*trans*-isomerase (Kepler *et al.*, 1971). The conformation structure of LA may assist in binding to the enzyme active site and hence, higher degradation rates are observed compared to OA and SA.

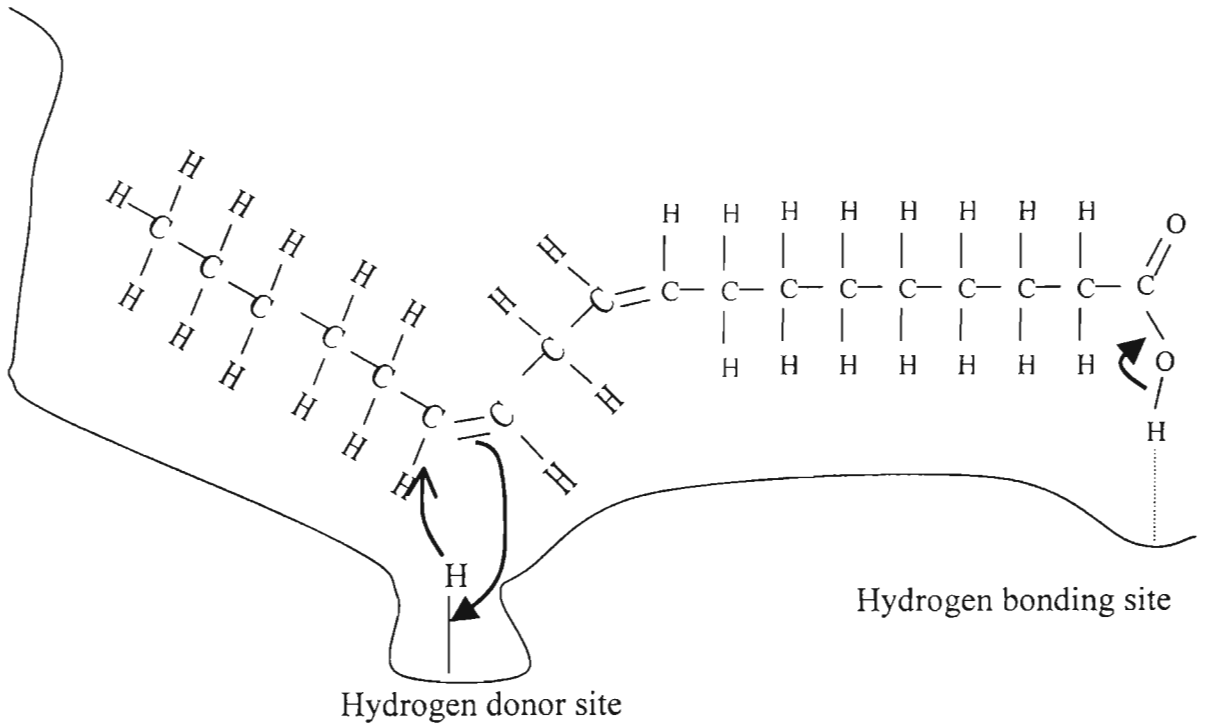
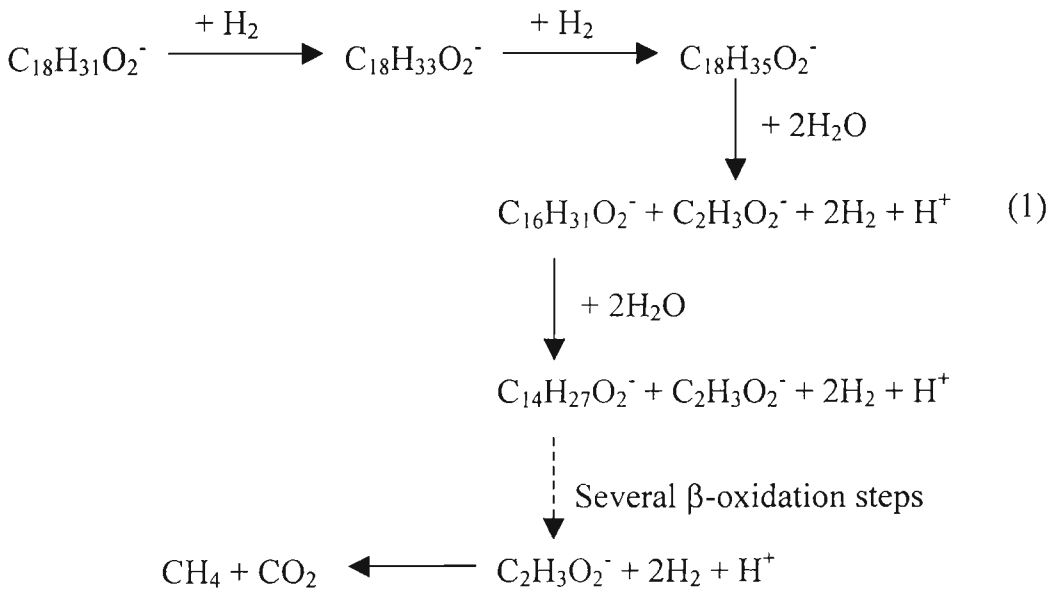


Figure 1.2 Interaction between linoleic acid and the active sites as reported by Harfoot (1978).

LCFAs are degraded via the β-oxidation of a fatty acyl-CoA intermediate. Equation (1) shows the overall reaction of LA β-oxidation. The products of β-oxidation are acetic acid and an LCFA with two fewer carbon atoms than the original acid. Equation (1) assumes full saturation of LA to SA prior to β-oxidation. This cycle is repeated until complete conversion of the fatty acid molecule to acetic acid is accomplished. Fatty acids contain more energy per carbon atom than other biological molecules (Gunstone, 1967). The oxidation of one palmitate (C_{16}) molecule, for example, has a net yield of 129 ATPs compared to glucose which only produces 2 ATPs in anaerobic glycolysis and 38 ATPs in aerobic metabolism (Voet & Voet, 1995).



(1)

During the β-oxidation, the electron produced is deposited on a suitable electron acceptor such as H^+ to produce hydrogen gas. The electrons from hydrogen oxidation can enter into several pathways. For example, Weng and Jeris (1976) suggest hydrogenotrophic methanogens used H_2 to form CH_4. Another intermediate of the β-oxidation of fatty acids is acetyl coenzyme A. Upon hydrolysis, acetyl co A produces

acetic acid which is degraded by aceticlastic methanogens to methane and carbon dioxide (Equation 2).

$$C_2H_4O_2 \rightarrow CH_4 + CO_2 \quad (2)$$

When treated anaerobically, lipids are hydrolyzed to LCFA and glycerol (Hanaki *et al.* 1981). Although fat hydrolysis is not rate limiting (Hukelekian and Muller, 1958), lipids particles usually have slower hydrolysis rates in contrast to carbohydrates and proteins. On average, the first order hydrolysis constant is 3 times slower than that of protein and 5 times slower than that of carbohydrates (Christ *et al.* 1999). Accordingly the rate at which fat particles hydrolyze may affect the start-up time of anaerobic reactors treating wastewaters with a high fat content (Masse *et al.* 1999). To minimize this problem, several researchers have proposed the use of a two-phase anaerobic process (Hanaki *et al.* 1981; Komatsu *et al.* 1991). The use of such a system allows the removal of suspended solids and partial hydrolysis and acidification in the first stage while methanogenesis occurs in the second stage.

1.7 Carbohydrates Fermentation

Under anaerobic conditions, complex carbohydrates, after hydrolysis to simple sugars, are converted to volatile fatty acids (VFA). VFAs are further converted to acetate, CO_2, and H_2 by acetogens and finally, acetate is degraded by aceticlastic methanogens. Hydrogenotrophic methanogens convert H_2 and CO_2 to CH_4. Several clostridial species are able to ferment sugars to butyric acid. Equation (3) shows the overall reaction pathway for the conversion of glucose to butyrate.

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2 \quad (3)$$

Zigová and Šturdik (2000) listed 10 different species capable of producing butyrate. Of these species, strains of *Clostridium* sp are the most commonly isolated from wastewater. The metabolic pathways of *Clostridium*, which are obligate anaerobes, produce several products including acetic and butyric acids (Evans and Wang, 1990). In the formation of butyrate, two acetic acid molecules are converted to butyrate (Equation 4).

$$2C_2H_4O_2 + 2H_2 \rightarrow C_4H_8O_2 + 2H_2O \quad (4)$$

In contrast, acetogens are able to convert butyrate to acetate. Gujer and Zehnder (1983) reported that conversion of butyrate to acetate is thermodynamically unfavorable under standard conditions and at pH of 7. For this reaction to be favorable, the hydrogen produced must be readily utilized by hydrogenotrophic methanogens.

The production of propionate from glucose is also feasible. The bacteria mediating this reaction (Equation (5)) is of the genus *Propionibacterium* (Tyree *et al.* 1991). Mosey (1983) proposed, using a mathematical model, that, under shock loads, the concentration of propionate increases much more than that of acetate and butyrate.

$$1.5C_6H_{12}O_6 \rightarrow 2C_3H_6O_2 + C_2H_4O_2 + CO_2 + H_2O \quad (5)$$

Peck *et al.* (1986) reported that propionate-degrading bacteria were the most sensitive of the VFA-degrading bacteria when subjected to a temperature shock. In addition, propionate degradation is sensitive to the hydrogen partial pressure P_{H2}. Confirmation of this observation has been reported by Fuş and Wiesmann (1995). Supporting research by Mosey (1983) and Boone and Xun (1987) also showed that increasing hydrogen partial pressure inhibits propionate degradation.

Of the many microorganisms carrying out glucose fermentation, each population mediates the conversion of glucose to a different product. Because mixed cultures treating wastewater contain different microorganism populations, a variety of glucose by-products are produced. Figure 1.3 summarizes several glucose fermentation pathways which are of interest for this research.

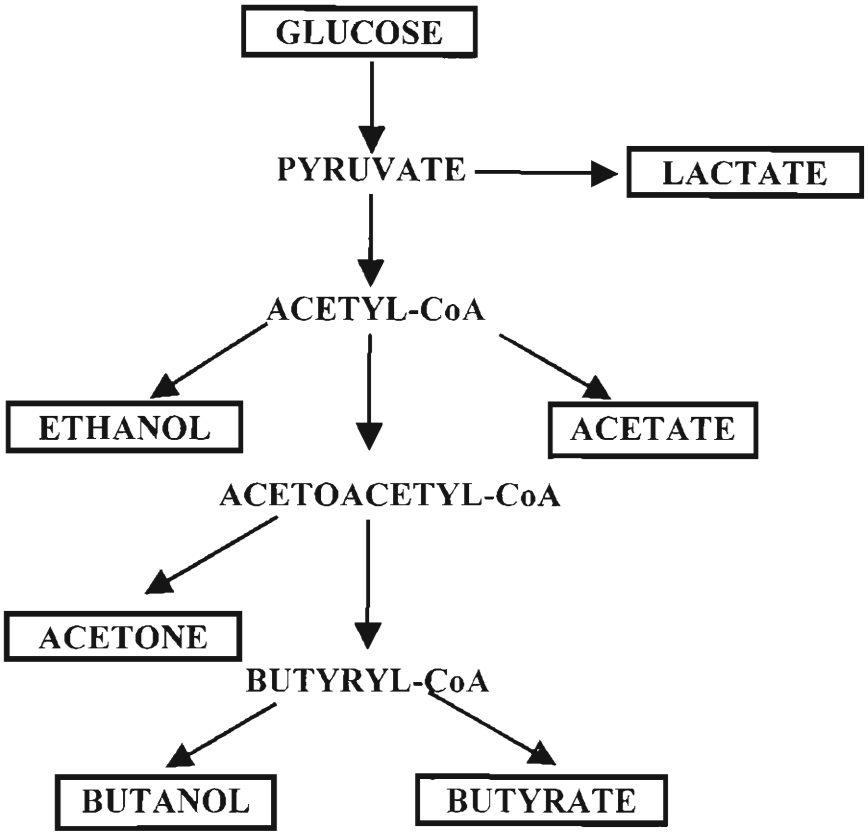


Figure 1.3. Pathways of glucose degradation (adapted from Zigová and Šturdik, 2000).

1.8 Problems Caused By LCFA Degradation

Although degradable, LCFA have been reported to cause two major problems during anaerobic treatment: biomass flotation with subsequent washout due to LCFA adsorption

onto the biomass and acute LCFA toxicity against both methanogens and acetogens (Alves *et al.* 2001).

1.8.1 Biomass Flotation

Biomass flotation and washout caused by the adsorption of LCFA onto granular sludge has been reported as the major reason for reactor failure (Lettinga and Hulshoff Pol, 1992). Hwu *et al.* (1998) investigated the effect of biosorption of LCFAs on the performance of upflow anaerobic sludge blanket (UASB) reactors. They observed that adsorption of oleic acid caused sludge flotation of granular sludge in a UASB reactor at concentrations well below the toxicity limits. Hwu *et al.* (1998) concluded that sludge flotation is caused by adsorption and depends on LCFA loading rates rather on LCFA concentrations. This is particularly important in anaerobic wastewater treatment systems such as high-rate UASB reactors. In fact, several high-rate reactors treating wastewaters from slaughterhouses containing high fat concentrations suffered severe operational problems (Martinez *et al.* 1995; Saxena *et al.* 1986; Sayed *et al.* 1988). Rinzema *et al.* (1993) also reported biomass flotation and total biomass washout in a UASB reactor when lipids loading rates exceeded 2,000 to 3,000 mg COD/L.

1.8.2 Inhibitory Effects of LCFAs

Fats and oils from animals and vegetables are known for their ability to inhibit microorganisms. Harris *et al.* (1932) observed increasing inhibition of microorganisms in foods with oils containing more unsaturated and longer-chain fatty acids. Nieman (1954) showed unsaturated fatty acids exerted antibacterial effects on gram-positive bacteria and

yeast but they did not have an effect on gram-negative bacteria. He concluded that the inhibitory effects of unsaturated fatty acids increased as the number of double bonds increased. Accordingly, Fuller and Moore (1967) observed that the inhibitory effect of LA was far greater than that of OA. In addition, several studies have shown that monoglycerides are active against gram-positive bacteria and certain fungi (Kabara *et al.* 1972, Kato and Shibasaki, 1975). LCFAs toxicity has been reported to be the mechanism of inhibition for suspended or flocculent sludge (Hwu *et al.* 1996). Several studies showed that LCFA are inhibitory, at low concentrations, to various microorganisms (Lalman and Bagley, 2000; Angelidaki and Ahring, 1990, Koster and Cramer, 1987; Hanaki *et al.*, 1981).

Research conducted by Hwu *et al.* (1998) has shown that adsorption of LCFA is prerequisite for their biodegradation. Moreover, Sayed *et al.* (1988) and Rinzema *et al.* (1993) suggested that fat adsorption to the surface of the anaerobic sludge may limit the transport of soluble substrates to the biomass and subsequently cause a decrease in the substrate conversion rate. Accordingly, Demeyer and Henderickx (1967) explained that unsaturated LCFAs toxicity is caused by adsorption onto the bacterial cell with subsequent alteration of the cell permeability.

Koster and Cramer (1987) studied the effect of four saturated fatty acids ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$) and one unsaturated fatty acid ($C_{18:1}$) on aceticlastic methanogens. They reported a toxicity threshold below which aceticlastic methanogenic acitivity was not affected by the presence of LCFAs. The greatest threshold concentration was 6.75 mM (975 mg/L) for $C_{8:0}$ and the lowest was 1.6 mM (320 mg/L) for $C_{12:0}$. Moreover, a mixture of lauric and myristic acids was reported to be more inhibitory than individual

LCFAs. This is of particular importance because in many cases effluents contain a mixture of LCFAs rather than individual acids. Therefore, consideration should be taken into when designing a reactor to treat wastewater with high fat contents since the presence of more than one LCFA may cause synergistic toxic effects.

Work reported by Hanaki *et al.* (1981) showed the effect of LCFAs on all stages of anaerobic digestion. They showed LCFAs are inhibitory to several bacterial populations. Particularly, LCFAs were shown to affect the amount of hydrogen produced by acetogenic organisms which are responsible for the β-oxidation of LCFA (Vidal *et al.* 2000). They monitored the degradation of acetate, n-butyrate, and glucose in the presence of oleate ($C_{18:1}$). In comparison, Hanaki *et al.* (1981) did not monitor glucose directly. Instead, acetate produced was used as an indicator of glucose fermentation. In addition to inhibiting its own β-oxidation, oleate was also inhibitory to H_2-producing acetogenic bacteria. Moreover, Hanaki *et al.* (1981) observed longer lag phases for methane production and concluded that LCFAs were not inhibitory to glucose fermentation.

Research conducted by Rinzema *et al.* (1994) showed that LCFA β-oxidation is a rate-limiting step. Because of its severe toxicity, the model substrate chosen for this work was capric acid ($C_{10:0}$). They observed lag phases in both capric acid degradation and methane production. However, once acclimation of the culture was achieved, capric acid was degraded. This study showed that a concentration ranging from 1150 mg/L to 1550 mg/L severely impacted aceticlastic methanogens. Similarly, Koster and Cramer (1987) observed a threshold concentration of 450 mg/L of capric acid caused inhibition. They concluded adaptation of aceticlastic methanogens was not possible once the LCFA concentration reached a threshold inhibitory level, contrary to the acetogenic bacteria

inhibited aceticlastic methanogens, but concentrations greater than 30mg/L of LA slightly inhibited hydrogenotrophic methanogens. Thus, in comparison to hydrogenotrophic methanogens, the inhibition of aceticlastic methanogens is expected to impair the performance of systems treating vegetable oil wastewater (Lalman and Bagley, 2000). Lalman and Bagley (2000) also reported the effects of oleic ($C_{18:1}$) and stearic acid ($C_{18:0}$) on aceticlastic and hydrogenotrophic methanogenesis at 21°C. They reported that SA did not inhibit aceticlastic methanogenesis even at 100 mg/L whereas 30 mg/L oleic acid was enough to inhibit acetic acid consumption and affect hydrogenotrophic methanogenesis. These findings support work by Galbraith *et al.* (1971) and Vidal *et al.* (2000) who reported that LCFA toxicity increased with the number of double bonds.

Beccari *et al.* (1996) examined the degradation of olive oil mill effluent (OME) under various process conditions. They found that a pH of 8.5 was optimum for lipids degradation and all lipids (initial OME concentration was 10,000 mg COD/l) were degraded compared to pH 6 where virtually no degradation of lipids occurred. In addition, acidogenic and methanogenic yields (VFA production/methane production) decreased with increasing OME concentration with methanogenic activity being more sensitive than that of acetogenesis. They concluded, under optimal conditions (pH, temperature, and maximum concentrations) OME can be degraded with high conversion yields. However, because acidogens and methanogens have different conversion yields and different degrees of tolerance to inhibitory compounds, Beccari *et al.* (1996) concluded that a two-stage reactor was more suitable for OME wastewater.

2.0 MATERIALS AND METHODS

2.1 Experimental Plan

Experiments were designed to investigate the effects of LCFA concentration on glucose fermentation. The experimental plan was divided to examine two objectives. In objective 1, studies were conducted using linoleic, oleic and stearic acids (Table 2.1). The conditions examined are as follows: LCFAs controls containing no glucose, LCFAs plus 1000 mg/L glucose, and LCFAs plus 2000 mg/L glucose. The LCFAs concentrations used in all the conditions examined are shown in Tables 2.1, 2.2, and 2.3.

Table 2.1. Experimental Plan For Degradation Study.

LCFAs conc. mg/L	Glucose Conc. mg/L		
50	0	1000	2000
100	0	1000	2000
300	0	1000	2000
500	0	1000	2000
700	0	1000	2000
1000	0	1000	2000

The individual acids used are: linoleic, oleic, and stearic acids.

Table 2.2. Inhibition Studies For LA/OA, LA/SA, and OA/SA Mixtures.

Individual LCFA Concentration, mg/L	Total LCFA Concentration, mg/L	Glucose Concentration, mg/L
25	50	2000
50	100	2000
150	300	2000
250	500	2000
350	700	2000
500	1000	2000

LA = Linoleic Acid, OA = Oleic Acid, SA = Stearic Acid.

Table 2.3 Inhibition Studies For The LA/OA/SA Mixture.

Individual LCFA Concentration, mg/L	Total LCFA Concentration, mg/L	Glucose Concentration, mg/L
17	50	2000
33	100	2000
100	300	2000
167	500	2000
233	700	2000
333	1000	2000

LA = Linoleic Acid, OA = Oleic Acid, SA = Stearic Acid.

For objective 2, (Tables 2.2 and 2.3) cultures received mixtures of the three LCFAs acids along with 2000 mg/L glucose. All controls and bottles containing LCFAs (individual and mixtures) were prepared in triplicates. All bottles were monitored for glucose, butyrate, propionate, acetate, methane, and LCFAs. A summary of the objectives of this study, together with the experimental procedures are summarized in table 2.4.

2.2 Reagents

Butyric (99+%) (Lancaster Synthesis, Pelham, NH), propionic (99+%), acetic (99+%) acids (Spectrum Chemicals, Gardena, CA), and D-(+)-glucose anhydrous (99+%) (Lancaster synthesis, Pelham, NH) were used to calibrate the Dionex DX-600 ion chromatograph (IC). Standards containing linoleic (99%), oleic (99+%), stearic (99%), palmitic (95%), myristic (98%), lauric (98%), capric (99%), caprylic (98%) acids (Lancaster Synthesis, Pelham, CA) in hexane (Spectrum, Inc. New Brunswick, NJ) were used to calibrate the gas chromatograph.

Standard grade methane (99.99%) (Altech Associates Inc., Deerfield, IL) was used to calibrate the GC. Hexane and methyl tertiary butyl ether (MTBE) (Spectrum, Inc. New Brunswick, NJ) were HPLC grade (Spectrum). All other chemicals used were

reagent grade. Carrier gases used were helium (99.99%) and nitrogen (99.99%) (UHP grade, Air Liquide American Corp.).

Table 2.4 Scope of Experiment and Procedures to Fulfill the Study Objectives

Experiment	Action	Relation to objectives
Batch reactor operation	• Feed 6 g glucose (2000 mg/L) • Monitor glucose and VFA • Calculate glucose initial degradation rates	• Investigate if there was a change in the glucose degrading organisms population over the duration of the study
Effects of LA, OA, and SA on glucose degradation	• Add to the serum bottles 1000 mg/L glucose • Add to the serum bottles the following individual LCFA concentrations: 0, 50, 100, 300, 500, 700, and 1000 mg/L • Monitor glucose, VFA, LCFA, and CH_4 • Calculate glucose initial degradation rates • Calculate LCFA initial degradation rates	• Examine the effects of LCFA on glucose initial degradation rates • Examine if the presence of glucose enhanced LCFA β-oxidation
Inhibition kinetics of LA, OA, and SA on glucose degradation	• Add to the serum bottles 2000 mg/L glucose • Add to the serum bottles the following individual and mixtures LCFA concentrations: 0, 50, 100, 300, 500, 700, and 1000 mg/L • Monitor glucose, VFA, LCFA, and CH_4 • Calculate Vmax, K_M, and K_I	• Examine the effects of LCFA on Vmax, K_M, and K_I • Model the inhibition mechanisms • Examine if mixtures of LCFA exerted synergistic effects on glucose degradation

2.3 Batch Reactors

Unacclimated anaerobic digester sludge from the Stillwater wastewater treatment plant was used as a seed culture. The culture was maintained in a 4-L semi-continuous reactor (Reactor A1) with a 3-L liquid volume. Reactor A1 was maintained at 21°C with approximately 0.9% (9,000 mg/L) volatile suspended solids (VSS). Inoculum from A1

was diluted to 2,000 mg/L (0.2%) VSS into a second 4-L semi-continuous reactor (Reactor A2) using basal media (Table 2.4). Biomass from A2 served as an inoculum source for the 160 mL serum bottles.

2.4 Inoculum Reactors Operation

Reactors A1 and A2 were operated in batch mode and acclimatized to 2,000 mg/L glucose at 21 °C. To avoid shock load, feeding was initiated with 500 mg/L and gradually increased in 500 mg/L increments to a final concentration of 2,000 mg/L glucose. Feeding (after acclimation) with 2000 mg/L glucose was repeated when acetate and gas production measurements indicated that all glucose and byproducts were consumed (within 5 to 6 days). A concentrated glucose feed solution (30,000 mg/L) for both reactors (A1 and A2) was prepared in basal media. The basal media composition used in this study was adapted from Lalman and Bagley (2000) and had a pH of 7.6 to 7.8 and contained the following constituents (as mg/L): $NaHCO_3$, 6,000; NH_4HCO_3, 70; KCl, 25; K_2HPO_4, 14; $(NH_4)_2SO_4$, 10; yeast extract, 10; resazurin, 1; $MgCl_2.4H_2O$, 9; $FeCl_2.4H_2O$, 2; EDTA, 1; $MnCl_2.4H_2O$, 0.5; $CoCl_2.6H_2O$, 15; Na_2SeO_3, 0.1; $(NH_4)_6MoO_7.4H_2O$, 0.09; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $NiCl_2.6H_2O$, 0.05; and $CuCl_2.2H_2O$, 0.03.

2.5 Serum Bottles Preparation

All studies were conducted in 160 mL serum bottles with a 100 mL total liquid volume at 21 °C. The atmosphere headspace was 80%/20% N_2/CO_2. The bottles were prepared inside a Coy® anaerobic chamber (Coy Laboratory Products, Inc.) having the same percentage of gas as described previously. Varying amounts of biomass volumes

were added, taking into consideration the glucose and LCFA volumes needed. LCFA and glucose stock solutions were 5,000 mg/L and 20,000 mg/L respectively. To maintain reducing conditions, 75 mg/L ferrous chloride and 75 mg/L sodium sulphide were added to the culture. Resazurin (1 mg/L) was used to determine the anaerobic conditions.

After inoculation, the bottles were sealed with Teflon® lined septa and aluminum crimp caps and pressurized with 20 mL of gas mixture (80%/20% N_2/CO_2) to avoid negative headspace pressure during sampling. The bottles were placed on an orbital shaker (Lab-Line Instruments, Inc. model No.3520) equipped with an opaque chamber to prevent photosynthetic reactions at 200 rpm. Upon completion of each study, the bottles were sacrificed to measure the pH, alkalinity, TSS, and VSS. Measurements were performed according to *Standard Methods*.

2.6 Gas Measurement

A 20 μL GASTIGHT® syringe (Hamilton Co.) was used to remove liquid samples from the serum bottles. An Agilent 6890 gas chromatograph equipped with a thermal conductivity detector (TCD) was used for methane analysis. The analytical column was a 30 m × 0.53 mm Carboxen™ 1006 Fused Silica Capillary Column (Supleco). The total run time was 2.0 minutes and the analysis was isothermal at 100 °C; the carrier gas was nitrogen at 10mL/min. Methane was detected at 1.1 minutes and the detection limit was 0.8 μM.

Calibration standards for the GC were prepared in 160 mL serum bottles purged with nitrogen (UHP grade). All bottles were sealed with Teflon® lined septa and capped with aluminum crimp seals, and known quantities of methane were injected into each

bottle. Triplicate samples (15 μL) were removed and analyzed for methane. Calibration standards were used in all headspace analysis to insure the instrument remained calibrated over the duration of this research.

2.7 VFA Measurement

One mL samples for VFAs analysis were withdrawn from the serum bottles and diluted with de-ionized water. After dilution the samples were centrifuged for 5 minutes, then the centrate was removed and filtered using OnGuard™-H cartridges (Dionex). The filtered samples were analyzed using a Dionex DX-600 ion chromatograph (IC). The IC was equipped with an AS40 automated sampler, an LC20 liquid chromatograph, a GP50 multi-gradient pump, and an ED50 electrochemical detector. For VFA analysis, a conductivity detector was used with a 24 cm × 4mm IonPac® AS11 column, an ASRS-ULTRA® (4-mm) anion self-regenerating suppressor, and an IonPac® ATC-1 cartridge. The eluents used consisted of A (dionized water), B (5 mM NaOH), and C (50 mM NaOH) at a total flow of 2 mL/min. The percentage of each eluent is as shown in Table 2.5.

Table 2.5: Ion chromatography eluent concentration gradient

Time (min)	Condition
0 to 2	93% A, 7% B
2 to 6	Decrease A from 93% to 0% and increase B from 7% to 100 %
6 to 9	Decrease B from 100 % to 50 % and increase C from 0% to 50%
9 to 9.99	50 % B and 50 % C
10 to 26	93% A and 7% B

This method provided detection of acetic (C2), propionic (C3), and butyric (C4) acids. The effective detection limits were 0.1 mg/L for acetate, 0.2 mg/L for propionate, and 0.4 mg/L for butyrate.

Triplicate standards for VFA analysis were prepared using a 5000 mg/L VFAs stock solution. The stock solution contained acetic, propionic, and butyric acids and was prepared in de-ionized water. Several standards were analyzed during each sample sequence and compared against the standard curve to ensure instrument calibration.

2.8 Glucose Measurement

For glucose analysis, sample preparation was similar to that for VFA measurement. The sample was diluted with de-ionized water and filtered using OnGuard™-H cartridges (Dionex). A Dionex DX-600 IC equipped with a 25 cm × 4mm CarboPac™ MA1 column was used for the analysis. The run time was 25 minutes and a 614 mM NaOH solution was used as an eluent. The detection time was 19 minutes and the detection limit was 0.1 mg/L using a flow rate of 0.5 mL/min.

Triplicate standards for glucose analysis were prepared using a 10,000 mg/L glucose stock solution. Several standards used as quality control samples were analyzed during each sequence to ensure instrument calibration.

2.9 LCFA Delivery Method

For straight-chain aliphatic hydrocarbons, hydrophobicity is a function of the number of methylenic groups. As the number of methylene groups increases, the degree of hydrophobicity increases. Short-chain saturated fatty acids (below C_8) are miscible with water; however as the number of carbon atoms increases, the solubility decreases rapidly. All saturated acids above lauric acid are considered insoluble in water according to Bloor (1943). The solubility of saturated fatty acids ranges from 680 mg/L for C_8 to 3

mg/L for C_{18} at 20 °C (Ralston and Hoerr, 1942). Table 2.6 shows the aqueous solubility of C_2 to C_{18} LCFAs in mg/L.

Table 2.6 Solubility of C_2 to C_{18} acids (mg/L) at 20°C

Acid	C_2	C_3	C_4	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{18:0}$
Solubility mg/L	Miscible[1]	Miscible[1]	Miscible[1]	680[2]	150[2]	55[2]	20[2]	7.5[2]	3[2]

[1]The National MSDS Repository (2001), [2]Ralston and Hoerr (1942)

Because of LCFAs aqueous insolubility, a delivery method was used to increase the amount available to the microorganisms. This is of particular importance since the substrate utilization is dependent on the physical state of the hydrocarbon. Wodzinki *et al.* (1972) have shown that naphthalene degrading microorganisms utilized dissolved naphthalene and did not utilize the solid directly. Several approaches have been used by researchers to disperse LCFAs in aqueous solution. One approach is to use dispersing agents such as diethyl ether, dimethyl formamide, dimethyl sulfoxide, acetone, and ethanol. These agents work by dispersing the LCFAs into solution and thus increase the substrate surface area. However, a major problem associated with these compounds is their toxicity to microorganisms (Lalman and Bagley, 2000 Sikkema *et al.* 1995).

To avoid solvent toxicity, a delivery method developed by Angelidaki and Ahring (1992) consisted of adding the LCFAs as sodium salts. The LCFAs were melted *au bain-marie* and added to a hot and vigorously stirred NaOH solution. The quantities of sodium hydroxide used (expressed as g of NaOH per g of LCFA) are provided in Table 2.7.

Table 2.7 Quantity of hydroxide used for LCFA stock solution preparation

LCFA	Sodium Hydroxide used (g/g LCFA)
Caprylic	0.278
Capric	0.233
Lauric	0.200
Myristic	0.175
Palmitic	0.156
Stearic	0.141
Oleic	0.142
Linoleic	0.143

Amounts are expressed as g NaOH per g of LCFA

2.10 LCFA Extraction

Since LCFAs are insoluble in water, an extraction method is needed to ensure accurate measurement of the aqueous samples removed from the serum bottle. The extraction method is adapted from Lalman (2000) and is described in section 2.11. Figure 2.1 shows the percent recovery of C_8 to $C_{18:2}$ for the lowest concentration (50 mg/L) and the highest concentration (1000 mg/L) used. The LCFA having the lowest extraction efficiency is a C_8 compound ranging from 82% for 50 mg/L and 86% at 1000 mg/L. Higher LCFAs (C_{10} to $C_{18:2}$) had extraction efficiencies ranging from 89% to 96%.

2.11 LCFA Measurement

The method for LCFA analysis is adapted from Lalman and Bagley (2000) and is described below. One mL samples were withdrawn from the serum bottles and placed in a 5 mL vial containing 2 mL of 50:50 hexane:MTBE, 0.05g NaCl, and 2 drops of 50% H_2SO_4. The vial was sealed with Teflon® lined septa and capped with aluminum crimp seals and placed on an orbital shaker (Lab-Line Instruments, Inc. model No. 3520) for 20 min at 200 rpm. Next, the vial was centrifuged for 5 min at 1750 g. The organic phase

3.0 Batch Reactor Operation

3.1 Experimental Results

3.1.1 Glucose Consumption

Glucose degradation profile for reactor A2 is shown in Figure 3.1. Reactor A2 was fed with 6g glucose to maintain a concentration of 2,000 mg/L (reactor volume was maintained at 3 L). Undetectable levels of glucose were achieved within 240 minutes. The biomass concentration used in reactor A2 was approximately 2,000 mg VSS/L.

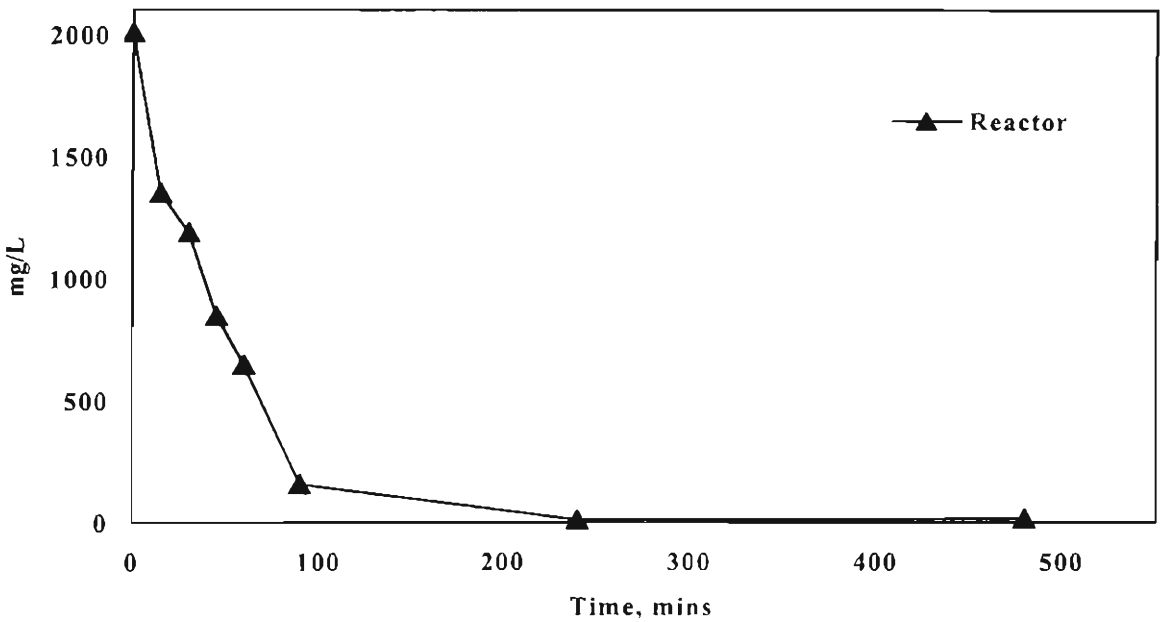


Figure 3.1: Glucose degradation profiles for reactor A2.

Tables 3.1, 3.2, and 3.3 show glucose initial degradation rates, maximum reaction velocity (V_{max}), and the binding affinity (K_M) for serum bottle controls and for reactor A2 respectively. Based on the Tukey's paired comparison procedure (Steel *et al.*, 1997), no statistical differences in glucose degradation rates between the serum bottle controls were

observed. Also no statistical difference was observed between the serum bottle controls and reactor A2. Similarly, no statistical differences were observed for V_{max} and K_M.

Table 3.1: Glucose degradation rates $\left(\mu g \cdot mgVSS^{-1} \cdot \min^{-1}\right)$ for control cultures receiving 2000 mg/L glucose.

Ctr #1	Ctr #2	Ctr #3	Ctr #4	Ctr #5	Ctr #6	Ctr #7	Reactor
14.57±0.39	15.19±0.56	15.21±0.46	15.02±0.22	15.01±0.34	14.49±0.35	14.41±0.66	14.29±0.78

Average and standard deviation for triplicate samples are shown. Ctr = control

Table 3.2: Maximum reaction velocity (V_{max} $mg.L^{-1}.hr^{-1}$) for control cultures receiving 2000 mg/L glucose.

Ctr #1	Ctr #2	Ctr #3	Ctr #4	Ctr #5	Ctr #6	Ctr #7	Reactor
31.2 ±1.32	30.9±1.32	31.8±1.40	31.5±2.15	29.9±1.16	30.4±2.58	32.0±1.40	31.3±1.21

Average and standard deviation for triplicate samples are shown. Ctr = control

Table 3.3: Binding affinity (K_M $mg.L^{-1}$) for control cultures receiving 2000 mg/L glucose.

Ctr #1	Ctr #2	Ctr #3	Ctr #4	Ctr #5	Ctr #6	Ctr #7	Reactor
474.7±32.1	496.9±36.4	511.7±28.6	476.9±32.5	517.2±31.4	492.5±33.4	499.7±29.6	532.2±51.1

Average and standard deviation for triplicate samples are shown. Ctr = control

3.1.2 VFA Degradation

Acetic and propionic acid production profiles are shown in Figures 3.2 and 3.3 for reactor A2. A maximum concentration of acetate was achieved within 4 days and 86% removal was observed within 20 days. For propionate (Figure 3.3) the maximum concentration was achieved within 2 days and complete removal was observed after day 20.

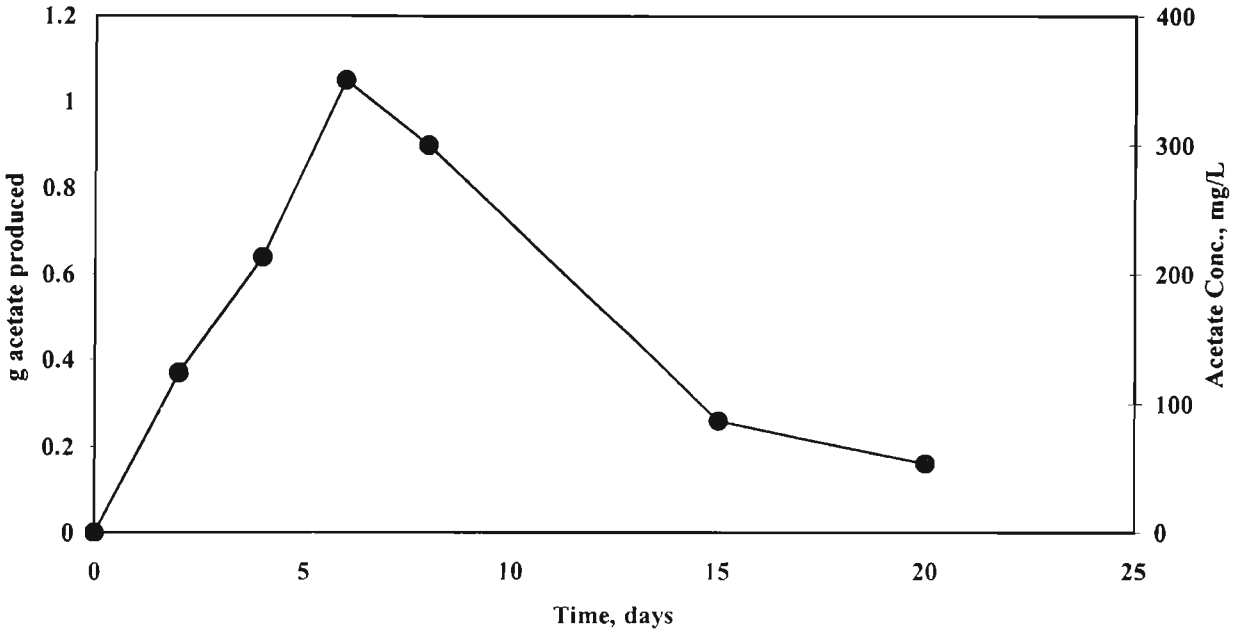


Figure 3.2: Acetate degradation profile for reactor A2.

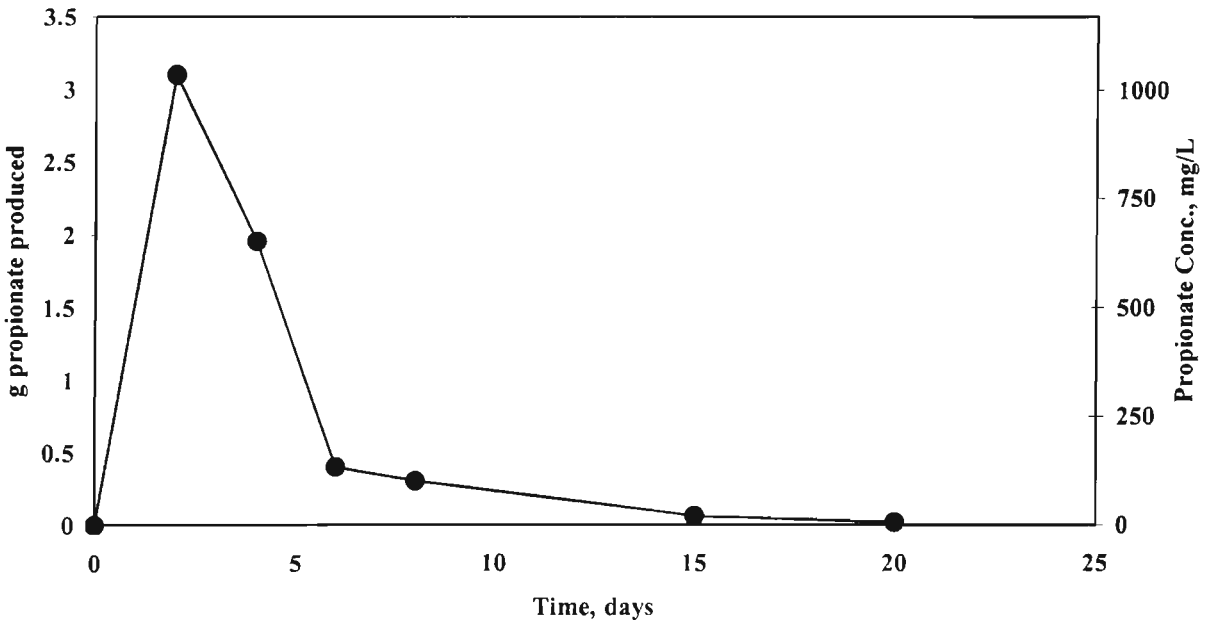


Figure 3.3: Propionate degradation profile for reactor A2.

3.1.3 Gas Production

The total gas production profile for reactor A2 is shown in Figure 3.4. Complete conversion of glucose was achieved within 20 days. The total gas produced was assumed to be composed of methane and carbon dioxide with a ratio of 1:1 (mole:mole) according to equation 6.

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \qquad (6)$$

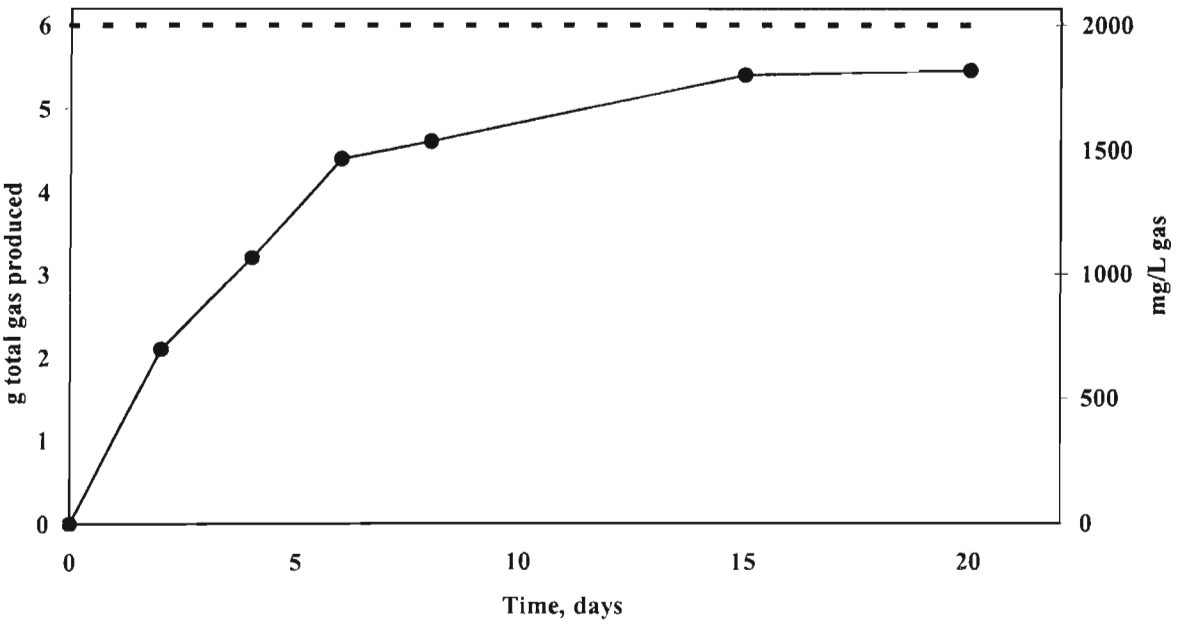


Figure 3.4: Gas production profile for reactor A2.
--------- Theoretical gas produced

3.1.4 Mass Balance

The mass balance profile for reactor A2 in Figure 3.5 also show the horizontal line which represents the total carbon derived from glucose. Error bars represent standard deviation ranging from 3% to 11%.

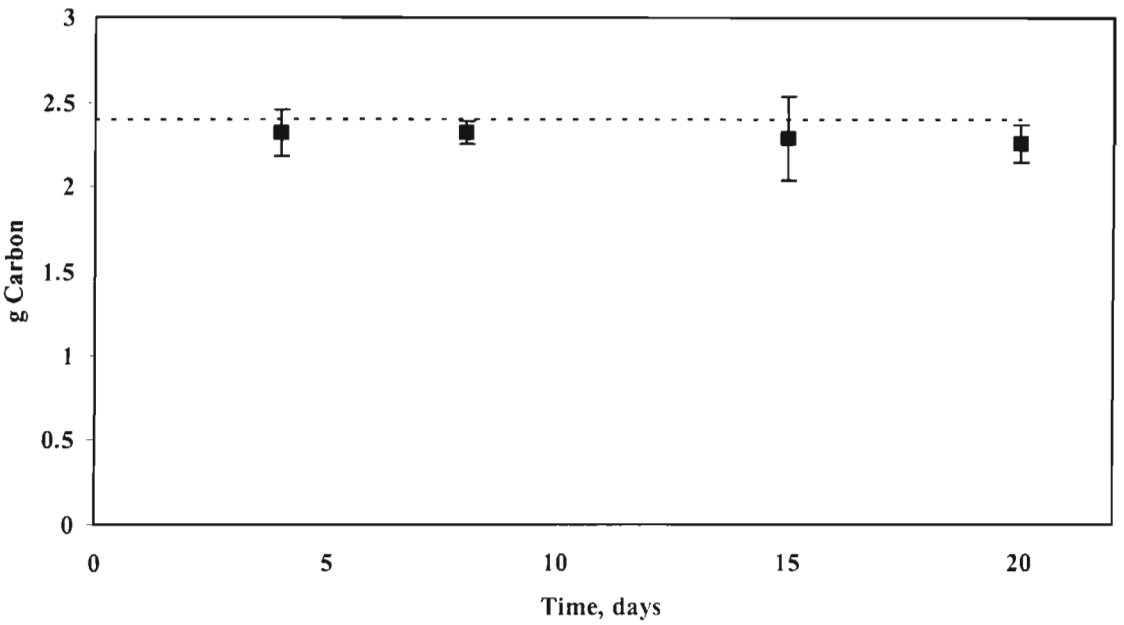


Figure 3.5: Carbon mass balance profile for reactor A2.
--------- Theoretical carbon balance

3.2 Discussion of Results

3.2.1 Glucose Degradation

Statistical comparison of Vmax, K_M, and initial degradation rates for control cultures and reactor A2 suggests that there were minimal changes in the glucose degrading organism population over the duration of the study. Glucose anaerobic fermentation using mixed cultures produces a mixture of several products (see Figure 1.2). Hitchener *et al.* (1979) have shown that lactate and ethanol are produced in glucose-limited conditions. On the other hand, Grau (1983) reported that ethanol, acetate, formate

and lactate were end products of glucose fermentation by *Brochothrix thermosphacta.* Grau (1983) also showed that increasing glucose concentrations had a significant effect on the end product. For example at high glucose concentration more lactate conversion and less ethanol conversion was observed. In addition, at low pH values more acetate and formate were observed. Moreover, very little carbon dioxide was observed when the pH varied between 6.4 and 7.0. In this research, the end products of glucose fermentation were acetate and propionate with propionate concentrations higher than acetate. Moreover, Ueno *et al.* (2001) showed that product formation from carbohydrate fermentation was affected by the predominant microorganisms in the seed culture. In addition, their work showed that the microorganisms are strongly interrelated and any disturbance of a population at one level affects the entire community and causes an imbalance which affects the product distribution.

3.2.2 VFA Degradation

The anaerobic production and degradation of VFAs is strongly affected by the characteristics of the feed substrate and by the operational conditions such as pH value, temperature, hydrogen partial pressure, and available trace minerals (Ueno *et al.* 2001). Hydrogen plays an important role in the anaerobic energy transfer. For example, H^+ is used as an electron acceptor in several reactions, and hydrogen gas is produced. Moreover, hydrogen gas can be used by hydrogenotrophic methanogens to form CH_4. In fact, the consumption of hydrogen is critical to the overall performance of an anaerobic fermenter. Gujer and Zehnder (1983) have shown that conversion of butyrate to acetate is thermodynamically unfavorable unless the hydrogen produced can be readily utilized by

hydrogenotrophic methanogens. In addition, propionate degradation is very sensitive to the hydrogen partial pressure, Fuş and Wiesmann (1995) reported that an increase in hydrogen may inhibit propionate degradation.

3.2.3 Gas Production

Gas produced in reactor A2 approached 96% of the theoretical quantity within 15 to 20 days. Ueno *et al.* (2001) reported a steady methane formation implies the stable production and consumption of intermediate metabolites. In addition, research by Tabassum and Rajoka (2000) showed that methane production depends on substrate composition. For example, methane production was optimized at pH 7.5 when α-cellulose was used and at pH 6.0-6.5 when glucose was the substrate.

3.2.4 Mass Balance

Carbon mass balance for reactor A2, based on conversion to g of carbon per bottle, was within 10% of the theoretical amount of carbon. An accountability of all the CO_2 produced by fermentation and the amount added to the head space gas caused an error in the carbon mass balance. In particular, because CO2 is aqueous soluble, the fraction produced by fermentation will be in equilibrium with aqueous carbonate species and another fraction will remain in the gas phase. In the presence of hydrogenotrophic methanogens, a fraction of aqueous CO_2 will be converted to methane gas. The conversion of CO_2 to organic and inorganic carbon species caused some difficulties in accounting for all the carbon species derived from glucose fermentation.

4.0 Effects Of Linoleic ($C_{18:2}$), Oleic ($C_{18:1}$), And Stearic ($C_{18:0}$) Acid On Glucose Degradation

4.1 Experimental Results

4.1.1 Glucose Consumption

Glucose degradation profiles for cultures receiving 1000 and 2000 mg/L are shown in Figures 4.1 to 4.6. Undetectable levels of glucose were achieved within 240 minutes in all the control cultures. In the presence of individual LCFAs at 50 or 100 mg/L, similar removal times were observed. Approximately 25% of the glucose substrate remained undegraded for cultures receiving equal or greater than 300 mg/L.

For cultures receiving less than 100 mg/L LA, glucose removal was accomplished within approximately less than 250 min. Glucose residual was also observed in cultures receiving equal or greater than 300 mg/L OA. The amount of residual was OA concentration dependent. At equal or less than 100 mg/L OA, complete glucose degradation was accomplished within approximately 250 min. A similar trend was observed for cultures receiving SA, however, less glucose residual was observed at equal or greater than 300 mg/l SA.

In the cultures receiving 500, 700, or 1000 mg/L LCFAs, the amount of glucose removed after 100 minutes was less in comparison to the control cultures. No detectable levels of glucose were observed after 24 hours (Data not shown).

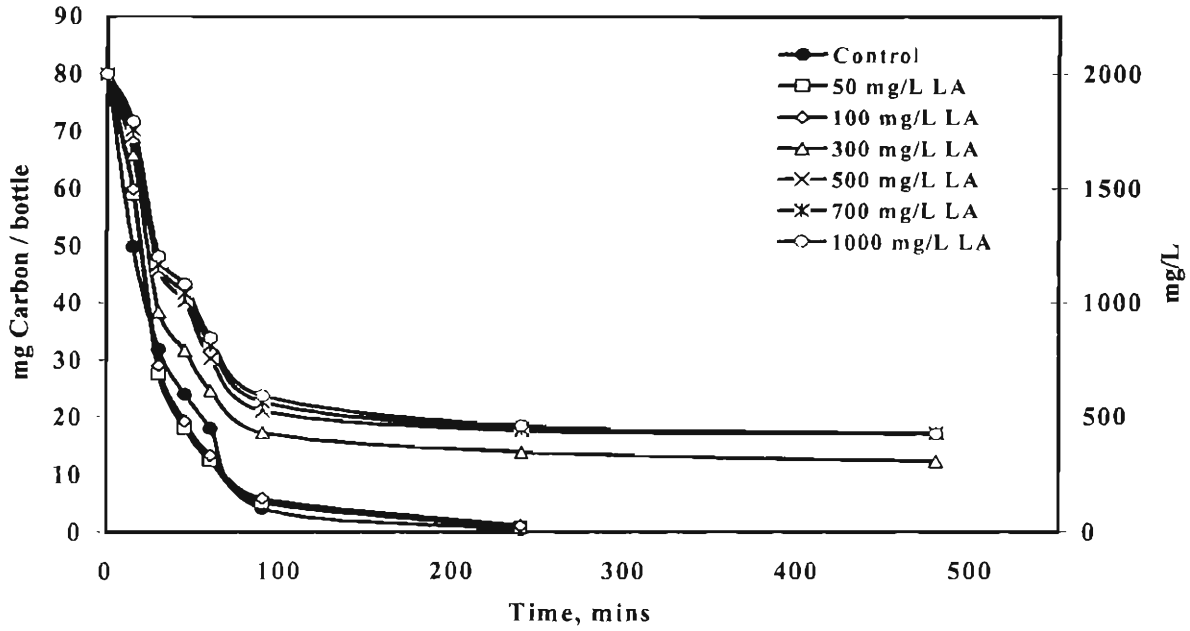


Figure 4.1: Glucose degradation profiles for cultures receiving linoleic acid plus 2000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are shown).

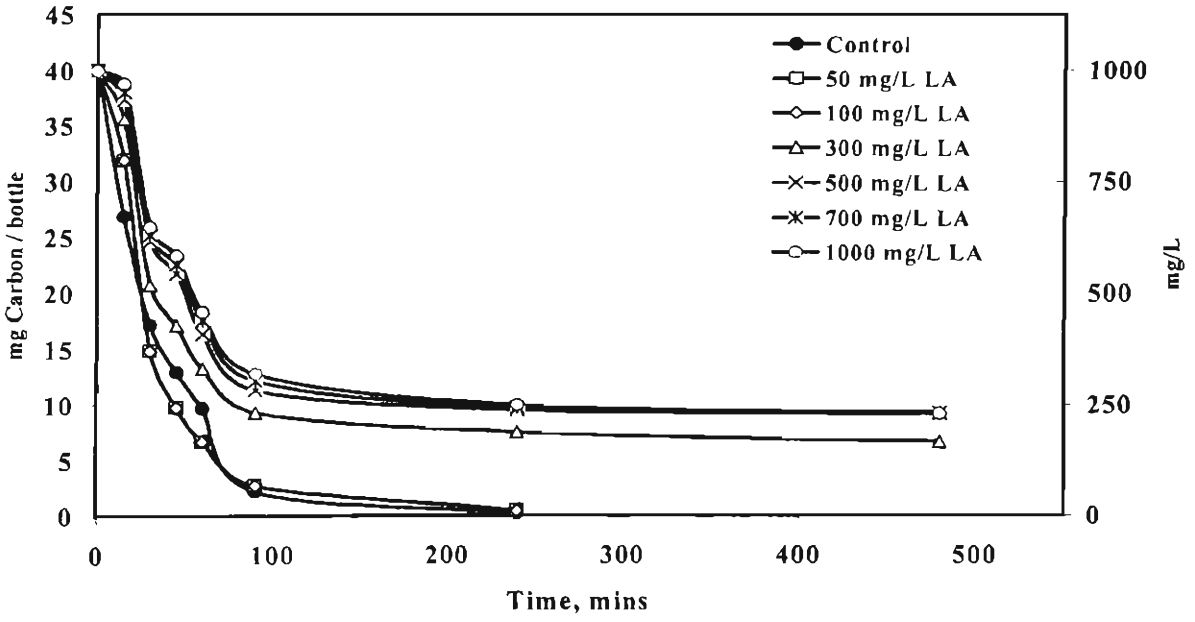


Figure 4.2: Glucose degradation profiles for cultures receiving linoleic acid plus 1000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are shown).

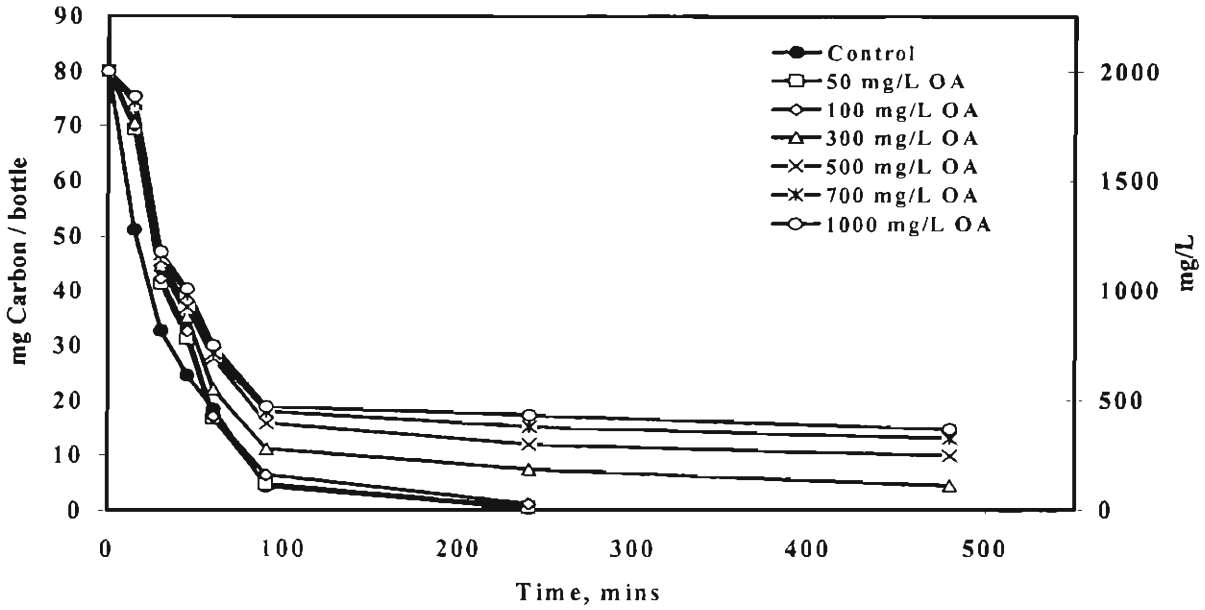


Figure 4.3: Glucose degradation profiles for cultures receiving oleic acid plus 2000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown).

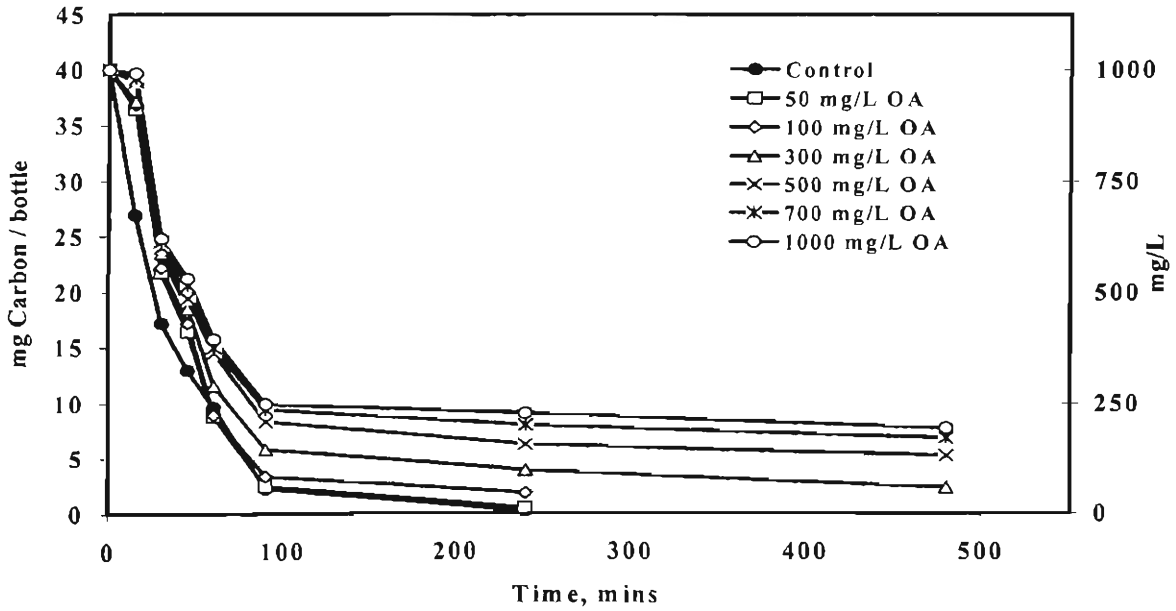


Figure 4.4: Glucose degradation profiles for cultures receiving oleic acid plus 1000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown).

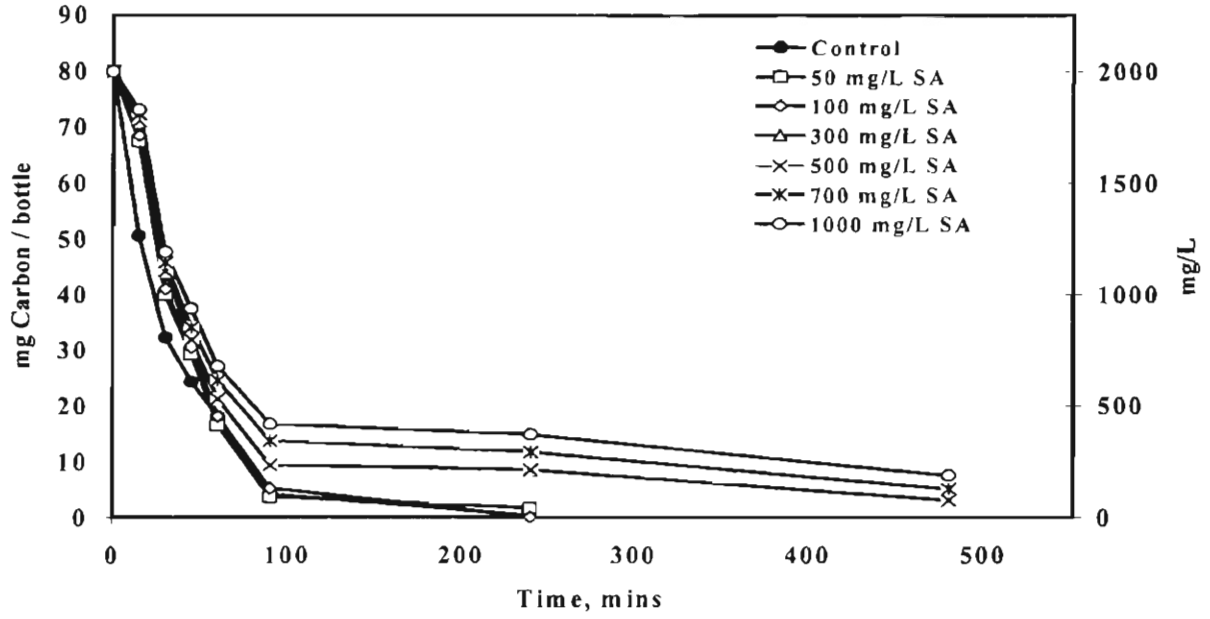


Figure 4.5: Glucose degradation profiles for cultures receiving stearic acid plus 2000 mg/L glucose. (SA = stearic acid, average for triplicate samples are shown).

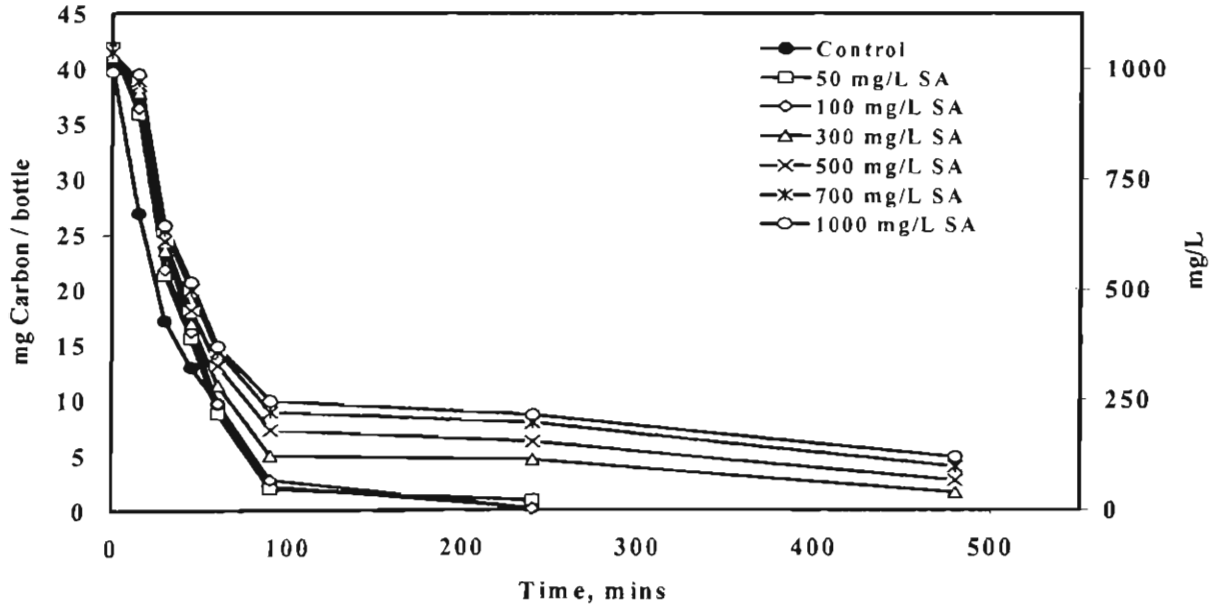


Figure 4.6: Glucose degradation profiles for cultures receiving stearic acid plus 1000 mg/L glucose. (SA = stearic acid, average for triplicate samples are shown).

Degradation rates of glucose in cultures receiving linoleic, oleic, and stearic acids plus 1000 and 2000 mg/L glucose are shown in Tables 4.1 and 4.2 respectively. Statistical comparison between each data set was performed using the Tukey's paired comparison procedure (Steel *et al.* 1997).

Table 4.1: Glucose degradation rates $\left(\mu g \cdot mgVSS^{-1} \cdot \min^{-1}\right)$ for cultures receiving linoleic, oleic, and stearic acids plus 1000 mg/L glucose.

	LCFA Concentration						
	0 mg/L	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
LA	8.01 ± 0.24[a]	6.08 ± 0.33[b]	5.83 ± 0.17[c]	4.41 ± 0.23[d]	3.91 ±0.22[e]	3.63 ±0.11[e]	3.37 ±0.41[e]
OA	8.83 ± 0.15[a]	8.79 ± 0.16[a]	7.85 ± 0.24[a]	5.52 ± 0.39[d]	4.82 ±0.16[e]	4.56 ±0.29[e]	4.15 ±0.17[e]
SA	8.51 ± 0.14[a]	8.55 ± 0.52[a]	7.91± 0.36[a]	5.46 ± 0.16[d]	4.76 ±0.14[e]	4.12 ±0.64[e]	3.93 ±0.32[e]

Average and standard deviation for triplicate samples are shown. $C_{18:2}$ = linoleic acid (LA), $C_{18:1}$ = oleic acid (OA), $C_{18:0}$ = stearic acid (SA). a, b, c, d, and e = means followed by the same letter are not statistically different within rows.

Table 4.2: Glucose degradation rates $\left(\mu g \cdot mgVSS^{-1} \cdot \min^{-1}\right)$ for cultures receiving linoleic, oleic, and stearic acids plus 2000 mg/L glucose.

	LCFA Concentration						
	0 mg/L	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000
LA	14.57± 0.39[a]	11.06± 0.26[b]	10.18± 0.34[c]	8.63± 0.36[d]	7.88±0.54[e]	7.11±0.24[e]	6.87 0.21[e]
OA	15.19± 0.56[a]	14.01± 0.34[a]	13.38± 0.48[a]	10.44± 0.34[d]	9.61±0.29[e]	9.19±0.18[e]	8.78 0.25[e]
SA	15.21± 0.46[a]	14.89± 0.45[a]	13.97± 0.42[a]	10.64± 0.54[d]	9.58±0.29[e]	8.89±0.34[e]	8.46 0.29[e]

Average and standard deviation for triplicate samples are shown. $C_{18:2}$ = linoleic acid (LA), $C_{18:1}$ = oleic acid (OA), $C_{18:0}$ = stearic acid (SA). a, b, c, d, and e = means followed by the same letter are not statistically different within rows.

In comparison to control cultures and those receiving LA, the glucose degradation rates for cultures receiving 50 or 100 mg/L LA were statistically different. However, in the case of oleic and stearic acids, there was no statistical difference between control cultures and those receiving 50 or 100 mg/L OA or SA. Moreover, there was no statistical difference in the glucose degradation rates between cultures receiving 50 and 100 mg/L of the same LCFA.

In the cultures receiving 300 mg/L of any LCFA, the degradation rates were different from the controls and those cultures receiving 50 or 100 mg/L of any LCFA. In cultures receiving greater than 500 mg/L of any LCFA, the glucose degradation rates were statistically different than those receiving 300 mg/L of the same LCFA. No difference in the degradation rates between the cultures receiving 500, 700, 1000 mg/L of the same LCFA was observed.

In addition, there was a statistical difference in the glucose degradation rate between cultures receiving 500 mg/L LA and those receiving 500 mg/L OA or 500 mg/L SA. No difference in glucose degradation rates between the cultures receiving 500 mg/L OA and those receiving 500 mg/L SA was observed. Similarly for the cultures receiving LCFA concentrations higher than 500 mg/L, no statistical difference was observed.

4.1.2 LCFAs Degradation

LCFA degradation and their LCFAs by-products profiles are shown in Figures 4.7 to 4.11. In the cultures receiving 50 mg/L LA or OA almost all the LCFA was removed within 20 days. However, removal of SA was slower even in the cultures receiving 50 mg/L. In the cultures receiving greater than 300 mg/L LCFA, very little removal was observed. Palmitic acid ($C_{16:0}$), a saturated LCFA, was observed only in the cultures receiving LA or OA.

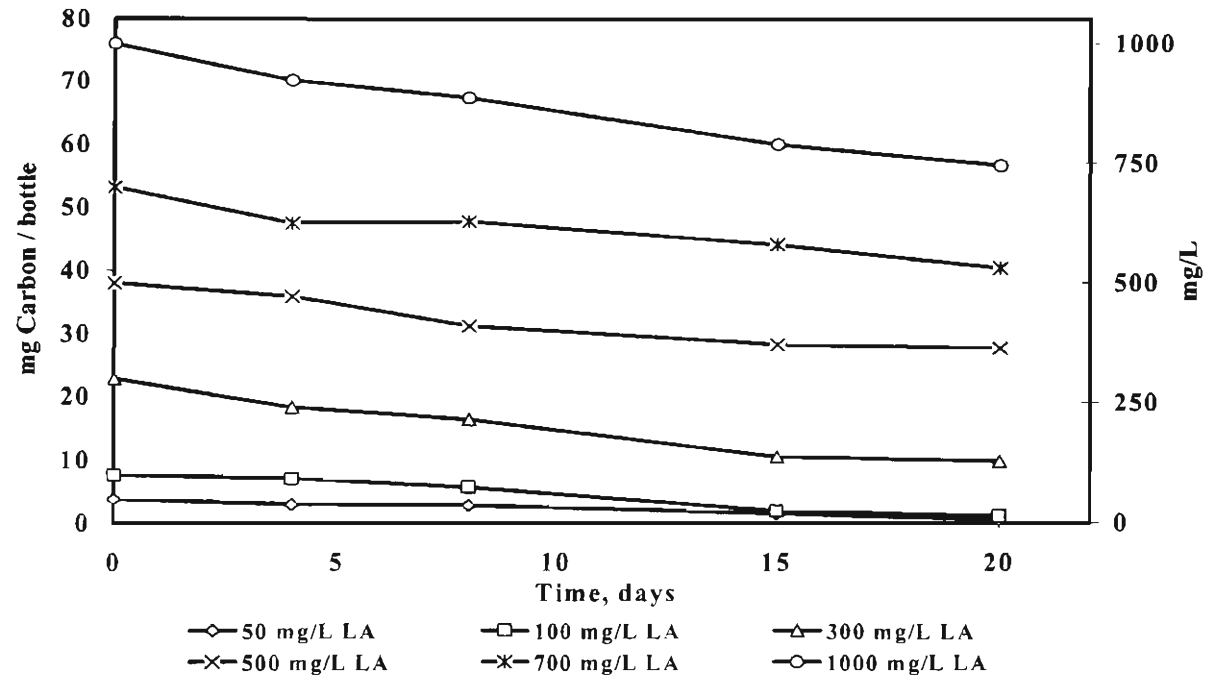


Figure 4.7: Linoleic acid degradation profiles for cultures receiving 1000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are shown).

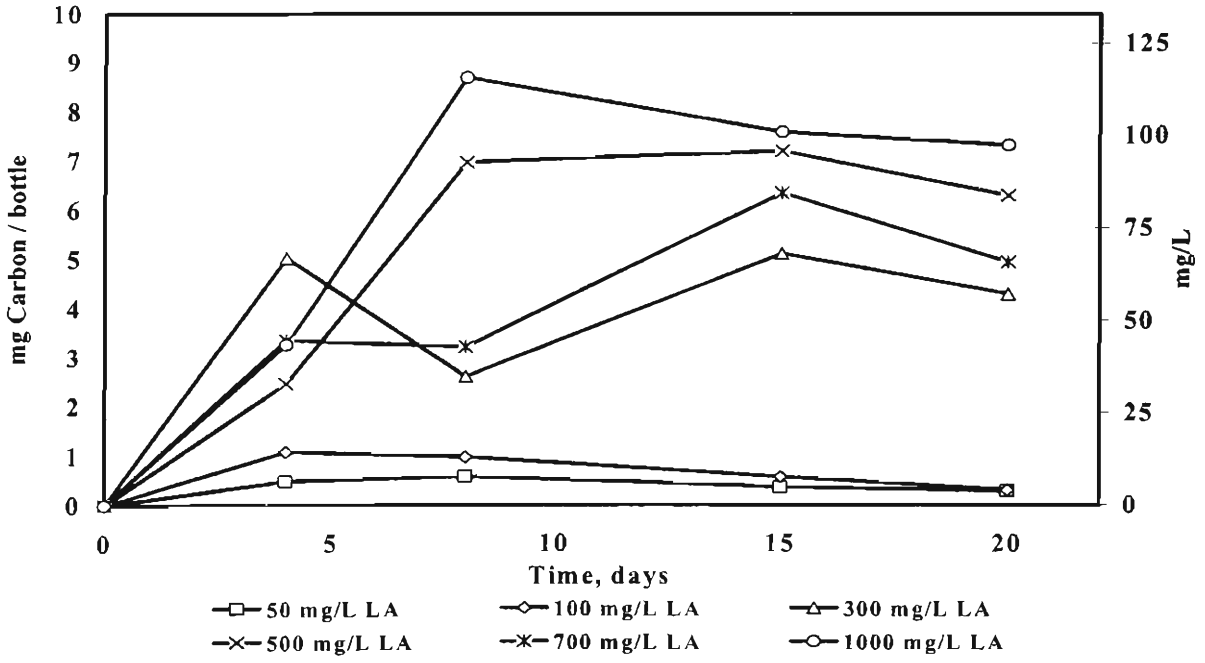


Figure 4.8: Palmitic acid production profiles for cultures receiving linoleic acid plus 1000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are shown).

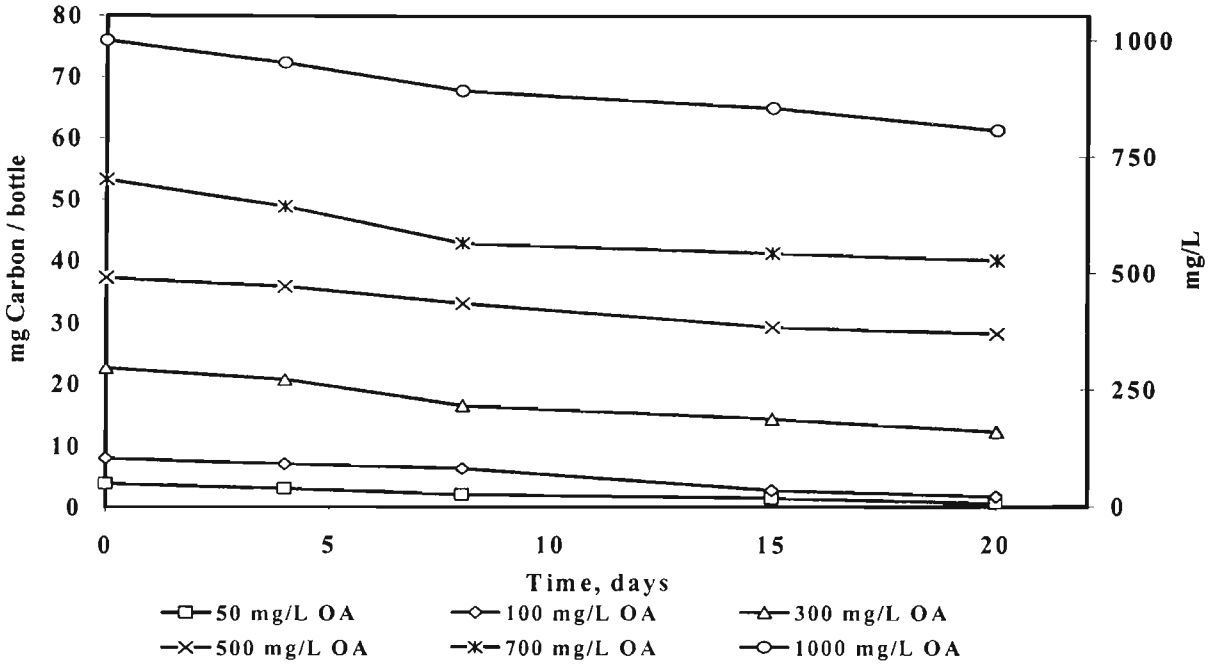


Figure 4.9: Oleic acid degradation profiles for cultures receiving 1000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown).

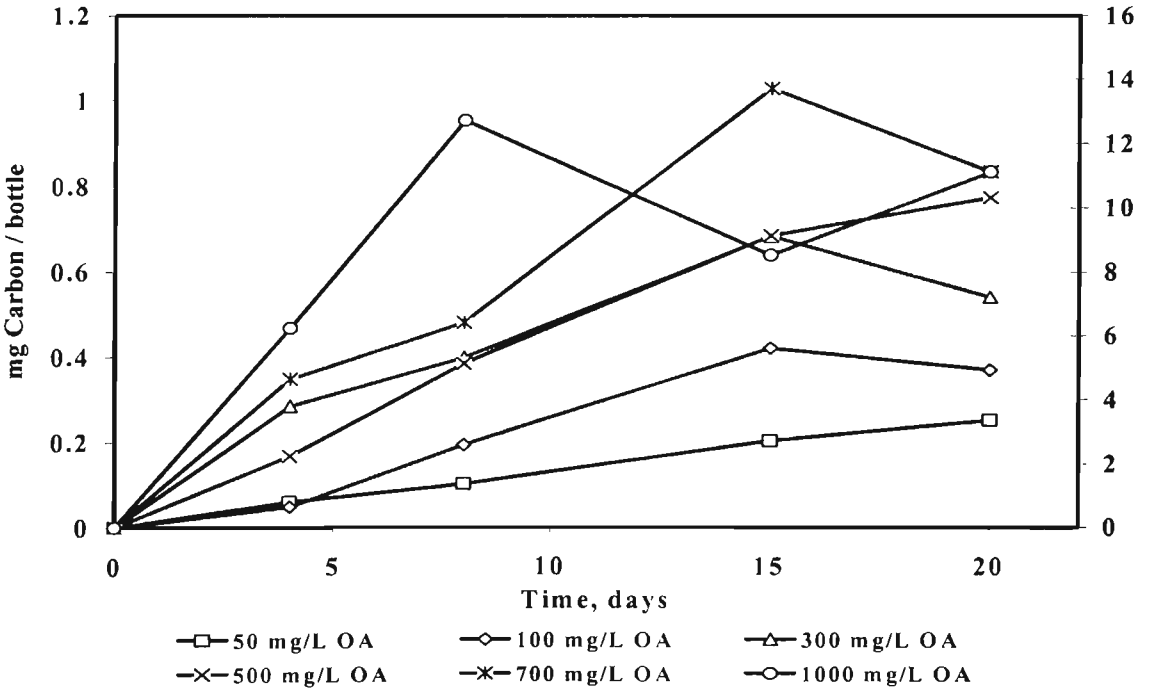


Figure 4.10: Palmitic acid production profiles for cultures receiving oleic acid plus 1000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown).

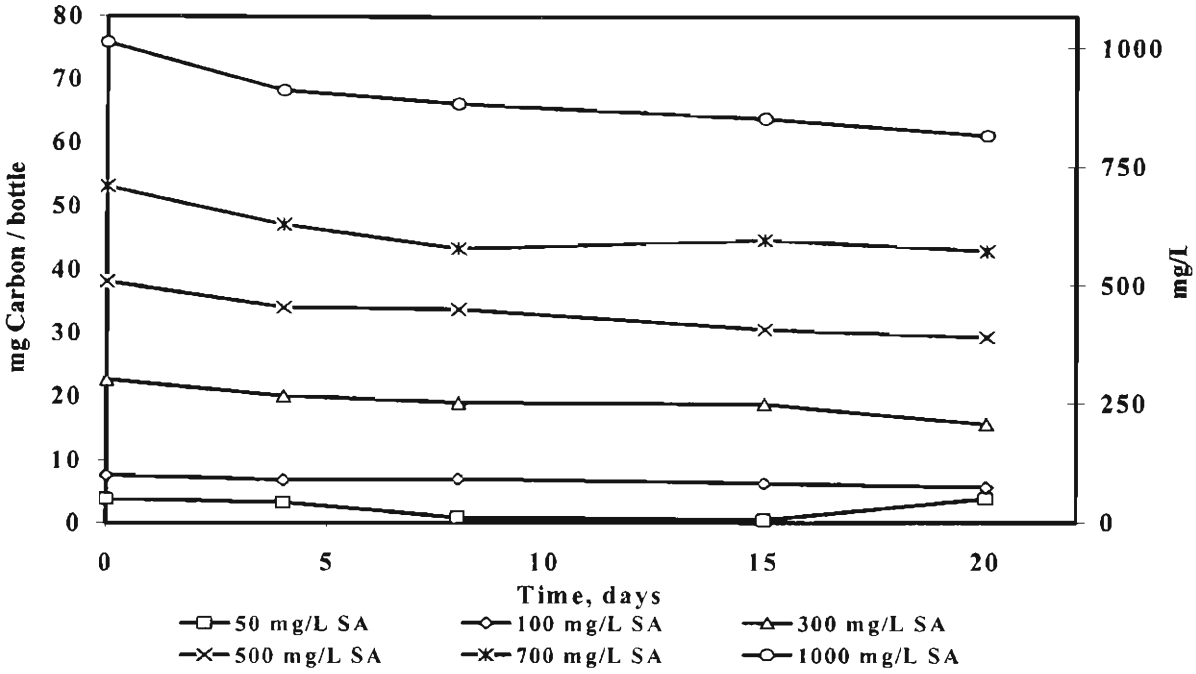


Figure 4.11: Stearic acid degradation profiles for cultures receiving 1000 mg/L glucose. (SA = stearic acid, average for triplicate samples are shown).

Degradation rates for LCFA mixtures and their LCFAs by-products profiles are shown in Figures 4.12 to 4.24. In the cultures receiving 50 or 100 mg/L of mixtures of LA/OA, LA/SA, OA/SA, or LA, OA, and SA approximately all LA and OA were removed within 20 days but SA was degraded slower and was detected after 20 days.

In the culture receiving 300 mg/L of LA/OA, LA/SA, or OA/SA mixtures, LA removal was incomplete after 20 days. In cultures receiving 300 mg/L of a mixture of LA, OA, and SA (100 mg/L each), removal was also incomplete.

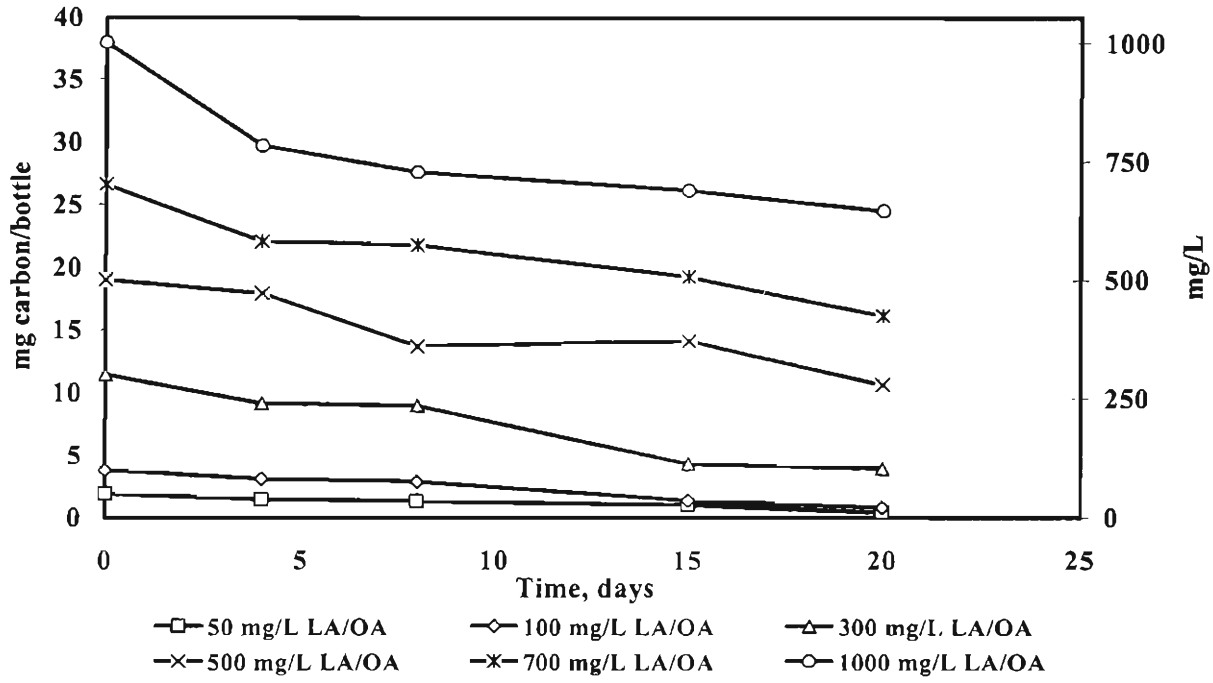


Figure 4.12: Linoleic acid degradation profiles for cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, average for triplicate samples are shown).

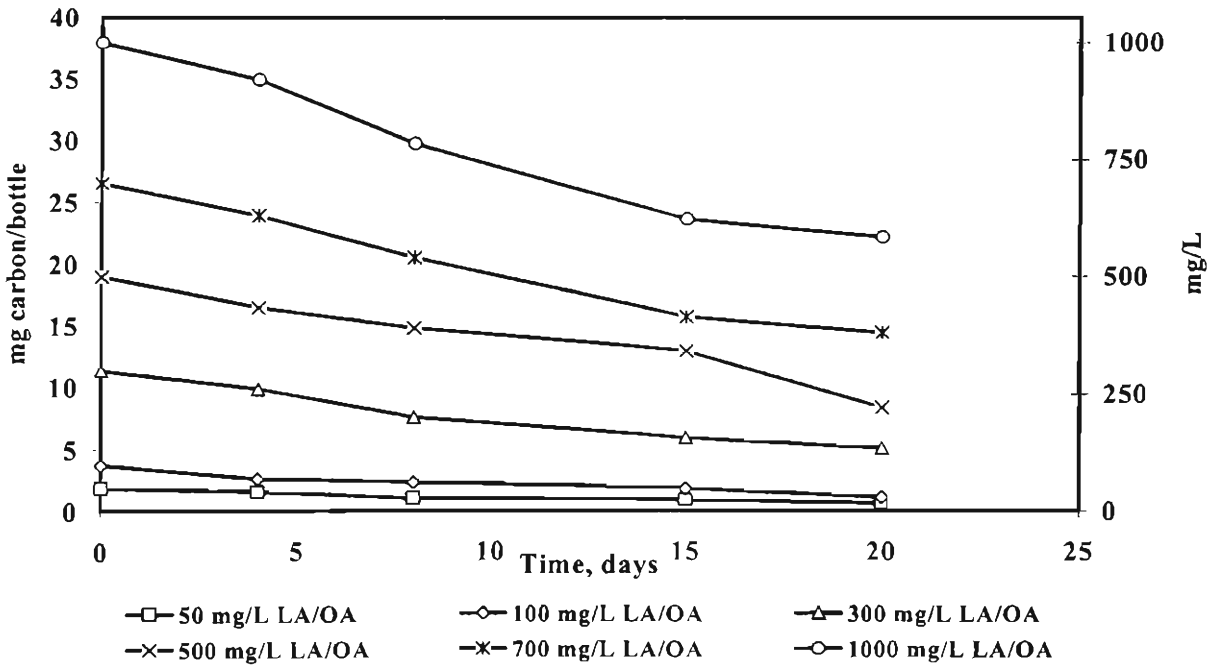


Figure 4.13: Oleic acid degradation profiles for cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA oleic acid, average for triplicate samples are shown).

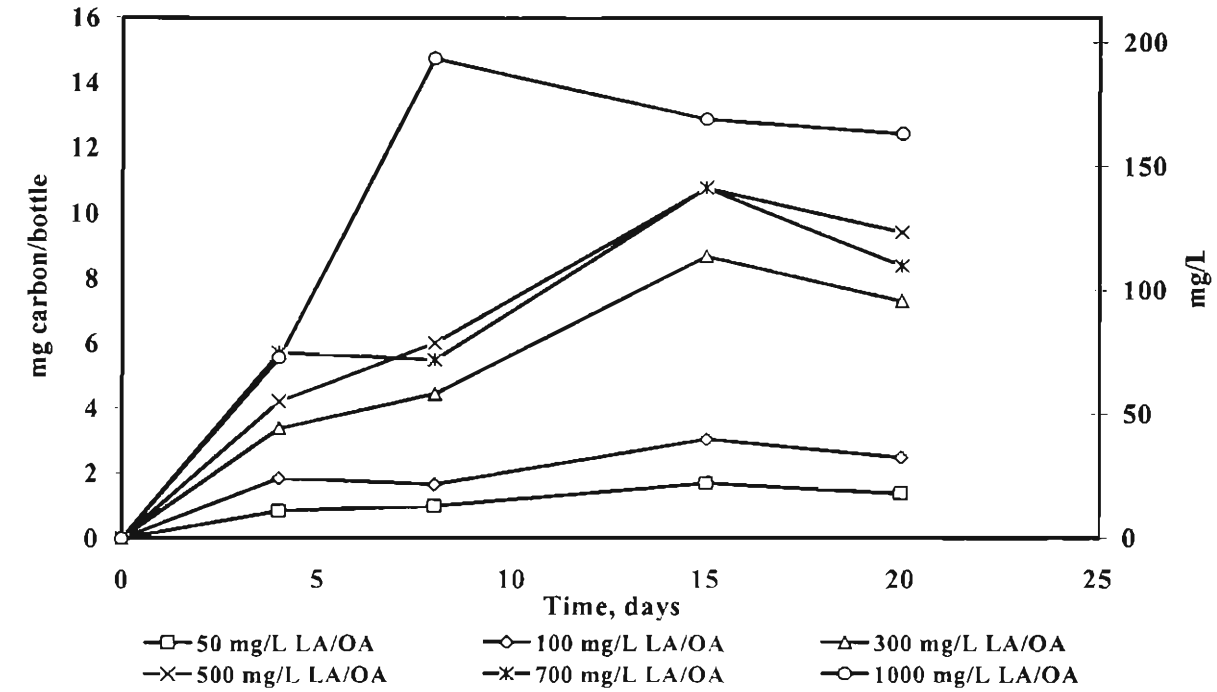


Figure 4.14: Palmitic acid production profiles for cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, average for triplicate samples are shown).

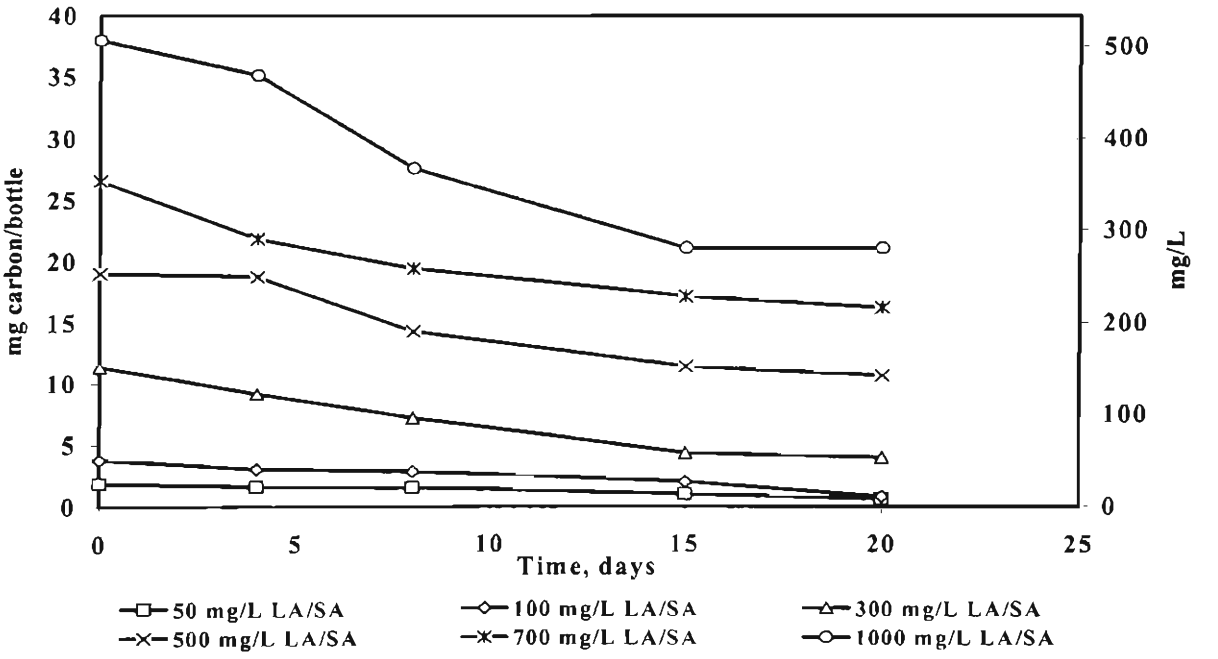


Figure 4.15: Linoleic acid degradation profiles for cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are shown).

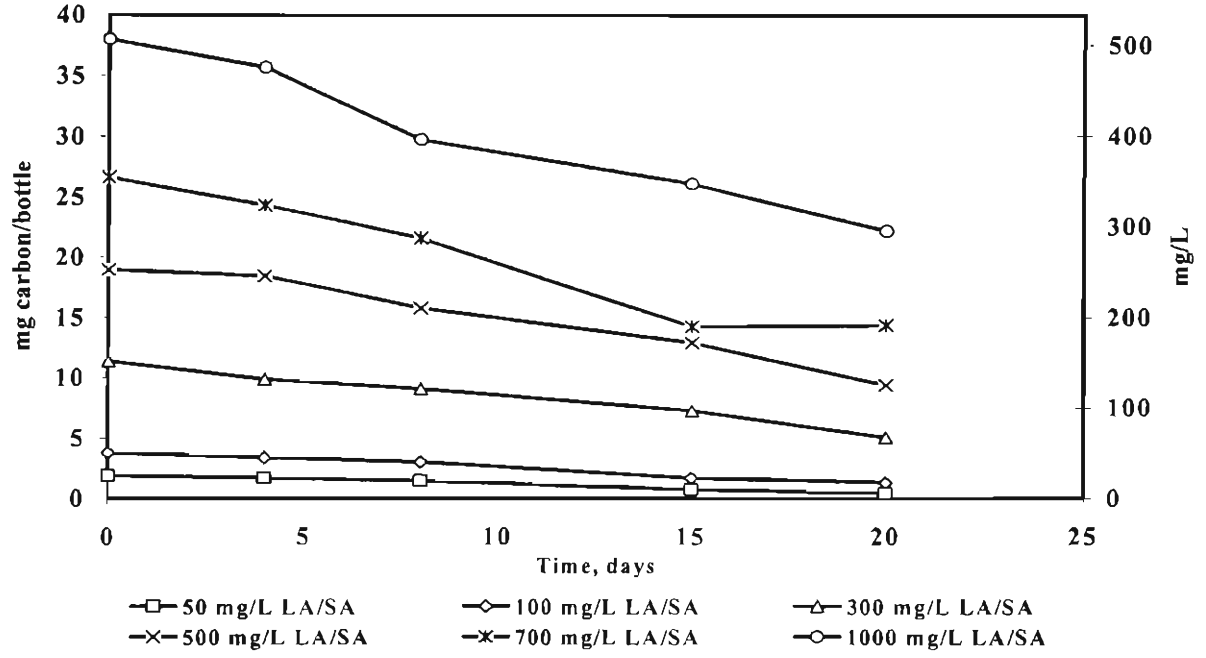


Figure 4.16: Stearic acid degradation profiles for cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are shown).

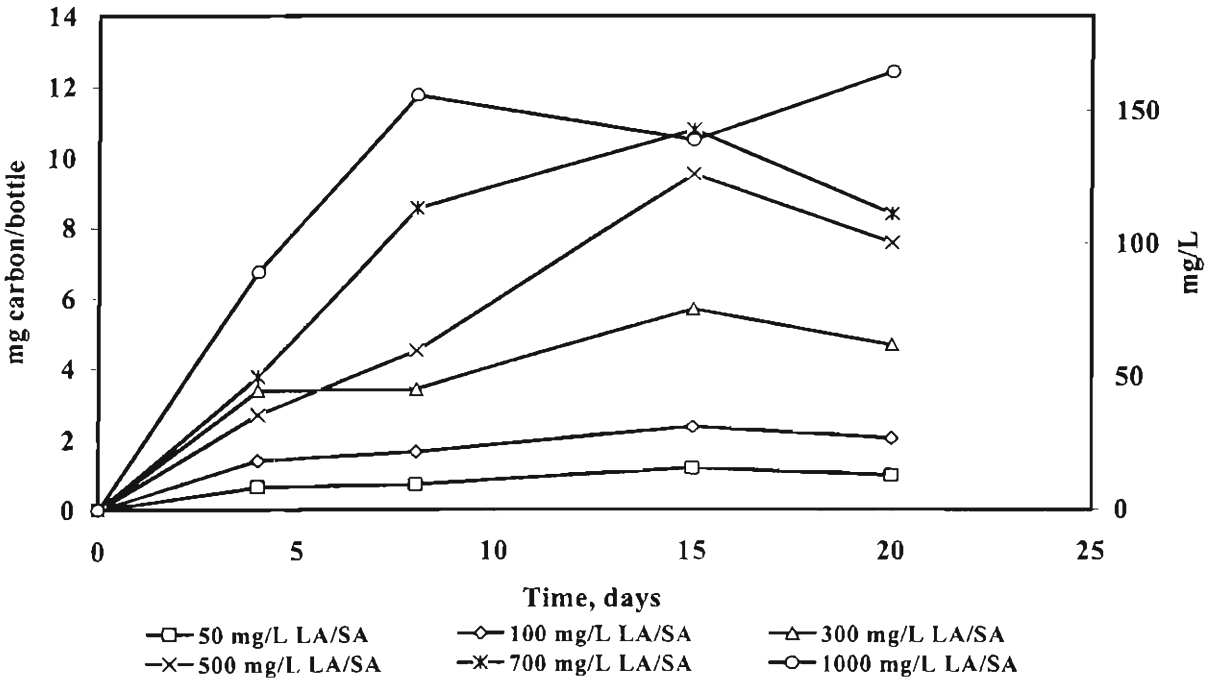


Figure 4.17: Palmitic acid production profiles for cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are shown).

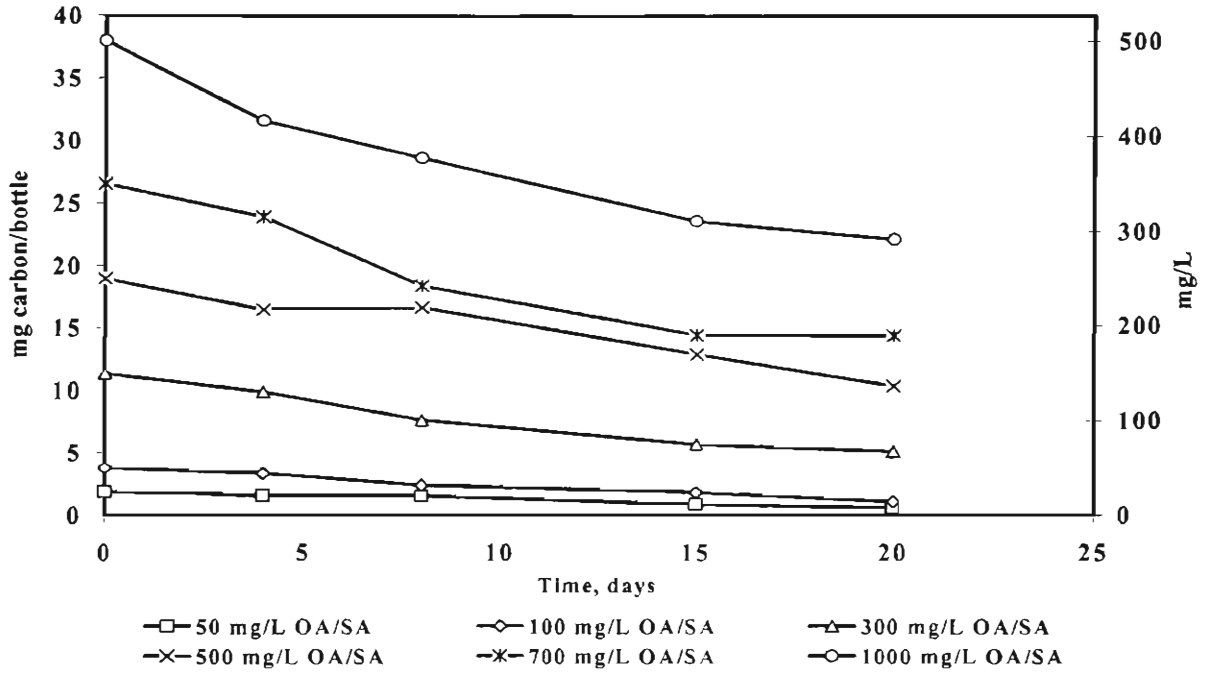


Figure 4.18: Oleic acid degradation profiles for cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

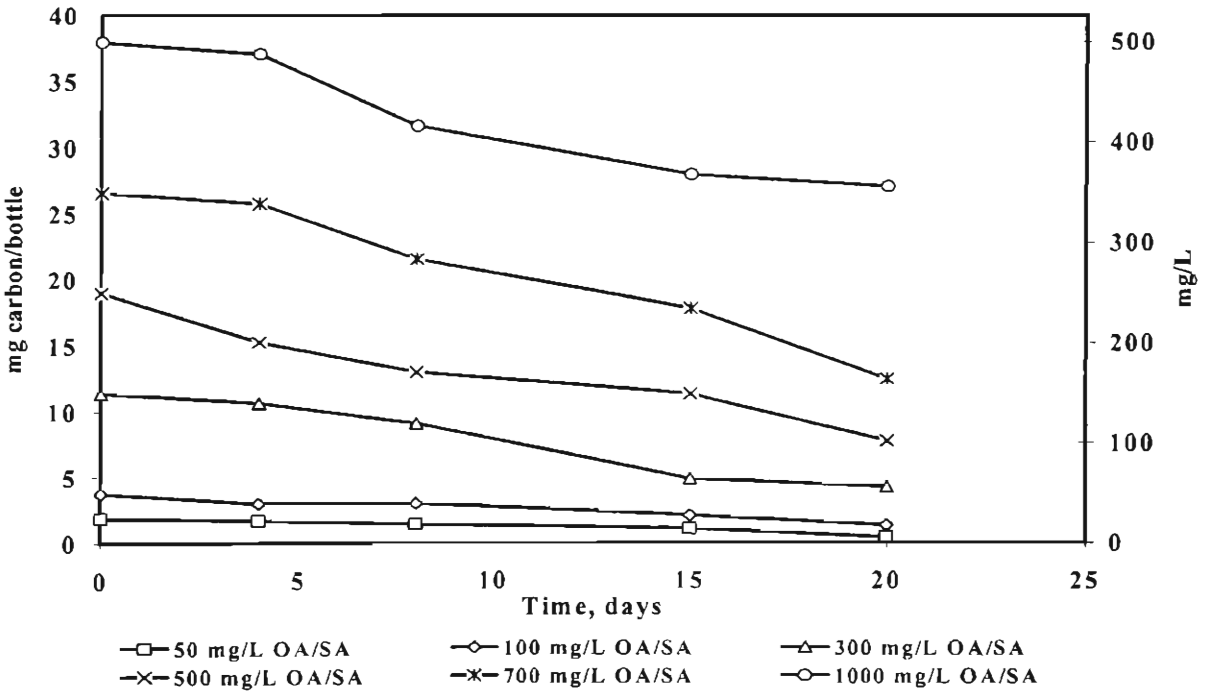


Figure 4.19: Stearic acid degradation profiles for cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

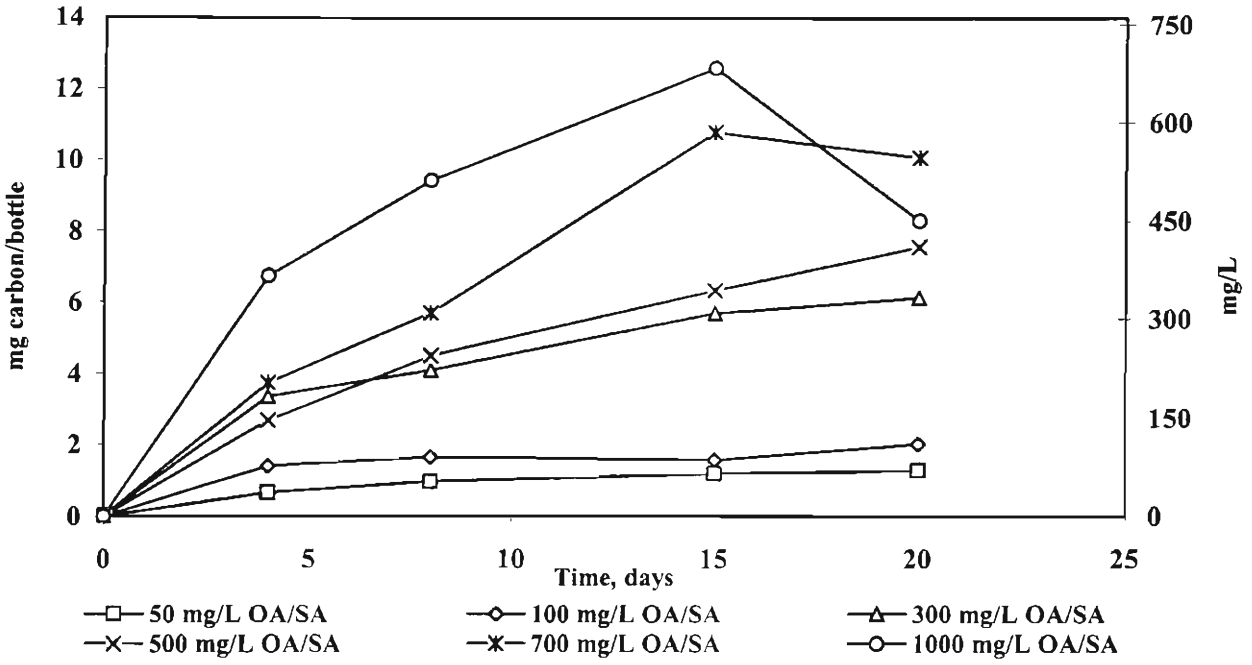


Figure 4.20: Palmitic acid production profiles for cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

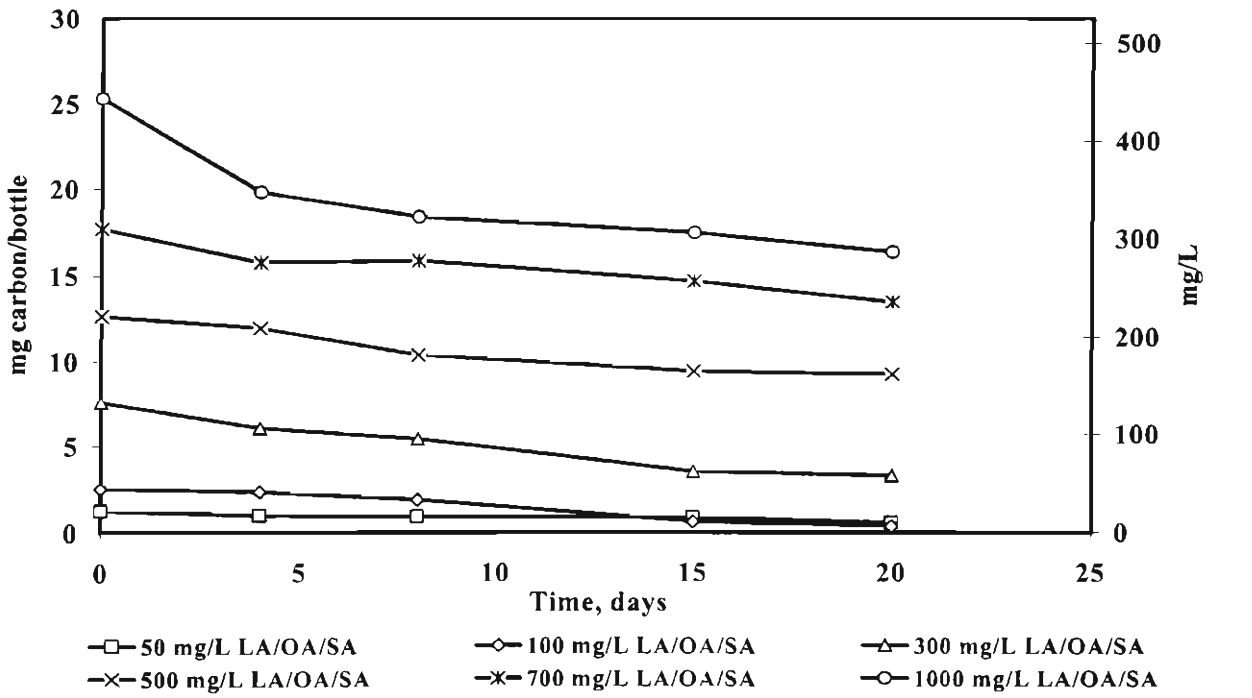


Figure 4.21: Linoleic acid degradation profiles for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

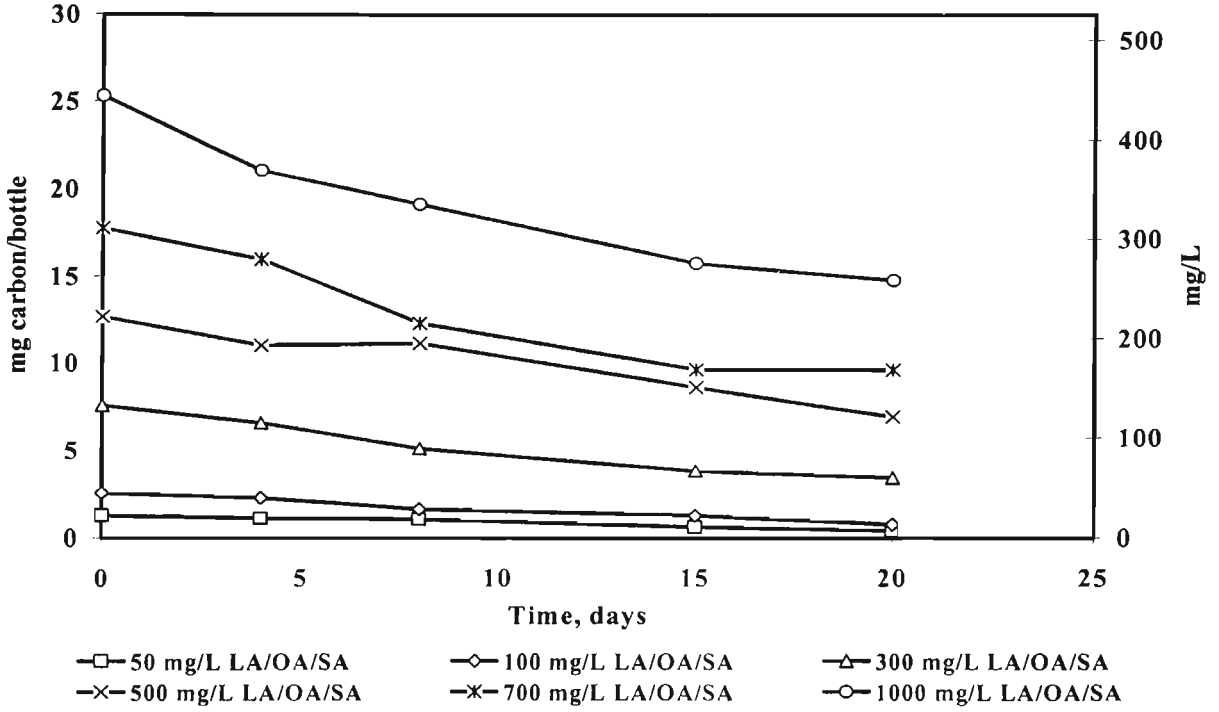


Figure 4.22: Oleic acid degradation profiles for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

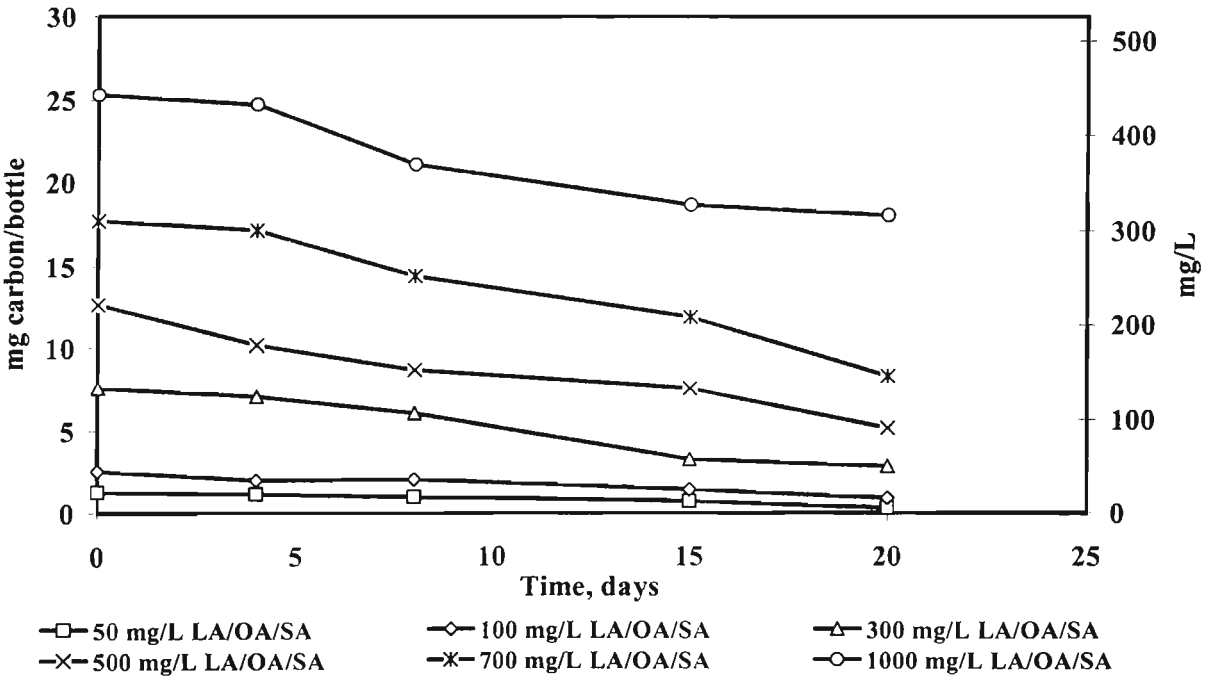


Figure 4.23: Stearic acid degradation profiles for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

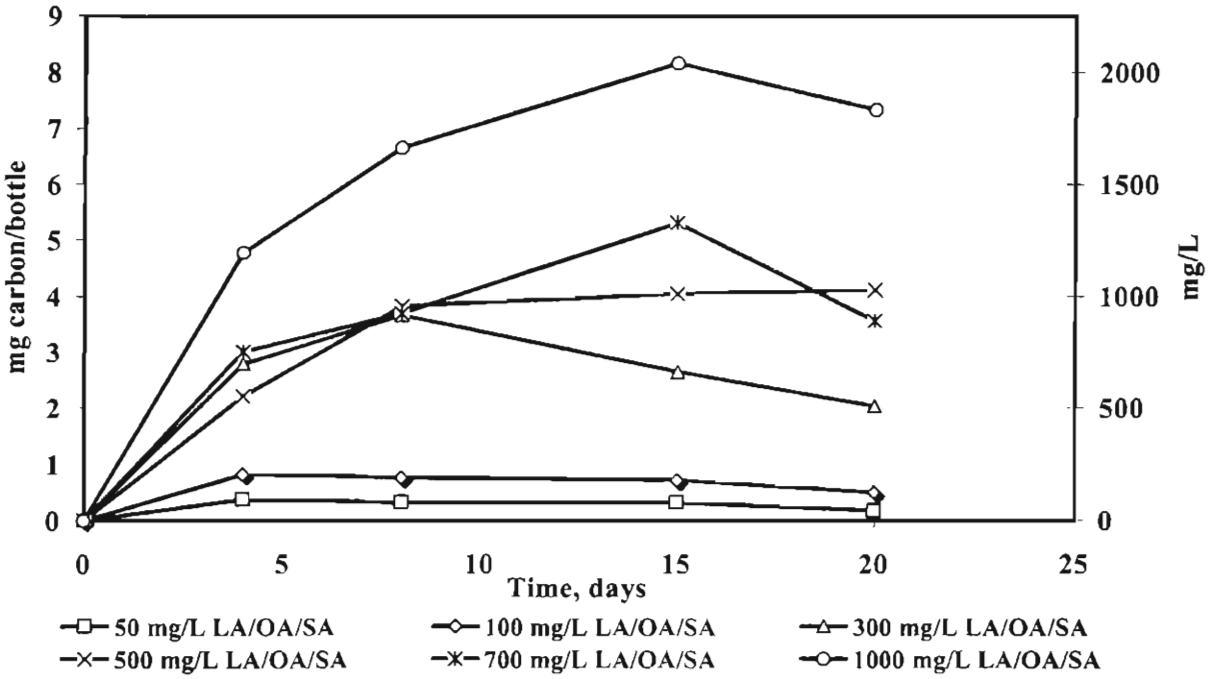


Figure 4.24: Palmitic acid production profiles for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are shown).

Linoleic, oleic, and stearic acid degradation rates for control cultures (without glucose) and in cultures receiving 1000 mg/L glucose are shown in Tables 4.3 and 4.4 respectively. Based on the Tukey's paired comparison procedure (Steel, 1999), the degradation rates for cultures receiving LA were statistically different than those receiving OA or SA. Also degradation rates for cultures receiving OA were different than those receiving SA. In addition, the degradation rates for cultures receiving 50 mg/L and 100 mg/L and 300 mg/L were statistically different but no difference was observed for cultures receiving concentrations greater than 300 mg/L LA. Similar results were observed for OA and SA. In addition, there was no difference in degradation rates between the cultures receiving LCFA (without glucose) and those receiving LCFA and 1000 mg/L glucose.

Table 4.3: LCFA Degradation rates $\left(\mu gLCFA \cdot mgVSS^{-1} \cdot d^{-1}\right)$ for cultures receiving linoleic, oleic, and stearic acid.

	LCFA concentration					
	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
LA	2.32 ± 0.21	3.33 ± 0.3	12.05 ± 0.51	11.81 ± 0.09	11.86 ± 0.22	12.16 ± 0.33
OA	0.97 ± 0.17	2.12 ± 0.11	8.01 ± 0.33	8.91 ± 0.17	6.11 ± 0.61	9.22 ± 0.16
SA	0.31 ± 0.19	0.5 ± 0.03	1.43 ± 0.2	2.62 ± 0.15	2.88 ± 0.44	3.11 ± 0.13

Average and standard deviation for triplicate samples are shown. $C_{18:2}$ = linoleic acid, $C_{18:1}$ = oleic acid, $C_{18:0}$ = stearic acid.

Table 4.4: LCFA Degradation rates $\left(\mu gLCFA \cdot mgVSS^{-1} \cdot d^{-1}\right)$ for cultures receiving linoleic, oleic, and stearic acid plus1000 mg/L glucose.

	LCFA concentration					
	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
LA	2.07 ± 0.14	3.69 ± 0.33	12.52 ± 0.09	8.05 ± 0.29	11.4 ± 0.19	11.89 ± 0.24
OA	1.13 ± 0.1	2.40 ± 0.23	7.95 ± 0.24	8.41 ± 0.23	9.21 ± 0.36	9.59 ± 0.21
SA	0.25 ± 0.11	0.46 ± 0.16	1.13 ± 0.15	2.21 ± 0.43	1.72 ± 0.11	2.97 ± 0.17

Average and standard deviation for triplicate samples are shown. $C_{18:2}$ = linoleic acid $C_{18:1}$ = oleic acid, $C_{18:0}$ = stearic acid.

Degradation rates of linoleic, oleic, and stearic acids (mixtures) in cultures receiving 2000 mg/L glucose are shown in Tables 4.5, 4.6, 4.7, and 4.8 respectively. Degradation rates for LA were statistically different from OA or SA in all mixtures.

Table 4.5: LCFAs degradation rates $\left(\mu gLCFA \cdot mgVSS^{-1} \cdot d^{-1}\right)$ for the cultures receiving 50 and 100 mg/L (total concentration) LCFA Mixtures.

	50			100		
	LA	OA	SA	LA	OA	SA
LA/OA	1.09±0.04	0.55±0.01		2.22 ± 0.19	1.02 ± 0.11	
LA/SA	1.12±0.06		0.18±0.07	2.52 ± 0.05		0.33± 0.08
OA/SA		0.42±0.03	0.17±0.02		0.89 ± 0.09	0.31± 0.1
LA/OA/SA	0.58±0.03	0.36±0.02	0.15±0.02	1.13±0.09	0.71±0.11	0.26±0.06

Average and standard deviation for triplicate samples are shown. LA = linoleic acid, OA = oleic acid, SA = stearic acid.

Table 4.6: LCFAs degradation rates $\left(\mu gLCFA \cdot mgVSS^{-1} \cdot d^{-1}\right)$ for the cultures receiving 300 mg/L (total concentration) LCFA Mixtures.

	300		
	LA	OA	SA
LA/OA	3.13±0.27	1.62±0.21	
LA/SA	3.26±0.31		0.55±0.23
OA/SA		1.32±0.15	0.47±0.26
LA/OA/SA	3.69 ± 0.13	2.07 ± 0.08	0.81 ± 0.33

Average and standard deviation for triplicate samples are shown. LA = linoleic acid, OA = oleic acid, SA = stearic acid.

Table 4.7: LCFAs degradation rates $\left(\mu gLCFA \cdot mgVSS^{-1} \cdot d^{-1}\right)$ for the cultures receiving 500 and 700 mg/L (total concentration) LCFA Mixtures.

	500 mg/L			700 mg/L		
	LA	OA	SA	LA	OA	SA
LA/OA	5.35±0.19	2.48±0.21		10.88±1.62	9.01±0.41	
LA/SA	5.59±0.22		1.01±0.07	11.01±1.43		2.21±0.11
OA/SA		2.11±0.19	0.76±0.1		8.92±1.01	2.44±0.09
LA/OA/SA	5.72±0.37	2.01±0.16	0.92±0.08	7.99±1.21	5.24±0.66	1.83±0.07

Average and standard deviation for triplicate samples are shown. LA = linoleic acid, OA = oleic acid, SA = stearic acid.

Table 4.8: LCFAs degradation rates $\left(\mu gLCFA \cdot mgVSS^{-1} \cdot d^{-1}\right)$ for the cultures receiving 1000 mg/L (total concentration) LCFA Mixtures.

	1000 mg/L		
	LA	OA	SA
LA/OA	11.62 ± 1.11	8.52 ± 0.09	
LA/SA	11.71 ± 1.13		2.54 ± 0.07
OA/SA		9.02 ± 0.62	2.42 ± 0.15
LA/OA/SA	11.12±1.03	8.02±0.41	2.11±0.22

Average and standard deviation for triplicate samples are shown. LA = linoleic acid, OA = oleic acid, SA = stearic acid.

Also the degradation rates for OA were statistically different than SA. In addition, there was no difference in degradation rates between cultures receiving 50 mg/L individual LCFAs (Tables 4.3 and 4.4) and those receiving 100 mg/L (total) mixtures of

linoleic, oleic and stearic acid (Table 4.5). Similarly, there was no difference between the cultures receiving 500 mg/L individual LCFAs (Table 4.3 and 4.4) and those receiving 1000 mg/L mixtures of LCFAs (Table 4.8). Also, no statistical difference between the cultures receiving 100 mg/L individual LCFAs and and those receiving 300 mg/L LA/OA/SA (Table 4.6).

4.1.3 VFAs Production

Acetate, propionate, and butyrate production profiles are shown in Figures 4.25 to 4.31. Acetate and propionate were observed in all cultures receiving glucose. Butyrate, however, was only observed in cultures receiving greater than 300 mg/L LA.

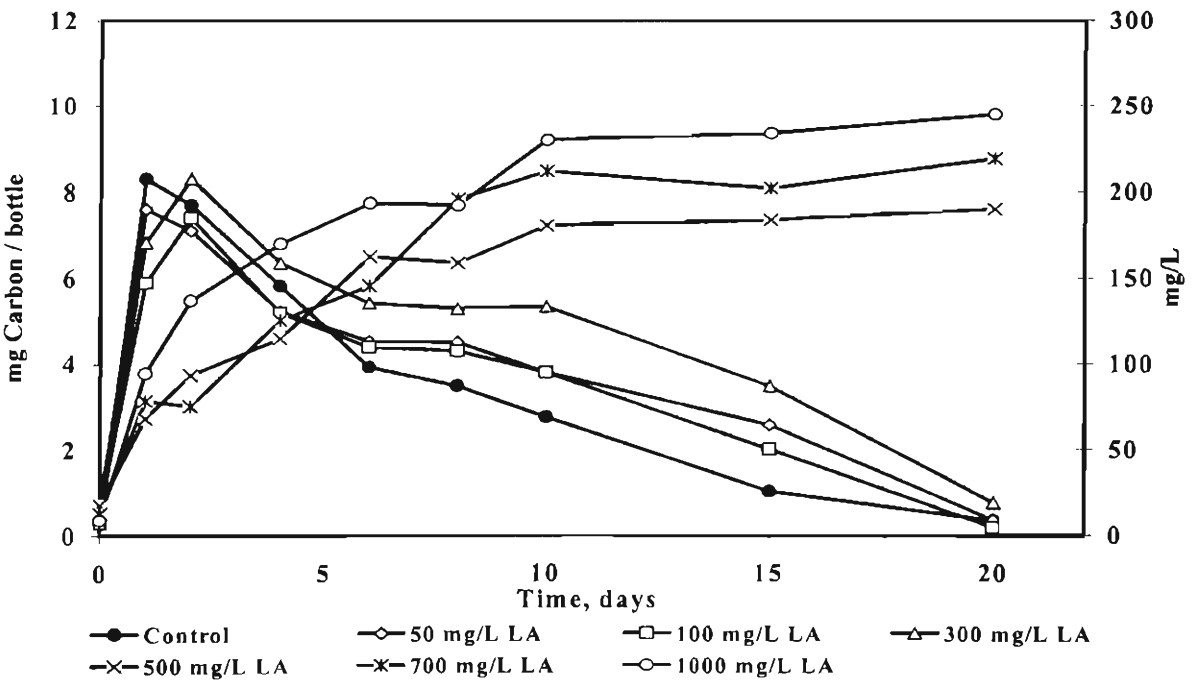


Figure 4.25: Acetic acid production profiles for cultures receiving linoleic acid plus 1000 mg/L glucose. (LA = linoleic acid, average of triplicate samples are shown).

In control cultures, the maximum concentration of acetate was achieved within a day and complete removal was observed within 15 to 20 days (Figures 4.25, 4.26, and 4.27). Similar removal times were observed in cultures receiving 50 or 100 mg/L of each LCFA. In the cultures receiving 300 mg/L SA, acetate removal times were similar to control cultures. However, in the cultures receiving 300 mg/L LA or OA (Figures 4.25 and 4.26) more accumulation was observed, compared to the control cultures, but the removal time was similar to the controls. In the cultures receiving 500, 700, or 1000 mg/L of LCFAs the maximum acetate concentration was achieved after a lag phase of 6 to 8 days and some removal was observed after 10 days (Figures 4.25 and 4.26) only in cultures fed with OA and SA. In contrast, acetate concentrations increased after day 10 for cultures fed with equal or greater than 500 mg/L LA.

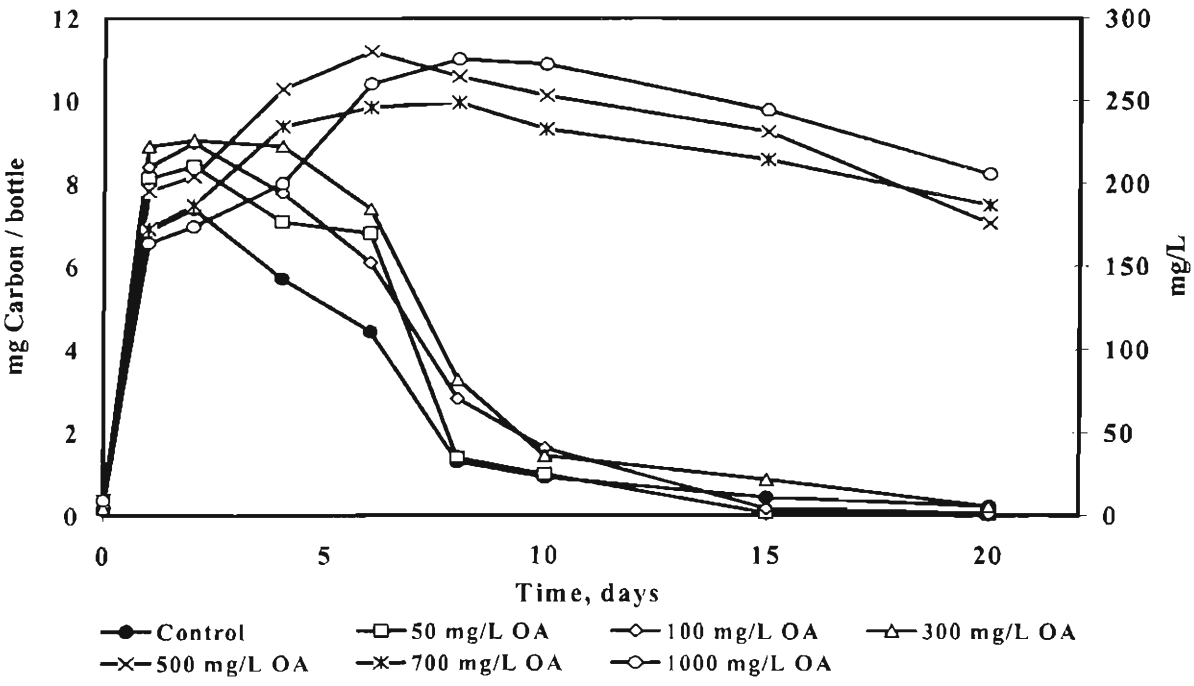


Figure 4.26: Acetic acid production profiles for cultures receiving oleic acid plus 1000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown).

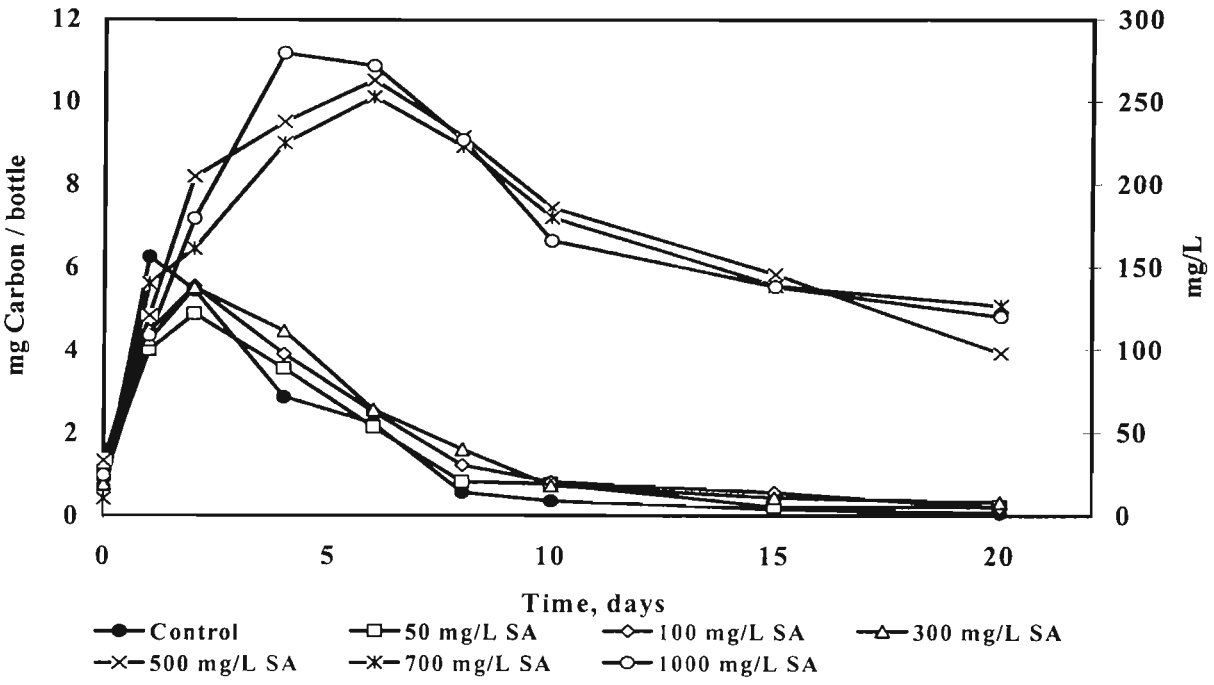


Figure 4.27: Acetic acid production profiles for cultures receiving stearic acid plus 1000 mg/L glucose. (SA = stearic acid, average of triplicate samples are shown).

Inhibition of acetate degradation was observed under threshold LCFA concentration. In cultures receiving 50 or 100 mg/L, no significant inhibition was observed compared to the controls. Maximum acetate concentrations were achieved within approximately 1 day and complete removal was observed within 20 days. These results suggest that none of the LCFAs tested were inhibitory between 50 to 100 mg/L.

Although acetate removal in cultures receiving 300 mg/L LA was approximately the same as the control cultures, complete removal was also achieved in 20 days. A similar pattern was observed in the cultures receiving 300 mg/L OA but the inhibition was less than for cultures receiving 300 mg/L LA.

In the cultures receiving greater than 500 mg/L LA, the maximum acetate accumulation was achieved in 10 days. However, additional acetate production seemed to

have ceased after 10 days. Similar patterns were observed for cultures receiving 700 and 1000 mg/L. For oleic acid, the maximum acetate accumulation was observed after 6 days and some acetate removal was observed thereafter.

In the case of SA, the addition of greater than 500 mg/L also caused inhibition to acetate degradation. Maximum accumulation of acetate varied between 4 to 6 days and acetate degradation was observed after approximately 6 days.

In control cultures, the maximum propionate concentration was achieved within a day and complete removal was achieved within 10 to 15 days (Figures 4.28, 4.29, and 4.30). Similar removal times were observed for cultures receiving 50 or 100 mg/L LCFAs. In cultures receiving 300 mg/L OA or SA (Figures 4.29 and 4.30) removal times were similar to the control cultures. However, removal of propionate in cultures receiving 300 mg/L LA (Figure 4.28) was less than those receiving similar concentrations of OA or SA (Figures 4.29 and 4.30) with residual propionate observed after 20 days. In cultures receiving 500, 700, or 1000 mg/L of the LCFAs, the maximum propionate concentration was achieved in 6 to 8 days and propionate removal was significantly slower than in the control cultures. Although propionate was observed in cultures receiving SA, greater amount was removed compared to cultures receiving LA or OA.

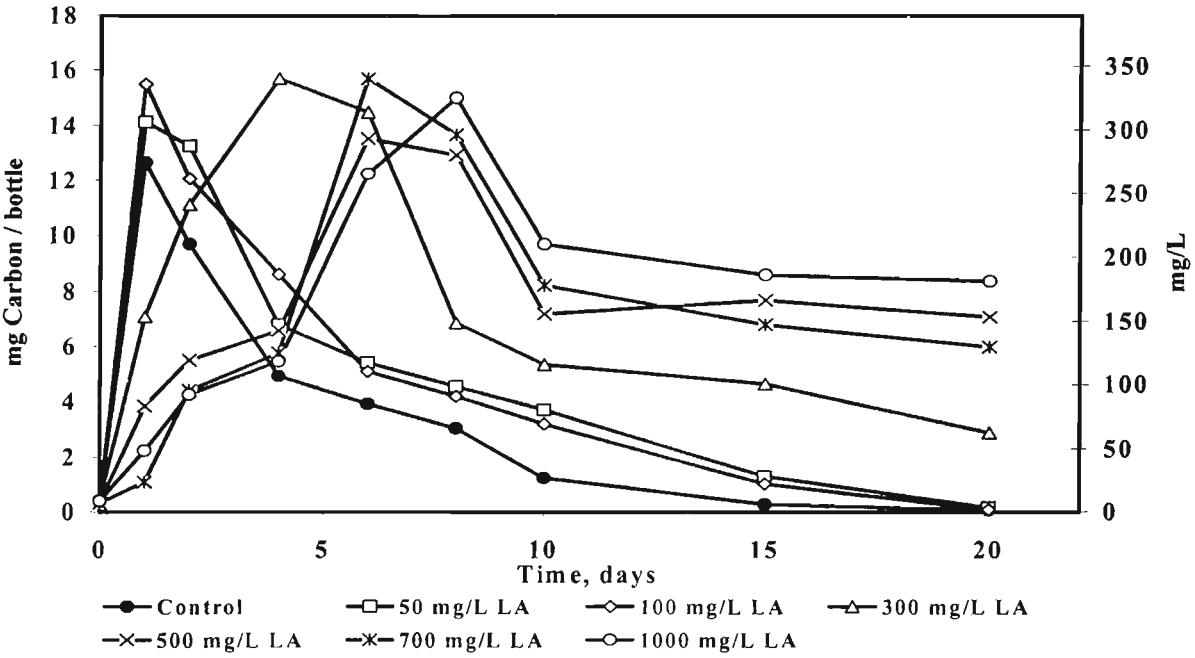


Figure 4.28: Propionic acid production profiles for cultures receiving linoleic acid plus 1000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are shown).

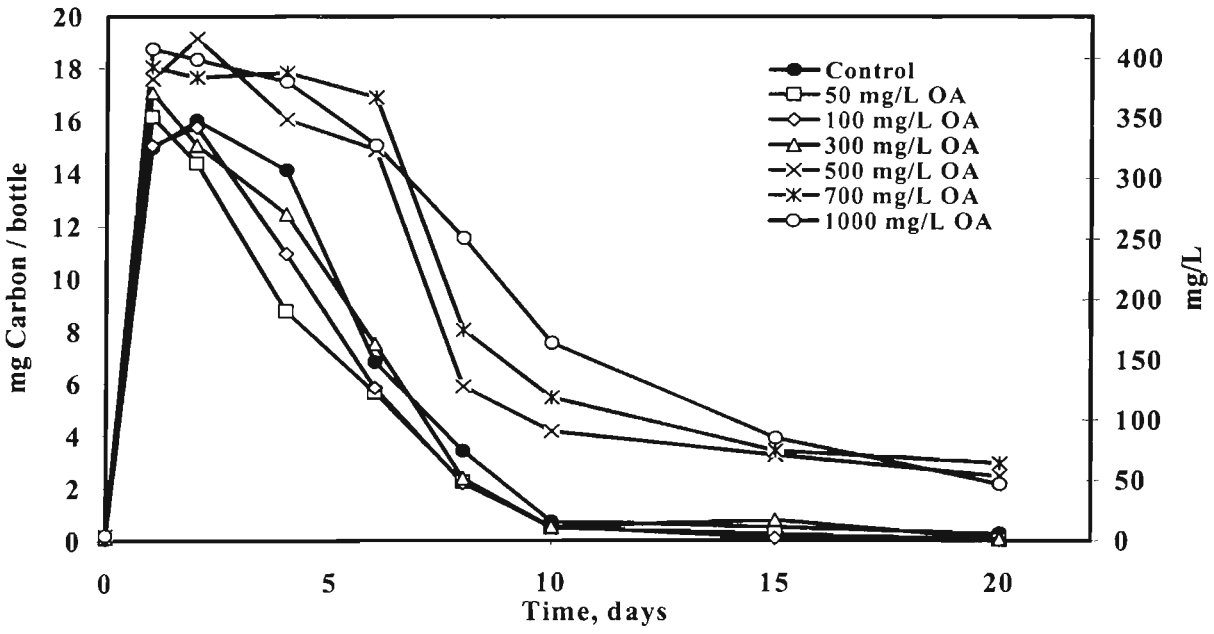


Figure 4.29: Propionic acid production profiles for cultures receiving oleic acid plus 1000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown).

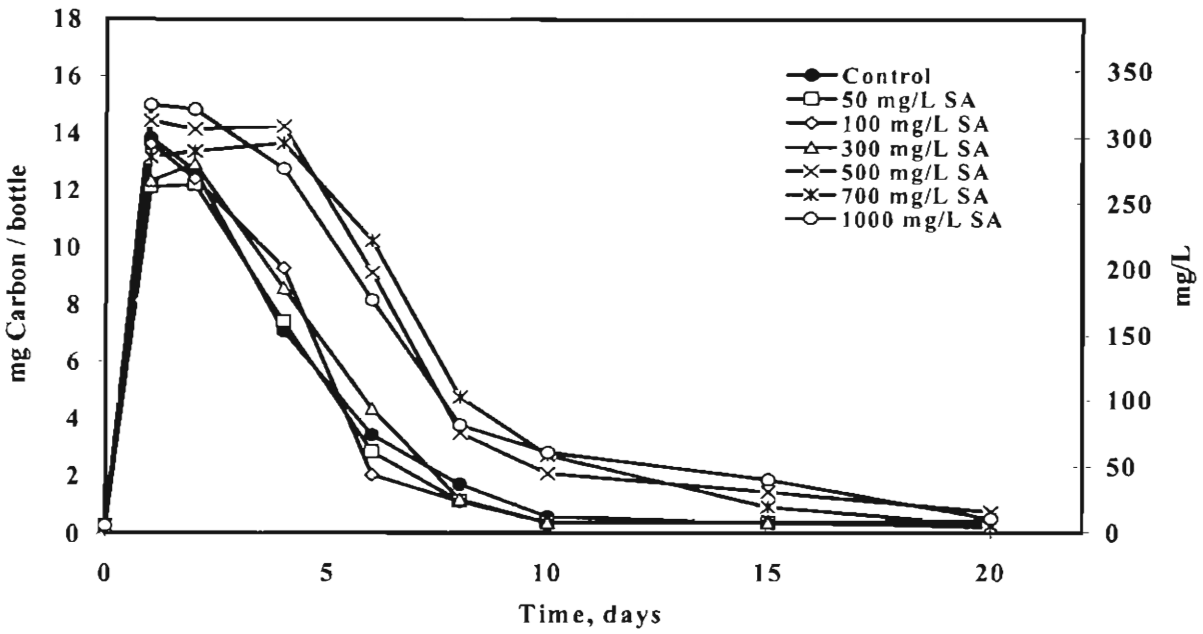


Figure 4.30: Propionic acid production profiles for cultures receiving stearic acid plus 1000 mg/L glucose. (SA = stearic acid, average for triplicate samples are shown).

Inhibition profiles for propionate were similar to those observed for acetate. Maximum propionate accumulation was observed within 1 day for control cultures and cultures receiving 50 or 100 mg/L linoleic, oleic, or stearic acids. However, in the cultures receiving 50 or 100 mg/L LA, larger quantities of propionate accumulation were observed in comparison to the controls. This pattern was not observed in the cultures receiving similar concentrations of OA and SA. In cultures receiving 300 mg/L LA, the maximum concentration accumulated was greater than the controls. Inhibition was observed but after 2 days of contact time, propionate removal increased and most of the propionate was removed within 20 days. For cultures receiving equal or greater than 500 mg/L LA, an initial lag phase was observed up to approximately 3 days. The peak propionate concentration was observed between 6 to 7 days with a subsequent decrease to between approximately 130 and 180 mg/L.

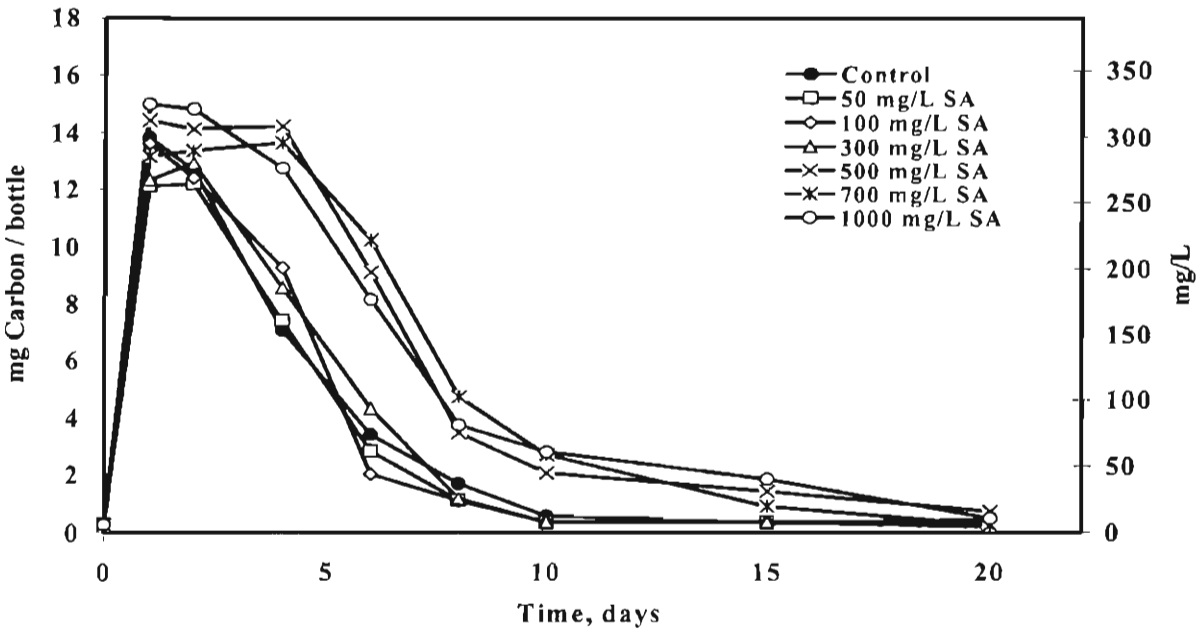


Figure 4.30: Propionic acid production profiles for cultures receiving stearic acid plus 1000 mg/L glucose. (SA = stearic acid, average for triplicate samples are shown).

Inhibition profiles for propionate were similar to those observed for acetate. Maximum propionate accumulation was observed within 1 day for control cultures and cultures receiving 50 or 100 mg/L linoleic, oleic, or stearic acids. However, in the cultures receiving 50 or 100 mg/L LA, larger quantities of propionate accumulation were observed in comparison to the controls. This pattern was not observed in the cultures receiving similar concentrations of OA and SA. In cultures receiving 300 mg/L LA, the maximum concentration accumulated was greater than the controls. Inhibition was observed but after 2 days of contact time, propionate removal increased and most of the propionate was removed within 20 days. For cultures receiving equal or greater than 500 mg/L LA, an initial lag phase was observed up to approximately 3 days. The peak propionate concentration was observed between 6 to 7 days with a subsequent decrease to between approximately 130 and 180 mg/L.

Propionate inhibition caused by 300 mg/L OA or SA was less than that of LA. In fact, no lag in the maximum accumulation was observed and the removal pattern was similar to the cultures receiving 50 or 100 mg/L of the same acid. The addition of 500 mg/L of LA affected propionate production and degradation severely. Maximum accumulation varied between 6 and 8 days. However degradation activity was restored after 8 days, a pattern that was not observed in acetate degradation for the same LCFA concentration. For the cultures receiving equal or greater than 500 mg/L OA or SA, no lag phase was observed. Propionate accumulation in cultures fed with stearic acid lasted until approximately 4 days and complete removal within 20 days.

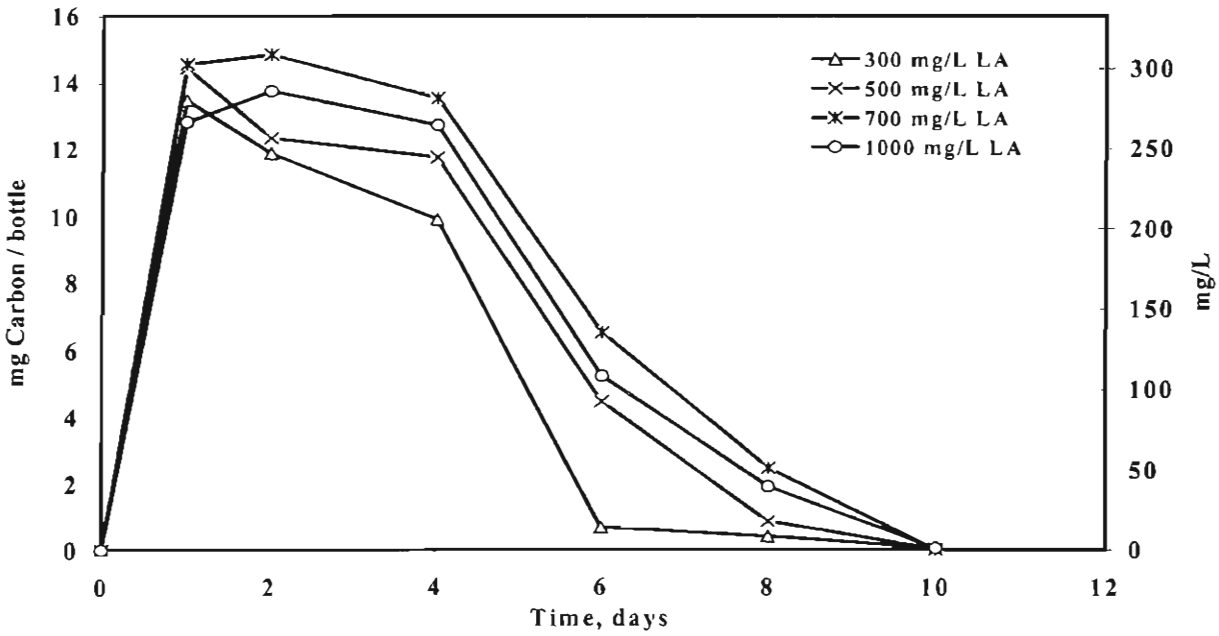


Figure 4.31: Butyric acid production profiles for cultures receiving linoleic acid plus 1000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are shown).

Butyric acid was not observed in cultures receiving OA or SA and was only observed in the cultures receiving equal or greater than 300 mg/L LA (Figure 4.31). The maximum concentration of butyric acid was achieved within 1 to 2 days and complete

removal was achieved within 8 to 10 days. A stationary phase was observed but based on the data available no comparative evaluation can be made on the toxicity effects of the three LCFAs examined.

4.1.4 Methane Production

Methane production profiles are shown in Figures 4.32 to 4.34. In the control cultures, complete conversion of glucose was achieved within 20 days. In the cultures receiving 50 or 100 mg/L LA, methane production was slightly less than the controls but after 20 days, total methane production was similar to the controls. In the cultures receiving 50 or 100 mg/L OA or SA, total methane production was slightly greater than the controls. In the cultures receiving equal or greater than 300 mg/L LA, methane production was less than control cultures. However, in the cultures receiving 300 mg/L OA or SA, the total methane production was greater than that of the controls (Figures 4.33 and 4.34).

In cultures receiving 500, 700, or 1000 mg/L LCFA the total amount of methane produced was less than the control cultures. Cultures receiving LA produced the least amount of methane. In addition, lag-phases of up to 4 days were observed, however in the cultures receiving concentrations greater than 500 mg/L SA, the total methane production exceeded that of the control cultures (Figure 4.34)

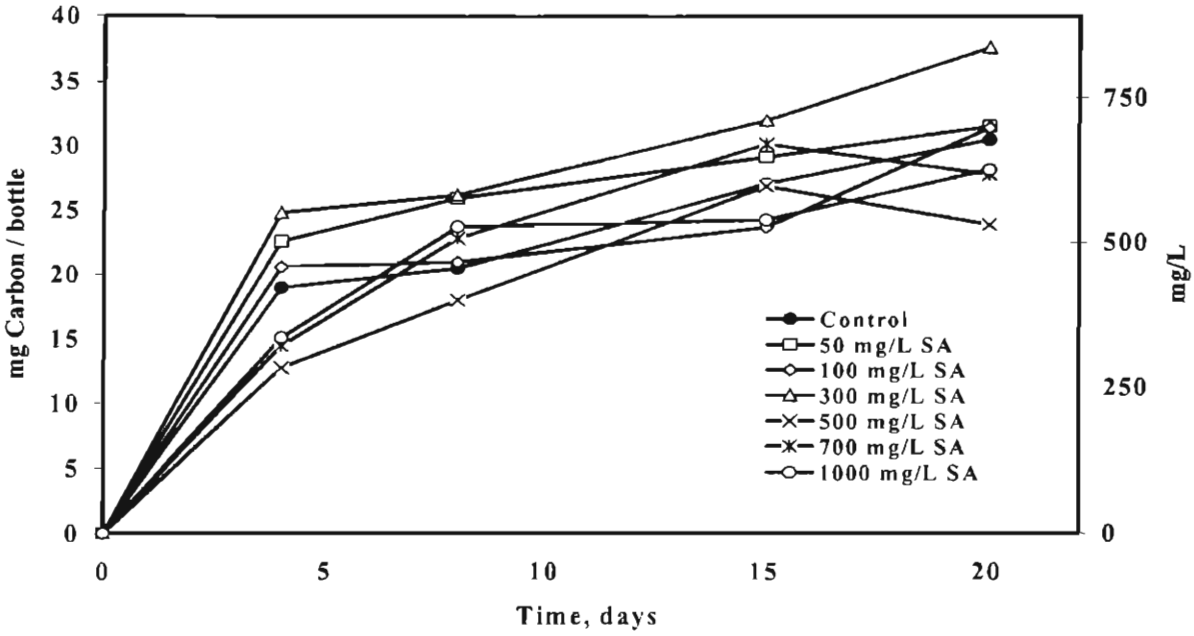


Figure 4.34: Methane production profiles for cultures receiving stearic acid plus 1000 mg/L glucose. (SA = stearic acid, average for triplicate samples are).

In cultures receiving greater than 300 mg/L LA lag-phases were observed between 7 to 15 days. However, during this period the culture adapted and methane production was observed. In the cultures receiving 300 mg/L OA lag-phases were also observed for up 4 days but methane production quickly surpassed the amount produced by control cultures. Although LA and OA significantly inhibited methane production at greater than 300 mg/L, OA was less inhibitory and its inhibition was not permanent in comparison to cultures receiving LA. The addition of concentrations higher than 500 mg/L LA caused a significant reduction in methane production. Lag-phases were observed for up to 4 days. After day 4, total methane production was significantly lower than that of the controls in the cultures receiving LA. However, inhibition was not permanent because methane production was greater than the controls after day 10. In the cultures receiving concentrations greater than 500 mg/L SA, methane production was slightly lower than

that of the controls for up to 4 days. After day 4, methane production was greater than the controls. Thus, indicating that although slightly inhibitory, SA did not completely inhibit methane production even at concentrations greater than 500 mg/L.

4.1.5 Mass Balance

Mass balances for control cultures and those receiving LCFAs, expressed as mg carbon per bottle, are shown in Figures 4.35, 4.36, and 4.37. Mass balances for control cultures and those receiving 50 or 100 mg/L LCFA were within approximately 10% of the theoretical amount of carbon. In cultures receiving equal or greater than 300 mg/L LCFA the error was slightly larger than controls. For the cultures receiving equal or greater than 500 mg/L LCFA, the error was larger than the control.

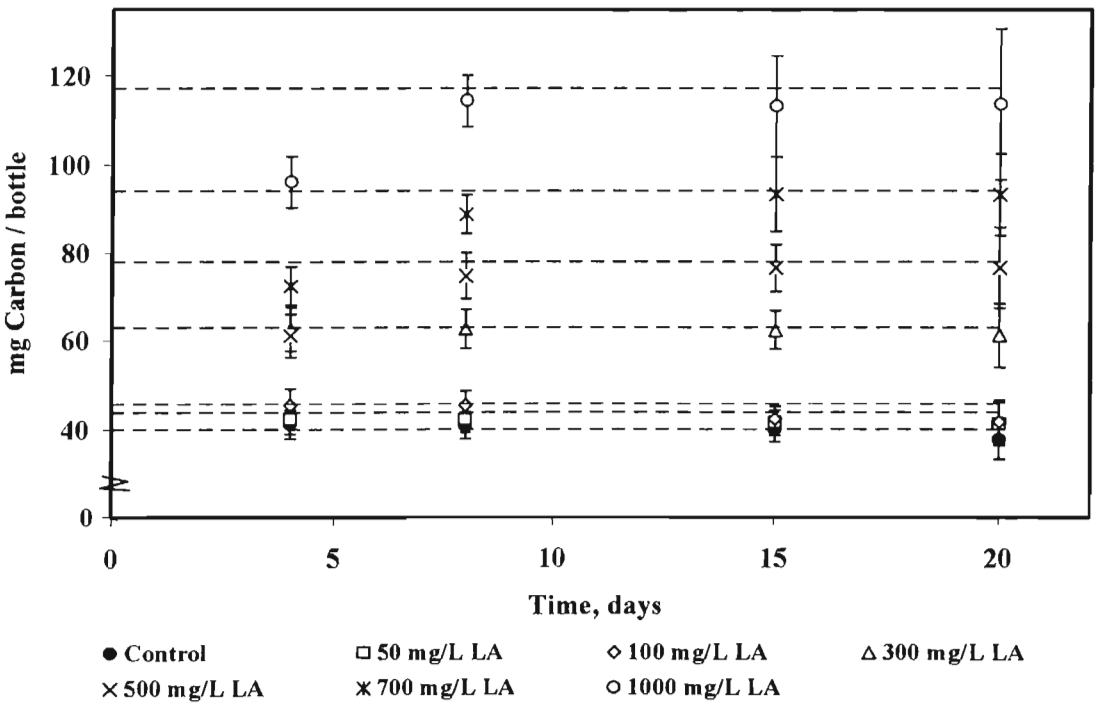


Figure 4.35: Mass balance for cultures receiving linoleic acid plus1000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are used, error bars are standard deviation). --------- Theoretical mass balance.

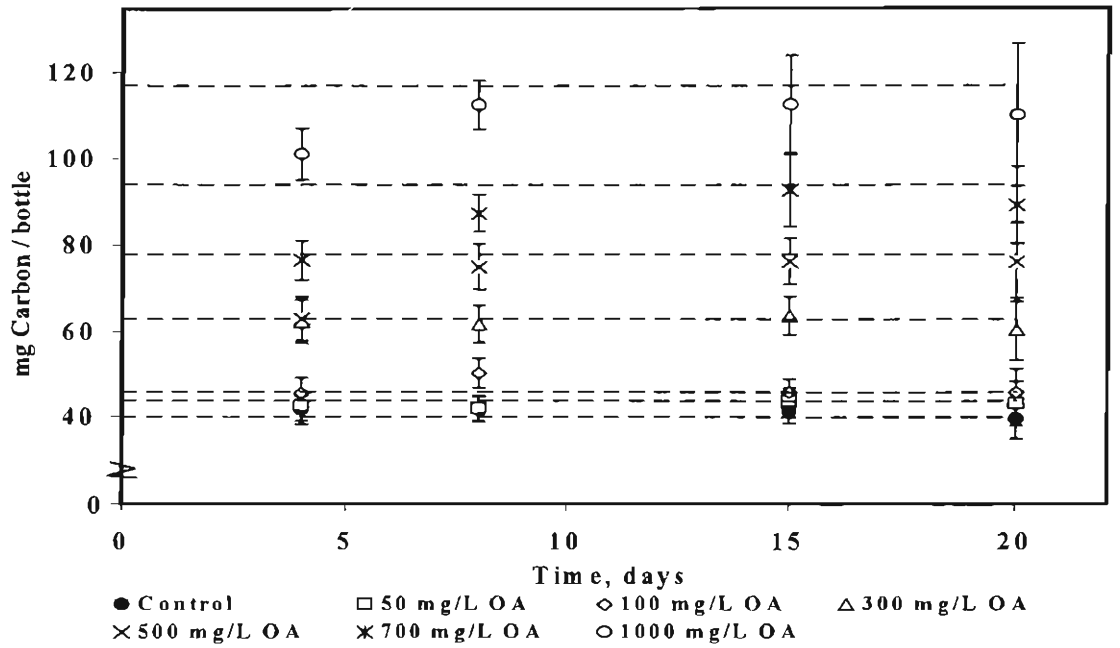


Figure 4.36: Mass balance for cultures receiving oleic acid plus 1000 mg/L glucose. (OA = oleic acid, average for triplicate samples are shown, error bars are standard deviation). --------- Theoretical mass balance.

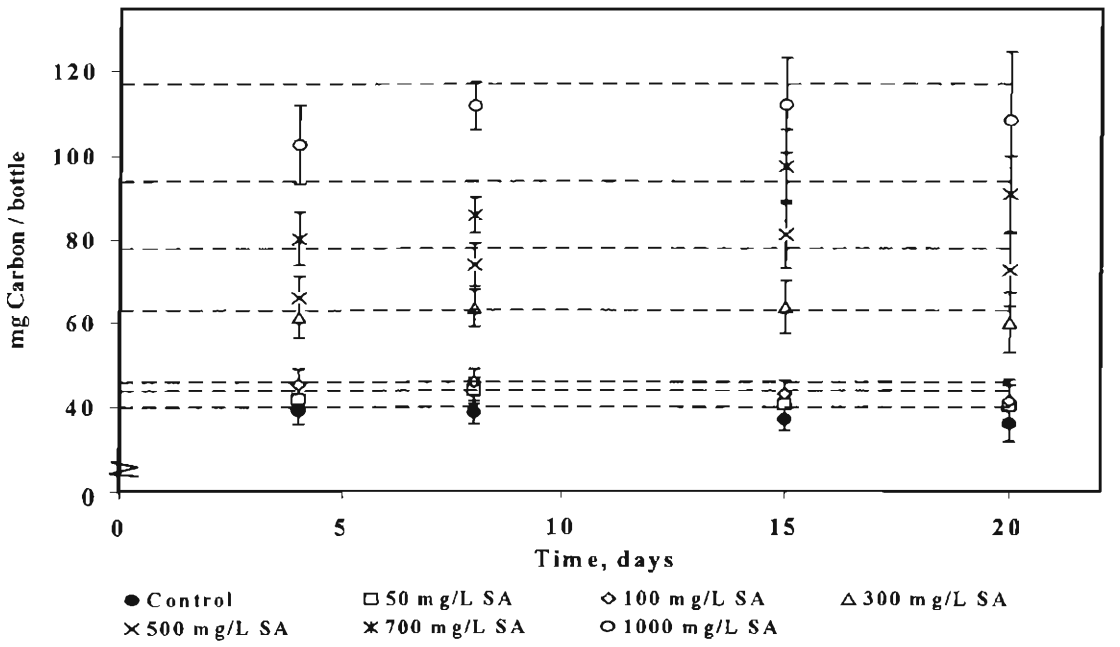


Figure 4.37: Mass balance for cultures receiving stearic acid plus 1000 mg/L glucose. (SA = stearic acid, average of triplicate samples are shown, error bars are standard deviation). --------- Theoretical mass balance.

4.2 Discussion of Results

4.2.1 Glucose degradation

Between the three LCFAs examined, LA was the most inhibitory to glucose degradation. Glucose initial degradation rates were affected by the addition of 50 mg/L LA but the removal times were similar to the control cultures. The addition of 50 or 100 mg/L OA or SA did not affect the initial glucose degradation rate in comparison to control cultures. Moreover, the reduction in degradation rates, due to inhibition, caused by LA was greater than that caused by oleic and stearic acids. The addition of a second double bond had a significant effect on the degradation of glucose. For example, in comparison to control cultures, the addition of 300 mg/L LA caused a 45% decrease in the degradation rates compared to 37% for OA and 36% for SA. This decrease suggests that addition of a second double bond in LA affected glucose degradation. These findings are in agreement with data reported by Lalman and Bagley (in press). In their work, LCFA unacclimated sludge (1500 mg/L VSS) was fed with 500 mg/L glucose. They reported that the addition of 100 mg/L LA affected glucose degradation rates compared to cultures receiving the same amount of oleic or stearic acids.

In comparison to controls, glucose degradation rates decreased by approximately 57% in cultures receiving 300 mg/L LA but no further significant decrease was observed in cultures receiving higher concentrations. This may suggest there is an inhibition threshold above which no further reduction in the degradation rates is observed. Similar threshold were observed in the cultures receiving oleic and stearic acids.

Research on the impact of LCFAs on glucose fermentation is limited except for research reported by Lalman and Bagley (in press) and Hanaki *et al.* (1981). Hanaki *et al.*

(1981) reported no inhibition of glucose degradation occurred when LCFAs concentrations of up to 2000 mg/L (as oleic acid) were added to an acclimated culture. Hanaki *et al.*(1981) used acclimated sludge and this may have reduced, if not eliminated, the inhibitory effects of LCFAs. Their work used acetic acid production/removal as an indication of glucose removal. Two major problems may arise from such an experimental approach. First, acetate is a by-product of both glucose anaerobic fermentation and LCFA β-oxidation. Second, although no research has reported on the effects of LCFAs on glucose degradation prior to research by Hanaki *et al.* (1981), many studies have shown that LCFA inhibited acetate removal even at low LCFA concentration and significant acetate accumulation has been observed in the presence of LCFAs (Koster and Kramer 1986, Angelidaki and Ahring 1992, Rinzema *et al.* 1994, Vidal *et al.* 2000). Given these observations, acetate is not a proper indicator of glucose degradation.

4.2.2 LCFAs β-Oxidation

A β-oxidation product observed from LA and OA was palmitic acid. If double bond hydrogenation is not the first reaction step, then the expected β-oxidation product from LA and OA could be a $C_{16:2}$ compound in the case of LA and a $C_{16:1}$ compound in the case of OA degradation. Neither of theses LCFA by products were detected during this research. This is in contrast to research reported by Lalman and Bagley (2000) and Canovas-Diaz *et al.* (1991) who observed hydrogenation prior to β-oxidation. Moreover, Lalman and Bagley (2000) detected palmitoleic acid (a 16-carbon acid with a single double bond) during LA degradation. This observation may suggest that β-oxidation of LCFAs is culture dependent.

A possible explanation for such observation arises by a consideration of the free energies involved in the β-oxidation process. The production of palmitic acid from LA is more energetically favorable than the production of OA from LA (-106.4 kJ/mol for the production of palmitic acid vs. –78.6 kJ/mol for the production of OA, equations 7 and 8 respectively).

$$C_{18}H_{31}O_2^- + 2\,H_2O \rightarrow C_{16}H_{31}O_2^- + Ac^- + H^+ \qquad \Delta G^{\circ\prime} = -106.4 kJ/mole \qquad (7)$$

$$C_{18}H_{31}O_2^- + H_2 \rightarrow C_{18}H_{33}O_2^- \qquad \Delta G^{\circ\prime} = -78.6 kJ/mole \qquad (8)$$

In addition, based on reactions (7) and (8) the production of OA is dependent on the hydrogen partial pressure while the production of palmitic acid is independent (see Appendix B for calculation and references). However, the production of SA and the production of palmitic acid from OA are both dependent on the hydrogen partial pressure (equations 9 and 10 respectively). Moreover, the production of SA from OA is more energetically favorable than the production of palmitic acid from OA (-78.6 kJ/mol for SA production versus –27.8 kJ/mol for OA production see appendix B).

$$C_{18}H_{33}O_2^- + H_2 \rightarrow C_{18}H_{35}O_2^- \qquad \Delta G^{\circ\prime} = -78.6 kJ/mole \qquad (9)$$

$$C_{18}H_{33}O_2^- + 2H_2O \rightarrow C_{16}H_{31}O_2^- + Ac^- + H_2 + H^+ \qquad \Delta G^{\circ\prime} = -27.8 kJ/mole \qquad (10)$$

The production of palmitic acid from SA is not energetically favorable (50.8 kJ/mol) under standard conditions (equation 11). However, when the hydrogen partial pressure reaches a critical value, the free energy of reaction (11) is favorable and the reaction proceeds from left to right.

$$C_{18}H_{35}O_2^- + 2\,H_2O \rightarrow C_{16}H_{31}O_2^- + Ac^- + 2H_2 + H^+ \qquad \Delta G^{\circ\prime} = 50.8 kJ/mole \qquad (11)$$

Glucose was examined as a co-substrate for LCFA degradation. However, there is no conclusive evidence from this study that the presence of glucose enhanced the

degradation of the LCFAs. This observation is in contrast to the work made by Beccari *et al.* (1996) who reported that glucose enhanced the co-digestion of OA.

No statistical differences in LCFA degradation rates were observed between controls (without glucose) and cultures receiving 1000 mg/L glucose. Moreover, for cultures receiving no glucose higher degradation rates were observed for LA in comparison to cultures receiving SA. In addition, degradation rates increased with increasing concentrations up to 300 mg/L for all LCFAs. At concentrations higher than 300 mg/L, lower degradation rates were observed thus, indicating LCFAs might be inhibitory to their own degradation.

4.2.3 VFA Degradation

The accumulation of VFAs during of this research supports previous research by Lalman and Bagley (2001) who showed that OA was inhibitory at low concentration while SA was not. Moreover, Angelidaki and Ahring (1992) reported that oleate was more toxic than stearate with inhibitory concentrations of 100 to 200 mg/L for oleate and 500 mg/L for stearate. However, in contrast to this research, Angelidaki and Ahring (1992) reported that aceticlastic methanogenic activity could not be restored to cultures fed with 500 mg/L oleate. One possible explanation is the difference in operating temperature of culture used by Angelidaki and Ahring (1992) and this research. Angelidaki and Ahring (1992) used a culture that was acclimated at 55 °C while in this study the culture was adapted at room temperature (21 °C). The microorganisms used by Angelidaki and Ahring (1992) may have a reduced LCFAs tolerance (i.e. more inhibition) at higher temperatures.

Propionate-degrading microorganisms appeared to be less sensitive to the LCFAs examined than acetate-degrading microorganisms since propionate degradation activity was restored in all cases when greater than 500 mg/L of LCFAs were used. Angelidaki and Ahring (1992) reported that oleic and stearic acids inhibited acetate, propionate, and butyrate degradation but they did not compare the LCFA inhibitory effects. Hanaki *et al.* (1981) showed that LCFAs inhibited acetate and butyrate degradations. Thus, since butyrate and propionate degradation is dependent on the hydrogen partial pressure, hydrogen accumulation is expected to inhibit the degradation of these VFAs.

A consideration of the reaction free energies will assist in explaining the experimental observations. Equations 12, 13, and 14 show the degradation of butyrate and propionate and the consumption of hydrogen respectively (see appendix B for calculation and references).

$$C_4H_7O_2^- + 2H_2O \rightarrow 2Ac^- + H^+ + 2H_2 \quad (12)$$

$$C_3H_5O_2^- + 3H_2O \rightarrow Ac^- + HCO_3^- + H^+ + 3H_2 \quad (13)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (14)$$

The degradation of butyrate and propionate are hydrogen producing (48.3 kJ/mol and 69.8 kJ/mol respectively). Hence, the degradation of butyrate or propionate does not proceed unless the hydrogen partial pressure can be reduced by the hydrogenotrophic methanogens. The conversion of CO_2 and hydrogen to methane acetate is hydrogen consuming (-130.75 kJ/mol). Figures 4.37 and 4.38 shows the effect of hydrogen partial pressure on the free energy of butyrate degradation and propionate degradation reactions.

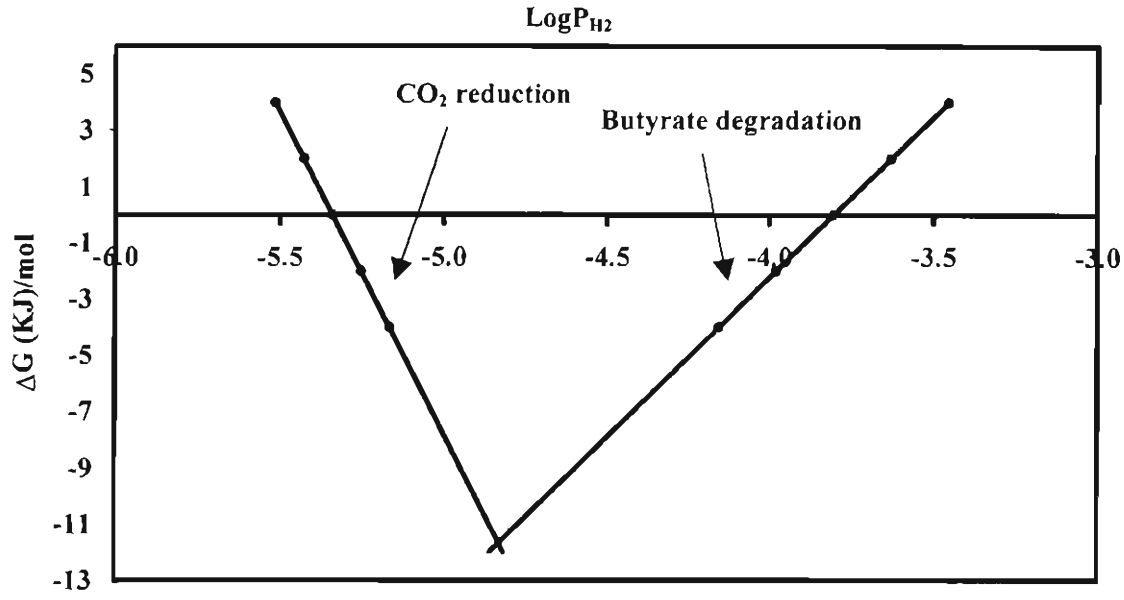


Figure 4.37: The effect of hydrogen partial pressure on the free energy of butyrate degradation reaction.

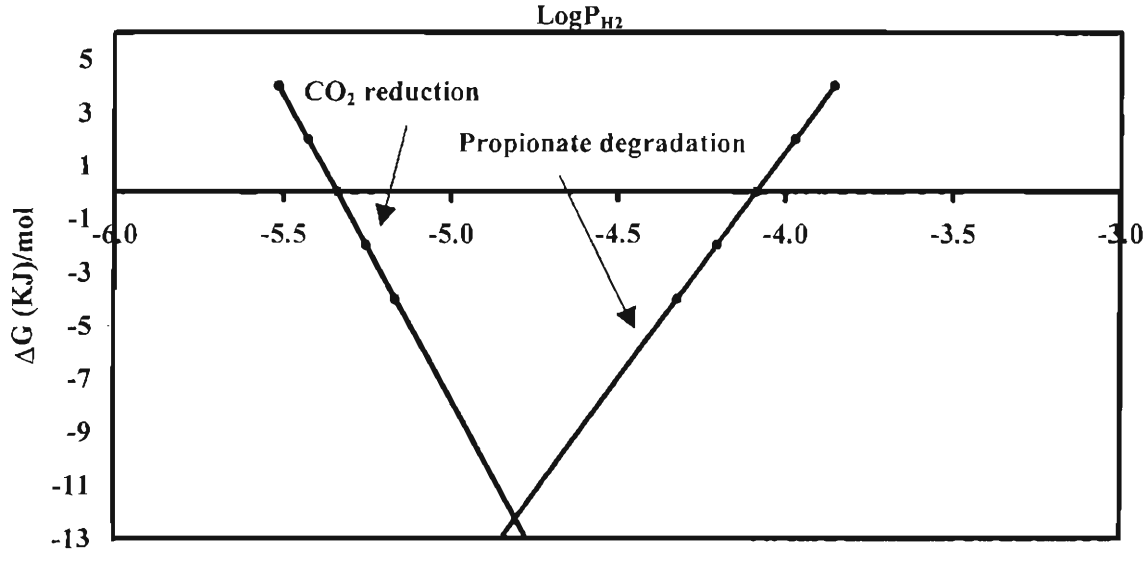


Figure 4.38: The effect of hydrogen partial pressure on the free energy of propionate degradation reaction.

According to the relationships in Figures 4.25 and 4.26, butyrate degradation is favorable when hydrogen partial pressure is less than 15.4×10^{-5} atm and propionate degradation is favored when hydrogen partial pressure is less than 8.3×10^{-5} atm.

4.2.4 Methane Production

The addition of LA, OA, and SA to the cultures inhibited methanogenesis. Between the three LCFAs tested, LA was the most inhibitory to methane production. At 50 or 100 mg/L LA inhibition was minimal but not permanent. In addition, OA was slightly inhibitory at these concentrations and the cultures adapted much faster than those receiving LA. Methane production in the cultures receiving SA indicates that this LCFA is not inhibitory at the concentrations examined. Moreover, methanogenic bacteria were severely affected when concentrations equal or greater than 500 mg/L LA were added. However, no further decrease in methane production in the cultures receiving concentrations greater than 500 mg/L LA was observed. The results suggest that a threshold condition may have been reached for LA inhibition. In the cultures receiving concentrations higher than 500 mg/L OA, lag-phases were observed for up to 4 days indicating that initially OA inhibited the culture.

These observations agree with research reported by Lalman and Bagley (2000) who reported that inhibition was not permanent for cultures receiving greater than 30 mg/L LA. In comparison to control cultures, less methane was produced in cultures receiving greater than 300 mg/L LA. Lalman and Bagley (2001) also showed that no inhibition occurred until more than 100 mg/L OA was added and 100 mg/L SA was not inhibitory. In addition, Koster and Cramer (1987) observed threshold below which aceticlastic methanogenic activity was not affected by the presence of LCFAs. These observations are also supported in this research. Moreover, Koster and Cramer (1987) showed a mixture of lauric and myristic acids was more inhibitory than individual LCFAs. In contrast to the findings of Angelidaki and Ahring (1992), the inhibition effects

observed for OA and SA were significantly less than those reported by Angelidaki and Ahring (1992). They reported that the addition of 300 mg/L oleate cause a prolonged lag-phase and methane production was inhibited.

5.0 Inhibition Kinetics Of Linoleic ($C_{18:2}$), Oleic ($C_{18:1}$), And Stearic ($C_{18:0}$) Acid On Glucose Degradation

5.1 Experimental Results

5.1.1 Glucose Degradation.

Glucose degradation profiles are shown in Figures 5.1 to 5.4 for controls and cultures receiving mixed LCFAs. Undetectable levels of glucose were reached within 200 minutes in all control cultures. In the presence of 50 or 100 mg/L mixtures of LCFAs, the degradation of glucose was accomplished within 200 to 240 minutes. The addition of 300, 500, 700, or 1000 mg/L mixtures of LCFAs caused an increase in the removal time and little or no degradations were observed after approximately 240 minutes.

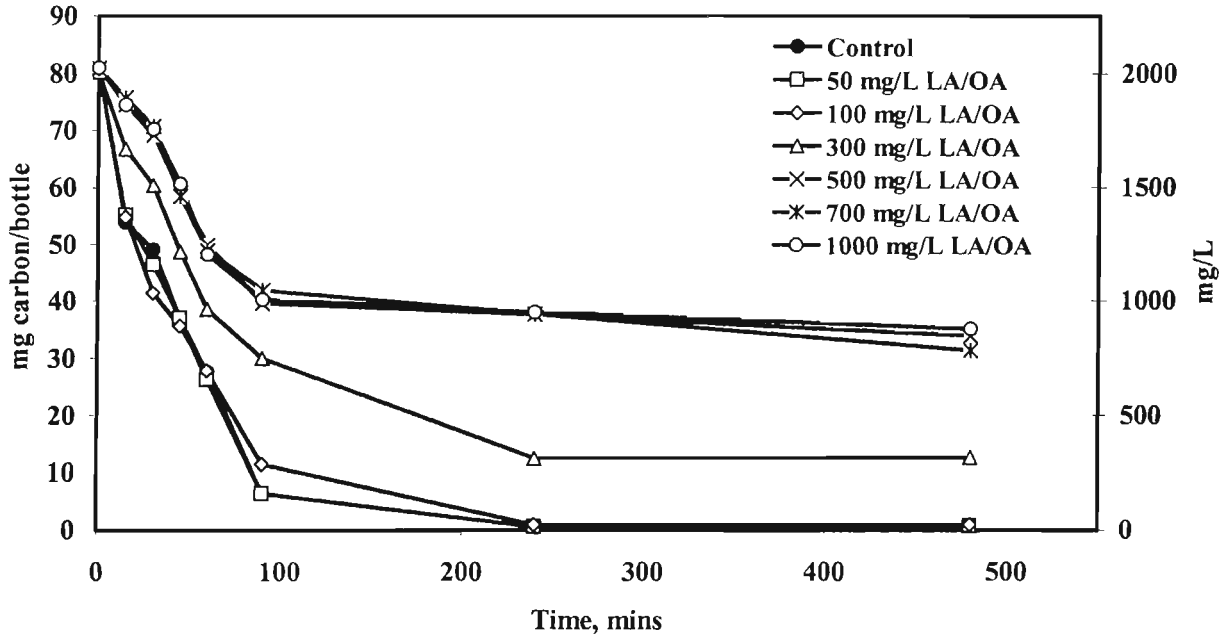


Figure 5.1: Glucose degradation profile for cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, average for triplicate samples are shown)

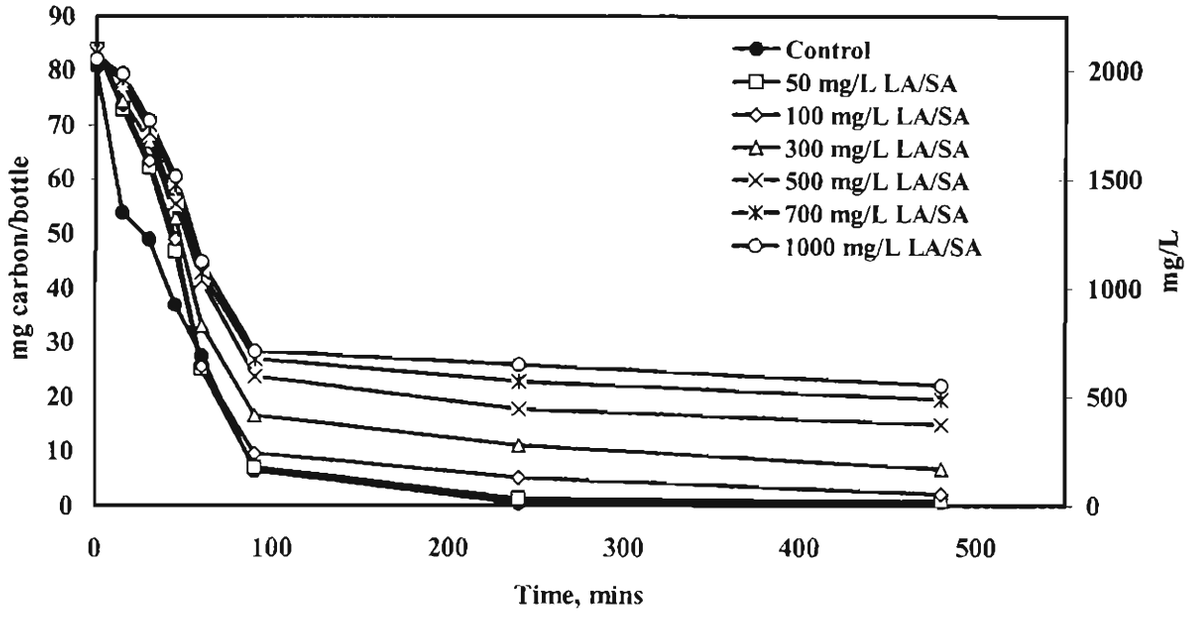


Figure 5.2: Glucose degradation profile for cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are shown)

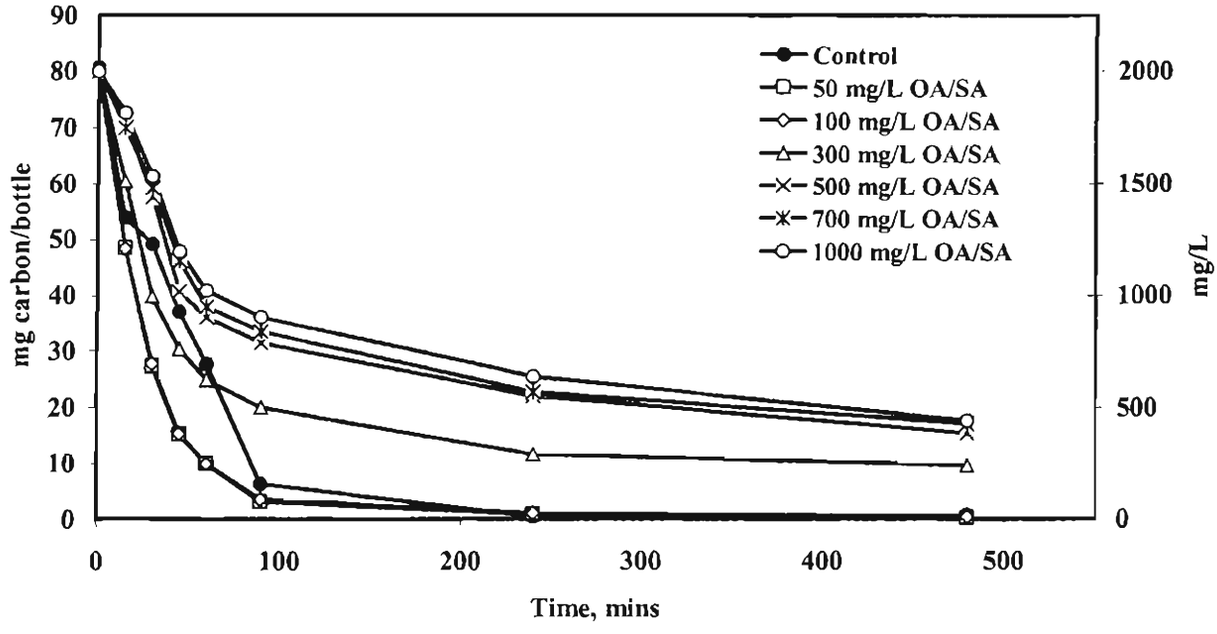


Figure 5.3: Glucose degradation profile for cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are shown)

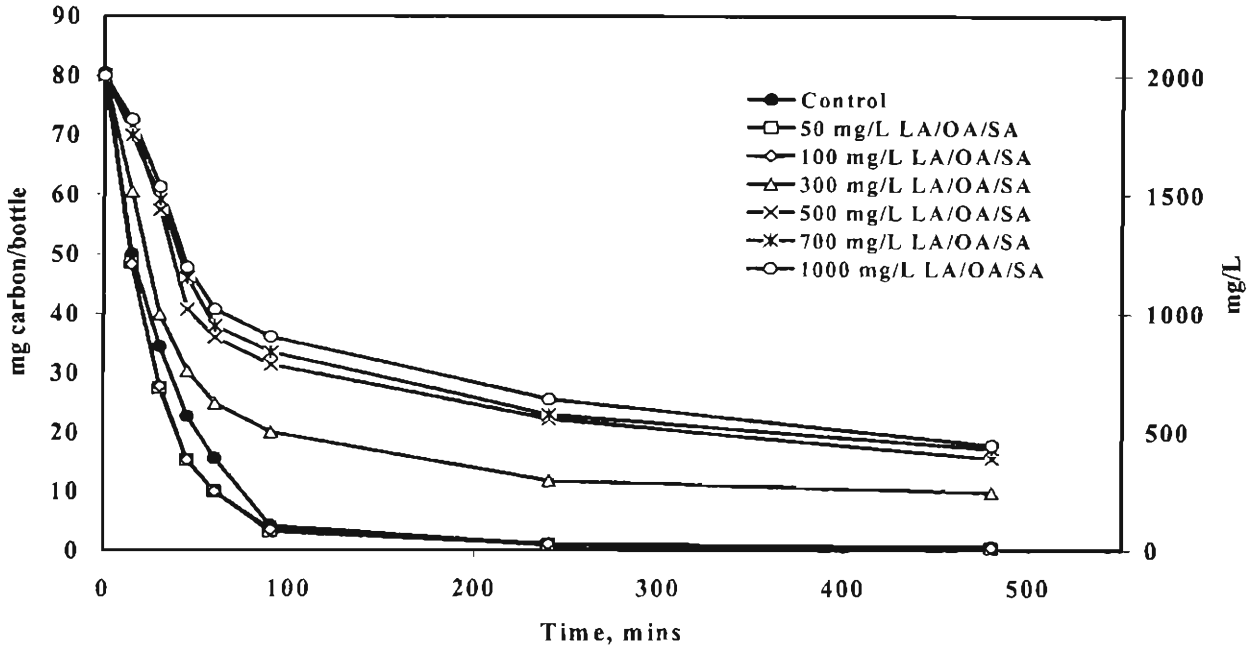


Figure 5.4: Glucose degradation profile for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose . (LA = linoleic acid, OA = oleic acid, SA = stearic acid average for triplicate samples are shown)

The maximum reaction velocity $V_{max,}$ (Table 5.1) and the binding affinity for both the substrate ($K_{M\ app}$, Table 5.2) and the inhibitor equilibrium constant (K_I, Table 5.3) were calculated from glucose degradation profiles using the Eadie-Hofstee plot. The kinetics constants for the individual LCFAs were calculated using glucose degradation data (2000 mg/L glucose) which was presented in chapter 4. The values denoted as bold font are those where the error was greater than 10%. Statistical comparison was conducted using the Tukey's paired comparison procedure (Steel *et al.* 1997).

Statistical analysis of V_{max} values for each LCFA condition examined showed no statistical difference between the control and the culture receiving invidual or mixed LCFAs. Also, there was no statistical difference in $K_{M\ app}$ values between the controls and the cultures receiving 50 or 100 mg/L individual or mixtures of LCFAs. However, the addition of greater than 300 mg/L caused a significant difference in $K_{M\ app}$ values of up to

500% compared to the controls. Statistical differences in $K_{M\ app}$ values were also observed for cultures receiving 300, 500, 700, or 1000 mg/L LCFA.

No statistical differences in K_I values were observed for cultures receiving equal or less than 100 mg/L individual or LCFA mixtures. However, there were significant statistical differences between cultures receiving less than 100 mg/L LCFA and those receiving greater than 300 mg/L individual or mixtures of LCFAs. Also, differences were observed between cultures receiving 300 mg/L and those receiving greater than 500 mg/L LCFA. No differences were observed for cultures receiving greater than 500 mg/L.

Table 5.1: Glucose maximum reaction velocity (V_{max}) for cultures receiving varying amounts of individual and mixed LCFAs.

LCFA	Control	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
LA	31.17±1.32	31.21±1.52	31.06±1.09	32.36±1.79	30.51±1.33	**32.73±4.35**	30.96±1.7
OA	32.95±1.62	32.97±2.19	32.46±1.73	31.75±1.5	32.23±2.27	32.84±2.13	31.23±2.02
SA	30.67±1.4	31.35±1.62	30.14±2.11	**30.31±3.72**	29.32±2.73	29.89±2.94	31.48±2.13
LA/OA	30.91±2.15	31.4±1.46	**30.39±3.67**	30.45±2.43	30.96±2.35	31.7±1.89	30.91±2.22
LA/SA	31.41±1.16	32.17±1.62	32.32±2.4	31.44±2.55	31.12±2.41	31.77±2.03	**30.55±4.96**
OA/SA	32.78±2.58	32.76±2.03	33.61±1.58	33.31±2.60	32.35±2.18	32.66±1.58	33.02±3.14
LA/OA/SA	31.05±1.4	31.81±1.62	31.06±2.11	**30.66±4.41**	30.00±1.73	30.49±3.01	**30.97±3.13**

Average and standard deviation for triplicate samples are shown. Control = 0 mg/L LCFA.

Table 5.2: Glucose binding affinity constant ($K_{M\ app}$) for cultures receiving varying amounts of individual and mixed LCFAs.

LCFA	Control	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
LA	496±32[a]	511±29[a]	491±28[a]	1136±95[b]	1786±117[c]	1791±114[c]	1811±140[c]
OA	475±36[a]	450±32[a]	467±31[a]	**973±108**[b]	1596±108[c]	1618±110[c]	1672±102[c]
SA	481±28[a]	490±30[a]	472±28[a]	992±89[b]	1532±122[c]	**1605±313**[c]	1636±128[c]
LA/OA	483±32[a]	519±40[a]	**503±86**[a]	946±80[b]	1609±84[c]	1590±158[c]	1608±197[c]
LA/SA	508±31[a]	513±33[a]	471±25[a]	991±92[b]	**1585±300**[c]	1601±110[c]	1604±145[c]
OA/SA	502±33[a]	456±29[a]	496±35[a]	**1068±147**[b]	1582±98[c]	1592±143[c]	1572±141[c]
LA/OA/SA	480±29[a]	432±30[a]	487±31[a]	1215±90[b]	1979±102[c]	1912±103[c]	**2001±361**[c]

All values are averages for triplicate samples. Control = 0 mg/L LCFA. a, b, And c, = means followed by the same letter are not statistically different within rows.

Table 5.3: LCFAs binding affinity constant (K_I) for cultures receiving varying amounts of individual and mixed LCFAs.

LCFA	50 mg/L	100 mg/L	300 mg/L	500 mg/L	700 mg/L	1000 mg/L
LA	1653±1366	-9920±2049	377±17[a]	268±15[b]	222±19[c]	192±14[c]
OA	-950±27	-5938±16	397±39[a]	291±16[b]	286±25[c]	**212±27**[c]
SA	2672±101	-5344±1205	416±21[a]	300±23[b]	**282±88**[c]	229±20[c]
LA/OA	671±21	2415±523	429±30[a]	305±32[b]	313±20[c]	**214±24**[c]
LA/SA	5080±56	-1373±55	464±28[a]	**325±62**[b]	316±20[c]	**236±27**[c]
OA/SA	-546±141	-8367±122	**469±90**[a]	322±22[b]	266±25[c]	**232±33**[c]
LA/OA/SA	-500±114	6857±501	316±14[a]	235±20[b]	**196±20**[c]	**160±77**[c]

All values are averages for triplicate samples. a, b, and c, = means followed by the same letter are not statistically different within rows.

No statistical differences in $K_{M\ app}$ values were observed for cultures receiving 300 mg/L of OA or SA, or mixtures of LA/OA, LA/SA, or OA/SA. However, a higher $K_{M\ app}$ value was observed for cultures receiving 300 mg/L LA. Also $K_{M\ app}$ for cultures

receiving 300 mg/L LA/OA/SA (1:1:1) was statistically different than $K_{M\ app}$ for cultures receiving 300 mg/L LA. Similar trends were observed for cultures receiving 500, 700, and 1,000 mg/L LCFA.

In cultures receiving 300 mg/L OA, SA, or mixtures of LA/OA, LA/SA, or OA/SA, there were no statistical differences in K_I values. For cultures fed with 500, 700, and 1,000 mg/L similar trends were observed. However, in cultures receiving 300 mg/L of LA, the K_I value was lower than those receiving 300 mg/L OA, SA, or mixtures of LA/OA, LA/SA, or OA/SA. Also in cultures receiving 300 mg/L of LA/OA/SA (1:1:1), the K_I was lower than cultures receiving 300 mg/L LA. For cultures fed with 500, 700, and 1,000 mg/L similar trends were observed.

5.1.2 VFAs Production

Butyric acid concentrations profiles for the culture receiving LA/OA are shown in Figure 5.5. Butyric acid was not observed in any other condition examined. The maximum concentration of butyric acid was achieved within a day and complete removal was achieved within 8 days in all cultures.

The effects of linoleic, oleic, and stearic acids (individuals and mixtures) on the production/removal of propionate are shown in Figures 5.6 to 5.9. In all conditions examined, propionate accumulation was observed. In control cultures, the maximum propionate concentration was achieved within a day and complete removal was achieved within 20 days. In cultures receiving 50 or 100 mg/L of LCFAs mixtures, complete removal was achieved within 20 days.

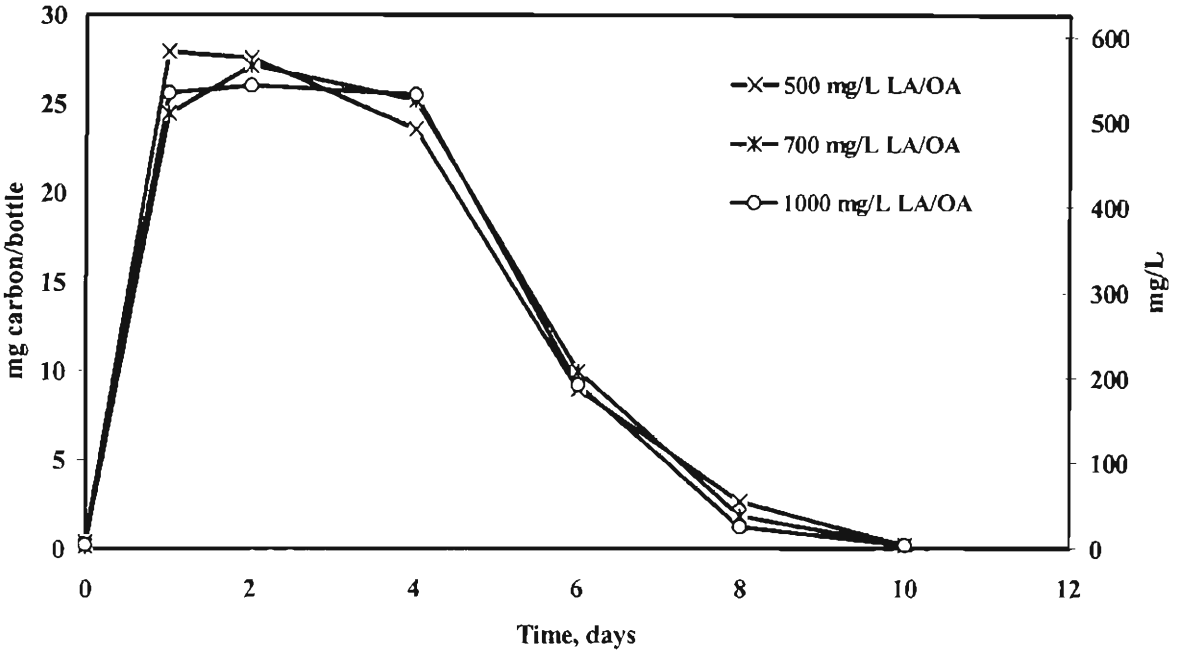


Figure 5.5: Butyrate production in cultures receiving mixtures of linoleic and oleic acids and 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, average for triplicate samples are shown)

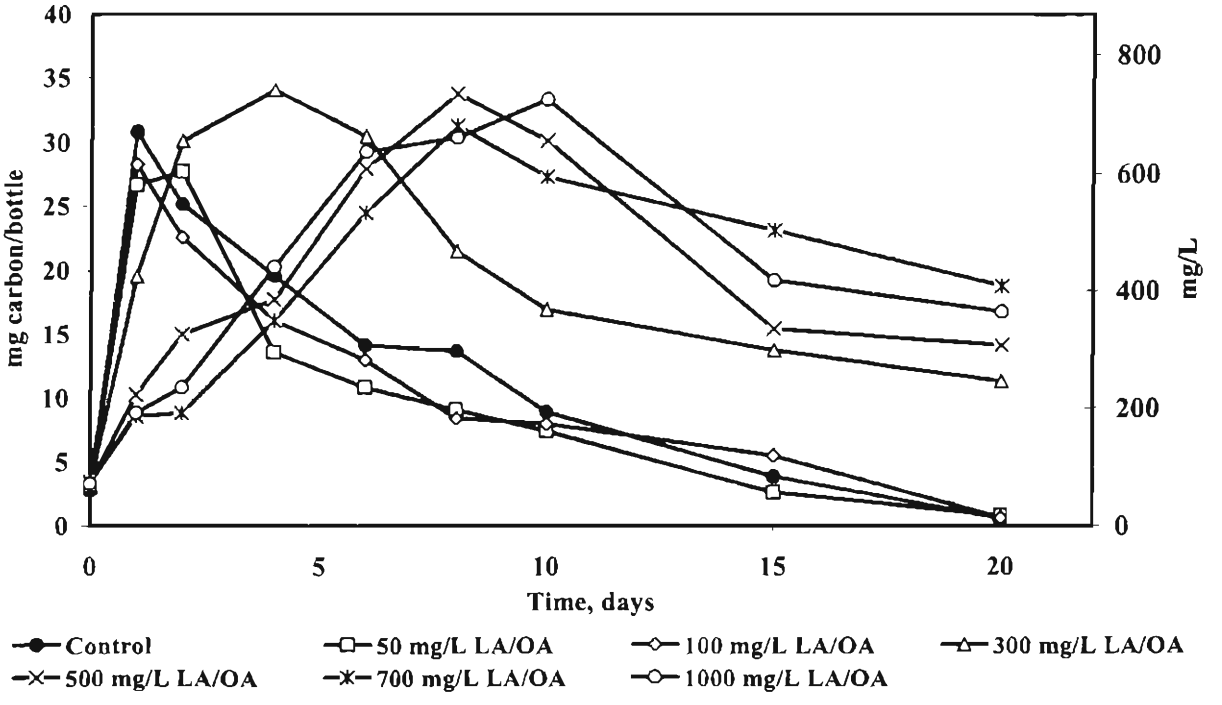


Figure 5.6: Propionate production in cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, average for triplicate samples are shown)

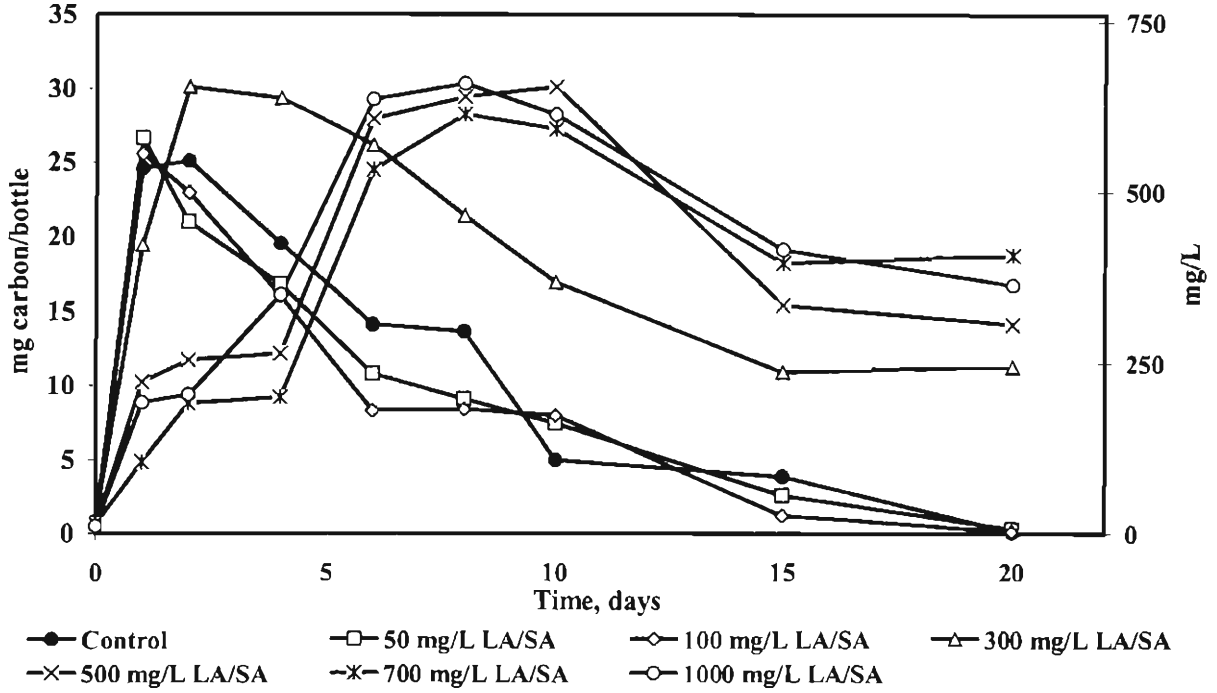


Figure 5.7: Propionate production in cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are shown)

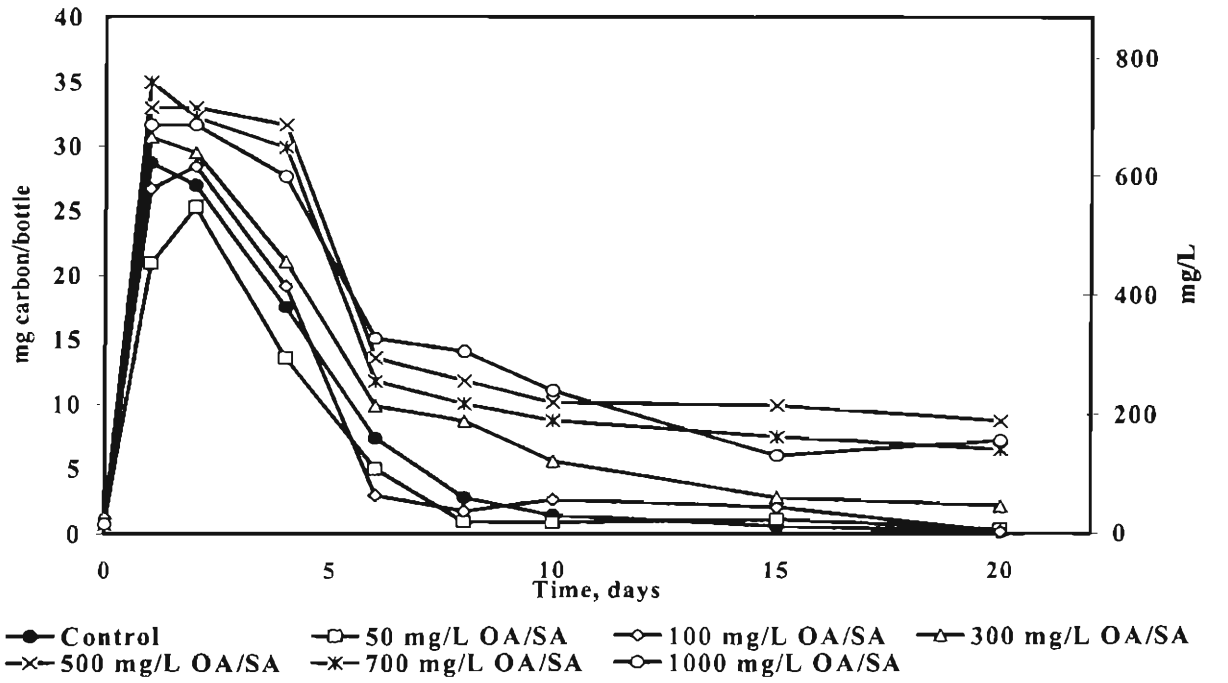


Figure 5.8: Propionate production in cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are shown)

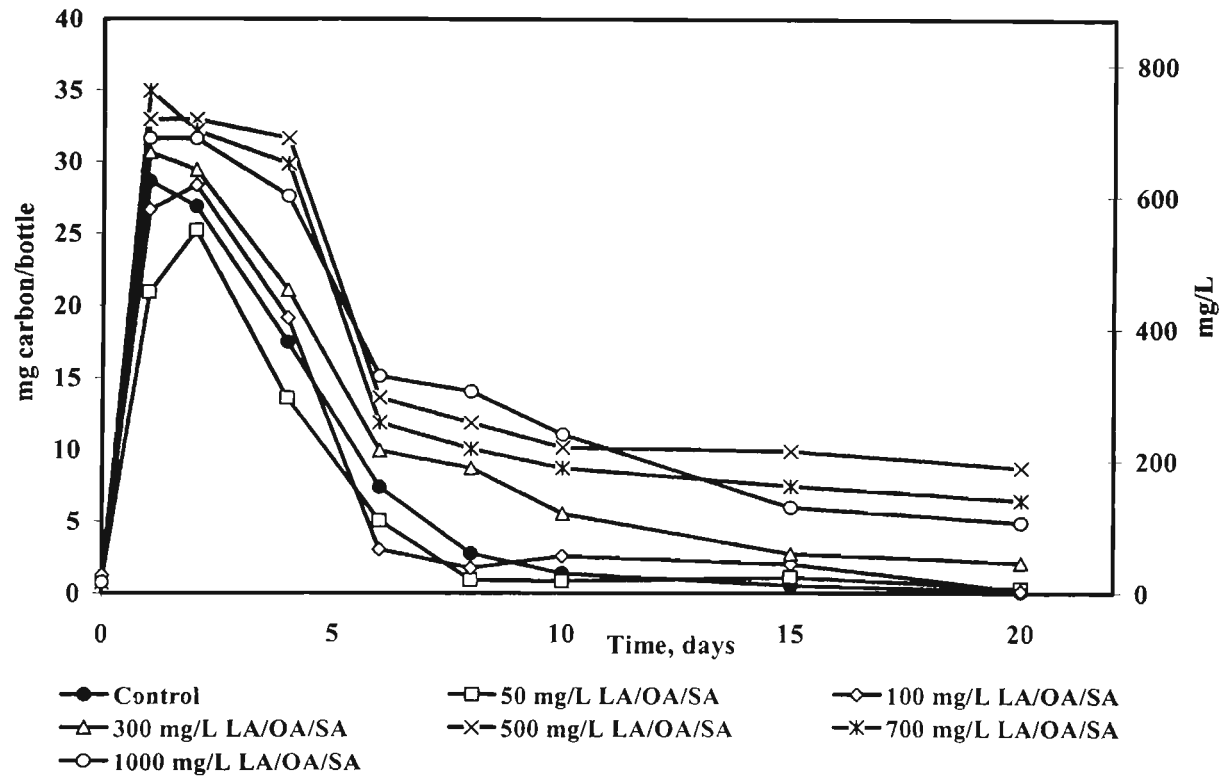


Figure 5.9: Propionate production in cultures receiving mixtures of linoleic, oleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are shown)

In cultures receiving 300 mg/L individuals or mixtures of LCFAs, the maximum propionate concentration was achieved within 4 days in all cases. However, the removal times were variable. In cultures receiving 500, 700, or 1000 mg/L of LCFA mixtures, the maximum propionate concentration was achieved in 6 to 8 days and propionate removal was significantly slower in comparison to control cultures.

Figures 5.10 to 5.13 show the production/removal of acetate in cultures receiving LCFA mixtures. In all cases, acetate accumulation was observed. In control cultures, the maximum concentration of acetate was achieved within a day and complete removal was achieved within 20 days. Similar removal times were observed in cultures receiving 50 or 100 mg/L of individual or mixtures of LCFAs.

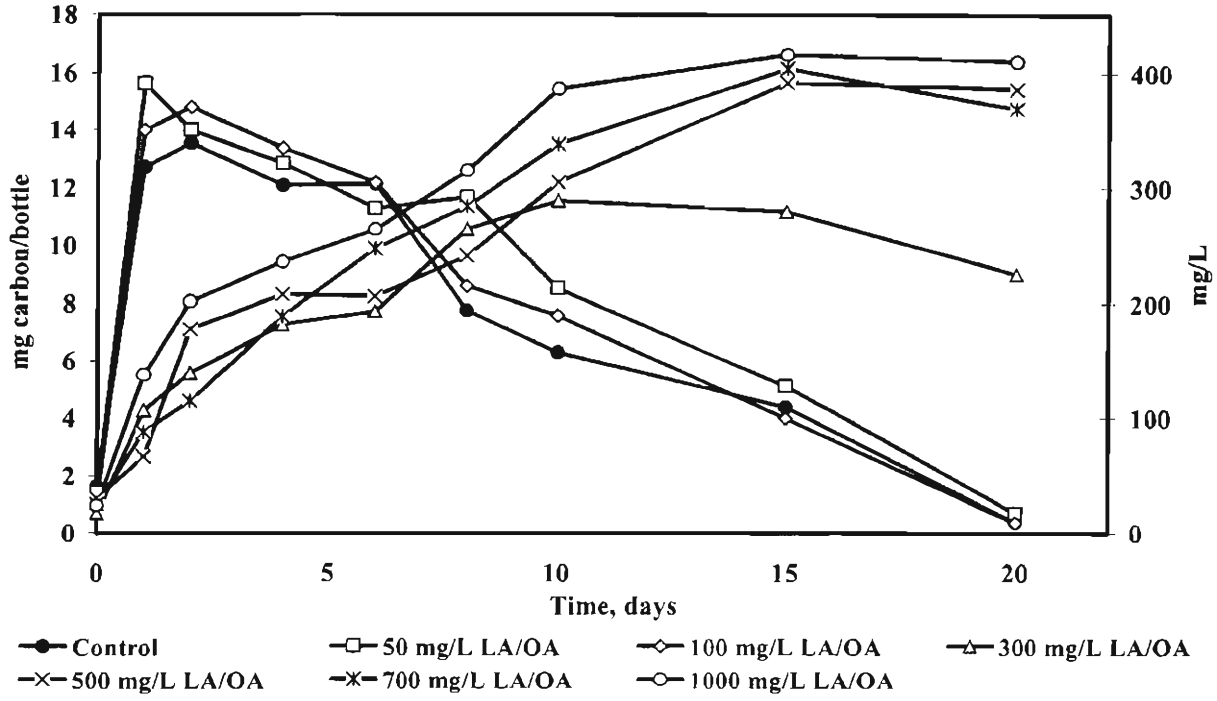


Figure 5.10: Acetate production in cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA – linoleic acid, OA = oleic acid, average for triplicate samples are shown)

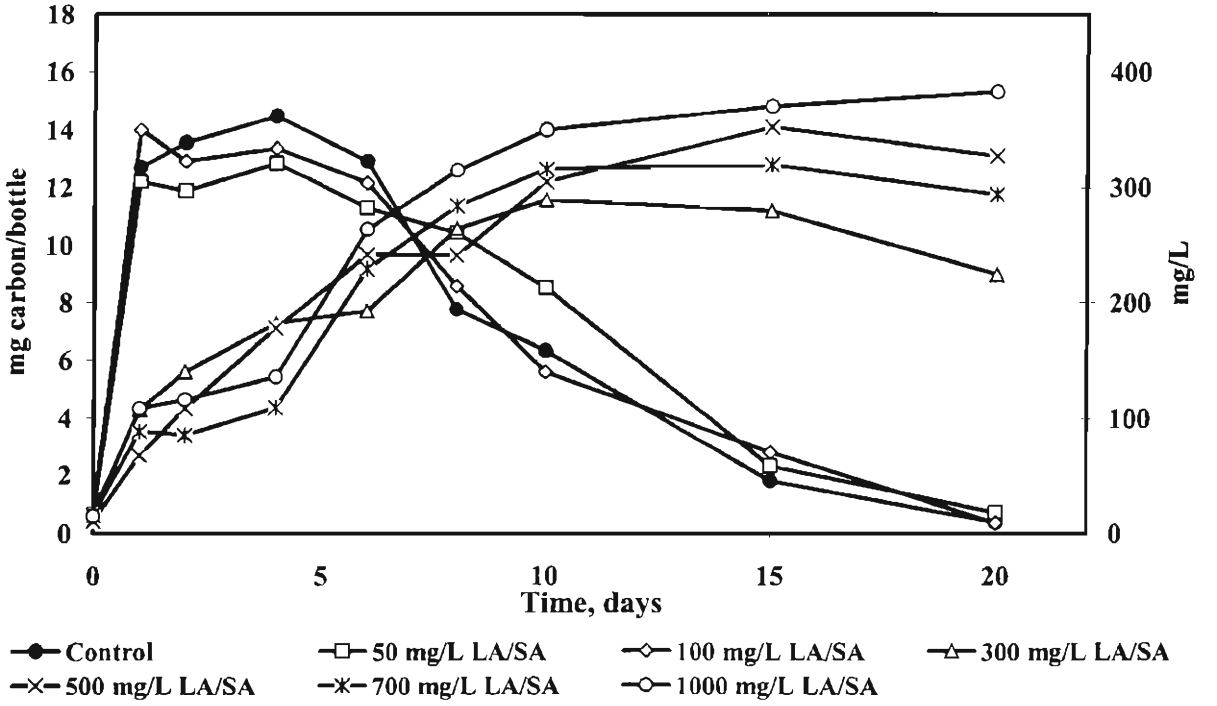


Figure 5.11: Acetate production in cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are shown)

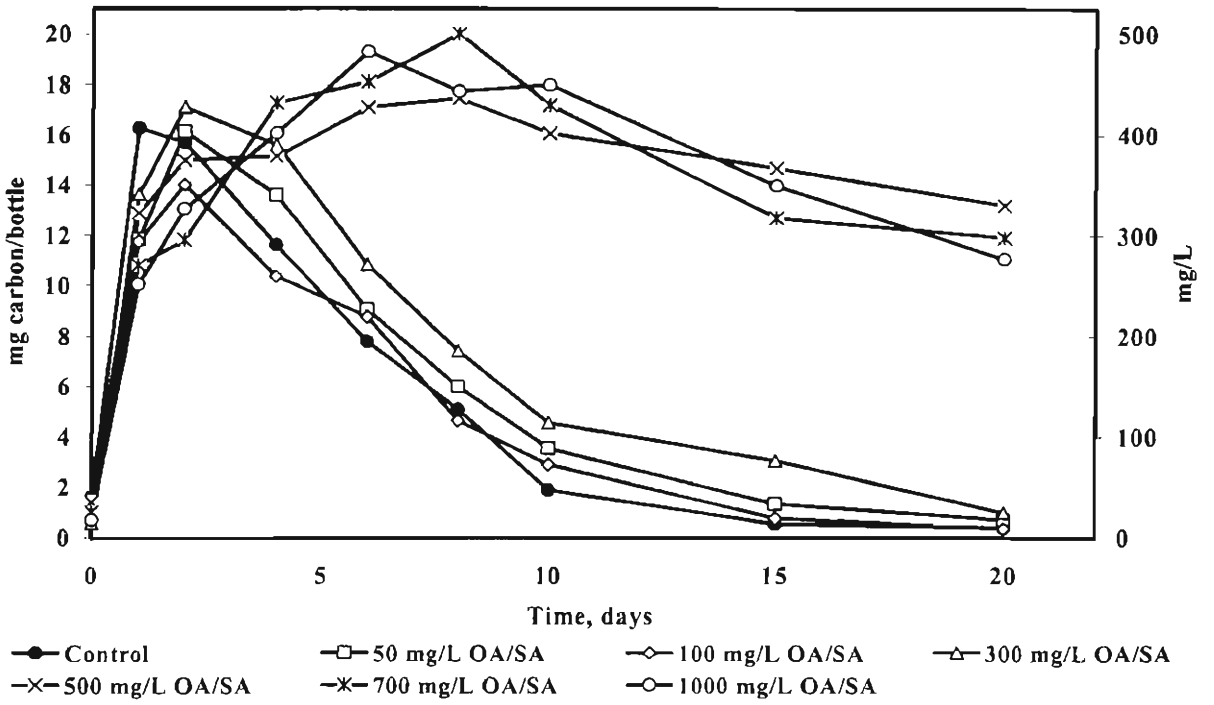


Figure 5.12: Acetate production in cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA – oleic acid, SA = stearic acid, average for triplicate samples are shown)

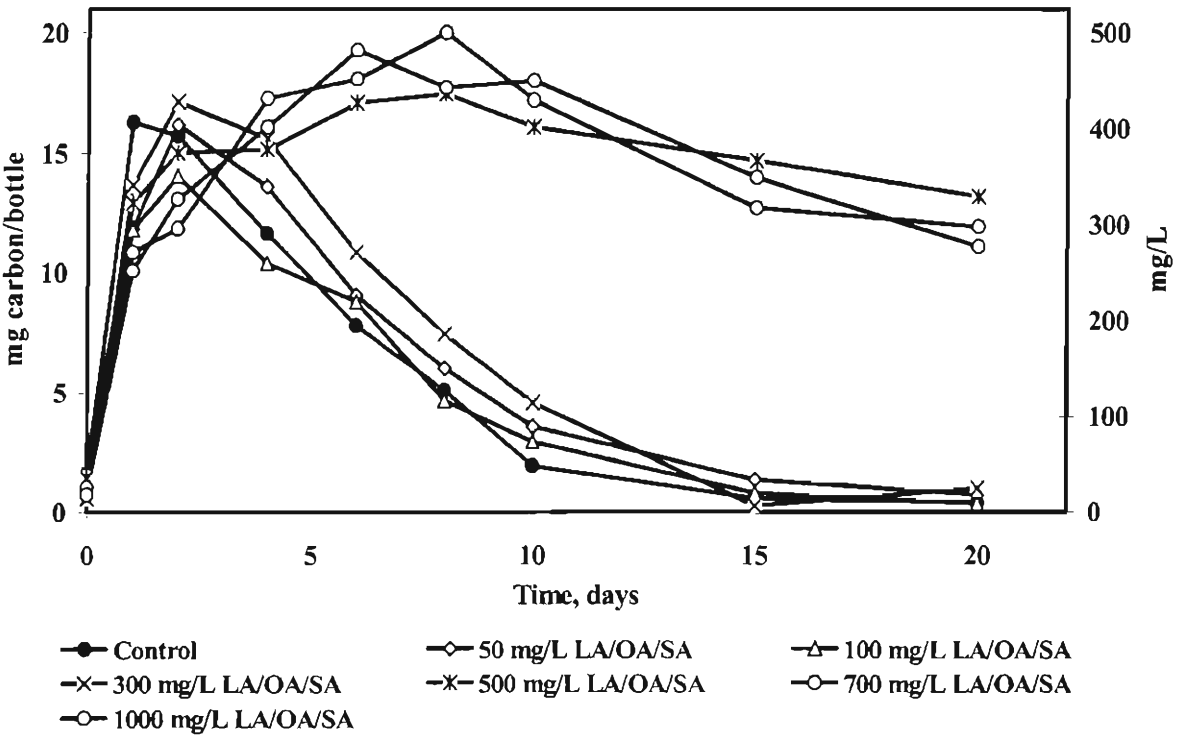


Figure 5.13: Acetate production in cultures receiving mixtures of linoleic, oleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid)

In the cultures receiving 300 mg/L of LA/OA or LA/SA less acetate removal was observed than those receiving the same concentration of OA/SA, or LA/OA/SA. In the cultures receiving 500, 700, or 1000 mg/L of LCFAs mixtures, the maximum acetate concentration was achieved within 6 to 10 days and little or no removal was observed after 10 days.

5.1.3 Methane Production

Methane productions profiles are shown in Figures 5.14 to 5.17. In the control cultures, complete conversion of glucose was achieved within 20 days. However, in the cultures receiving 50 or 100 mg/L LCFA mixtures, the total methane production was equal or greater than the control cultures. In contrast, in the cultures receiving 300 mg/L mixtures of linoleic and oleic acids or mixtures of linoleic and stearic acids, methane production was significantly lower than the controls. In the cultures receiving 300 mg/L mixtures of oleic and stearic acids, the total methane production was slightly less (OA/SA, Figure 5.16) than control cultures. In contrast, in cultures receiving 300 mg/L linoleic, oleic and stearic acids (Figure 5.17) methane production was greater than control cultures. A threshold LCFA concentration inhibiting methane production is observed in Figures 5.14 and 5.15 while no threshold is observed in Figures 5.16 and 5.17. In cultures receiving concentrations greater than 500 mg/L of mixtures of LCFAs, methane production was significantly lower than that of the controls for cultures receiving mixtures of linoleic and oleic acids or linoleic and stearic acids but was slightly lower for the cultures receiving oleic and stearic acids or mixtures of linoleic, oleic, and stearic acids.

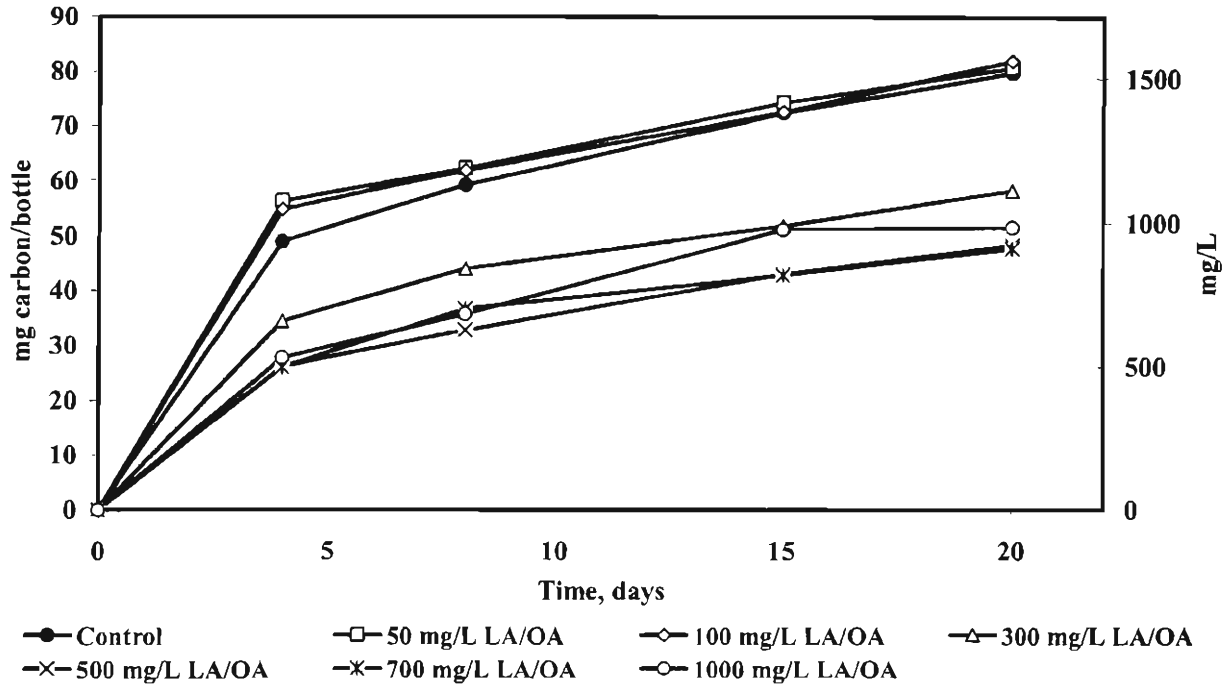


Figure 5.14: Methane production in cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid)

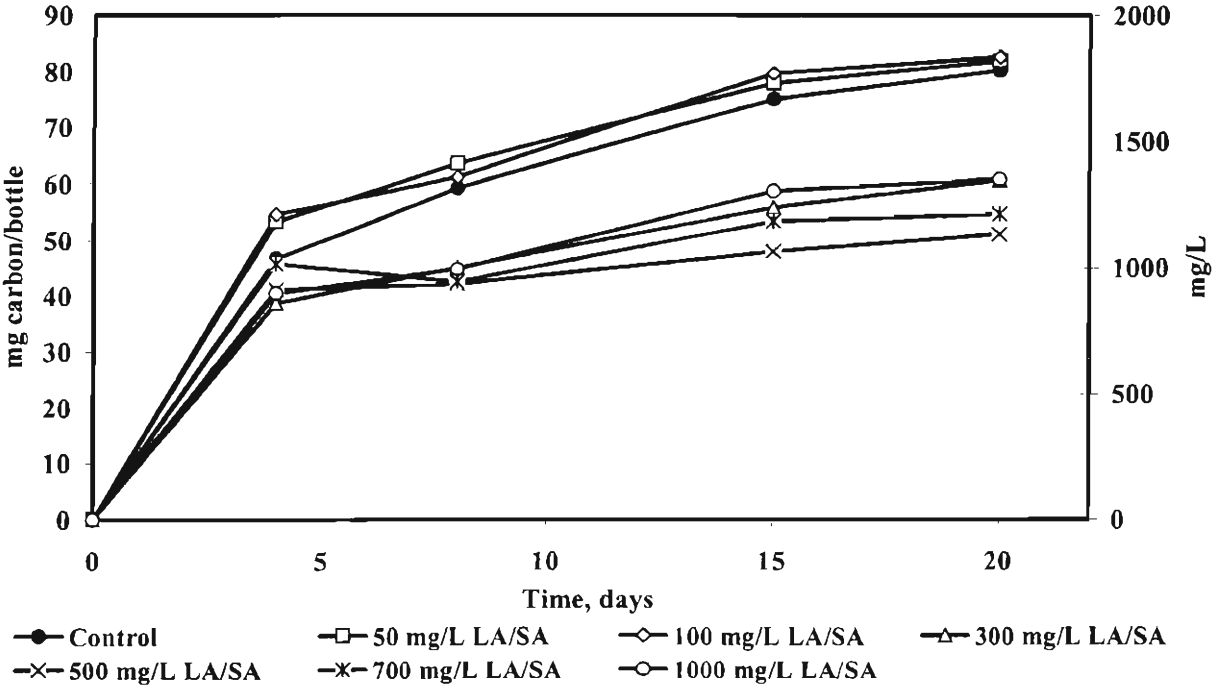


Figure 5.15: Methane production in cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid)

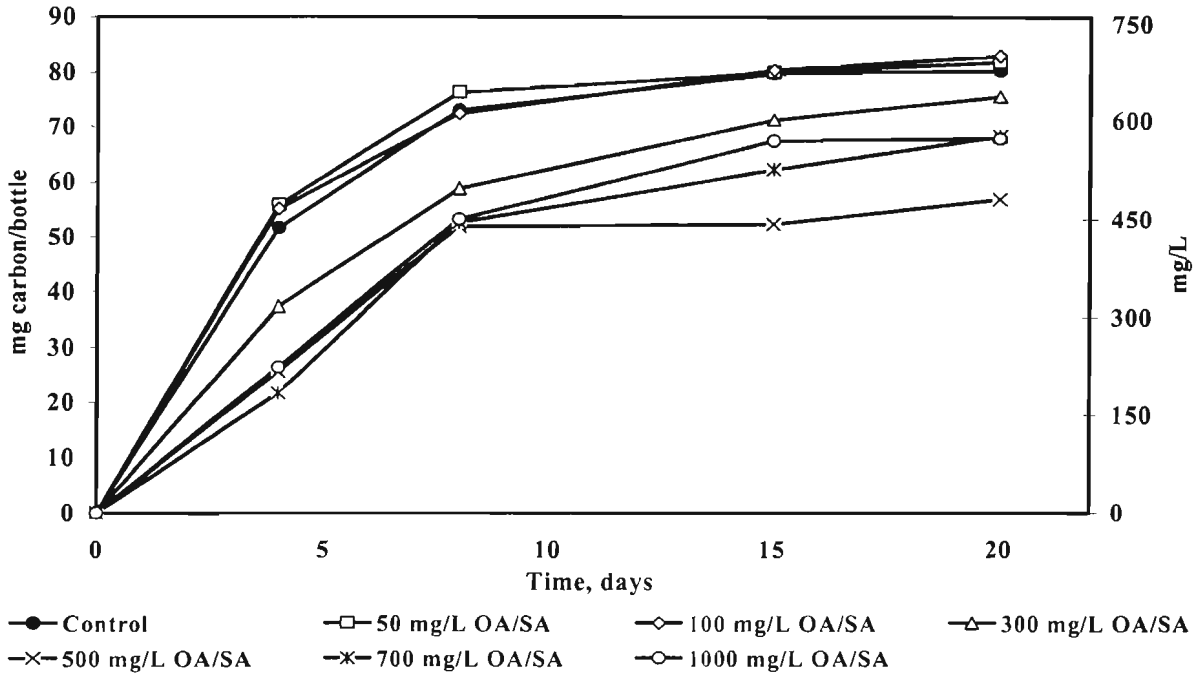


Figure 5.16: Methane production in cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid)

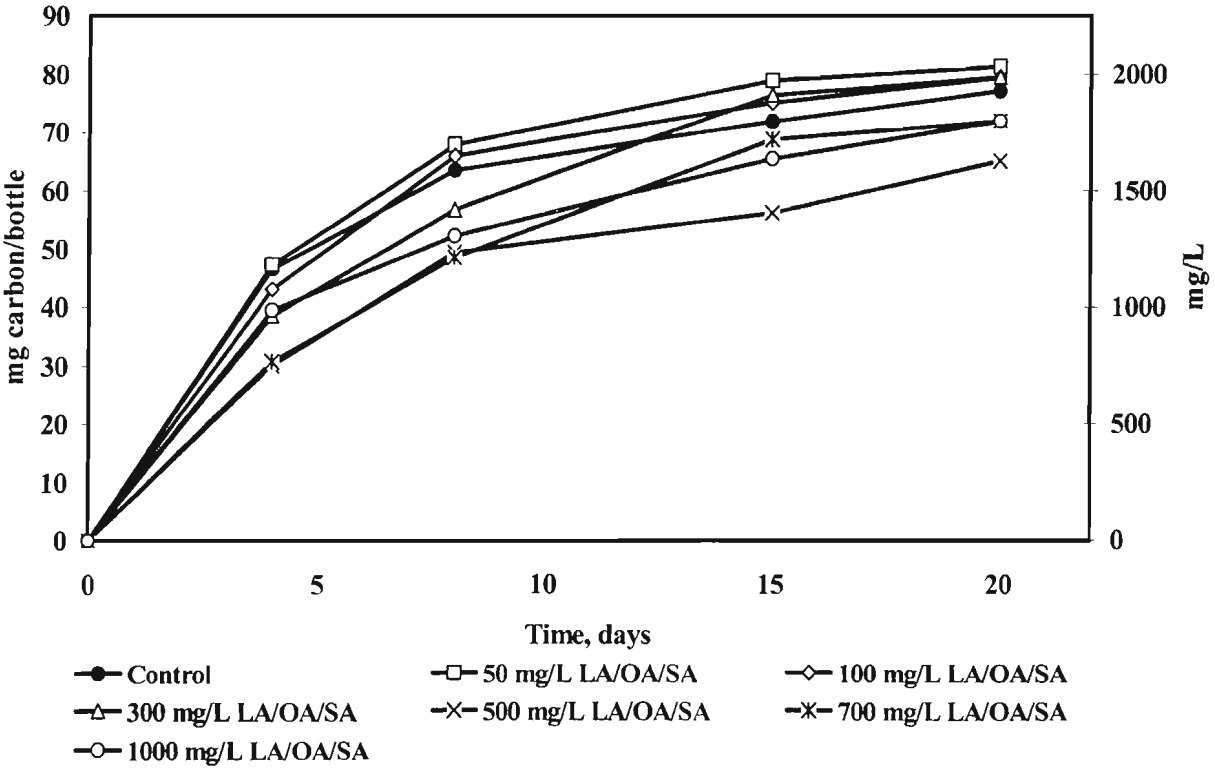


Figure 5.17: Methane production in cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid)

5.1.4 Mass Balance

Mass balances for control cultures and those receiving mixtures of LCFAs, expressed as mg carbon per bottle, are shown in Figures 5.18 to 5.21. Mass balances for control cultures and those receiving 50 or 100 mg/L mixtures of LCFAs were within approximately 10% of the theoretical amount of carbon. In cultures receiving equal or greater than 300 mg/L of mixtures of LCFAs, the error was slightly larger compared with the controls. For cultures receiving greater than 500 mg/L LCFA mixtures (except for LA/OA/SA, Figure 5.21) a larger standard deviation was observed after day 4.

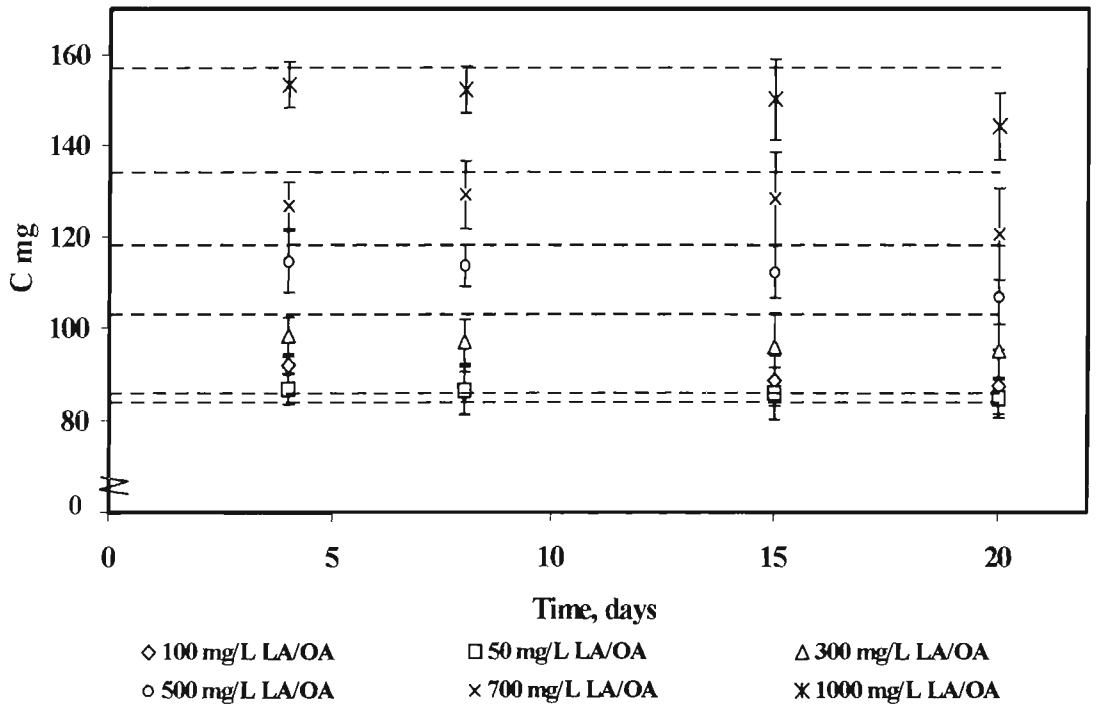


Figure 5.18: Mass balance for cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, average for triplicate samples are used, error bars are standard deviation). --------- Theoretical carbon balance.

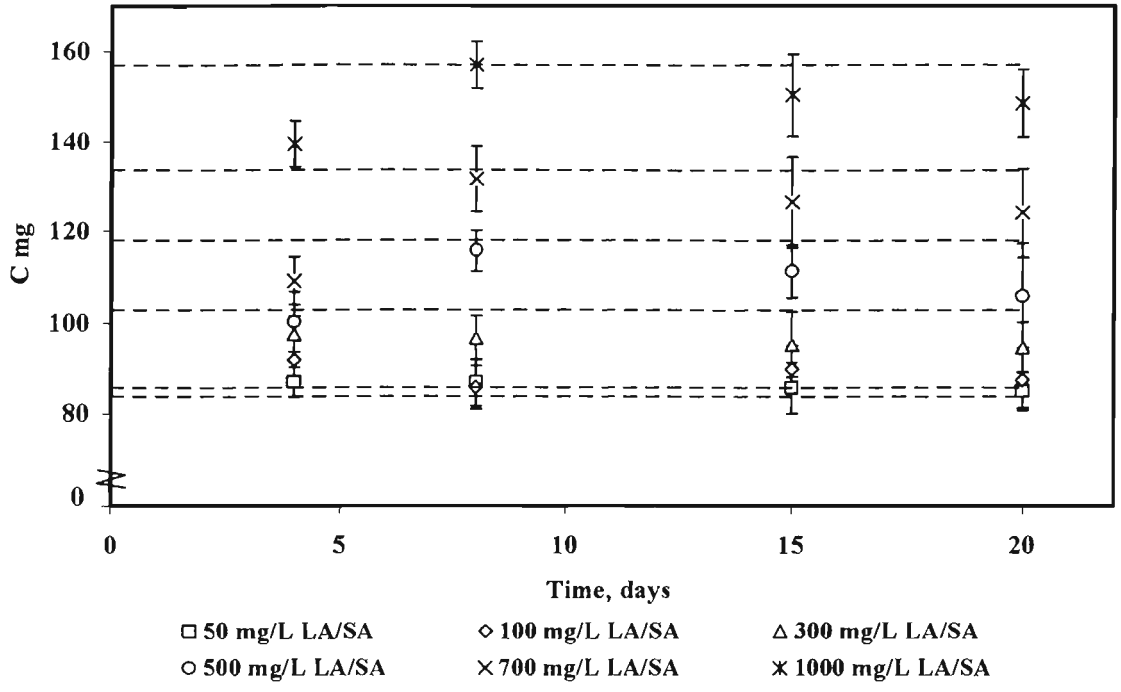


Figure 5.19: Mass balance for cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are used, error bars are standard deviation).
--------- Theoretical carbon balance.

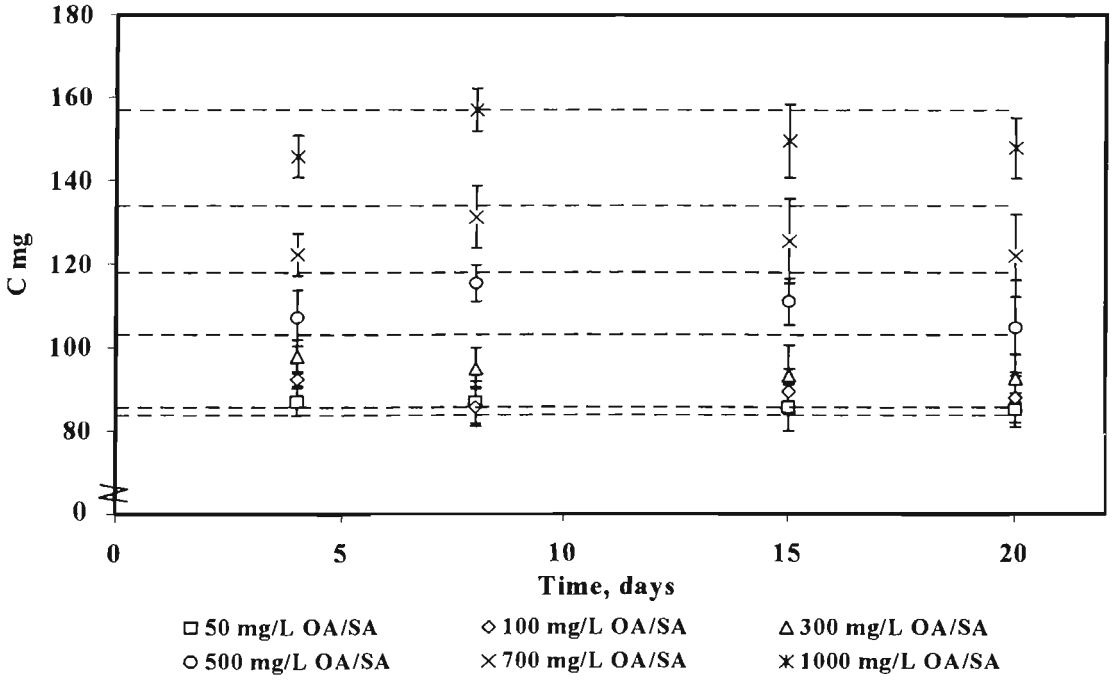


Figure 5.20: Mass balance for cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are used, error bars are standard deviation).
--------- Theoretical carbon balance.

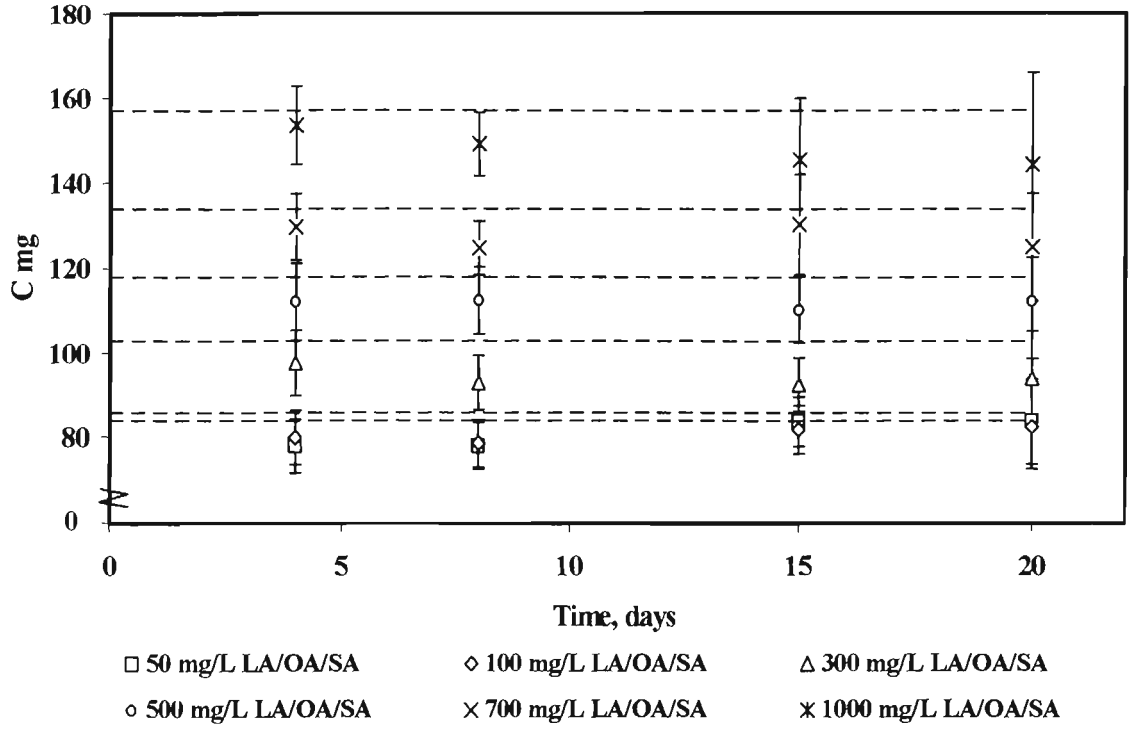


Figure 5.21: Mass balance for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are used, error bars are standard deviation). --------- Theoretical carbon balance.

5.2 Discussion Of Results

5.2.1 Glucose consumption

The addition of LCFAs mixtures did not affect the maximum reaction velocity. However, the binding affinity ($K_{M\ app}$) was affected. This observation provided evidence that the addition of the LCFAs severely affected enzymes or receptors responsible for glucose degradation or cell uptake. In addition, $K_{M\ app}$ values where the largest for cultures receiving mixtures of LA/OA/SA. A large $K_{M\ app}$ value observed for mixtures of the three LCFA support the hypothesis that the inhibitory effect of one LCFA may enhance the effect caused by the presence of another LCFA. Such synergism was not observed in cultures receiving mixtures of LA/OA, LA/SA, and OA/SA.

The lowest value of inhibitor binding affinity (K_I) was observed for cultures receiving mixtures of LA/OA/SA. This may suggest that the LA/OA/SA mixture might have caused a LCFA substrate to bind more tightly to enzyme or receptors in comparison to individual LCFAs.

In all the conditions examined, the V_{max} values were unchanged. In contrast, the $K_{M\ app}$ values increased with increasing LCFA concentration thus indicating the substrate was bound less tightly to an enzyme or receptor site. Based on Eadie-Hofstee plot for cultures receiving individual and mixtures of LCFAs (shown in Figures 5.22 to 5.28), a competitive type mechanism of inhibition is proposed. From Figures 5.22 to 5.28, the Y-intercept is V_{max}, the slope is $-K_{M\ app}$, and the X-intercept is $V_{max}/K_{M\ app}$.

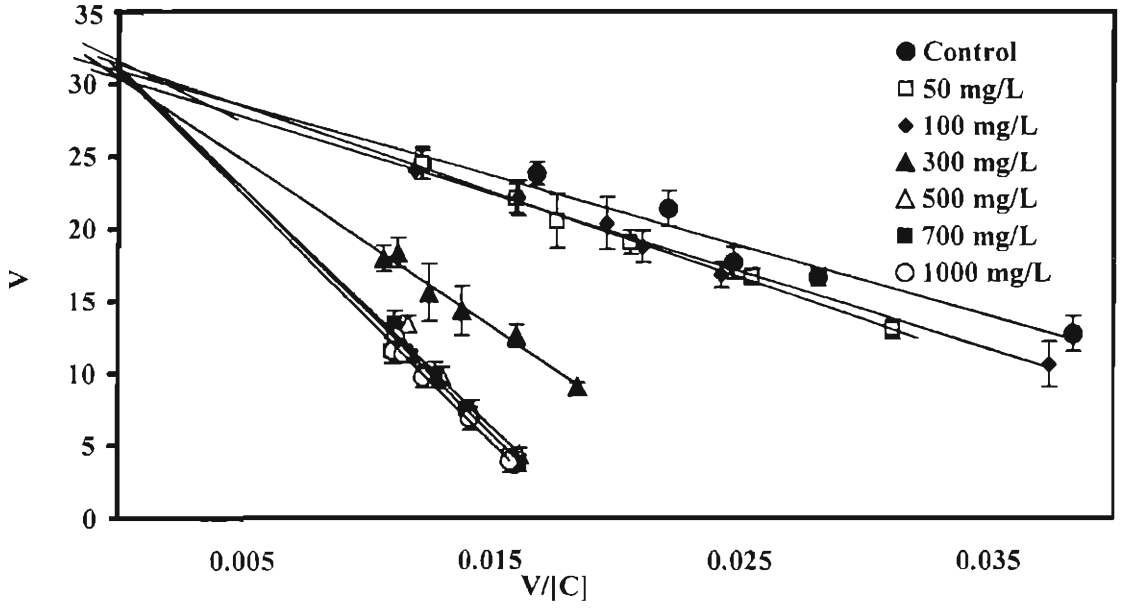


Figure 5.22: Eadie-Hofstee plot for cultures receiving mixtures of linoleic and oleic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid average for triplicate samples are used, error bars are standard deviation).

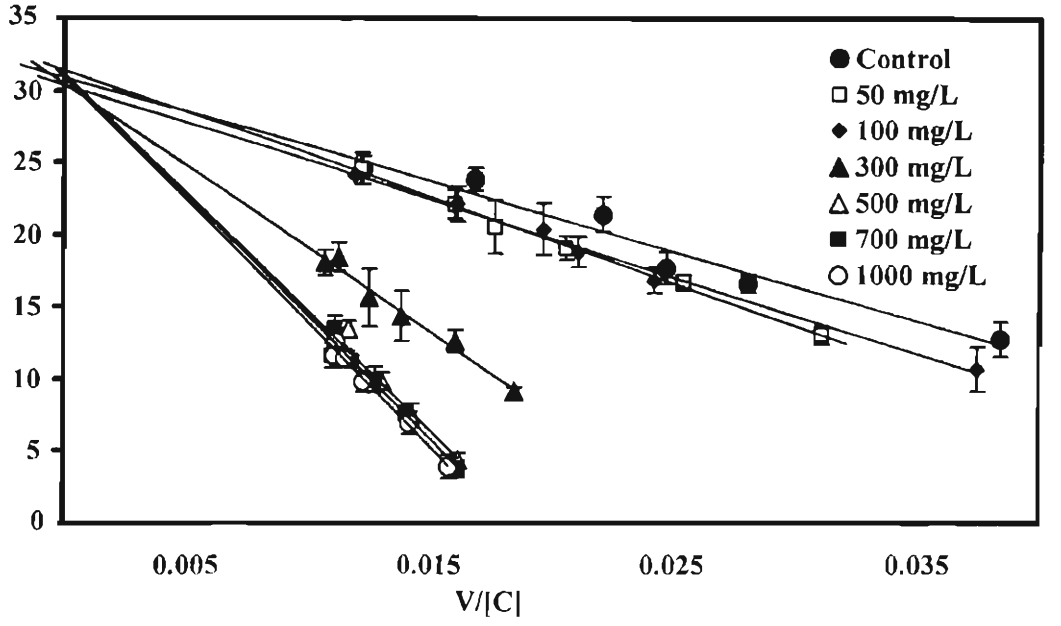


Figure 5.23 Eadie-Hofstee plot for cultures receiving linoleic acid plus 2000 mg/L glucose. (LA = linoleic acid, average for triplicate samples are used, error bars are standard deviation).

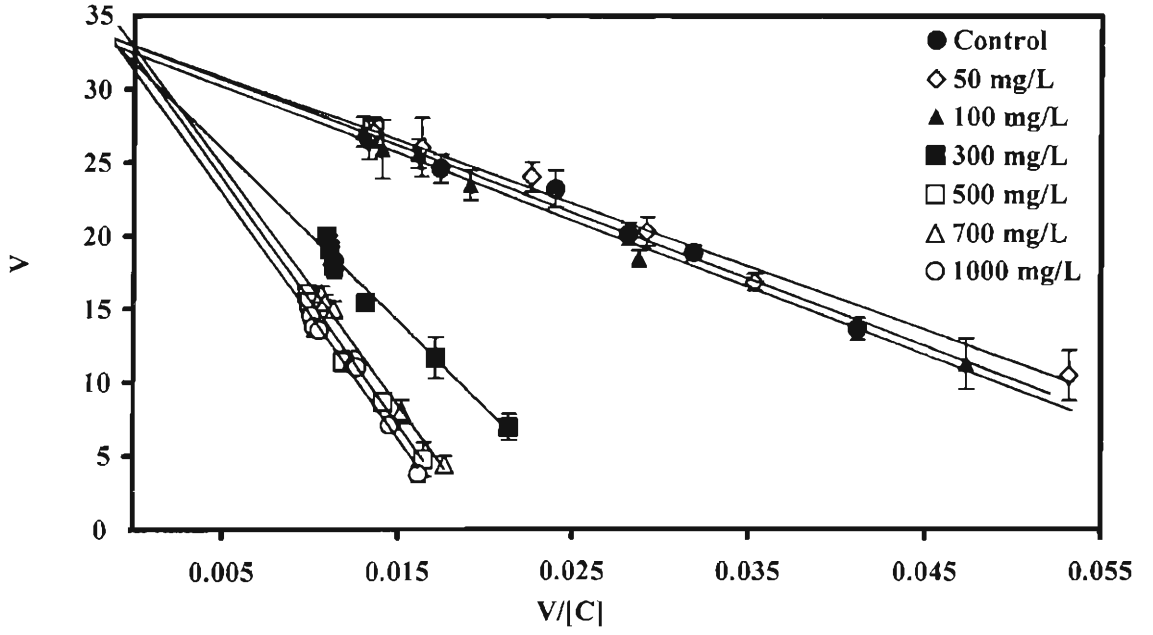


Figure 5.24 Eadie-Hofstee plot for cultures receiving oleic acid plus 2000 mg/L glucose. (OA = oleic acid, average for triplicate samples are used, error bars are standard deviation).

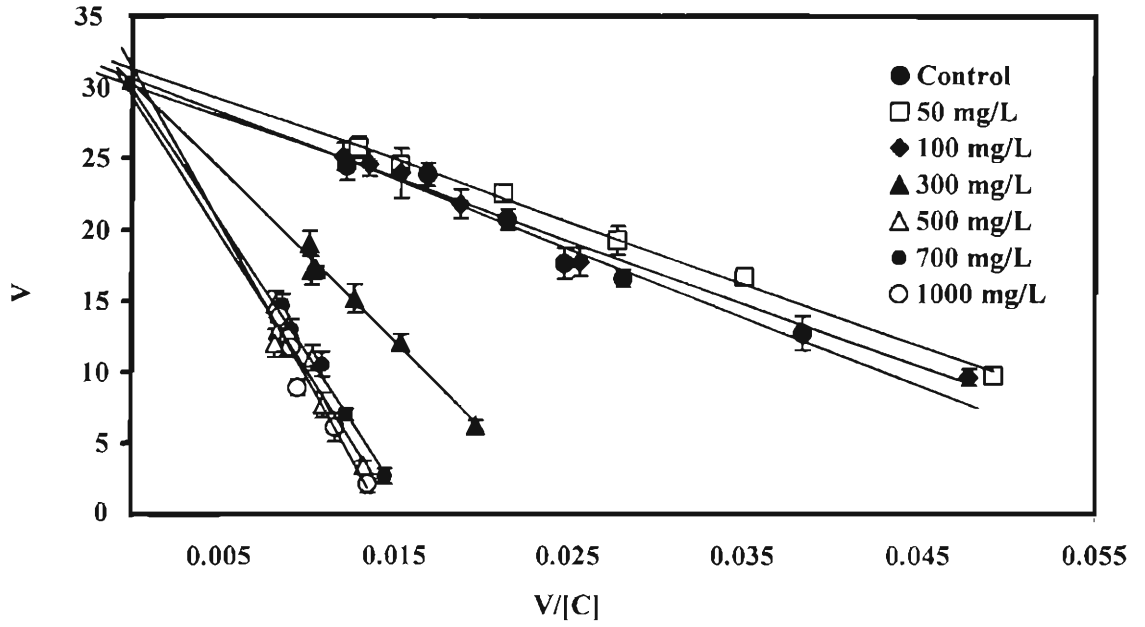


Figure 5.25 Eadie-Hofstee plot for cultures receiving stearic acid plus 2000 mg/L glucose. (SA = stearic acid, average for triplicate samples are used, error bars are standard deviation).

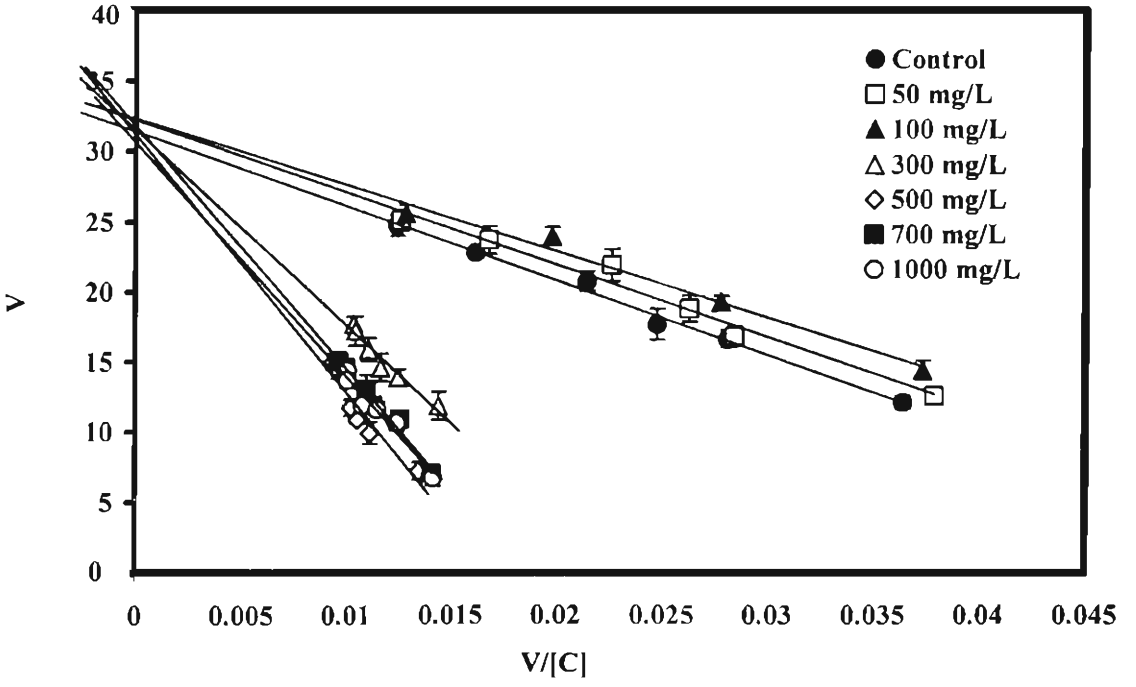


Figure 5.26 Eadie-Hofstee plot for cultures receiving mixtures of linoleic and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, SA = stearic acid, average for triplicate samples are used, error bars are standard deviation).

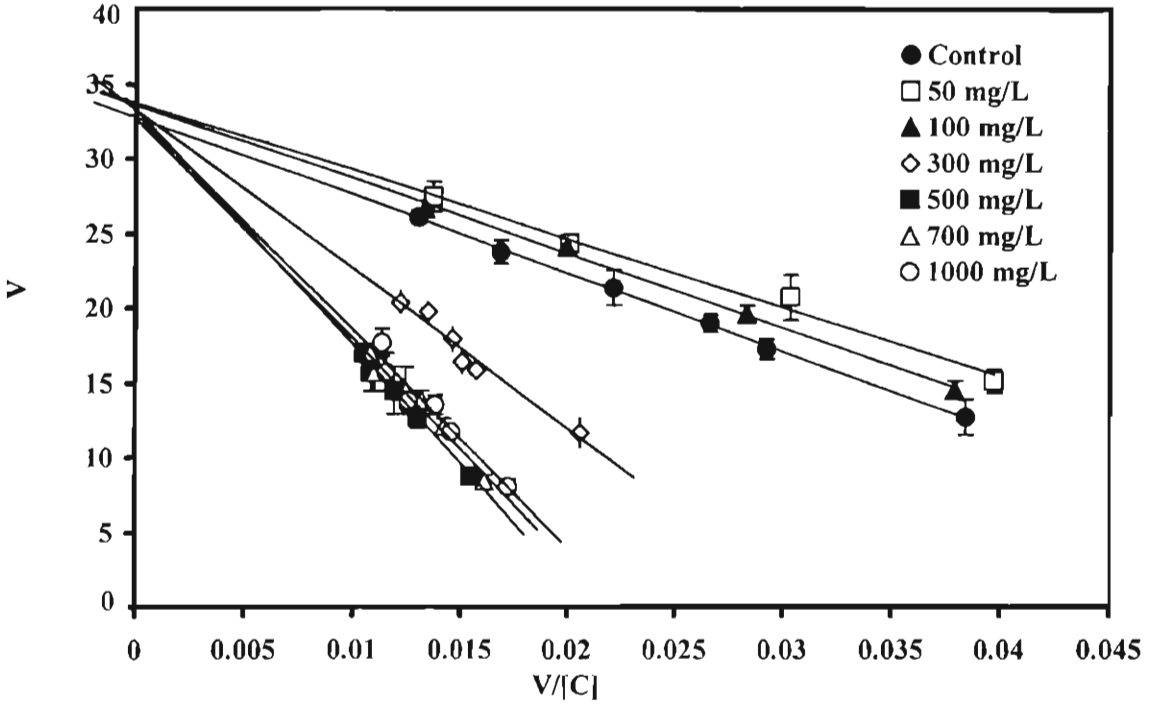


Figure 5.27 Eadie-Hofstee plot for cultures receiving mixtures of oleic and stearic acids plus 2000 mg/L glucose. (OA = oleic acid, SA = stearic acid, average for triplicate samples are used, error bars are standard deviation).

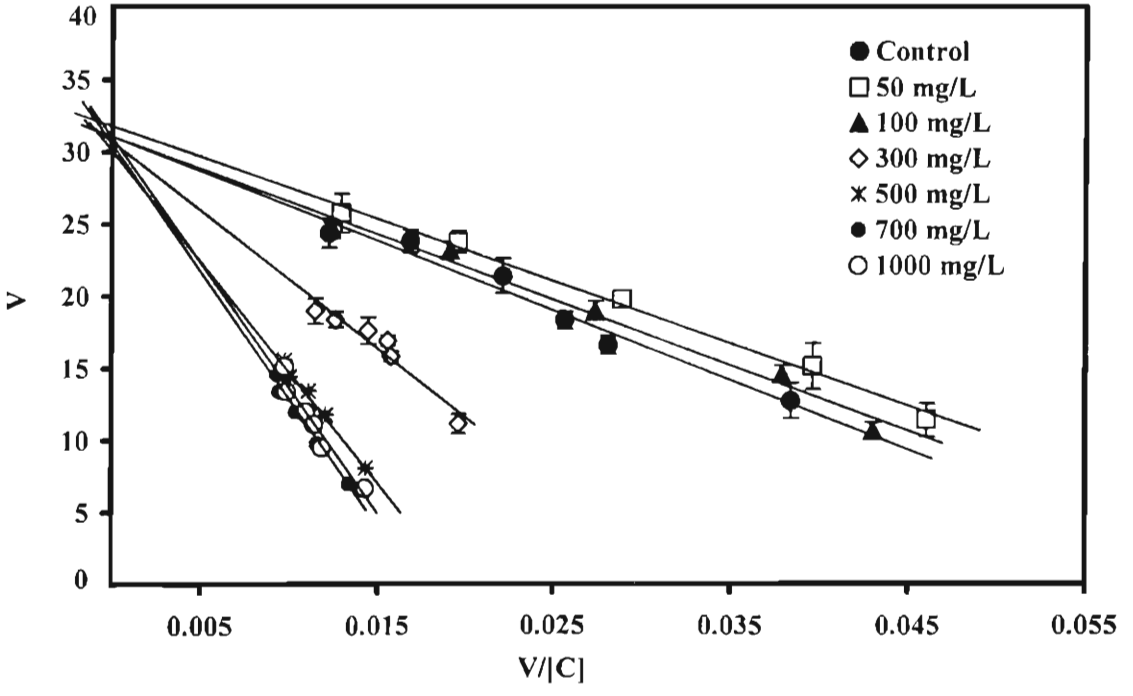


Figure 5.28 Eadie-Hofstee plot for cultures receiving mixtures of linoleic, oleic, and stearic acids plus 2000 mg/L glucose. (LA = linoleic acid, OA = oleic acid, SA = stearic acid, average for triplicate samples are used, error bars are standard deviation).

In competitive inhibition, the inhibitor binds to active sites of the enzymes thus reducing the amount of [E] available for the substrate by formation of [EI] complex. Since the inhibitor, in this case, does not affect the [ES] complex after it has formed, V_{max} will not change but K_M will increase. In the competitive inhibition mechanism, the maximum velocity ,V_{max}, can be reached if sufficient substrate is available because at high levels of substrate, all of the inhibitor is displaced by substrate.

5.2.2 VFAs Degradation

In cultures receiving 50 or 100 mg/L mixtures of LCFAs, no significant inhibition was observed compared to the controls. This suggests that none of the LCFAs mixtures added were inhibitory between 50 and 100 mg/L. The inhibitory effects due to mixtures of OA/SA and LA/OA/SA were less than that caused by LA/OA and LA/SA mixtures.

A similar inhibition pattern was observed for propionate production and degradation. As an overall comparison, propionate-degrading microorganisms appeared to be less sensitive for the LCFAs added than acetate-degrading microorganisms since propionate degradation activity was restored in all cases where concentrations higher than 500 mg/L mixtures of LCFAs were used.

5.2.3 Methane Production

The addition of 50 or 100 mg/L mixtures of LCFAs to the cultures did not cause inhibition to methane production. This may suggest the cultures were able to adapt to

degrading microorganisms appeared to be less sensitive to the LCFAs examined when compared to the aceticlastic methanogens since propionate degradation activity was restored in all cases examined.

The results of this research showed that LA was the most inhibitory to methane production. However, in contrast to the findings of Angelidaki and Ahring (1992), the inhibition effects observed for OA and SA were significantly lower in this work than those reported by Angelidaki and Ahring (1992). Also, inhibitory effects of cultures fed with C_{18} LCFAs increased with increasing number of double bonds.

This work also showed that inhibition of glucose by LCFA is competitive, the maximum reaction velocity, V_{max}, did not change. In contrast, the binding affinity $K_{M\ app}$, increased with increasing LCFA concentration. Moreover, $K_{M\ app}$ values were the lowest when mixtures of LA, OA, and SA were added indicating that there were synergistic inhibitory effects on glucose degradation.

7.0 Future Recommendations

In designing anaerobic treatment systems, several parameters should be taken into consideration. Solids retention time (SRT) should be selected based on the slowest degradation rate of components to be treated. Based on degradation data, SA has a degradation rate slower than OA and LA, thus SA will control the design SRT of a bioreactor. LA has the most inhibitory effects on glucose degradation rates in comparison to oleic and stearic acids. LA concentrations as low as 50 mg/L LA affected the glucose degradation rate. Moreover, based on kinetics data, LCFAs inhibited glucose degradation. Such inhibition does not affect the maximum velocity of the reaction but only affects the binding affinity of the substrate.

Data from this research also showed that LCFAs are inhibitory to VFA-degrading microorganisms. Propionate degradation is very sensitive to the hydrogen partial pressure P_{H2}. Mosey (1983) and Boone and Xun (1987) observed inhibition of propionate degradation by increasing hydrogen partial pressure. A two-stage reactor can be used as a possible way to eliminate the effects of hydrogen on VFA degradation. In the first stage, carbohydrates are degraded to VFAs and LCFAs which are β-oxidized to acetate and shorter chain LCFAs. VFAs would then be transferred to the second stage methanogenic reactor. Hanaki *et al.* (1981) proposed the use of a two-stage reactor as a way to eliminate the inhibitory effects of LCFAs, however to this date no one has investigated the use of such technology.

The effects of LCFAs on the maximum velocity of the reaction and the binding affinity of acetate, propionate, and butyrate are unknown. Kinetic studies are needed to

investigate the effects of LCFAs inhibition on V_{max} and K_M of acetate, butyrate, and propionate.

Angelidaki and Ahring (1992) used a culture operated at 55 °C and showed that concentrations greater than 500 mg/L OA cause permanent inhibitory effects. In this study a culture adapted at 21 °C was used. Data from these studies showed the inhibitory effects due to OA and SA on propionate and butyrate degradation took place at relatively high OA and SA concentrations. Therefore, work is needed to investigate the effects of temperature on LCFAs inhibition of glucose fermentation.

Finally, the effect of individual LCFAs on the fermentation of glucose at 2000 mg/L was not examined in this research. Additional research is required to further clarify the effects of individual LCFA on various glucose concentrations. In this research, no definite conclusion was drawn on the synergistic effects of LCFAs on VFA degradation. Results from additional experiments are required to confirm the synergistic effects of LA, OA, and SA on VFA degradation.

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Appendixes

Appendix A: Schematic Of The Batch Reactors

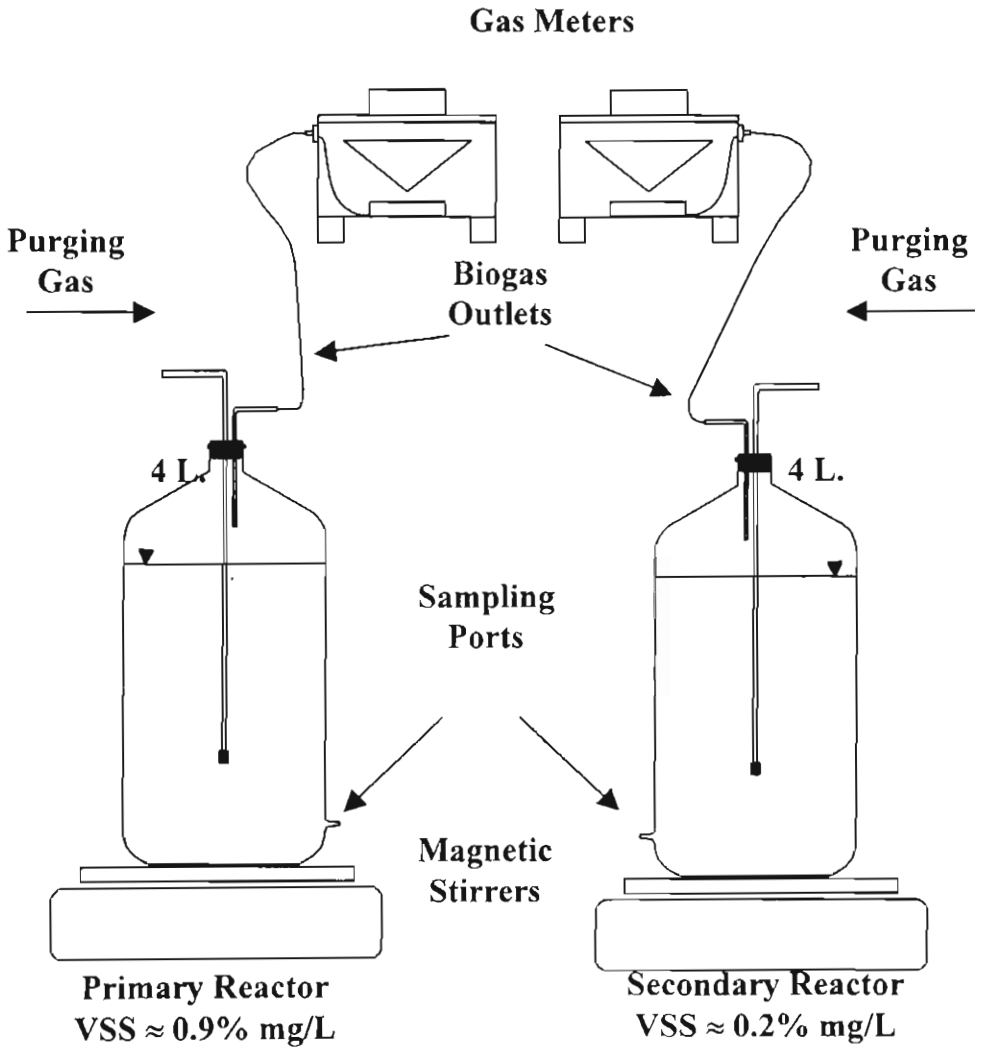

Figure A.1: Primary and secondary reactors schematic.

Appendix B: Free Energies (ΔG) For Some Reactions

Reaction:	ΔG(KJ/mole)
Glucose to acetate: $Glucose + 4H_2O \rightarrow 2Ac^- + 2HCO_3^- + 4H^+ + 4H_2$	-206[1]
Glucose to butyrate: $C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2$	-224[1]
Acetate to CH_4: $Ac^- + H_2O \rightarrow HCO_3^- + CH_4$	-31.0[1]
Acetate to Butyrate: $2Ac^- + H^+ + 2H_2 \rightarrow Bu^- + 2H_2O$	-48.1[1]
Butyrate to acetate: $Bu^- + 2H_2O \rightarrow 2Ac^- + H^+ + 2H_2$	48.3[1]
Propionate to acetate: $Pr^- + 3H_2O \rightarrow Ac^- + HCO_3^- + H^+ + 3H_2$	18.3[1]
CO_2 to CH_4: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-130.7[1]
Linoleic acid to palmitic acid: $C_{18}H_{31}O_2^- + 2\ H_2O \rightarrow C_{16}H_{31}O_2^- + Ac^- + H^+$	-106.4[2]
Oleic acid to palmitic acid: $C_{18}H_{33}O_2^- + 2\ H_2O \rightarrow C_{16}H_{31}O_2^- + Ac^- + H_2 + H^+$	-27.8[2]
Stearic acid to palmitic acid: $C_{18}H_{35}O_2^- + 2\ H_2O \rightarrow C_{16}H_{31}O_2^- + Ac^- + 2H_2 + H^+$	50.8[2]

[1] Thauer et al. (1977), [2] Lalman and Bagley, (2000)

ΔG(KJ/mole) vs. Hydrogen Partial Pressure (atm)	
CO_2 to CH_4:	$\Delta G = -22.8\left(\log\left[P_{H_2}\right] + 5.28\right)$
Butyrate to acetate = -(acetate to butyrate):	$\Delta G = 11.4\left(\log\left[P_{H_2}\right] + 3.81\right)$
Propionate to acetate:	$\Delta G = 17.1\left(\log\left[P_{H_2}\right] + 4.08\right)$

Appendix C: Example Calculations for Statistical Comparisons, Degradation Rates, and Mass Balances.

Statistical comparison is made using Tukey's w procedure: Two means are declared different if the difference between the two means is larger than w i.e. $|\overline{X}_1 - \overline{X}_2| \rangle w$ where $w = q_\alpha(t, df_w)\sqrt{\frac{Sw^2}{n_i}}$,

- t is the batch number or the number of cases, t = 7 (0, 50, 100, 300, 500, 700, and 1000 mg/L).
- n_i is the sampling number = 3 (sampling is done in triplicates).
- $df_w = n_i - 1$
- α is the confidence level, the default value is used (0.95)
- q_α: upper percentage points of the studentized range, values are pre-calculated and can be obtained from any statistical textbook.
- Sw^2

Degradation rates are calculated using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Example: glucose degradation profiles for the cultures receiving 1000 mg/L glucose. (see figure 4.1, control).

$$C\ mg/L = 1980\left(1 - e^{-0.485t}\right) \Rightarrow v = \frac{-dC}{dt} = 961.2 \cdot e^{-0.485t}$$

At t = 0 $\Rightarrow$ initial degradation rate = 961.2 $mg.L^{-1}.hr^{-1}$ = 16.2 $mg.L^{-1}.min$

Biomass concentration = 2000 mg $VSS.L^{-1}$ $\Rightarrow$

intial degradation rate = 8.01 $\mu g.mg\ VSS^{-1}.min$

Mass balance calculation is done using the following formula:

$\Sigma Substrate_{=0} = \Sigma Product_t + \Sigma Substrate_t$ Example: cultures receiving 1000 mg/L LA plus 1000 mg/L glucose (see figures 4.1, 4.7, 4.25, 4.28, and 4.32)

at t = 0, theoretical amount is 78 mg C (from LA) + 40 mg C (from glucose) = 118 mg C

at 20 days, C mass = 68 mg C (from LA) + 11 mg C (from acetate) + 11 mg C (from propionate) + 22 mg C (from CH_4) = 112 mg C

$\mathcal{V}$

VITA

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