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Microbial production of hydrogen under mesophilic conditions.

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MICROBIAL PRODUCTION OF HYDROGEN UNDER MESOPHILIC CONDITIONS

by
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ABSTRACT

Fermentative hydrogen production from biomass using mixed anaerobic cultures has a greater potential to be developed as a practical biohydrogen system than systems utilizing pure cultures. To optimize hydrogen production, it is important to inhibit hydrogen consumers during glucose fermentation. Long chain fatty acids (LCFAs) are inhibitors of aceticlastic methanogenic bacteria and these fatty acids could act as hydrogenotroph methanogenic inhibitor in fermentative hydrogen production.

Batch studies were conducted to assess the effects of two C18 LCFAs on microbial hydrogen production from glucose under mesophilic conditions. Experiments were conducted using different concentrations of linoleic acid (LA) and oleic acid (OA) at 37±1°C. The effects of initial pH in the presence of two C18 LCFAs on hydrogen production were assessed by controlling the initial pH. Glucose was re-injected on day 4 or day 5 to examine the combined effect of LCFA and volatile fatty acids (VFAs) on hydrogen production and also the inhibition time dependence.

In control experiments conducted with LCFA or glucose, no detectable amounts of hydrogen were observed in the headspace. In general, the amount of hydrogen produced was a function of the LCFA concentration and initial pH of the cultures. The maximum hydrogen yield (2.37 moles H_2.mole^{-1} glucose) was observed in cultures inoculated with 2,000 mg l^{-1} LA with an initial pH of 5.0. Hydrogen yield for cultures exposed to 2,000 mg l^{-1} LA and at a pH of approximately 7.6 was less than yields obtained at lower pH conditions.

To my parents

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NOMENCLATURE

ATP	adenosine tri-phosphate
COD	chemical oxygen demand
Fd	ferredoxin
GC	gas chromatography
HRT	hydraulic retention time
IC	ion chromatography
LA	linoleic acid
LCFAs	long-chain fatty acids
OA	oleic acid
SRT	solid retention time
TCD	thermal conductivity detector
TSS	total suspended solids
VFAs	volatile fatty acids
VSS	volatile suspended solids

CHAPTER 1

INTRODUCTION

1.1 Background

Energy consumption and technology development are measures of an advancing civilization and economic prosperity. At present, global energy requirements are dependent mainly on fossil fuels. Byproducts of fossil fuel combustion include oxides of carbon, sulfur and nitrogen and particulate matter (Gosselink, 2002). A well-established fact is that higher atmospheric levels of green house gases, such as CO_2 and raising global surface temperature are linked to the consumption of fossil fuels (IPCC, 2001). Extensive use of fossil fuels can accelerate climate change, increase the loss of small islands, accelerate extreme weather events, reduce agricultural productivity and water availability, and increase the loss of biodiversity (IEA, 2000).

The increasing rate of energy consumption and depletion of fossil fuel reserves have placed increasing demands on researchers to find alternative energy resources. In industrialized or first world countries, the share of imports in overall energy demand is projected to rise roughly from 56% to 72% by 2010 (IEA, 2000). A large fraction of the remaining fossil fuels reserves are located in politically unstable countries. If the governments of these countries are deposed and replaced by 'rogue' regimes, the flow of oil to many industrialized countries could be halted. The national security of many Western nations is dependent on a continuous supply of energy. Hence, to meet rising energy demands and simultaneously preserve and protect the environment requires developing sustainable and renewable energy sources.

Hydrogen is a promising alternative to fossil fuels, both from an economic and environmental standpoint. It is a clean and environmentally friendly fuel, which produces water instead of green house gases when combusted (Suzuki, 1982; Bockris, 1981). Furthermore, it has a high-energy yield (122 kJ g^{-1}) that is about 2.75 times greater than that of hydrocarbon fuels, and could be used directly to produce electricity by using fuel cells (Lay *et al.*, 1999; Mizuno *et al.*, 2000).

In addition, hydrogen could facilitate the transition from limited non-renewable stocks of fossil fuels to unlimited renewable energy sources. It will help in the "decarbonization" of global energy systems (Carl-Jochen, 2000), which is important in avoiding severe effects of climatic change. According to the World Energy Assessment, released in 2000 by several United Nations (U.N.) agencies and World Energy Council, "the strategic importance of hydrogen as an energy carrier" will result in the accelerated replacement of oil and other fossil fuels and it will help to reduce carbon emissions and avoid the doubling of pre-industrial carbon dioxide (CO_2) concentrations in the atmosphere – a level at which scientists expect major, and potentially irreversible, ecological and economic disruptions (UNDP *et al.*, 2000).

Over the last three decades a number of international programs have been implemented to investigate and develop hydrogen as a sustainable and renewable energy source. These programs include the National Hydrogen Program of Japan which belongs to the World Energy Program, WE-NET (WE-NET, 1997), India's Hydrogen Energy Program (Anon, 1998), the Program for Hydrogen Fuel for Collective Transport in Brazil (Brazil, 2002) and the Transport Energy Strategy in Germany (Bunger, 2001).

Now-a-days hydrogen is mainly produced from fossil fuels, water and biomass. The methods used to manufacture hydrogen from fossil fuels are grouped into the following four categories: (Rosen and Scott, 1998)

1. Steam reforming of natural gas
2. Thermal cracking of natural gas
3. Partial oxidation of heavier than naphtha hydrocarbons
4. Coal gasification

Other methods under development to produce hydrogen from water include (Rosen and Scott, 1998, Casper, 1978, Benemann, 1997):

1. Electrolysis
2. Photolysis
3. Thermochemical process
4. Direct thermal decomposition or thermolysis
5. Biological production

Hydrogen produced from biomass by pyrolysis or gasification generates a mixture of gases consisting of H_2, CH_4, CO_2, CO and N_2. Among these processes, nearly 90% of hydrogen is produced by processing of natural gas or light oil fractions and steam at high temperature. These industrial methods consume fossil fuels as well as hydroelectricity as an energy source (Rosen and Scott, 1996; Lodhi, 1987; Casper, 1978). However, both thermochemical and electrochemical hydrogen generation processes are energy intensive, uneconomical, and not always environmentally friendly. In comparison to the latter processes, biological hydrogen production methods are mostly operated at ambient temperatures and pressures. These processes are environmentally friendly and will lead

to opening a 'new avenue' of renewable energy resources, which are inexhaustible (Benemann, 1997; Tanisho *et al.*, 1983). In addition, wastewater and organic waste materials can be utilized as substrate in these processes. Biological hydrogen production processes under development is divided into the following:

1. Biophotolysis of water using algae and cyanobacteria.
2. Photodecomposition of organic compounds by photosynthetic bacteria.
3. Fermentative hydrogen production from organic compounds.
4. Hybrid systems using photosynthetic and fermentative bacteria.

Photosynthetic production of hydrogen is a biological process in which water is converted into hydrogen in the presence of sunlight. Cyanobacteria (also known as blue green algae) can synthesize and evolve hydrogen gas through photosynthesis. This process is light dependent and the conversion efficiencies are low (Hallenbeck and Benemann, 2002; Benemann, 1997). Fermentative hydrogen production from organic compounds has a greater potential to be developed as a practical biohydrogen system. Fermentative microorganisms have a relative high evolution rate of hydrogen and can produce constantly from organic substrates. These microorganisms have excellent growth rates to supply the required enzymes for hydrogen production systems. This makes the fermentative system more advantageous than photochemical evolution for large scale production of hydrogen by microorganisms (Tanisho *et. al.*, 1983; Tanisho *et. al.*, 1995).

A number of bench-scale experiments have demonstrated that higher hydrogen yields are obtainable using fermentative bacteria (Adams and Stiefel, 1998; Das and Veziroglu, 2001). Several researchers have attempted to optimize pH and substrate for mesophilic fermentative hydrogen production systems (Hawkes *et al.*, 2002). Inhibition

of hydrogen consuming bacteria during anaerobic digestion is essential to maximize hydrogen production. Lay (2000) used wet heat treatment (boiling for 15 minutes) of anaerobic digester sludge, whereas, Van Ginkel *et al.* (2001) used dry heat treatment (baking at 104°C for 2 hours) of compost and soil to inhibit methanogenic bacteria. The presence of fats and oils from animals and vegetables sources can also decrease the utilization of hydrogen by inhibiting hydrogen consumers in mixed anaerobic cultures (Hanaki *et al.*, 1981). In mixed culture systems, hydrogen is produced during the acidogenic and acetogenic phases. Hydrogen is subsequently consumed by hydrogen consumers to produce methane, hydrogen sulphide, ammonia and acetate. Inhibiting these reactions will prevent hydrogen consumption. The inhibitory effects of unsaturated fatty acids on microorganisms have been documented in the 1950s by Nieman (1954). Also, Koster and Cramer (1987) and Lalman and Bagley (2001, 2000) have reported the effects of C18 long chain fatty acids (LCFAs) on a variety of anaerobic organisms.

1.2 Objectives

The purpose of this study was to investigate the effects of two C18 long chain fatty acids (LCFAs) on hydrogen production during the fermentation of glucose at 37°C.

The specific objectives of this research were:

- To examine the effects of oleic acid (OA, $C_{18:1}$) and linoleic acid (LA, $C_{18:2}$) on hydrogen yields
- To assess the effect of intermittent sparging with nitrogen and the presence of volatile fatty acids (VFAs) and LCFAs
- To determine the effects of controlling the pH

CHAPTER 2

LITERATURE REVIEW

The biological generation of hydrogen from anaerobic fermentation of organic substrates could likely become an economical and sustainable technology for hydrogen production if conversion efficiencies can be increased (Benemann, 1997). Anaerobic fermentation has been previously used for producing methane and solvents. Hydrogen can be produced from waste materials containing high concentrations of organic material, such as municipal solid waste, industrial wastewater, and agricultural waste. Biohydrogen production is interesting because of its environmentally friendly and renewable nature (Das and Veziroglu, 2001; Hallenbeck and Benemann, 2002). Dark fermentation has a great potential to become a practical process for producing biohydrogen (Das and Veziroglu, 2001).

2.1 Anaerobic Biohydrogen Production

Dark hydrogen fermentation is of a ubiquitous nature in anaerobic digestion of organic compounds because of the absence of oxygen as an electron acceptor. During growth of heterotrophic bacteria, organic substrates are degraded by oxidation to provide building blocks and metabolic energy for growth. Oxidation reaction generates electrons, which need to be disposed. In aerobic or oxic environments, oxygen is reduced to water. In anaerobic or anoxic environments, other electron acceptors such as protons are reduced to molecular hydrogen (H_2) (Nandi and Sengupta, 1998). The biochemical pathway of conversion of renewable biomass to hydrogen via fermentation is depicted in Figure 2.1.

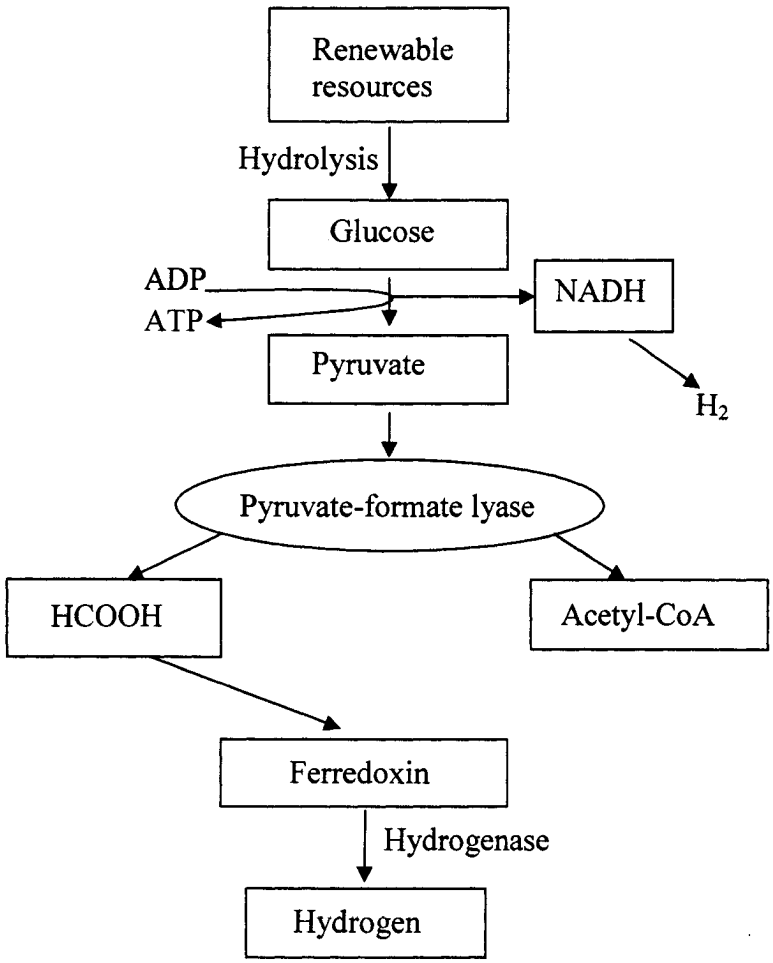

Figure 2.1: Biochemical decomposition of biomass into hydrogen via fermentation (Nath and Das, 2004)

During glucose fermentation to hydrogen, pyruvate is oxidized to acetyl-CoA and is subsequently converted to acetyl phosphate. From this latter product, acetate and ATP is formed. Pyruvate oxidation to acetyl-CoA is coupled to the ferredoxin (Fd) reduction. Reduced ferredoxin is oxidized by hydrogenase, which generates ferredoxin and releases electrons as molecular hydrogen (Hallenbeck and Benemann, 2002).

$$\text{Pyruvate} + \text{CoA} + 2\ \text{Fd (ox)} \longrightarrow \text{Acetyl-CoA} + 2\ \text{Fd (red)} + CO_2 \qquad (2.1)$$

$$2\ Fd\ (red) \longrightarrow 2\ Fd\ (ox) + H_2 \qquad (2.2)$$

Dark fermentation enables the production of hydrogen by a relatively simple process from a wide range of reduced substrates. In addition, fermentative hydrogen production generally yields higher reaction rate and does not rely on the availability of light sources. Carbohydrates, mainly as glucose, are the preferred carbon sources for the fermentation process which predominately give rises to acetic acid, butyric acid and hydrogen gas (Classen *et al.*, 1999).

Equations 2.3 and 2.4 are the two major pathways for hydrogen production through glycolysis and pyruvic acid decomposition. The maximum theoretical hydrogen yield is 4 moles $H_2 \cdot mole^{-1}$ glucose when acetic acid is the by-product (eq. 2.3) and the yield is halved when the byproduct is butyric acid (eq. 2.4). The end products of glucose fermentation by anaerobic and facultative anaerobic chemoheterotrophs, such as clostridia and enteric bacteria, are derived through pyruvate, a three carbon intermediate, during anaerobic degradation of carbohydrates.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad (2.3)$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad (2.4)$$

2.2 Anaerobic Degradation Process

Anaerobic degradation processes are widely used to treat wastewater and organic wastes. The process assists in maintaining the integrity between the health of the environment and that of human beings. During degradation, complex organic substrates are converted into simpler forms and byproducts of one reaction serve as substrates of other reactions in the sequence. The ultimate end products of anaerobic conversion of

biodegradable organic substrates are methane (CH_4), carbon dioxide (CO_2), water plus a small amount of culture biomass.

A consortium of microorganisms facilitates the different steps of anaerobic digestion of organic substrates. The anaerobic process shown in Figure 2.2 is divided into the following categories:

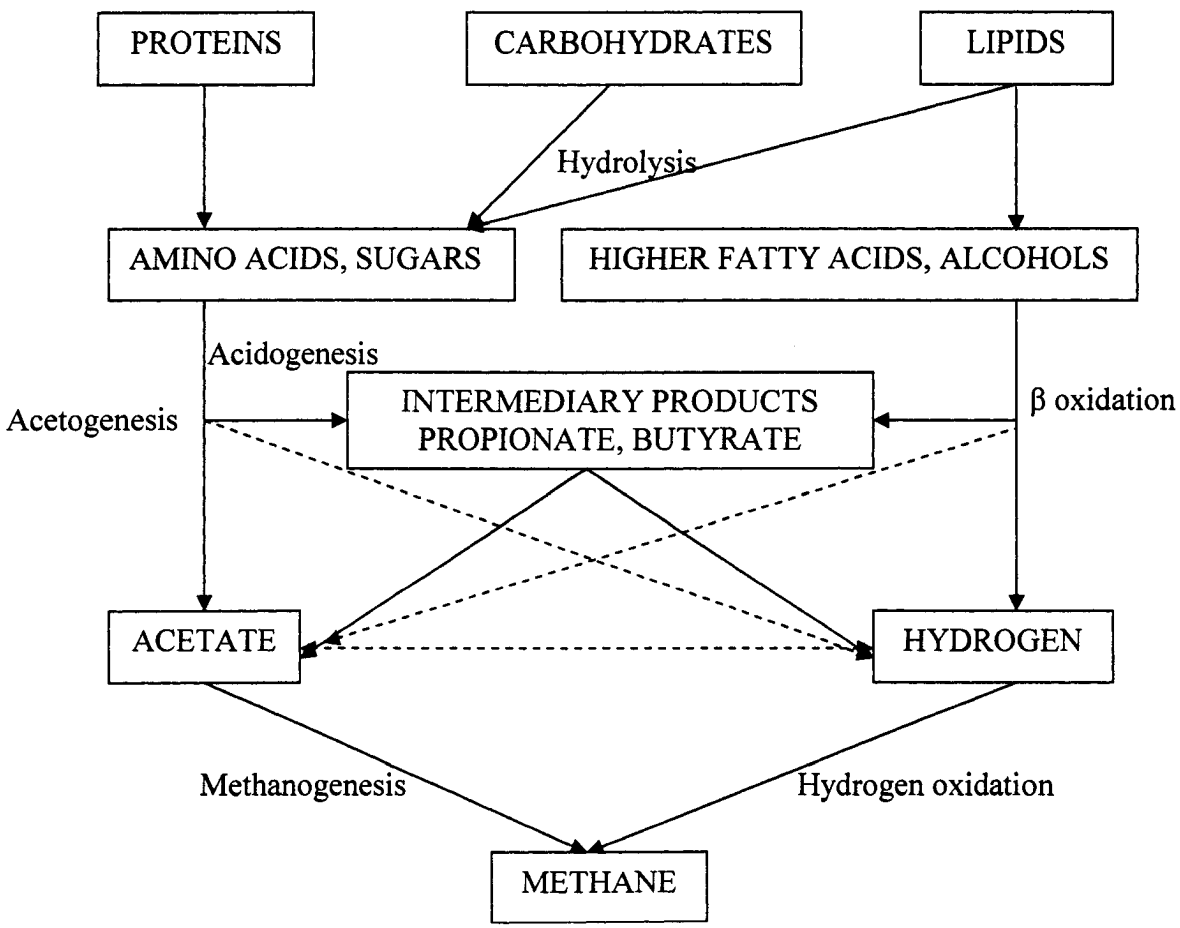

Figure 2.2: Pathway of anaerobic biodegradation (Gujer and Zehnder, 1983)

2.2.1 Hydrolysis

During hydrolysis, organic polymers are converted into monomers. Polysaccharides are easily hydrolyzed into monosaccharides by hydrolytic bacteria. However, lipids and large molecular weight proteins do not readily dissolve into the aqueous phase because of hydrophobic components. Proteins and lipids are degraded into LCFAs byproducts by proteases and lipases, respectively. The carbohydrate hydrolysis reaction is mediated by amylase (e.g. sucrase, glucoamylase) excreted by hydrolytic organisms (Veeken *et al.*, 2000).

2.2.2 Acidogenesis

Acidogenesis is the next step in the anaerobic reaction sequence where the compounds produced during hydrolysis are converted into volatile fatty acids (VFAs), hydrogen, alcohols and other intermediate products. Under certain conditions depending upon substrate, pH, temperature and culture, hydrogen is produced together with propionate and butyrate from glucose. The hydrogen partial pressure plays an important role in this step. When the hydrogen partial pressure increases, reduced VFAs are formed to reduce the amount of hydrogen. Acidogenesis is facilitated by a broad group of fermentative organisms which includes *Enterobacter aerogenes* and *Escherichia coli* (Malina and Pohland, 1992). *Clostridia* convert acidogenesis products into hydrogen and carbon dioxide during acetogenesis (Malina and Pohland, 1992).

2.2.3 Acetogenesis

VFAs, other than acetic acid that are formed during acidogenesis, are converted into acetic acid, hydrogen, and carbon dioxide. For complete degradation of VFAs

byproducts, a low hydrogen partial pressure is also very important. The balance between hydrogen production and consumption is maintained by a microbial symbiotic relationship which is not only biochemical, but also spatial (Gujer and Zehnder, 1983). For example, if hydrogenotrophs are inhibited, hydrogen accumulation is prevented by acidogens through the formation of reduced VFAs byproducts. The association between these organisms is termed syntropy and is found when two metabolically different types of bacterial species depend on each other for degradation of a certain substrate (Gujer and Zehnder, 1983).

2.2.4 Methanogenesis

During methanogenesis aceticlastic methanogens convert acetate into methane and carbon dioxide and hydrogenotrophic methanogens reduce carbon dioxide into methane. Most of the methane (approximately 70%) in the anaerobic degradation of organic matter is derived from decarboxylation of acetate by aceticlastic methanogens. Hydrogen generated at different stages is consumed by hydrogenotrophic methanogens (Speece, 1996). To increase the hydrogen yield using a mixed culture system, inhibition of hydrogenotrophs and other hydrogen consumers is essential. This approach can be accomplished by inhibiting the hydrogen-consuming bacteria.

2.3 End-Product Formation and Distribution

Industrial effluents containing a variety of organic compounds are degraded to shorter chain carbon compounds and eventually to gases such as methane, carbon dioxide. The presence of reduced chain carbon compounds in the form of volatile fatty acids (VFAs) and alcohols provides an indication of the process efficiency. For example,

if high levels of short chain fatty acids and alcohols are present during treatment, then this is an indication of inefficient hydrogen utilization.

VFAs that are generally found during anaerobic degradation include acetic acid, propionic acid, butyric acid, and iso-butyric acid (Rittmann and McCarty, 2001). Acetic acid is the most common product during the fermentation of carbohydrates and proteins; propionic acid is mostly formed from carbohydrates, and butyric acid is mainly generated during fermentation of proteins, lipids and carbohydrates (Elefsiniotis and Oldham, 1994). Typical products formed during glucose degradation are shown in Table 2.1:

Table 2.1: Products from glucose acidification (acidogenesis)

Reaction	**Equation Number**
$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2$	2.5
$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	2.6
$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O$	2.7
$C_6H_{12}O_6 \longrightarrow 2CH_3CHOHCOOH$	2.8
$3C_6H_{12}O_6 \longrightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2O$	2.9
$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$	2.10

(Source: IWA, 2002)

A maximum theoretical hydrogen yield of 4 moles $H_2 \cdot mole^{-1}$ glucose is achievable when the carbon by-product of glucose fermentation is only acetic acid (Table 2.1). In comparison, the yield is 2 moles $H_2 \cdot mole^{-1}$ glucose when butyric acid is the only

carbon by-product and it is zero when glucose is fermented to lactic acid, propionic acid or ethanol.

Fermentation reactions and byproducts of glucose acidification are shown in Table 2.2. Notice ethanol is formed during glucose acidification at a pH below 5.0 (IWA, 2002). However, at low hydrogen concentration, ethanol can be fermented to acetic acid and hydrogen. Lactic acid, an intermediate from pyruvate, is converted into propionic acid or into acetic acid plus hydrogen.

Table 2.2: Acetogenic reactions (acetogenesis)

Substrate	**Product**	**Reaction**	**Equation**
Lactic acid	Acetic acid	$CH_3CHOHCOOH + H_2O \longrightarrow CH_3COOH + CO_2 + 2H_2$	2.11
Butyric acid	Acetic acid	$CH_3CH_2CH_2COOH + 2H_2O \longrightarrow 2CH_3COOH + 2H_2$	2.12
Propionic acid	Acetic acid	$CH_3CH_2COOH + 2H_2O \longrightarrow CH_3COOH + CO_2 + 3H_2$	2.13
Ethanol	Acetic acid	$CH_3CH_2OH + H_2O \longrightarrow CH_3COOH + 2H_2$	2.14

(Source: Bagley and Brodkorb, 1999)

The product distribution during fermentation is a function of temperature, pH and organic loading (Zoetemeyer *et al.*, 1982A). Zoetemeyer and coworkers (1982B) reported higher ethanol concentrations are achievable in thermophilic systems than in mesophilic systems. Ethanol, butanol, propanol, n-propanol, n-butanol, 2, 3-butanediol

and glycerol are the predominant alcohols formed during acidification (Elefsiniotis and Oldham, 1994; Fabino and Perego, 2002).

Maximizing hydrogen yield to 4 moles H_2.mole^{-1} glucose requires the fermentation process be optimized for acetic acid formation. In methane producing anaerobic systems, acetic acid concentrations may rise due to a decrease in the activity of aceticlastic methanogenic activity (Kidby and Nedwell, 1991). However, experimental data on methane measurements in sewage sludge have shown that approximately 70% of the methane produced originates from acetate and one-third from hydrogen and carbon dioxide (Madigan *et al.*, 2000).

The products generated during the anaerobic digestion depend on hydrogen partial pressure in the liquid phase. Many hydrogen-producing reactions are not thermodynamically favorable under standard conditions. Butyric and propionic acids can be fermented only when hydrogen partial pressure is less than 32.04 or 40.34 Pa respectively, and ethanol and lactic acid are readily fermented at hydrogen partial pressures 2 to 3 time's higher (Fennell *et al.*, 1997). Less reduced products such as acetic acid are predominant at low hydrogen partial pressure; reduced organic products such as propionic and lactic acids are formed with increasing hydrogen partial pressure, which reduce the hydrogen yield (Bagley and Brodkorb, 1999). Vigorous stirring assist in the release of dissolved gases and can result in a three fold decrease in the ethanol to acetic acid ratio, as well as an increase in the amount of hydrogen produced (Lamed *et al.*, 1988).

2.4 Factors Influencing Biohydrogen Production

Hydrogen production is a growth-associated product and experimental results show the specific growth rate is linearly correlated with the specific hydrogen production (Kumar and Das, 2000). Therefore, environmental conditions that increase metabolism and growth rate should increase hydrogen yield, assuming that a biodegradable substrate is available and free from product inhibition. However, the maximum cell growth is not essential because the creation of new cells is an electron sink, leaving fewer electrons for hydrogen production (Nandi and Sengupta, 1998).

2.4.1 Temperature

Temperature is a major variable in the anaerobic digestion of organic matter because it controls microbial growth. The most favorable temperatures for growth of various *Enterobacter* species have been reported to be in the mesophilic range 32 to 40°C (Kumar and Das, 2000). Mesophilic temperatures have commonly been identified as suitable for microbial growth. Kumar and Das (2000) reported increasing hydrogen yields from 15 to 36°C, and decreasing yield above 36°C. In studies using sewage sludge microflora (dominated by *Clostridium* species), hydrogen was produced during anaerobic acidogenic conversion of glucose at ambient temperatures 15 to 34°C (Lin and Chang, 2004). Though the thermophilic state requires extra thermal energy to maintain a high reactor temperature, higher loading rates and faster reactions are achievable. Rittmann and McCarty (2001) reported the reaction rate was approximately doubled for each 10°C increase in temperature within an optimal range. Elevated temperature decreases the gas solubility (greater Henry's constant) and hence, reduce the hydrogen partial pressure. Temperature affects the acidification of substrates present in wastewater (Fang and Yu,

2001). However acidification is also related to the type of substrate and microorganisms involved.

All chemical reactions are controlled by change in free energy (ΔG), which is a function of temperature. The free energy (ΔG) of a reaction is an indicator of its ability to proceed. Hydrogen producing reactions are affected by temperature and the hydrogen partial pressure. The free energy of a reaction is given by:

$$\Delta G = \Delta G^{\circ} + RT \ln[\text{products}]/[\text{reactants}] \quad (2.15)$$

where, ΔG is the change in free energy, ΔG° is the standard-state free energy of reaction, R is the universal gas constant, and T is the temperature (Kelvin). Equation 2.15 is used to predict the free energy change under non standard conditions.

2.4.2 pH

Microbial reaction rates are affected by pH and typically the optimum is approximately 7. Since pH affects the growth rate, changes in pH may cause shifts in the relative numbers of different microbial species in a heterogeneous population in a acidogenic reactor (Horiuchi *et al.*, 1999). Many aspects of microbial metabolism are influenced by variations in pH over the range within which the microorganisms can grow. These aspects include utilization of carbon and energy sources, efficiency of substrate degradation, synthesis of proteins and various types of storage material, and release of metabolic products from cells (Bailey and Ollis, 1986).

Researchers have reported an optimum pH range between 5.2 to 6.8 for producing VFAs and hydrogen, depending on the microorganisms or seed used. At an optimal pH of 5.5, a yield of 2.1 moles $H_2 \cdot mole^{-1}$ glucose was obtained using a mixed culture at 36°C

(Fang and Liu, 2002). In another study using a mixed culture at 36°C, both the maximum specific hydrogen production yield and the maximum specific hydrogen production rate were obtained at an alkaline pH of approximately 8.5 (Lee *et al.*, 2002). Hawkes *et al.* (2002) recommend using pH below 6.0 for producing hydrogen using mesophilic systems. In other studies, the initial pH had profound effect on hydrogen production potential and rate, and the highest specific hydrogen production potentials were reported at the lowest initial pH of 4.5 (Khanal *et al.*, 2004).

2.4.3 Hydrogen Partial Pressure

Anaerobic degradation is controlled by free energy changes, which depend on the hydrogen partial pressure (Guwy *et al.*, 1997). A high hydrogen partial pressure is thermodynamically unfavorable (Phelps *et al.*, 1985). Valentine *et al.* (2000) observed higher metabolic activity of the H_2-producing organisms than the H_2-consuming activity at low hydrogen partial pressure.

The conversion of butyrate to acetate, propionate to acetate, and acetate to hydrogen occurs according to the following reactions (Thauer *et al.*, 1977):

$$C_3H_7COO^- + 2H_2O \longrightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad (2.16)$$

$$\Delta G = +48.3 \text{ kJ mol}^{-1}$$

$$C_2H_5COO^- + 3H_2O \longrightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2 \qquad (2.17)$$

$$\Delta G = +18.3 \text{ kJ mol}^{-1}$$

$$CH_3COOH + 2H_2O \longrightarrow 4H_2 + 2CO_2 \qquad (2.18)$$

$$\Delta G = +106.4 \text{ kJ mol}^{-1}$$

Reactions (2.16 to 2.18) are thermodynamically unfavorable at moderate temperatures and the free energy is dependent on the hydrogen partial pressure (Classen *et al.,* 1999). Hydrogen evolution pathways are sensitive to H_2 concentrations and are subject to end-product inhibition. As the H_2 concentrations increase, H_2 synthesis decreases and metabolic pathways shift towards production of more reduced products, such as lactate, ethanol, acetone, butanol or alanine (Levin *et al.,* 2004). Continuous hydrogen synthesis requires a pH_2 of < 50 kPa at 60°C, < 20 kPa at 70°C, and < 2 kPa at 98°C (Van Niel *et al.,* 2003).

Gas sparging has been reported as a useful technique to reduce hydrogen partial pressure in the liquid phase and hence, to enhance hydrogen yield. Mizuno *et al.* (2000) observed that the specific hydrogen production rate increased from 1.4 ml H_2 min^{-1} g^{-1} biomass to 3.1 ml H_2 min^{-1} g^{-1} biomass under nitrogen sparging conditions. Hussy *et al.* (2005) also reported that nitrogen sparging improved the average daily hydrogen yields. They reported average daily hydrogen yields from 1.7 to 1.9 moles $H_2.mole^{-1}$ hexose for pure sucrose when sparged with nitrogen and 1.0 mole $H_2.mole^{-1}$ hexose for pure sucrose without sparging.

2.5 Effect of Microbial Source

Microorganisms play an important role during biological hydrogen production. Hydrogen production by different organisms is intimately related to their respective energy metabolisms. Electrons are released when substrates are oxidized. The reducing equivalents are captured by electron acceptors such as protons, carbon dioxide, nitrate, sulfate or organic intermediates. Hydrogen production is a specific mechanism by which hydrogen producing microorganisms dispose of excess electrons. Many experiments have

been conducted with pure and mixed cultures of fermentative bacteria to produce hydrogen.

2.5.1 Pure Cultures

Different strains of *Enterobacter aerogenes* are commonly used to produce hydrogen from carbohydrates under mesophilic conditions. Kumar and Das (2000) reported a maximum hydrogen yield of 2.2 moles $H_2 \cdot mole^{-1}$ glucose using *E. aerogenes* IIT-BT 08 under a pH of 6.0, 36°C, and a 5 hour HRT. Incomparision, Tanisho *et al.* (1983) reported using *E. aerogenes* E.82005 and observed a yield of 1.58 moles $H_2 \cdot mole^{-1}$ glucose at 38°C, a pH of 6.0 and a 20 hour HRT.

Clostridium species are also widely used because of their higher hydrogen yield capabilities compared to *Enterobacter* sp. (Hawkes *et al.*, 2002). However, the former organism requires specific conditions including nutrients and elimination of oxygen. In other studies conducted at 30°C using *Clostridium bacterium* SC-E1, a yield of 2.3 moles $H_2 \cdot mole^{-1}$ glucose was observed at a pH of 6.7 and an 8 hour HRT (Kataoka *et al.*, 1997). In comparison, using *Clostridium bacterium* CGS5, a yield of 2.78 moles $H_2 \cdot mole^{-1}$ sucrose was reported at a pH of 5.5 (Chen *et al.*, 2005). A higher hydrogen yield of 3.3 moles $H_2 \cdot mole^{-1}$ glucose was obtained using *Thermotoga elfi* at 65°C and a pH of 7.4 (Van Niel *et al.*, 2002). Schröder *et al.* (1994) reported 4.0 moles $H_2 \cdot mole^{-1}$ glucose using *Thermotoga maritima* in the batch cultures at 80°C and uncontrolled pH. Van Niel *et al.* (2002) also obtained a yield of 5.9 moles $H_2 \cdot mole^{-1}$ sucrose using *Thermotoga elfi* with a reactor operating at 70°C and the pH controlled at 7.0.

Pure cultures are commonly used in laboratory-scale experiments; however, disadvantages include the sensitivity of the culture to environmental and operational changes such as temperature, pH, nutrients, substrate loadings and oxygen. Pure cultures could also be contaminated by a variety of microbial populations present in substrate sources. Moreover, pure cultures require strict growth conditions to avoid contamination by other species.

2.5.2 Mixed Cultures

In comparison to pure cultures, mixed cultures are widely used for biological hydrogen production because of lower sensitivity to environmental conditions, ability to operate on non-sterile feedstock and the culture source can be obtained from an existing anaerobic facility (Hawkes *et al.*, 2002). Sources of mixed cultures include digested sewage sludge, soils, sludge composts, sludge drying beds and secondary settling tanks. Uneo *et al.* (1996) used an anaerobic microflora from sludge composting to produce hydrogen from a sugar based effluent at 60°C. They obtained a maximum yield of 2.59 moles H_2.mole^{-1} hexose at a 0.5 day SRT and at a pH of 6.8.

The presence of hydrogen consuming bacteria is a major disadvantage of using mixed cultures. Hydrogenotrophic methanogens consume hydrogen to produce methane. Malina and Pohland (1992) reported that approximately two thirds of the methane produced during anaerobic microbial activity is derived from acetic acid and one third is from hydrogen and carbon dioxide. Other potential hydrogen consumers include sulfur reducing bacteria and homoacetogens.

2.6 Microbial Inhibition

Microbial inhibition or the control of growth is accomplished using chemical and physical methods. Methanogenic activity is inhibited by 2-bromoethanosulfonic acid (Blach and Woelf, 1979; Rachman *et al.,* 1997; Hickey *et al.*, 1987), chloroform (Hickey *et al.*, 1987), trichloroacetic acid (Hickey *et al.*, 1987), and acetylene (Bomer *et al.*, 1985). High operational temperatures, oxygen and extreme pH can also inhibit methanogenic activity.

High VFAs and hydrogen levels may affect metabolism or indirectly create a response in the environment, such as a change in pH. Methanogenic inhibition has also been consumed by the presence of LCFAs (Harris *et al.,* 1932; Nieman, 1954; Lalman and Bagley, 2000). VFAs produced during anaerobic digestion are also inhibitory to methanogens but at relatively high concentrations (Sung *et al.*, 2002).

2.6.1 Heat Shock Treatment

Heat shock treatment of anaerobic digested sludge has been used as a method to inhibit hydrogen-consuming bacteria. Methanogens usually oxidize hydrogen as a source of energy to produce methane and for reductive assimilation of carbon dioxide into cellular carbon. Methanogens are non-spore forming bacteria and they cannot sustain growth at elevated temperatures. On the other hand hydrogen-producing bacteria such as *Clostridium* species can be sustained at extreme environmental conditions by forming spores. Heat shock treatment of anaerobically digested sludge involves either boiling the sludge for 15 minutes or baking at 104°C for two hours (Van Ginkel *et al.,* 2001).

2.6.2 Effect of pH

pH is an important factor for suppressing hydrogen consumers and to obtain an enriched culture consisting of hydrogen producers. Fermentative hydrogen production takes place in the acidogenic phase of anaerobic degradation; however, the accumulation of the organic acids causes a pH decrease (Chen *et al.*, 2002, Yokoi *et al.*, 1995). Methanogens grow slower compared to acidogenic bacteria and they prefer a pH range of 6.5 to 8.2 (Speece, 1996). In comparison acidogenic bacteria metabolize at pH values below 6.5. In order to obtain dominant microorganisms for hydrogen production and to prevent hydrogen removal by methanogenesis, the pH has to be controlled at an optimum value (approximately pH 5.5). However, very low pH can inhibit hydrogen production and at values lower than a pH of 6.3, methanogenesis decreases or stops (Chen *et al.*, 2002).

2.6.3 Microbial Inhibition due to VFAs

Methanogens are commonly considered to be the most sensitive of all microbial populations in anaerobic degradation (Speece, 1983). Inhibition of methanogens can be attained by the presence of high volatile fatty acids (VFAs) (Hawkes *et al.*, 2002). The toxicity caused by VFAs is pH dependent since, only the non-ionized forms exhibit microbial toxicity (Huesemann and Papautsakis, 1986; Kashket *et al.*, 1980). Herrero (1983) and Finean *et al.* (1984) reported that the movement of non-ionized acids into cells alters the pH and disrupts the proton pump. This disruption causes changes in membrane and intracellular processes. For example activity of aceticlastic methanogens is also affected by high acetate concentrations. Yang and Okos (1987) reported methanogenic inhibition at 6000 mg l^{-1} acetate, 37°C and pH 7.0. But no inhibition was

observed at 3,000 mg l^{-1} between 35 to 37°C and at pH 7.0 (Yang and Okos, 1987; Huser *et al.*, 1982; Hobson and Shaw, 1976).

2.6.4 Microbial Inhibitory Effects of Long Chain Fatty Acids (LCFAs)

LCFAs are toxic to a variety of microbial population during anaerobic treatment. Harris *et al.* (1932) observed increasing inhibition of microorganisms in foods containing oils bearing unsaturated and longer-chain fatty acids. According to Nieman (1954) unsaturated fatty acids have antibacterial effects on gram-positive bacteria but not on gram-negative bacteria. Nieman (1954) also reported that the inhibitory effects of unsaturated fatty acids increased as the number of double bonds increased. LA has a greater inhibitory effect on gram-positive bacteria than OA (Fuller and Moore, 1967). Several studies reported that LCFAs are inhibitory, at threshold concentrations, to various microorganisms (Lalman and Bagley, 2000; Angelidaki and Ahring, 1992; Koster and Cramer, 1987; Hanaki *et al.*, 1981).

Hwu *et al.* (1996) also reported that the adsorption of LCFAs is a prerequisite for biodegradation. Sayed *et al.* (1988) and Rinzema *et al.* (1993) demonstrated that fat adsorption to the surface of anaerobic sludge could limit the transport of soluble substrates to the cells and subsequently cause a decrease in the substrate conversion rate. Accordingly, Demeyer and Hendrickx (1967) explained that unsaturated LCFAs toxicity is caused by adsorption onto the bacterial cell with subsequent alteration of the cell permeability.

Hanaki *et al.* (1981) observed the effect of LCFAs on several stages of anaerobic digestion and also reported inhibitory effects on many bacterial populations. Angelidaki and Ahring (1992) reported that 500 mg l^{-1} oleate inhibited both VFA degradation and

methane production and the inhibitory effect of oleate (100 to 200 mg l^{-1}) was higher than that of stearate (500 mg l^{-1}). In addition, the lag phase for methane production increased with increasing OA and SA concentrations. Lalman and Bagley (2000) examined the impact of LA on aceticlastic and hydrogenotrophic methanogens at 21°C. They reported a threshold of 30 mg l^{-1} LA inhibited aceticlastic methanogens, but concentrations greater than 30 mg l^{-1} slightly inhibited hydrogenotrophic methanogens. Again, Lalman and Komjarova (2004) reported that elevating the temperature from 21°C to 37°C increased both the substrate degradation rate and the inhibitory effects caused by LCFAs.

LCFAs are inhibitory to gram-positive bacteria at low concentrations but not gram-negative bacteria (Cherrington *et al.*, 1991). Gram-positive bacteria have cell walls similar to methanogens and hence, are expected to be susceptible to LCFA inhibition. LCFAs also act as membrane disrupting agents causing leakage of proteins or ions from gram-positive bacteria (Greenway and Dyke, 1979). After entering into cytoplasm, LCFAs dissociate carboxylic acids and cause acidification of cytoplasm (Baird-Parker, 1980). The overall impact is a reduction in the ΔpH across the membrane. At pH values less than 7 to 8 within the cell, most enzymes become inactivated and lose their activity (Lehninger *et al.*, 1999).

Wastewater arising from food processing industries contains significant amount of fats, oils, and carbohydrates, though the composition of these effluents depends on the processing industries. Several researchers have reported the inhibitory effects of LCFAs on aceticlastic and methanogenic microorganisms in anaerobic digestion processes (Nieman, 1954; Hanaki *et al.*, 1981; Koster and Cramer, 1987; Angelidaki and Ahring,

1992; Lalman and Bagley, 2000). Hence, in this study LCFAs will be utilized as inhibitors of hydrogen consumption in mixed anaerobic cultures.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Plan

The experiments were divided into three phases to achieve the research objectives. The first set of control experiments was conducted to examine hydrogen production from LCFAs degradation over an 8 day period and in 160 ml serum bottles maintained at 37°C and a pH of 7.8. The batch studies were conducted in 160 ml serum bottles with varying LCFAs concentrations ranging from 0 to 2,000 mg l^{-1} (Table 3.1). The second set of experiments examined the effect of OA and LA on hydrogen accumulation during glucose degradation (Table 3.2). The third series of experiments assessed the effect of OA and LA at varying initial pH on hydrogen production during glucose degradation (Table 3.3).

Table 3.1: Hydrogen production from LCFA degradation

LCFA	Concentrations, mg l^{-1}
OA	0, 300, 500, 1000, 1500, 2000
LA	0, 300, 500, 1000, 1500, 2000

Table 3.2: Hydrogen production from glucose in the presence of OA or LA

Glucose Conc., mg l^{-1}	LCFA Conc., mg l^{-1}
0	0
5000	0
5000	500
5000	1000
5000	1500
5000	2000

Table 3.3: Hydrogen production from glucose in the presence of OA or LA at different initial pH conditions

Glucose Conc., mg l^{-1}	LCFA Conc., mg l^{-1}	PH
0	0	0
5000	0	7.6
5000	2000	7.6
5000	0	6.0
5000	2000	6.0
5000	0	5.0
5000	2000	5.0

For each condition examined, the serum bottles were prepared in triplicate. Gas and liquid samples were removed for analysis by ion chromatography (IC) and gas chromatography (GC), respectively. A summary of the objectives of this study, together with the experimental procedures are given in Table 3.4.

3.2 Experimental Details

A 4-L semi-continuous fed batch reactor (Reactor B) maintained under mesophilic conditions was used to provide inoculum (2,000 mg l^{-1} VSS) for the serum bottles. All the experiments were conducted under mesophilic (37°C) conditions.

In phase-I, experiment assessed the effect of inoculating the culture with only LCFAs (Table 3.1). The controls prepared for this study consisted of only adding culture to the serum bottles. Headspace samples were removed and analyzed for hydrogen and methane where liquid samples were removed and analyzed for VFAs.

In phase II experiments, glucose and a LCFA were injected into the culture to assess the effects of LCFA on hydrogen production during glucose degradation at a constant pH of approximately 7.6 and 37°C (Tables 3.2 and 3.4). Two sets of controls were prepared for each experiment. In one control, no substrate was added, while in another only glucose was added.

Table 3.4: Experimental plan

Experimental Phases	Objectives	Action
Semi-continuous fed batch reactor operation	To acclimate cultures under mesophilic conditions. To provide source of inoculum for batch studies.	- Feeding glucose at 5,000 mg l^{-1} - Monitoring total gas production - Sampling for VFAs
Phase I	To assess the effect of LCFAs.	- Adding 0 to 2,000 mg l^{-1} OA or LA - Sample for H_2, CH_4, CO_2 and VFAs - Monitoring head-space pressure
Phase II	To assess the effects of LA or OA on hydrogen production.	- Adding 5,000 mg l^{-1} glucose, and 0 to 2,000 mg l^{-1} OA or LA - Sample for H2, CH_4, CO_2, VFAs, and glucose degradation - Re-injected 5,000 mg l^{-1} glucose on day 4 - Headspace purged after every 24 hours after re-injecting glucose
Phase III	To assess the effects of pH on hydrogen production in the presence of OA or LA.	- Adjusting the initial pH 7.6 to 5.0 - Adding 5,000 mg l^{-1} glucose, and 0 or 2,000 mg l^{-1} LCFA - Sample for degradation, pH, H_2, CH_4, CO_2, and VFAs - Re-adjust pH and re-inject 5,000 mg l^{-1} glucose on day 4

For cultures fed with glucose, liquid samples were removed every 2 hours for 8 hours; every 4 hours for the next 8 hours and at 24 hours . Twelve hours after the first glucose injection, headspace samples were removed and analyzed for expected

byproducts (hydrogen, methane and carbon dioxide). Samples for VFAs, hydrogen, methane and carbon dioxide analysis were removed again every 24 hours over a 4 day period. On day 4, the bottles were opened and the headspace was sparged with N_2 gas (Praxair, 99.999%) for 2 to 3 minutes. Next, glucose was injected again and liquid and headspace samples were removed for VFAs, hydrogen, methane and carbon dioxide analysis every 24 hours over a 3 day period. Samples were not removed for glucose analysis.

The effect of pH was investigated in phase III (Table 3.3 and 3.4). The glucose and LCFA concentration were constant (Table 3.3) and the pH conditions examined were 5.0, 6.0 and 7.6. This experiment was similar to the phase II experiment, except the pH was adjusted to the initial value on each occasion when the serum bottle headspace was purged with N_2. The pH was adjusted to the initial value using 1 M HCl or 1 M NaOH. The pH in each serum bottle was monitored over the duration of the experiment inside the anaerobic glove box. The serum bottles were not purged again with N_2 on days 5 and 6.

3.3 Reagents

Hydrogen (99.99%), and carbon dioxide (99.99%) gases (Praxair, ON) and methane (99.99%, Alltech, IL, USA) were used to calibrate the gas chromatograph (GC) and nitrogen (99.99%, Praxair, ON) was used as the carrier gas.

Glucose (ACP Chemicals, Montreal, Quebec) degradation was monitored using a Dionex 600 ion chromatograph (IC). Ethanol (95%), iso-propanol (99.9%), propanol (99%), iso-butanol (99%), and butanol (99.4%) (BDH Chemicals, Toronto, ON) were analyzed using the Dionex IC configured with the electrochemical detector.

The IC was also calibrated for acetic (C_2) acid (99.8%), propionic (C_3) acid (99.8%), and n-butyric (C_4) acid (99.9%) analysis (Fisher Scientific, Toronto, ON). In this case, the Dionex IC was equipped with a conductivity detector CD 20.

Sodium linoleate ($C_{18:2}$) (99%) and sodium oleate ($C_{18:1}$) (>99%) were prepared *au-bain marie* and dissolved in hot NaOH solution (50°C) using the acid from of each acid and used in the serum bottle studies (Angelidaki and Ahring, 1992). LA and OA were purchased from TCI America.

3.4 Batch Reactors

3.4.1 Inoculum Source

Unacclimated anaerobic digester sludge from an Ethanol facility (Chatham, ON) was used as the seed culture. The seed culture was maintained in a semi-continuous fed batch reactor (Reactor A) at a 3-L liquid volume. Reactor A was maintained at 37±2 °C using a heating tape (TP FG STD, Omegalux, USA) and controlled by a variable transformer (Staco, Inc. Ohio). The volatile suspended solids (VSS) concentration in Reactor A was 20,000 mg l^{-1}. Inoculum from Reactor A was diluted to 6,000 mg l^{-1} VSS using basal medium (Table 3.5) at pH 7.6 to 7.8 into a 4-L semi-continuous fed batch reactor (Reactor B). This reactor was heated in a similar manner as Reactor A. Reactor B was also maintained at a 3-L liquid volume and at 37±2°C. Biomass from the Reactor B was used as an inoculum source for the serum bottle studies (160 ml).

Table 3.5: Basal medium characteristics

Parameter	Concentration, mg l^{-1}
K_2HPO_4	14
$(NH_4)_2SO_4$	10
$NaHCO_3$	6000
NH_4HCO_3	70
$MgCl_2.4H_2O$	9
KCl	25
H_3BO_3	0.05
$FeCl_2.4H_2O$	2.0
$ZnCl_2$	0.05
$MnCl_2.4H_2O$	0.5
$CuCl_2.2H_2O$	0.03
$(NH_4)_6MoO_7.4H_2O$	0.09
$CoCl_2.6H_2O$	0.15
$NiCl_2.6H_2O$	0.05
Na_2SeO_3	0.1
EDTA	1.0
Resazurin	1.0
Yeast extract	10

(Adapted from Lalman and Bagley, 2000)

3.4.2 Operation of Inoculum Reactors

Reactors A and B were operated in semi-continuous fed batch mode and acclimatized to 5,000 mg l^{-1} glucose at 37±2 °C. Both reactors were fed when acetate and gas production measurements indicated that all glucose and byproducts were consumed (within 5 to 6 days). After feeding glucose from a stock solution, the reactor headspace was purged with nitrogen (99.99%) for 2 to 3 minutes. The operational stability for both the reactors was determined using gas production, VFA measurement, pH, and alkalinity (as $CaCO_3$). The serum bottles were prepared after determining the pH, total suspended solids (TSS) and volatile suspended solids (VSS) of Reactor B.

3.4.3 Serum Bottle Preparation

All experiments were conducted in 160 ml serum bottles with a 50 ml total liquid volume maintained at 37±1 °C. The batch reactors were prepared inside a Coy® anaerobic chamber (COY Laboratory Products, Inc., Michigan) containing 70% nitrogen, 20% carbon dioxide and 5% hydrogen (gas mixture, Praxair, Canada). Serum bottles were prepared by adding a known amount of biomass and varying the amount of basal media. The total liquid volume in the serum bottles was maintained at 50 ml. A 50,000 mg l^{-1} LCFA stock solution and a 100,000 mg l^{-1} glucose solution were used to inoculate the serum bottles. Resazurin (1 mg l^{-1}), an indicator, was used to determine the anaerobic condition of the serum bottles.

After preparing the 160 ml serum bottles, they were sealed with 20 mm Teflon® lined septa and aluminum crimp caps and over pressurized with 20 ml of the gas mixture from the anaerobic chamber to avoid negative headspace pressure during sampling. The serum bottles were placed on an orbital shaker (Lab-Line Instruments, Inc.) at 200 rpm and maintained 37±1 °C in an environmental chamber for 1 day prior to the initiation of each experiment to remove any residual hydrogen from the glove box. The pH, total suspended solids (TSS) and VSS were measured following the *Standard Methods for the Examination of Water and Wastewater* (APHA, AWWA, and WEF, 1998).

3.5 Analytical Methods

3.5.1 VFA Measurement

Volatile fatty acids (VFAs) (acetic acid, propionic acid and butyric acid) were analyzed using a DX-600 Ion Chromatograph (IC) (Dionex, California, USA) equipped

with an AS40 automated sampler, a GP50 multi-gradient pump, a LC10 liquid chromatography oven and a CD20 conductivity detector. An IonPac® AS11-HC analytical column (Dionex, California, USA) protected by an IonPac® AG11-HC guard column (Dionex, California, USA) and an ASRS-ULTRA® (4 mm) anion self-regenerating suppressor were used to analyze for the VFAs. The injection loop size was 10 µl. The three eluents used in the method were: eluent A, Milli-Q water (Millipore, Nepean, ON); eluent B, 5 mM NaOH and eluent C, 50 mM NaOH. The total eluent flow rate was 1.0 ml min^{-1} and the individual eluent flow rates were as follows: 0 - 2 mins., 93% A, 7% B; 2 – 6 mins., A ramped from 93% to 0%, B ramped from 7% to 100%; 6 – 9 mins., B ramped from 100% to 50%, C ramped from 0% to 50% and then held until 9.99 mins.; and from 10 – 17 mins., 93% A, 7% B. The regenerant (12.6 mM solution of H_2SO_4) flow rate was set at approximately 3.0 ml min^{-1}. All of the eluents and the regenerant solutions were prepared with Milli-Q grade water.

During each experiment, 0.5 ml of mixed liquor samples was periodically withdrawn from serum bottles. The samples were diluted with deionized water (4.5 ml) and then centrifuged at 1,750 g for 5.0 minutes. The centrate was removed, filtered through 0.45 µm nylon filter-paper (Osmonics Inc., USA) and a resin (Bio-Rad Laboratories, CA) to remove heavy metals. The filtered samples were recovered and analyzed using the Dionex IC.

The calibration standards were prepared in basal media and analyzed in triplicate. The detection limit was 0.5 mg l^{-1} for all the VFAs. To check the instrument calibration, VFA standards prepared in basal media were analyzed. A standard followed by deionized water was placed at every 15 to 18 samples interval.

3.5.2 Glucose and Alcohol Measurement

Glucose and alcohols (ethanol, iso-propanol, propanol, iso-butanol and butanol) were measured using a Dionex DX-600 Ion Chromatograph (IC) (Dionex, California, USA) equipped with as AS40 automated sampler, a GP50 multi-gradient pump, a LC10 liquid chromatography oven and an ED50 electrochemical detector. Sample preparation was the same as for VFA analysis. A 4.0 mm × 250 mm CarboPac^{TM} MA1 (Dionex, CA) analytical column protected by a 4 mm × 50 mm CarboPac^{TM} MA1 (Dionex, CA) guard column was used with a 10 µl injection loop and 480 mM NaOH solution. The total analysis time was 36 minutes. Glucose was detected at approximately 25 minutes, and iso-propanol, ethanol, propanol, iso-butanol and butanol were detected at 7.38, 7.68, 8.98, 10.46 and 11.53 minutes, respectively.

For the calibration graphs, standards were prepared and analyzed in triplicate. The detection limits for glucose and alcohols were 1 and 5 mg l^{-1}, respectively. Standard for analysis were the same as for VFAs.

3.5.3 Headspace Gas Measurement

Headspace samples were withdrawn from the serum bottles by using a 50 µL Hamilton Gastight syringe (Fisher Scientific, Canada). The gas samples were analyzed using a CP-6400 gas chromatograph (Varian Inc. USA) equipped with a thermal conductivity detector (TCD) and a Carbon Shin column (2 m × 2 mm, stainless steel, Restek, USA). A 25 µL gas sample was injected manually into the GC. The analysis time was 1.75 minutes and conducted isothermally at 100 °C. The inlet, detector and oven temperatures were set at 100 °C, 200 °C and 200°C, respectively. Nitrogen gas (99.99%) was used as a carrier gas at a flow rate of 20 ml min^{-1}. The retention times of

hydrogen, methane and carbon dioxide were 0.6 minute, 1.0 minute and 1.56 minute, respectively.

The instrument was calibrated for hydrogen (99.99%), methane (99.99%) and carbon dioxide (99.99%). A known amount of each analyte was injected into 160 ml serum bottles after purging with nitrogen (99.99%) for 2 to 3 minutes. The 160 ml serum bottles were sealed with Teflon lined septa and capped with aluminum crimp seals. The method detection limits were 0.1085 kPa, 0.1267 kPa, and 0.1587 kPa for hydrogen, methane and carbon dioxide, respectively.

3.5.4 LCFA Delivery Method

Straight-chain aliphatic hydrocarbons are hydrophobic in nature and the degree of hydrophobicity increases as the number of methylene groups increases. The solubility of fatty acids decreases rapidly as the number of carbon atoms increases. All LCFAs possessing greater than 12 carbons are considered insoluble in water according to Bloor (1943). The solubility of saturated fatty acids ranges from 680 mg l^{-1} for C_8 acids to 3 mg l^{-1} for C_{18} acids at 20 °C (Ralston and Hoerr, 1942).

Due to the insolubility of LCFAs in aqueous solution, a delivery method was used to increase the amount of LCFAs available to the microorganisms. This is of particular importance since the substrate utilization is dependent on the physical state of hydrocarbon. Wodzinski *et al.* (1972) have shown that naphthalene-degrading microorganisms utilized dissolved naphthalene instead of solid naphthalene directly. Researchers have used several approaches to disperse LCFAs into aqueous solution. Diethyl ether was used to disperse LCFAs into aqueous solution (Lalman and Bagley, 2004). This organic solvent assisted in dispersing the LCFAs into aqueous solution and

thus, increases the substrate availability. However, a major problem associated with this solvent is its 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 into a hot and vigorously stirred NaOH solution. The quantities of sodium hydroxide used (expressed as g of NaOH g^{-1} of LCFA) are provided in the Table 3.6.

Table 3.6: Amount of NaOH used for LCFA stock solution preparation

LCFA	NaOH (g g^{-1} LCFA)
Oleic	0.142
Linoleic	0.143

Amounts are expressed as g NaOH per g of LCFA.

3.5.5 Solids Measurement

The total suspended solids (TSS) (5 ml) and volatile suspended solids (VSS) (5ml) of the batch reactors were measured at the beginning of each experiment. The analyses were conducted in duplicate following the *Standard Methods for the Examination of Water and Wastewater* (APHA, AWWA, and WEF, 1998).

CHAPTER 4

BATCH REACTORS

4.1 Batch Reactor Operation

Two semi-continuous fed batch reactors A and B were maintained at 37 ± 2°C with approximately 20,000 mg l^{-1} and 6,000 mg l^{-1} VSS, respectively. The average alkalinity in the reactors was 4,500 ± 300 mg l^{-1} (as $CaCO_3$) and the pH range was 7.4 to 7.6. A schematic of reactors A and B is shown in Figure 4.1.

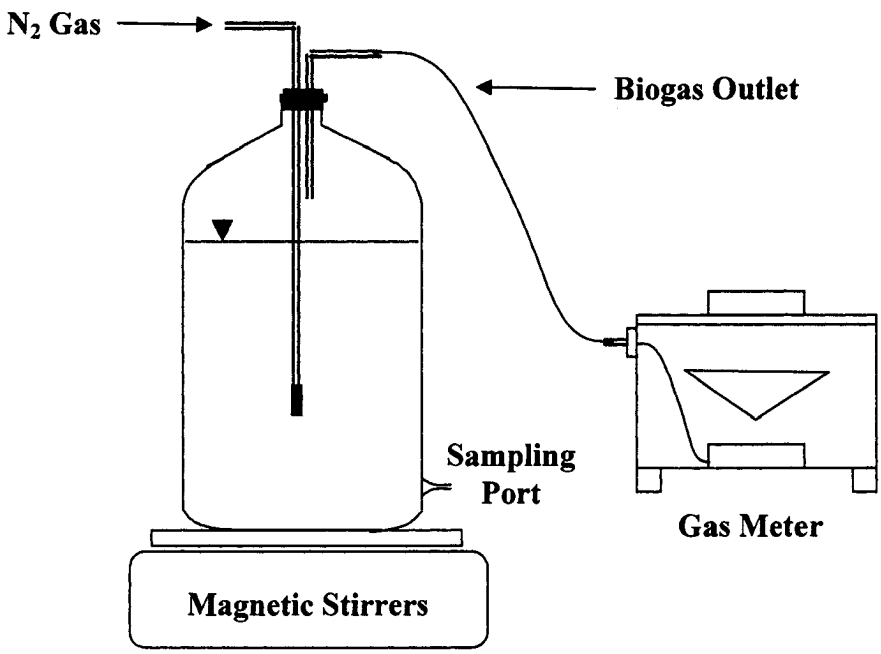

Figure 4.1: Reactor B schematic (similar for reactor A).

4.2 Experimental Results

Both reactors A and B were fed with 5,000 mg l^{-1} of glucose every 6 to 7 days. The total gas production and VFAs production profiles of reactor B are shown in Figures 4.2 and 4.3, respectively.

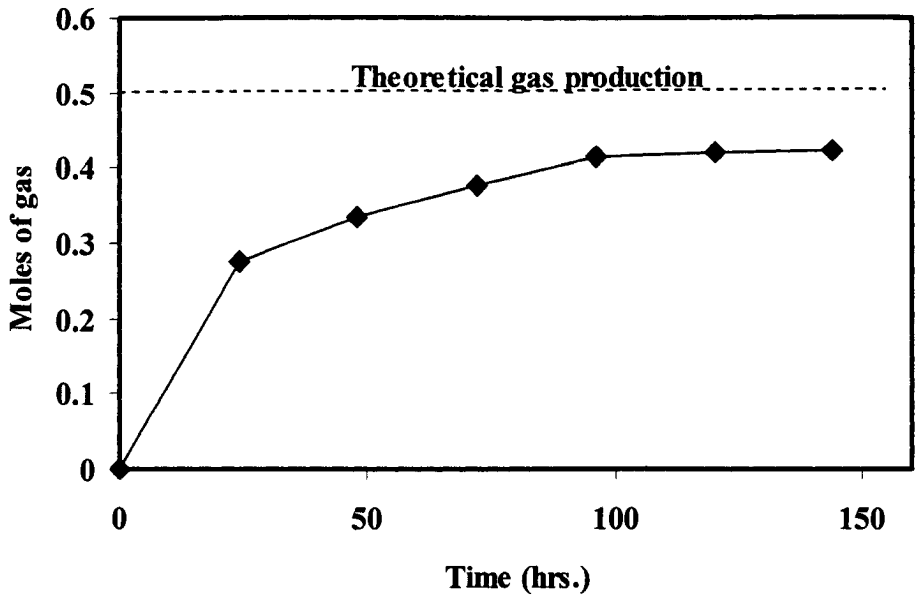

Figure 4.2: Gas production profile for reactor B.
---------- Theoretical gas production

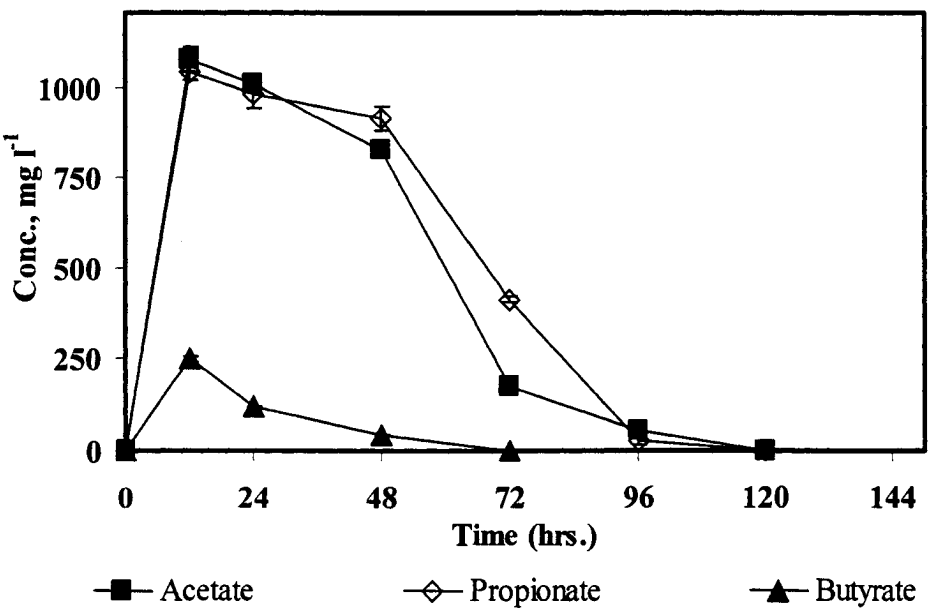

Figure 4.3: VFA production profiles for reactor B (average with SD for triplicate samples are shown).

The total gas production was calculated assuming the composition of methane and carbon dioxide was a ratio of 1:1 (mole:mole). Most of the glucose added to the reactor was converted into gas at end of 6 days. The total gas production in the reactor B was approximately 82% of theoretical gas production. The major VFAs detected during glucose degradation were acetate, propionate, and butyrate (Figure 4.3). The acetate concentrations in the reactor peaked (approximately 1,100 mg l^{-1}) at 12 hours after adding glucose. The maximum amount of propionate, approximately 1,000 mg l^{-1} was observed 12 hours after adding glucose, whereas, the butyrate concentration was only 250 mg l^{-1}. After 120 hours VFAs were undetectable.

CHAPTER 5

EFFECTS OF LINOLEIC ($C_{18:2}$) ACID ON HYDROGEN PRODUCTION FROM GLUCOSE

5.1 Experimental Results

5.1.1 LA or Glucose Degradation in Control Cultures

In control experiments conducted with LA or glucose, no detectable amounts of hydrogen were observed in the headspace. Acetate accumulation in cultures fed with 500, 1,000, 1,500 and 2,000 mg l^{-1} LA was observed (data not shown). In cultures receiving glucose, the byproducts observed included methane, acetate, propionate and butyrate.

5.1.2 Hydrogen and Methane Production

The effects of adding LA on hydrogen and methane production from glucose degradation are shown in Figures 5.1 and 5.2, respectively. Notice hydrogen only accumulated in the cultures receiving LA and in the controls none was detected.

Increasing quantities of hydrogen was observed in cultures receiving increasing LA concentrations. In cultures fed with 500 or 1,000 mg l^{-1} LA, peak hydrogen levels were recorded after 12 hours, whereas, a 12-hour lag was observed in cultures receiving 1,500 and 2,000 mg l^{-1} LA. Hydrogen reached a maximum level at approximately 24 hours in the cultures inoculated with 1,500 or 2,000 mg l^{-1} LA. In general, the amount of hydrogen produced was a function of the LA concentration.

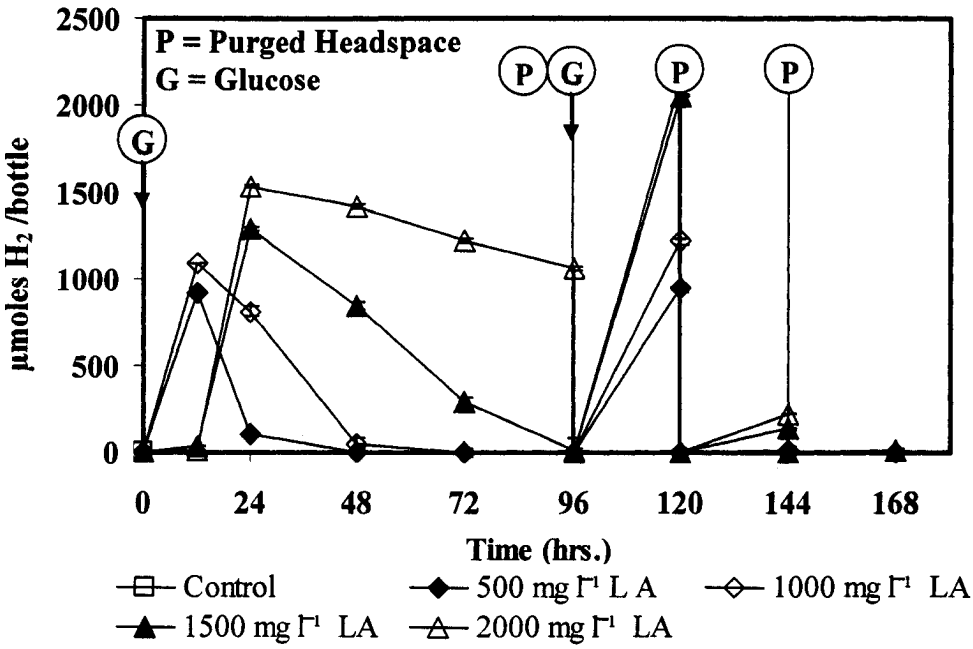

Figure 5.1: Effect of varying LA concentration on hydrogen production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

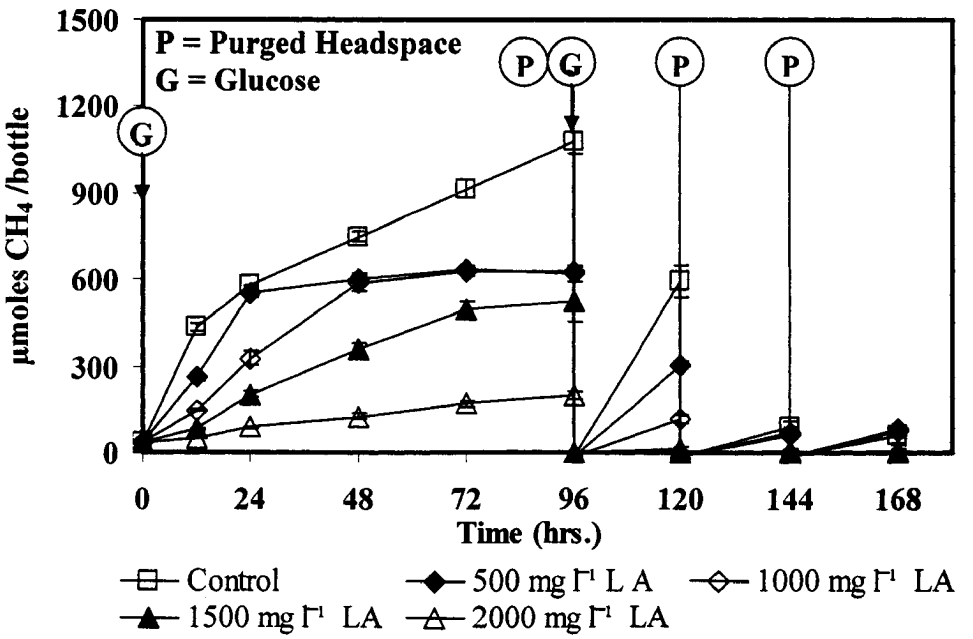

Figure 5.2: Effect of varying LA concentration on methane production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

The maximum amount of hydrogen produced, after the first injection of glucose, was 1.1 moles H_2.mole^{-1} glucose (Table 5.1) in cultures inoculated with 2,000 mg l^{-1} LA.

After reaching peak levels, the hydrogen concentration decreased in all cultures inoculated with LA. Notice the hydrogen removal rates decreased with increasing LA concentrations. On day 4 following the first glucose injection, no detectable amounts of hydrogen were observed in the cultures except those inoculated with 2,000 mg l^{-1} LA, where approximately 80% of the peak amount remained.

The quantity of hydrogen produced after the second glucose injection was greater than the first. A maximum yield of 1.6 moles H_2.mole^{-1} glucose (Table 5.1) was observed in cultures receiving 2,000 mg l^{-1} LA. This represents a 45% increase over the peak amount observed during the first glucose injection. These findings suggest that a combination of LCFAs and VFAs might be more inhibitory to the hydrogen consuming microorganisms than LA alone. More than 90% of the total hydrogen was produced within the first 24 hours following the injection. An additional 5% to 10% of the total hydrogen was produced on days 5 and 6.

The methane production trend (Figure 5.2) from the first glucose injection was opposite to the trend observed for hydrogen production. A decrease in methane production was observed in cultures receiving increasing amounts of LA. In the control cultures (no LA), methane accumulated until day 4. The total amount of methane on day 4 in cultures receiving 2,000 mg l^{-1} LA was approximately 20% of the control cultures. A similar pattern of methane accumulation was also observed in the cultures after the second glucose injection. In the control cultures, methane reached a maximum level at 24 hours after receiving glucose again. The total amount of methane observed in cultures

inoculated with LA at 72 hours after re-injection was less than the amounts observed after the first glucose addition.

Table 5.1: Hydrogen yield (mole H_2.mole^{-1} glucose) from glucose (5,000 mg l^{-1}) injected into cultures receiving LA (0 to 2,000 mg l^{-1})

	Linoleic Acid Concentration (mg l^{-1})				
	0	**500**	**1000**	**1500**	**2000**
1st Injection	ND	0.67 ± 0.02	0.79 ± 0.11	0.93 ± 0.02	1.11 ± 0.03
2nd Injection	ND	0.68 ± 0.06	0.87 ± 0.17	1.48 ± 0.08	1.55 ± 0.20

Note: Average with standard deviation for triplicate samples is shown.
ND = Not detected

5.1.3 VFA Production

The major VFAs detected during glucose degradation were acetate, propionate, and butyrate (Figures 5.3, 5.4 and 5.5). Acetate was produced in all the cultures after glucose addition. In the control cultures, the maximum acetate concentration was recorded on days 3 and 6 after injecting glucose into the cultures on days 0 and 4, respectively. In general, the acetate concentration in cultures inoculated with LA, reached maximum levels on day 3 after the first glucose injection and increased after the second glucose injection until day 7. Before the second glucose injection on day 4, the acetate concentration in the cultures inoculated with LA ranged from approximately 600 mg l^{-1} to 800 mg l^{-1} and on day 7, the lowest quantity was 1,300 mg l^{-1} and the maximum was 1,700 mg l^{-1}.

Propionate was also produced in the cultures after glucose addition. In the controls, the maximum propionate concentrations were recorded on days 4 and 6 after

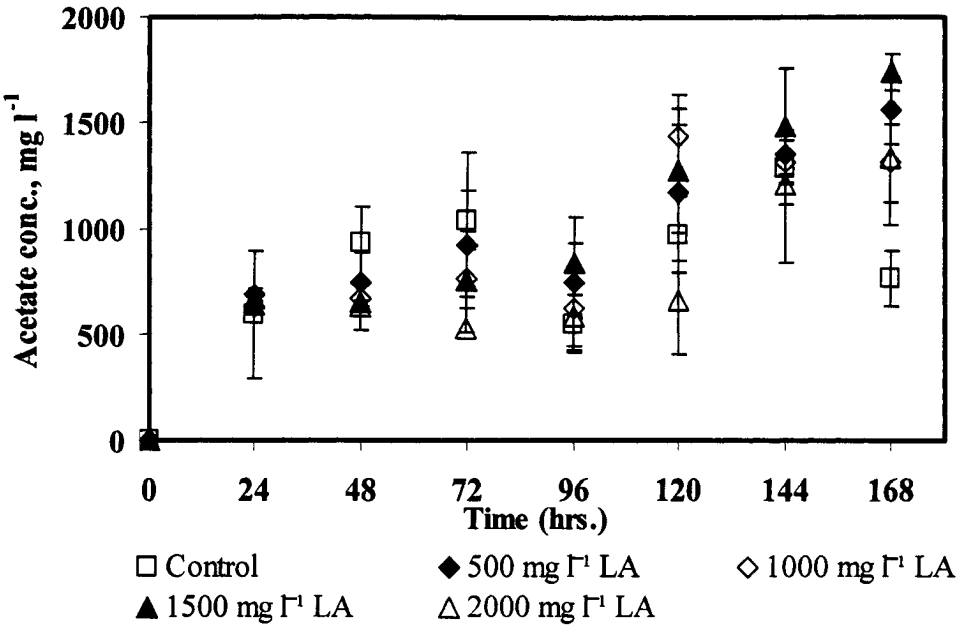

Figure 5.3: Effect of varying LA concentration on acetate production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

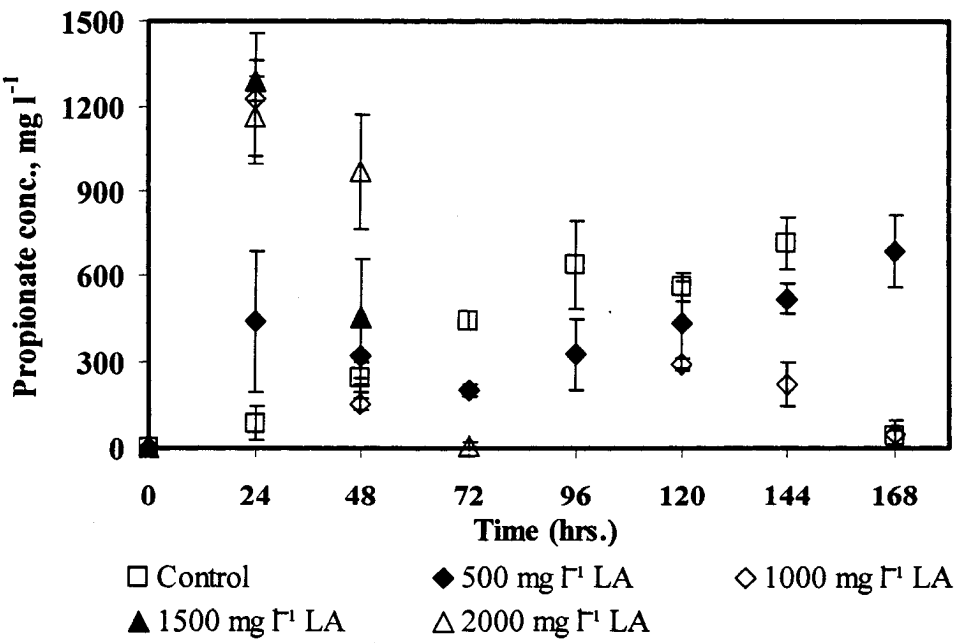

Figure 5.4: Effect of varying LA concentration on propionate production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

glucose was added on days 0 and 4, respectively. In general, the propionate concentration in the cultures inoculated with LA, reached maximum levels within 1 day of the first glucose injection and not much propionate was produced after the second glucose injection on day 4. The changes in propionate production after second glucose addition are likely due to a change in microbial community in the cultures.

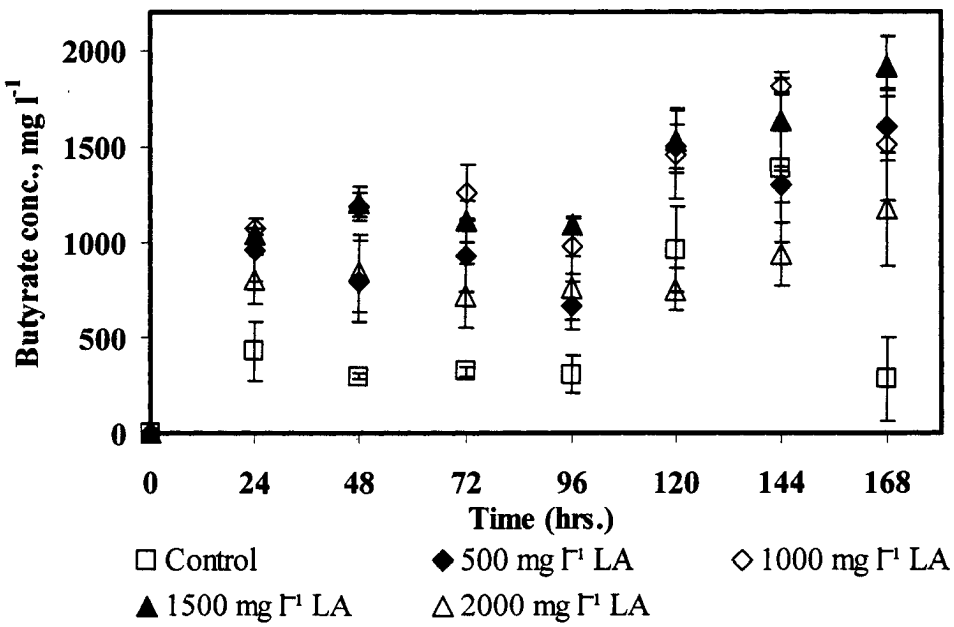

Figure 5.5: Effect of varying LA concentration on butyrate production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

Butyrate was produced in all the cultures after glucose addition. In the control cultures, the butyrate concentration reached peak levels on days 1 and 6 after glucose injection into the cultures on days 0 and 4, respectively. The maximum butyrate concentration was recorded in cultures inoculated with LA, on day 3 after the first glucose injection and increased after the second glucose injection until day 7. Before the

second glucose injection on day 4, the butyrate concentration in the cultures inoculated with LA ranged from approximately 300 mg l^{-1} to 1,000 mg l^{-1} and on day 7, the lowest quantity was 1,100 mg l^{-1} and the maximum was 1,700 mg l^{-1}. In general the maximum butyrate levels were associated with increasing LA concentrations.

5.1.4 Glucose Degradation

Glucose degradation in cultures receiving 5,000 mg l^{-1} and varying amount of LA is shown in the Figure 5.6. Glucose degradation was inhibited in the cultures inoculated with LA. In the control cultures most of the glucose was degraded within 8 hours, whereas an 8-hour lag was observed in the cultures receiving 1,500 or 2,000 mg l^{-1} LA. The glucose initial degradation rates (Table 5.2) were affected by the presence of LA however, glucose was degraded to undetectable levels between 16 to 24 hours.

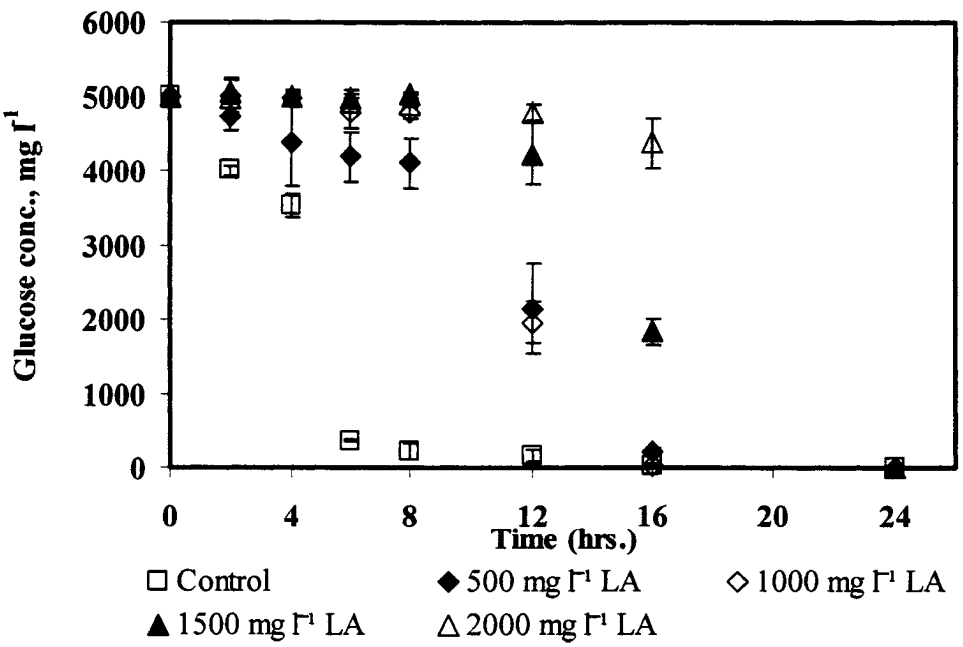

Figure 5.6: Effect of varying LA concentration on glucose degradation in cultures receiving 5,000 mg l^{-1} glucose. (glucose added at 0 hr, average with SD for triplicate samples are shown)

Table 5.2: Glucose initial degradation rates ($\mu g.mgVSS^{-1}.min^{-1}$) for cultures receiving LA and 5,000 mg l^{-1} glucose

Linoleic Acid Concentrations (mg l^{-1})				
0	**500**	**1000**	**1500**	**2000**
7.68±0.07	2.68±0.38	1.93±0.28	1.64±0.12	1.32±0.05

Note: Average with standard deviation for triplicate samples is shown.

5.2 Discussion of Results

Inhibition of methanogens is important in attaining a high hydrogen yield from carbohydrates degradation. The presence of LA assisted in redirecting the reducing equivalents for proton reduction. Hydrogen gas does not ordinarily accumulate to such high concentrations in mixed culture anaerobic reactors because of hydrogen consumption by methanogens and other hydrogen consuming microbes. The inhibitory effects of LCFAs on aceticlastic and hydrogenotrophic methanogens have been reported by many researchers (Lalman and Bagley, 2001, 2002; Angelidaki and Ahring, 1992; Koster and Cramer, 1987; Hanaki *et al.*, 1981; and Demeyer and Hendrickx, 1967). However, no study has reported the hydrogen production potential when using LCFAs during glucose fermentation is reported.

In general an increasing amount of hydrogen was observed in cultures inoculated with elevated amounts of LA. Approximately 1.11 to 1.55 moles $H_2.mole^{-1}$ glucose was observed in the cultures inoculated with 2,000 mg l^{-1} LA at 37°C. The glucose to hydrogen conversion efficiency of 28% to 39% is based on a theoretical maximum of 4 moles $H_2.mole^{-1}$ glucose (reaction 2.3). The conversion efficiencies of 28% to 39% obtained in this study is greater than the 23% (0.92 moles $H_2.mole^{-1}$ glucose) reported by Logan *et al.* (2002) using heat shocked mixed cultures from soil at 26°C and a pH of 6.0.

In a two-phase anaerobic system, Kraemer and Bagley (2005) obtained 1.38 moles H_2.mole^{-1} glucose (35% conversion efficiency) at the first stage using mixed cultures at 35°C and pH 5.5 with a 10 hour HRT. Higher hydrogen yields have also been reported using acclimated mixed cultures or pure cultures. In batch tests, Schröder *et al.* (1994) observed 4.0 moles H_2·mol^{-1} glucose using *Thermotoga maritima* at 80°C and a system without pH control. In continuous culture tests, using an acclimated mixed culture from sewage sludge at 35°C and a pH of 5.7, Chen *et al.* (2001) reported 1.7 moles H_2.mole^{-1} glucose (43% conversion efficiency). In this work, a comparatively higher hydrogen yield was observed in the cultures after the second glucose addition in the presence of LCFAs and VFAs. These finding are similar to Sung *et al.* (2002), who reported operating a continuous flow system at 37°C using heat treated mixed cultures with relatively high VFA concentrations. Previous studies have shown gas sparging is an effective method to increase the hydrogen yield by reducing hydrogen partial pressure in the liquid phase. Hussy *et al.* (2005) observed an improved hydrogen yield from 1.0 mole H_2.mole^{-1} glucose to 1.9 moles H_2.mole^{-1} glucose using nitrogen sparging in a continuous reactor maintained with mixed microflora at pH 5.2 and 32°C. However, intermittent nitrogen sparging was not successful to increase hydrogen production in this study, likely because the degradation of butyrate and propionate were inhibited by LA (Kim *et al.*, 2004). The methane production data observed in this experiment is strongly evidence that the hydrogenotrophic methanogens were inhibited by LCFAs.

Hydrogen production from carbon substrates is usually accompanied by short chain fatty acid production. The production of elevated intermediate levels reflects changes in the metabolic pathway of the microorganisms involved in carbohydrates

degradation. There are two common types of acidogenic reactions for hydrogen production: this includes acetate type and butyrate type. Acetate and butyrate accumulation were observed in the cultures inoculated with LA. Kim *et al.* (2004) reported that even 30 to 50 mg l^{-1} LA inhibited aceticlastic methanogens and propionate degradation. In this work, a sharp increase in the propionate concentration was observed after adding glucose in cultures inoculated with LA. This is likely due to the high hydrogen partial pressure and the impact of LA inhibition on propionate degrading organisms. Mykhaylovin *et al.* (2005) reported inhibition on butyrate degradation in the presence of 50 mg l^{-1} LA. Approximately 15% to 20% butyrate (on carbon basis) and 12% to 17% acetate (on carbon basis) were observed in the cultures inoculated with LA. The yield of 1.11 to 1.55 moles H_2.$mole^{-1}$ glucose observed in this work indicates butyrate type fermentation was dominant in the cultures rather than the acetate type.

Glucose degradation was inhibited in the cultures inoculated with LA. An increasing inhibitory effect was observed in cultures receiving increasing amounts of LA. Alosta *et al.* (2004), and Lalman and Bagley (2002) also reported inhibitory effects of LA on glucose degradation using mixed cultures. However, Zheng and Yu (2004) observed glucose (5,000 mg l^{-1}) was degraded rapidly and depleted within 12 hours in the cultures operating at 30°C and at a pH of 8.0.

CHAPTER 6

EFFECTS OF OLEIC ($C_{18:1}$) ACID ON HYDROGEN PRODUCTION FROM GLUCOSE

6.1 Experimental Results

6.1.1 OA or Glucose Degradation in Control Cultures

Control experiments were conducted with OA or glucose and hydrogen was not detected in the headspace. Acetate produced (data not shown) in cultures inoculated with 500, 1,000, 1,500 and 2,000 mg l^{-1} OA. Methane, acetate, propionate and butyrate were observed as byproducts in the control cultures receiving glucose.

6.1.2 Hydrogen and Methane Production

Hydrogen and methane production from glucose degradation in the cultures inoculated with OA are shown in Figures 6.1 and 6.2, respectively. Hydrogen accumulation was observed in cultures inoculated with OA. In cultures receiving elevated OA concentrations increasing amount of hydrogen was observed. The maximum amount of hydrogen was recorded at 12 hours in cultures inoculated with 500 mg l^{-1} OA, whereas a 12-hour lag was observed in cultures receiving more than 1,000 mg l^{-1} OA. In cultures inoculated with 1,000, 1,500 and 2,000 mg l^{-1} OA, hydrogen reached a maximum at approximately 24 hours. The maximum amount of hydrogen produced, after the first glucose injection was 1.0 mole $H_2 \cdot mole^{-1}$ glucose in cultures inoculated with more than 1,000 mg l^{-1} OA (Table 6.1). No increment of hydrogen yield was obtained in cultures inoculated with more than 1,000 mg l^{-1} OA.

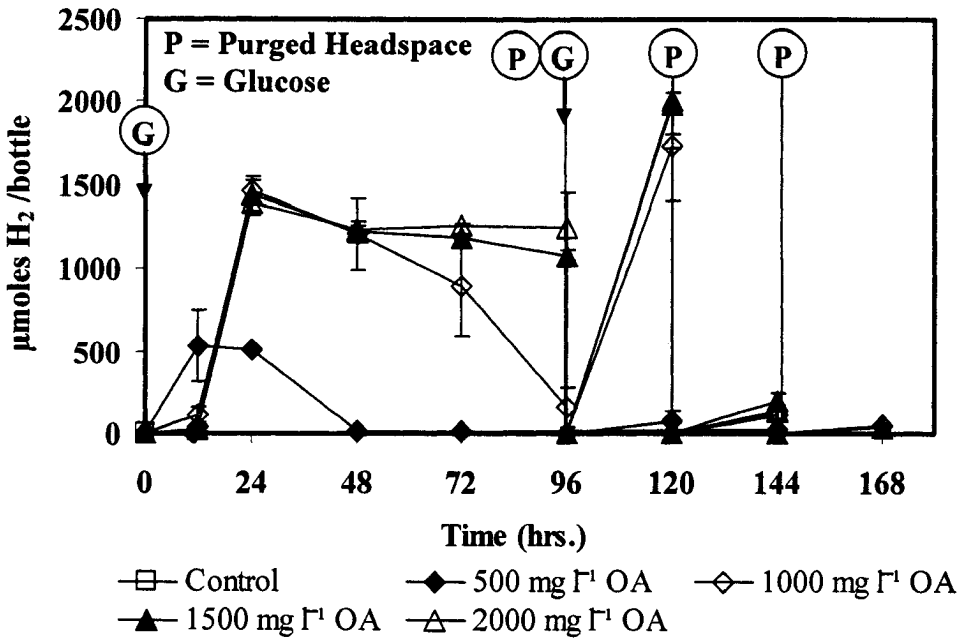

Figure 6.1: Effect of varying OA concentration on hydrogen production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

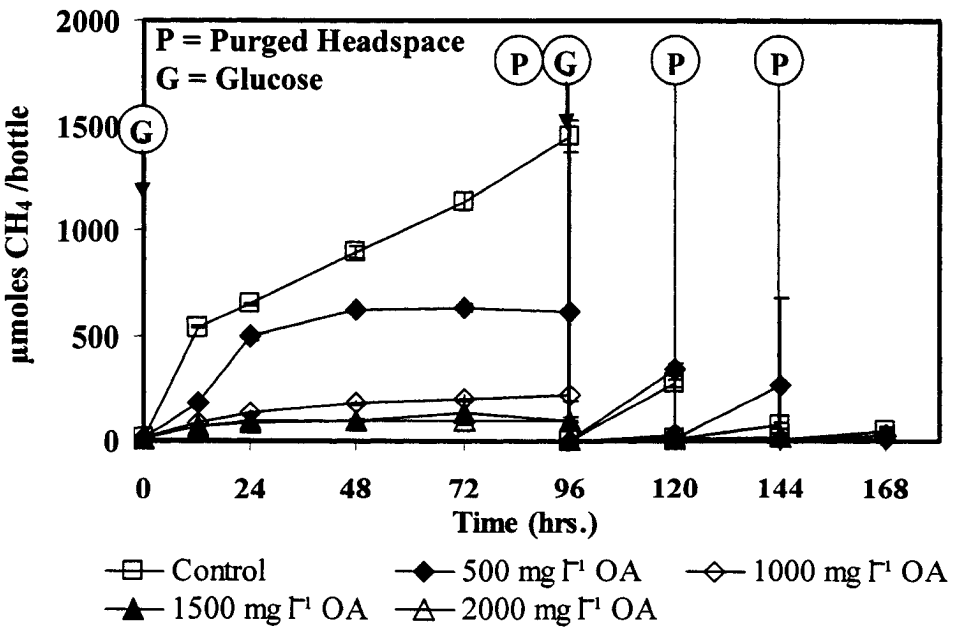

Figure 6.2: Effect of varying OA concentration on methane production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

Table 6.1: Hydrogen yield (mole H_2.mole^{-1} glucose) from glucose (5,000 mg l^{-1}) injected into cultures receiving OA (0 to 2,000 mg l^{-1})

	Oleic Acid Concentration (mg l^{-1})				
	0	**500**	**1000**	**1500**	**2000**
1st Injection	ND	0.38 ± 0.16	1.05 ± 0.04	1.03 ± 0.08	1.00 ± 0.03
2nd Injection	ND	0.05 ± 0.04	1.24 ± 0.24	1.43 ± 0.19	1.44 ± 0.15

Note: Average with standard deviation for triplicate samples is shown.
ND = Not detected

The hydrogen concentration decreased after reaching peak levels. In general faster hydrogen consumption was observed in cultures containing decreasing amounts of OA. On day 4 following the first glucose injection, significant amounts of hydrogen remained in the cultures inoculated with 1,500 and 2,000 mg l^{-1} OA. Approximately 80% of the peak amount recorded on day 1 remained on day 4.

After the second glucose injection, the maximum yield was approximately 1.44 moles H_2.mole^{-1} glucose (Table 6.1) in cultures receiving 1,500 or 2,000 mg l^{-1} OA. This was a 40% increase in hydrogen production compared to the peak amount observed during the first glucose injection. This increase in hydrogen production is likely due to the presence both of LCFAs and VFAs, which might be more inhibitory to the hydrogen consumers than OA alone. Most of the hydrogen was produced within the first 24 hours following the glucose injection. Only an additional 5% to 10% of the total hydrogen was produced on day 2 after the second injection, even though the headspace was sparged with N_2.

A decreasing trend in methane production was observed in cultures receiving increasing amounts of OA. In the controls (no OA), methane accumulated until day 4. The total amount of methane recorded on day 4 in cultures receiving 2,000 mg l^{-1} OA

was approximately 15% of the control cultures. The methane production pattern was similar after the second glucose injection. The total amount of methane produced by 72 hours following the glucose re-injection was less than the amounts observed after the first glucose addition.

6.1.3 VFA Production

The major VFAs detected during glucose degradation were acetate, propionate, and butyrate (Figures 6.3, 6.4 and 6.5). Acetate accumulated in cultures after receiving glucose. The acetate concentration was greater in cultures inoculated with OA than the control cultures (no OA). The maximum acetate concentrations were recorded on days 3 and 7 after glucose injection into cultures on days 0 and 4, respectively. Before the second glucose injection on day 4, the acetate concentration in the cultures inoculated with OA ranged from approximately 700 mg l^{-1} to 1,100 mg l^{-1} and on day 7, the lowest quantity was 1,700 mg l^{-1} and the maximum was 2,300 mg l^{-1}. A similar pattern in acetate accumulation was observed in cultures inoculated with LA. These findings indicate the inhibition of aceticlastic methanogens by LA or OA.

Propionate accumulated in cultures after injecting glucose especially after the second glucose addition on day 4. The propionate concentration was greater in the control cultures compared to the cultures inoculated with OA. On day 7, the maximum propionate concentration was approximately 1,300 mg l^{-1} in the control cultures, whereas it was 700 to 1,000 mg l^{-1} in the cultures receiving OA. The propionate accumulation pattern in cultures inoculated with OA was different than in the cultures inoculated with LA. In experiment with LA, the propionate concentrations were greater on day 1 but some degradation was observed.

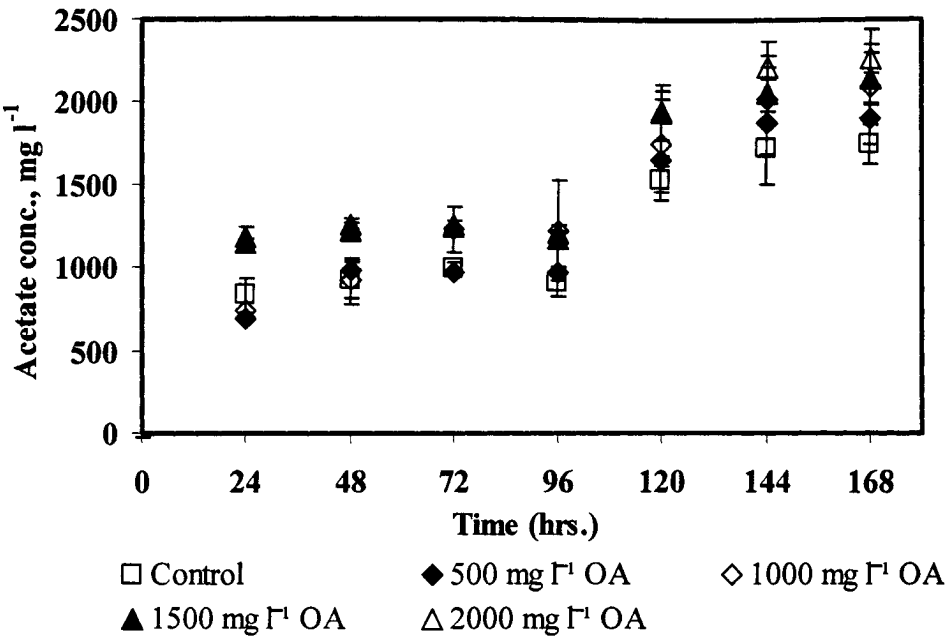

Figure 6.3: Effect of varying OA concentration on acetate production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

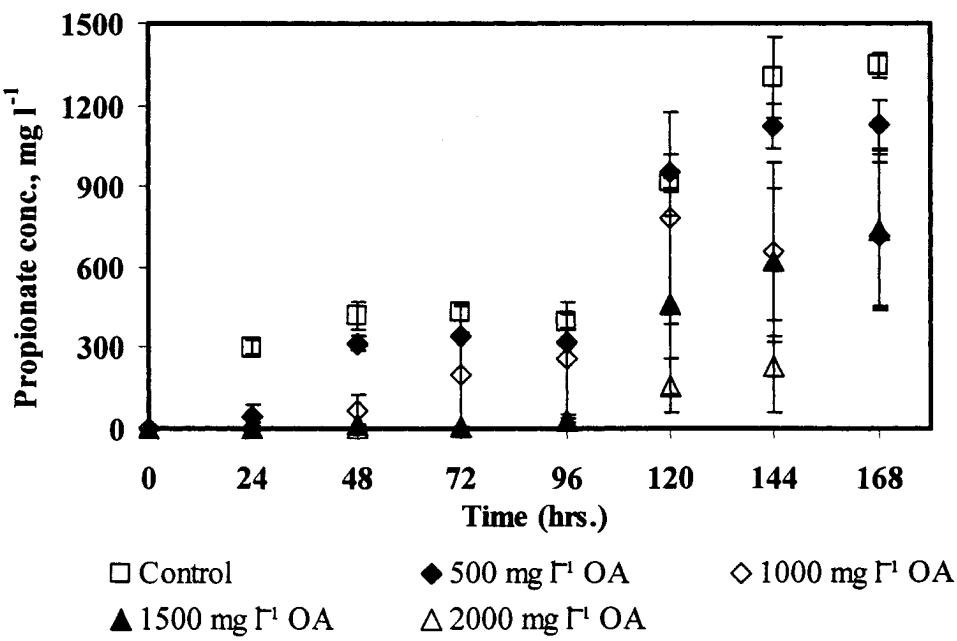

Figure 6.4: Effect of varying OA concentration on propionate production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr, average with SD for triplicate samples are shown).

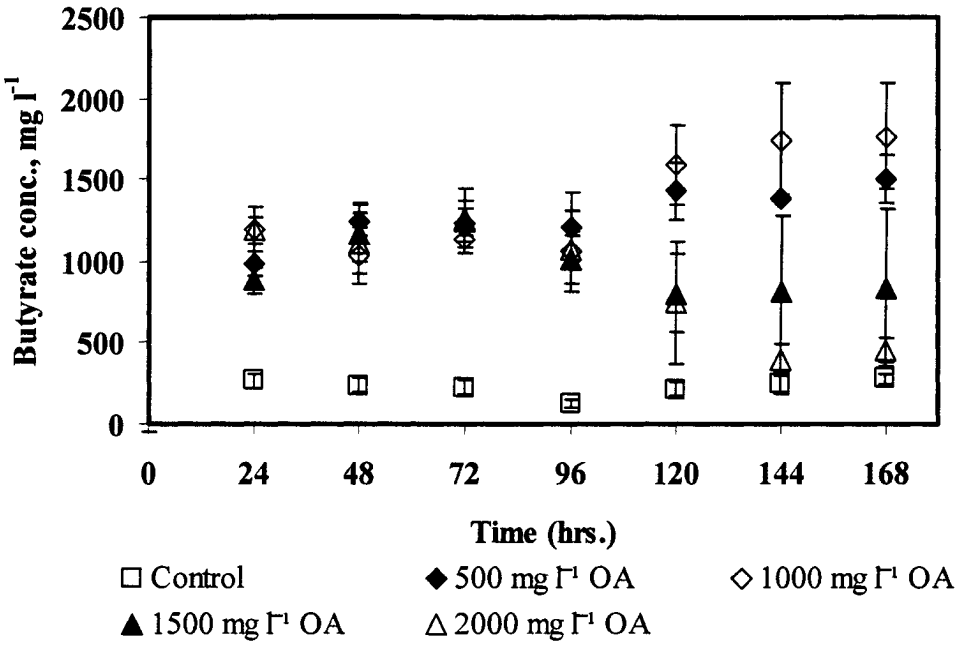

Figure 6.5: Effect of varying OA concentration on butyrate production in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr. average with SD for triplicate samples are shown).

In the control cultures, the butyrate concentration was less than 300 mg l^{-1} throughout the experiment. Butyrate accumulated in the cultures inoculated with 500 and 1,000 mg l^{-1} OA, and the maximum butyrate concentrations were recorded on days 3 and 7 after glucose injection into the cultures on days 0 and 4, respectively. In cultures inoculated with 1,500 and 2,000 mg l^{-1} OA, the butyrate concentration reached maximum levels on day 3 after the first glucose addition and not much accumulated after the second glucose addition. Before the second glucose injection on day 4, the butyrate concentration in cultures inoculated with OA ranged from approximately 900 mg l^{-1} to 1,200 mg l^{-1}. On day 7, the lowest amount was 300 mg l^{-1} in the control cultures while a maximum of 1,600 mg l^{-1} was observed in cultures inoculated with OA.

6.1.4 Glucose Degradation

Glucose degradation in the cultures receiving 5,000 mg l^{-1} glucose and varying amounts of OA is shown in Figure 6.6. Glucose degradation was inhibited in cultures inoculated with OA (Table 6.2). The data indicated that OA was inhibitory to glucose degradation. In the control cultures, most of the glucose was degraded by 6 hours, whereas only 10% to 25% of the glucose was degraded in the cultures inoculated with OA by the same time. Glucose was degraded to undetectable levels between 16 to 24 hours in the cultures inoculated with OA.

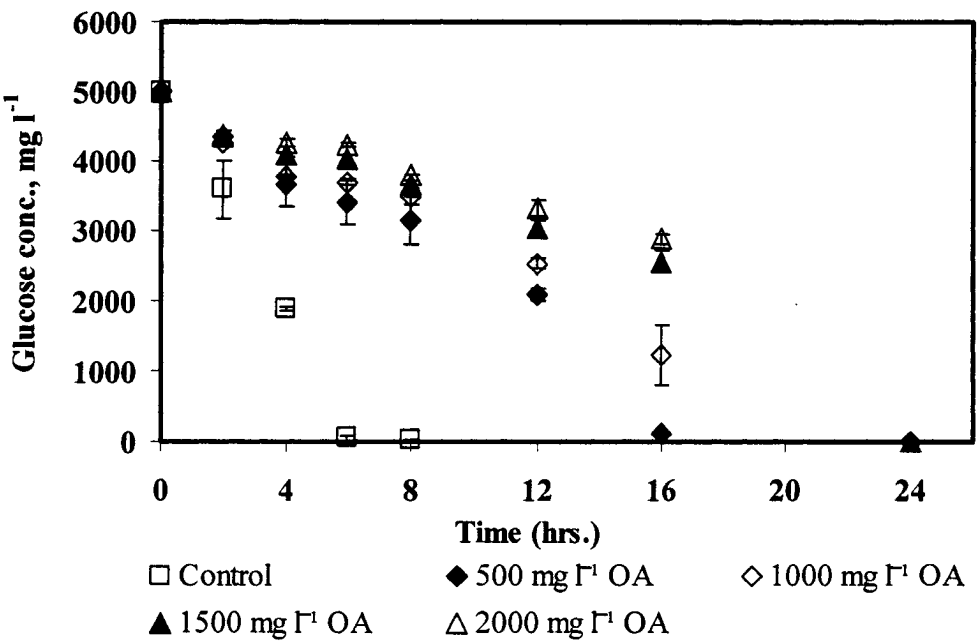

Figure 6.6: Effect of varying OA concentration on glucose degradation in cultures receiving 5,000 mg l^{-1} glucose (glucose added at 0 hr, average with SD for triplicate samples are shown).

Table 6.2: Glucose initial degradation rates ($\mu g.mgVSS^{-1}.min^{-1}$) for cultures receiving OA and 5,000 mg l^{-1} glucose

Oleic Acid Concentrations (mg l^{-1})				
0	**500**	**1000**	**1500**	**2000**
7.71±0.49	2.82±0.31	1.79±0.14	1.58±0.04	1.56±0.03

Note: Average with standard deviation for triplicate samples is shown.

6.2. Discussion of Results

An increasing amount of hydrogen was observed in the cultures inoculated with 1,500 or 2,000 mg l^{-1} OA. The maximum hydrogen yield was approximately 1.44 moles $H_2.mole^{-1}$ glucose (36% glucose to hydrogen conversion efficiency based on a theoretical maximum of 4 moles $H_2.mole^{-1}$ glucose) in cultures inoculated with 1,500 or 2,000 mg l^{-1} OA. The maximum amount of hydrogen (1.44 moles $H_2.mole^{-1}$ glucose) in cultures fed with OA was less than the quantity (1.55 moles $H_2.mole^{-1}$ glucose) observed in the presence of LA. This is likely due to the greater inhibition effect caused by LA on hydrogenotrophic methanogens. The yield obtained in the presence of LA or OA was greater than the yield of 0.72 moles $H_2.mole^{-1}$ glucose reported by Roychowdhury *et al.* (1998). Logan *et al.* (2002) reported a 23% conversion efficiency (0.92 moles $H_2.mole^{-1}$ glucose) in batch tests using heat shocked mixed cultures from soil at 26°C and a pH of 6.0. Higher hydrogen yields have also been observed using mixed cultures at lower initial pH. Khanal *et al.* (2004) obtained 1.8 moles $H_2.mole^{-1}$ glucose (45% conversion efficiency) in the batch tests using heat shocked mixed cultures at 37°C and an initial pH of 4.5. In continuous cultures, Chen *et al.* (2001) measured 3.47 moles $H_2.mole^{-1}$ sucrose (43% conversion efficiency based on a theoretical maximum of 8 moles $H_2.mole^{-1}$ sucrose) using an acclimated culture at 35°C and a pH of 6.7. In batch tests, Kumar and

Das (2000) reported 2.2 moles H_2.mole^{-1} glucose (55% conversion efficiency) using *Enterobacter cloacae* IIT-BT 08 at 36°C and a pH of 6.0. The conversion efficiency or hydrogen yield of a mixed anaerobic inoculum in the presence of LCFA can be increased by controlling the pH and using acclimated cultures in a continuous flow reactor. Though researchers have reported improved hydrogen yields with gas sparging, intermittent nitrogen sparging was not successful in increasing the hydrogen production in this study, likely because the degradation of butyrate and propionate were inhibited by OA.

Short chain fatty acids are the common byproducts during carbohydrates degradation. Two common types of acidogenic reactions producing hydrogen are acetate-type and butyrate-type. Kim *et al.* (2004) reported that 150 to 250 mg l^{-1} OA inhibited aceticlastic methanogens and propionate degradation. Mykhaylovin *et al.* (2005) observed inhibition on butyrate degradation in the presence of 50 mg l^{-1} OA. Though the acetate accumulated in the control cultures (no OA), hydrogen was undetectable. This was likely due to hydrogenotrophic activity in the control cultures. Approximately 18% to 22% acetate (on carbon basis) and 8% to 19% butyrate (on carbon basis) were observed in cultures inoculated with OA, which indicates acetate type fermentation was dominant rather than butyrate type. However, only 1.24 to 1.44 moles H_2.mole^{-1} glucose was observed in cultures inoculated with OA, where propionate accumulated.

Glucose degradation was inhibited in cultures inoculated with OA. An increasing inhibitory effect was observed in cultures receiving increasing amounts of OA. Alosta *et al.* (2004), and Lalman and Bagley (2002) also reported inhibitory effects of OA on glucose degradation using mixed cultures.

CHAPTER 7

EFFECTS OF LINOLEIC ($C_{18:2}$) ACID OR OLEIC ($C_{18:1}$) ACID ON HYDROGEN PRODUCTION FROM GLUCOSE AT DIFFERENT INITIAL pH

7.1 Experimental Results

To assess the effects of initial pH on hydrogen production from glucose experiments were conducted with 0 and 2,000 mg l^{-1} LCFA (LA or OA) at initial pH conditions of 7.6, 6.0, and 5.0.

7.1.1 Hydrogen and Methane Production

The effects of initial pH and the presence of LCFA on hydrogen and methane production from glucose are shown in Figures 7.1 to 7.4, respectively.

An increasing trend in hydrogen production was observed in cultures with reduced initial pH. In cultures inoculated with LCFA and at an initial pH of 7.6 and 6.0, hydrogen reached maximum levels within 24 hours, whereas, the cultures at an initial pH of 5.0 it took 72 hours to reach maximum hydrogen levels. The maximum yield after the first glucose addition was 1.58 to 1.43 moles $H_2.mole^{-1}$ glucose (Table 7.1) in cultures inoculated with LCFA at an initial pH of 5.0. After reaching maximum levels, the hydrogen concentration decreased in cultures with an initial pH value of 7.6 and 6.0, whereas, hydrogen consumption was insignificant in cultures with an initial pH of 5.0. The quantity of hydrogen produced after readjusting the pH and glucose addition in the cultures was greater compared to the first glucose injection.

The maximum yields were 2.37 and 2.14 moles $H_2.mole^{-1}$ glucose (Table 7.1) in the cultures inoculated with LA and OA, respectively, at an initial pH of 5.0. This was a

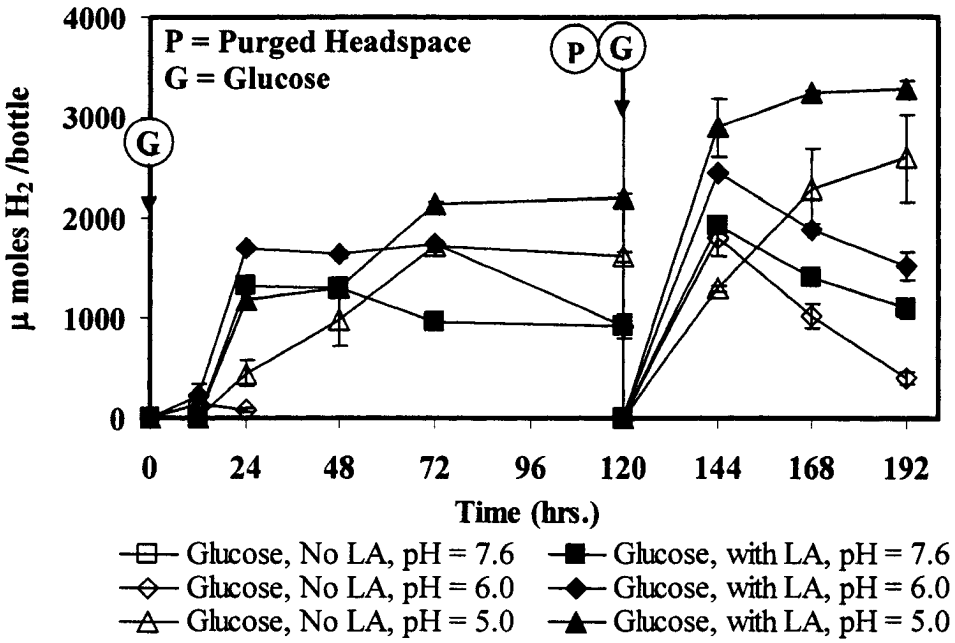

Figure 7.1: Effect of different initial pH on hydrogen production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

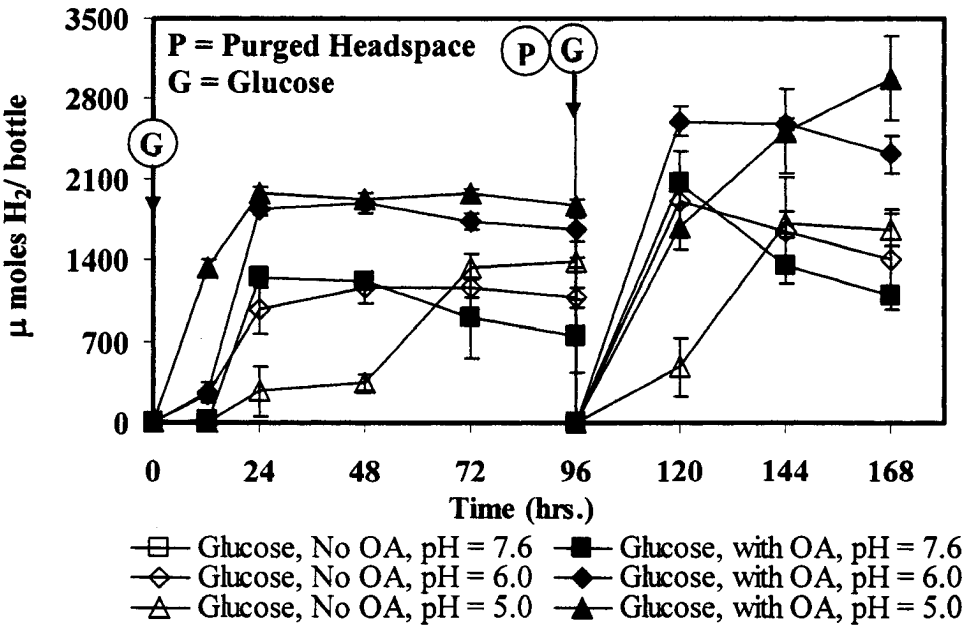

Figure 7.2: Effect of different initial pH on hydrogen production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

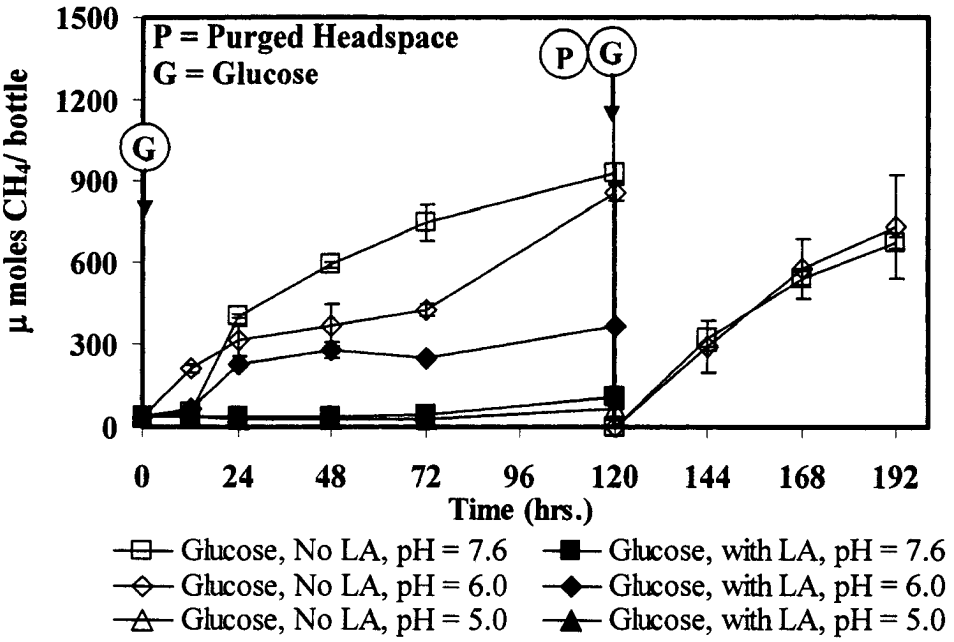

Figure 7.3: Methane production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

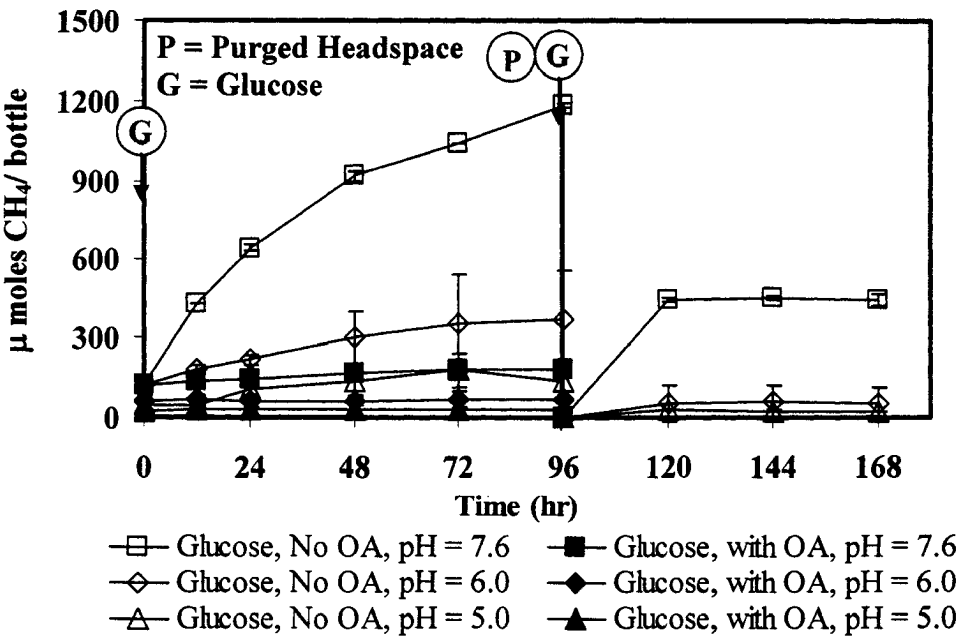

Figure 7.4: Methane production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

50% increase compared with the maximum amount observed during the first glucose addition. These findings indicate that the presence of LCFAs and VFAs at lower initial pH might be more inhibitory to the hydrogen consuming bacteria.

Table 7.1: Hydrogen yield (mole H_2.mole^{-1} glucose) from glucose (5,000 mg l^{-1}) injected into cultures at different initial pH and LCFA (0 or 2,000 mg l^{-1})

LCFA		0 mg l^{-1}			2000 mg l^{-1}		
		pH=7.6	pH=6.0	pH=5.0	pH=7.6	pH=6.0	pH=5.0
LA	1st Injection	ND	0.25±0.08	1.23±0.03	0.95±0.03	1.26±0.01	1.58±0.02
	2nd Injection	ND	1.30±0.13	1.87±0.31	1.38±0.05	1.76±0.01	2.37±0.11
OA	1st Injection	ND	0.84±0.06	0.99±0.03	0.90±0.01	1.36±0.02	1.43±0.02
	2nd Injection	ND	1.38±0.30	1.34±0.15	1.49±0.01	1.87±0.10	2.14±0.26

Note: Average with standard deviation for triplicate samples is shown.
ND = Not detected

The methane production trend in the cultures with different initial pH was opposite to the hydrogen production. A maximum amount of methane was observed in the controls not receiving any LCFA and at an initial pH of 7.6. Methane was below the detection limits in cultures with an initial pH of 5.0. The total amount of methane produced in cultures after the second glucose addition was less than the amount found after the first glucose injection.

7.1.2 VFA Production

The major VFAs detected during glucose degradation in cultures at different initial pH were acetate, propionate, and butyrate (Figures 7.5 to 7.10).

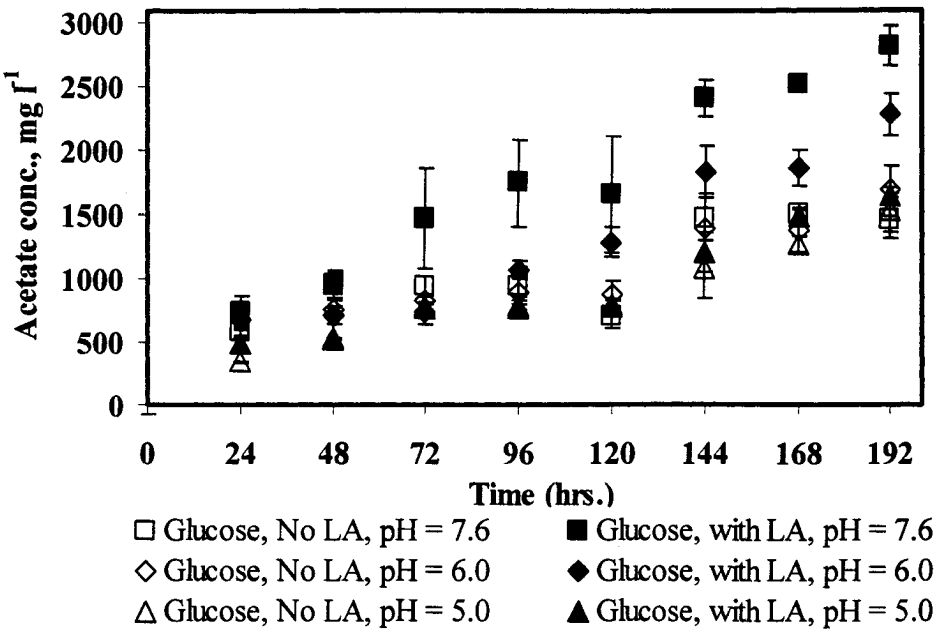

Figure 7.5: Acetate production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

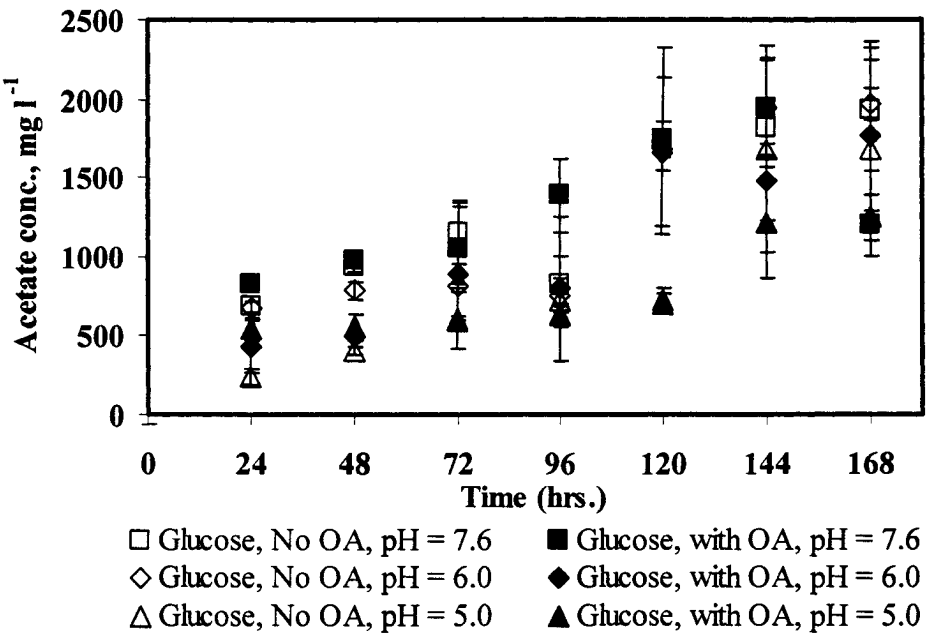

Figure 7.6: Acetate production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

Acetate was produced in all cultures after glucose addition. A high concentration of acetate was observed in the cultures inoculated with LCFA and an initial pH of 7.6 compared to the controls (no LCFA and pH = 7.6). In general, acetate accumulated in the cultures after the first glucose addition until day 4. Before the second glucose injection, the acetate concentration in cultures with different initial pH was approximately 600 to 1,600 mg l^{-1} and 500 to 1,300 mg l^{-1} in the presence of LA and OA, respectively. Three days after the second glucose addition, the acetate concentration was 1,400 to 2,900 mg l^{-1} in the cultures inoculated with LA, whereas it was 1,200 to 2,000 mg l^{-1} in the cultures inoculated with OA. A maximum acetate concentration of approximately 2,900 mg l^{-1} was observed in cultures inoculated with LA and at an initial pH of 7.6. In general, a greater amount of acetate accumulated in cultures inoculated with LA than those inoculated with OA.

Propionate was produced after the first glucose addition in cultures not inoculated with LCFA and at an initial pH of 7.6 and 6.0. Before the second glucose addition, the propionate concentration in the cultures with an initial pH of 7.6 and 6.0 were approximately from 300 to 400 mg l^{-1} and 100 to 200 mg l^{-1} in the presence of LA and OA, respectively. After the second glucose injection a sharp change in propionate production was observed in cultures with an initial pH of 7.6. Three days after the second glucose injection, the propionate concentration was 1,000 to 1,300 mg l^{-1} in the cultures with an initial pH of 7.6 inoculated with LA, whereas, it was 300 mg l^{-1} in the presence of OA. This variation in propionate production in cultures might be due to change in microbial community. Propionate was below the detection limit throughout the experiment in cultures at an initial pH of 5.0.

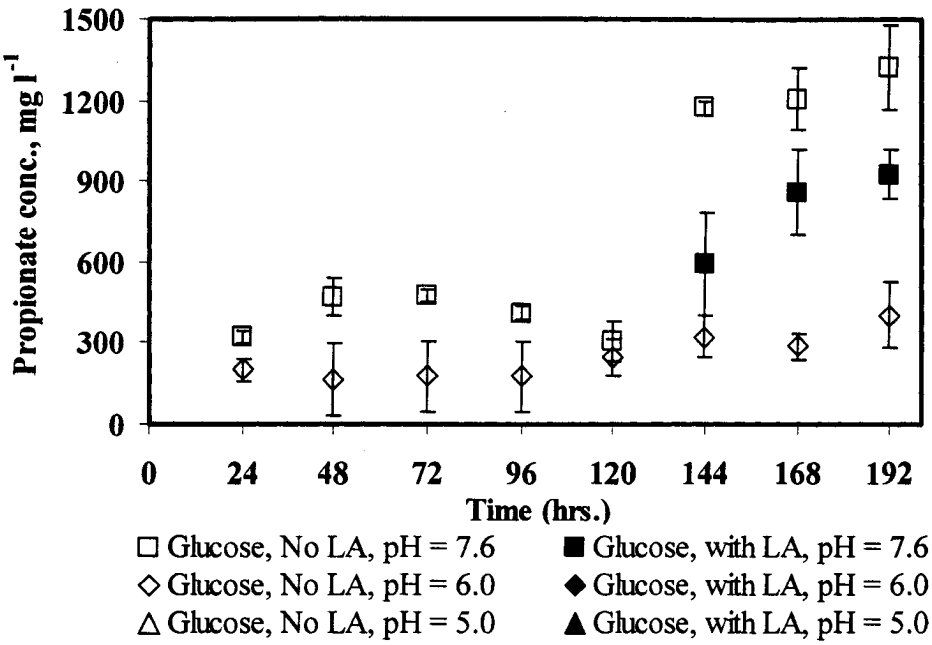

Figure 7.7: Propionate production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

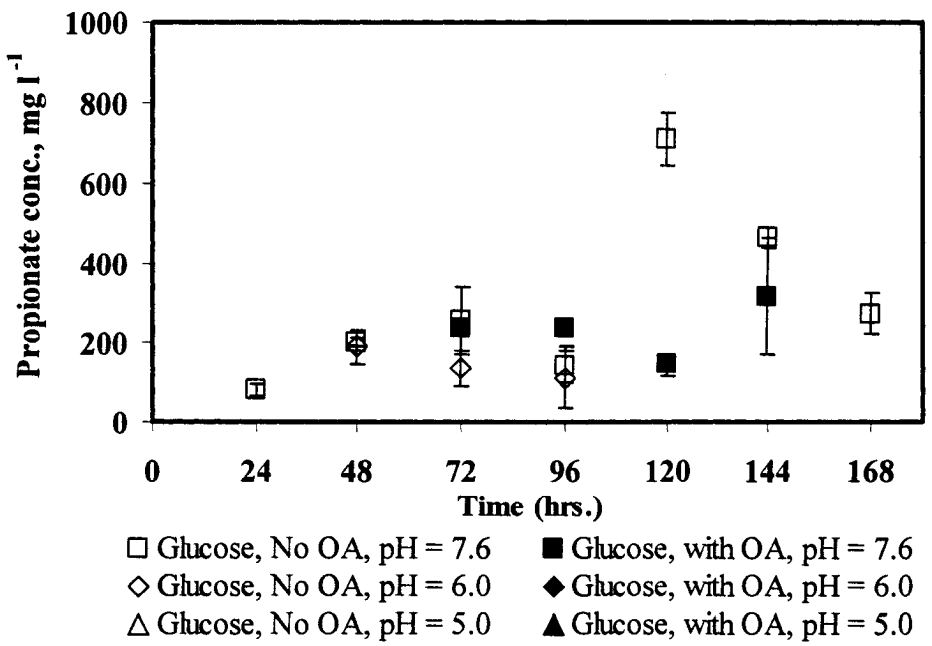

Figure 7.8: Propionate production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

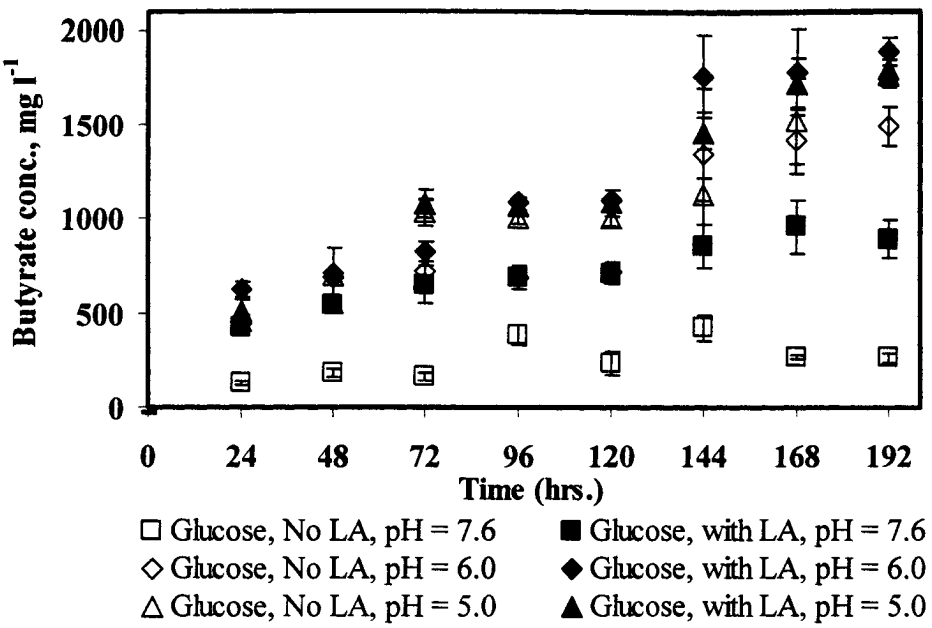

Figure 7.9: Butyrate production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

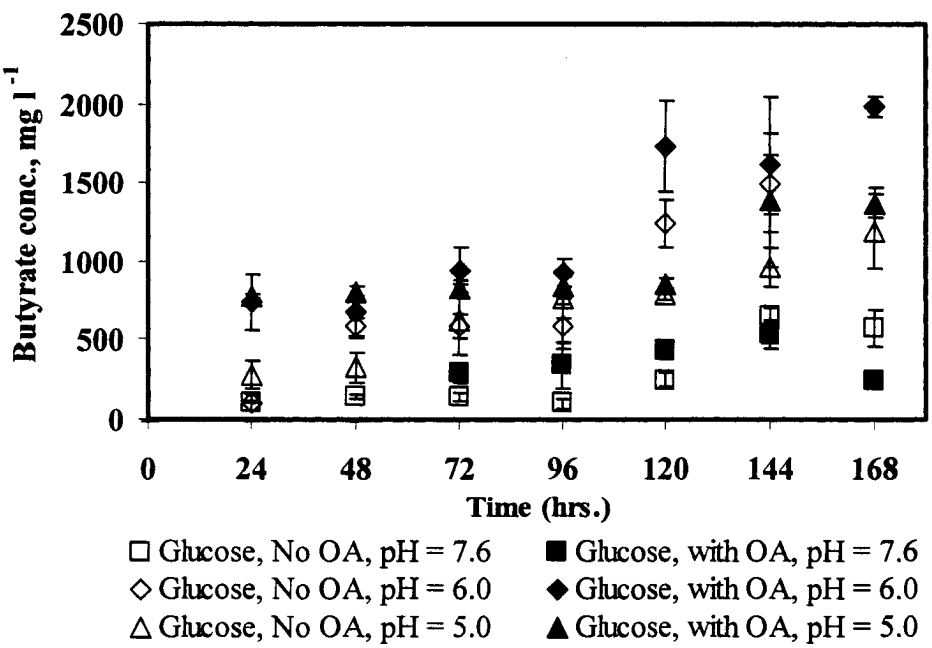

Figure 7.10: Butyrate production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

Butyrate was also produced in all the cultures after glucose addition. A small amount of butyrate was observed in the controls (no LCFA and pH = 7.6). In general, the butyrate concentration reached maximum levels on day 3 after the first glucose injection in cultures with initial pH values of 7.6, 6.0 and 5.0. The butyrate concentration was greater in cultures inoculated with LCFA and at initial pH of 6.0 and 5.0 compared to those at initial pH of 7.6. The butyrate concentration increased after the second glucose injection. On day 7 approximately 1,500 to 2,000 mg l^{-1} butyrate was observed in cultures with initial pH values of 6.0 and 5.0, whereas, it was 300 to 800 mg l^{-1} in cultures with an initial pH of 7.6.

7.1.3 Glucose Degradation

Glucose degradation in the cultures receiving 5,000 mg l^{-1} glucose at different initial pH in the presence of 0 and 2,000 mg l^{-1} LCFA (LA or OA) is shown in Figures 7.11 and 7.12. Glucose degradation was inhibited in cultures with reduced initial pH and inoculated with LCFA. The glucose initial degradation rates for cultures at initial pH values of 7.6, 6.0 and 5.0 are shown in Table 7.2. Notice the lower initial pH and LCFA reduced the glucose degradation rates in cultures. In the controls at an initial pH of 7.6 most of the glucose was depleted within 8 hours, whereas, it took more than 16 hours in cultures inoculated with LCFA. Glucose was degraded to undetectable levels between 16 to 24 hours in the cultures inoculated with LCFA at initial pH of 7.6 and 6.0. Even after 24 hours approximately 10% to 30% glucose was present in the cultures with an initial pH of 5.0.

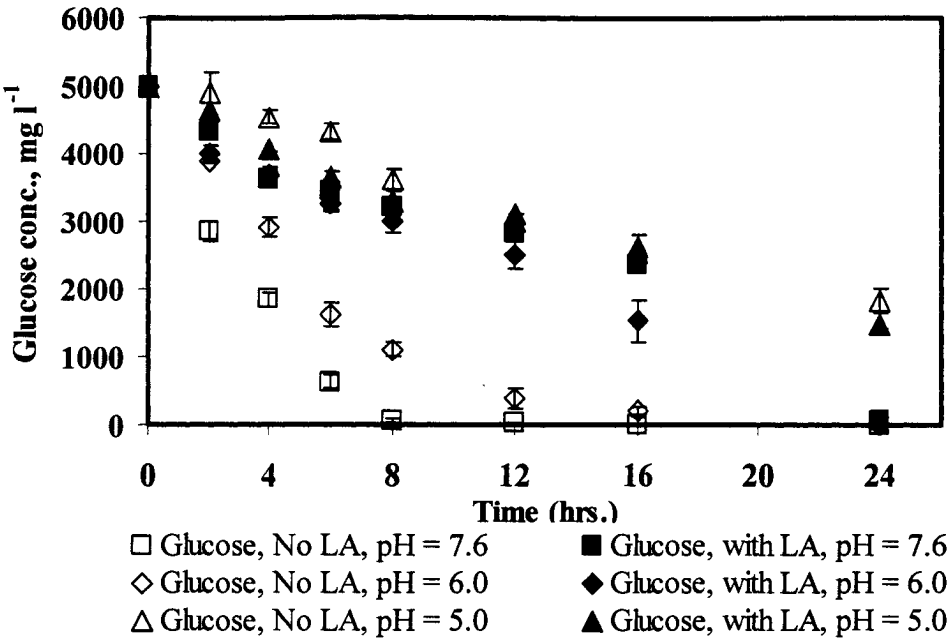

Figure 7.11: Glucose degradation in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr, average with SD for triplicate samples are shown).

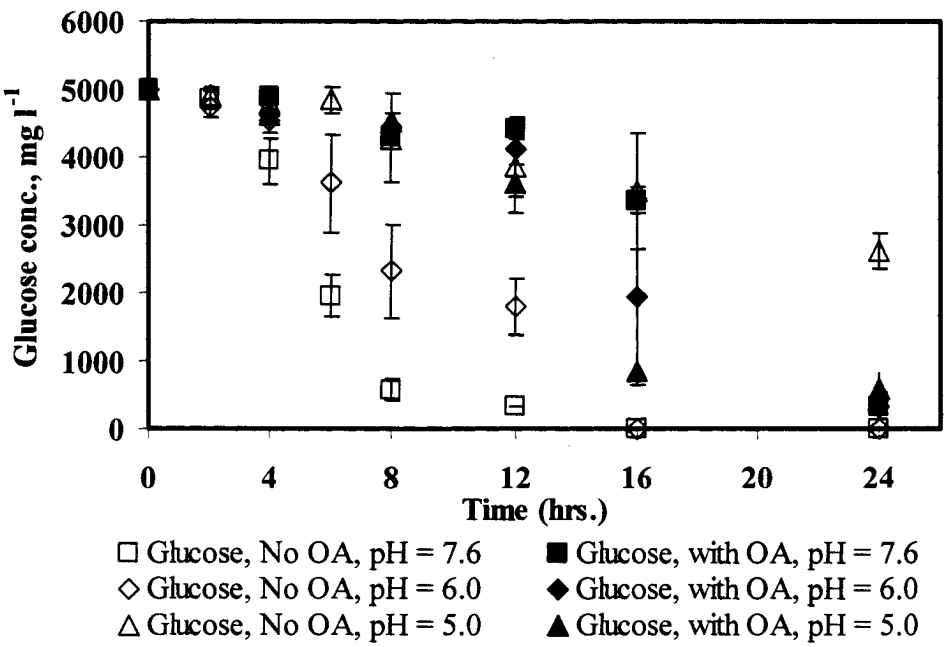

Figure 7.12: Glucose degradation in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr, average with SD for triplicate samples are shown).

Table 7.2 Glucose initial degradation rates ($\mu g.mgVSS^{-1}.min^{-1}$) for cultures receiving LCFA and 5,000 mg l^{-1} glucose at different initial pH

LCFA	0 mg l^{-1}			2000 mg l^{-1}		
	pH=7.6	pH=6.0	pH=5.0	pH=7.6	pH=6.0	pH=5.0
LA	7.84±0.62	7.29±0.55	1.67±0.12	1.53±0.05	1.80±0.22	1.76±0.27
OA	7.16±0.23	6.24±0.50	0.88±0.13	1.82±0.06	2.31±0.21	1.81±0.08

Note: Average with SD for triplicate samples is shown.

7.1.4 Alcohol Production

Ethanol, iso-propanol, propanol, iso-butanol, and butanol were also detected in the cultures after adding glucose at different initial pH. Alcohol production profiles are shown in Figures 7.13 to 7.21.

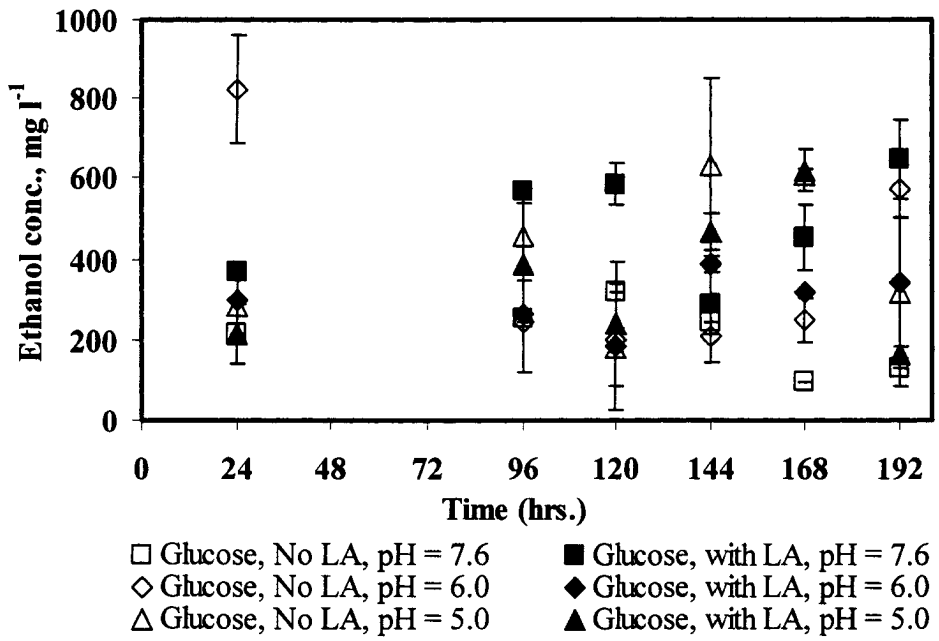

Figure 7.13: Ethanol production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

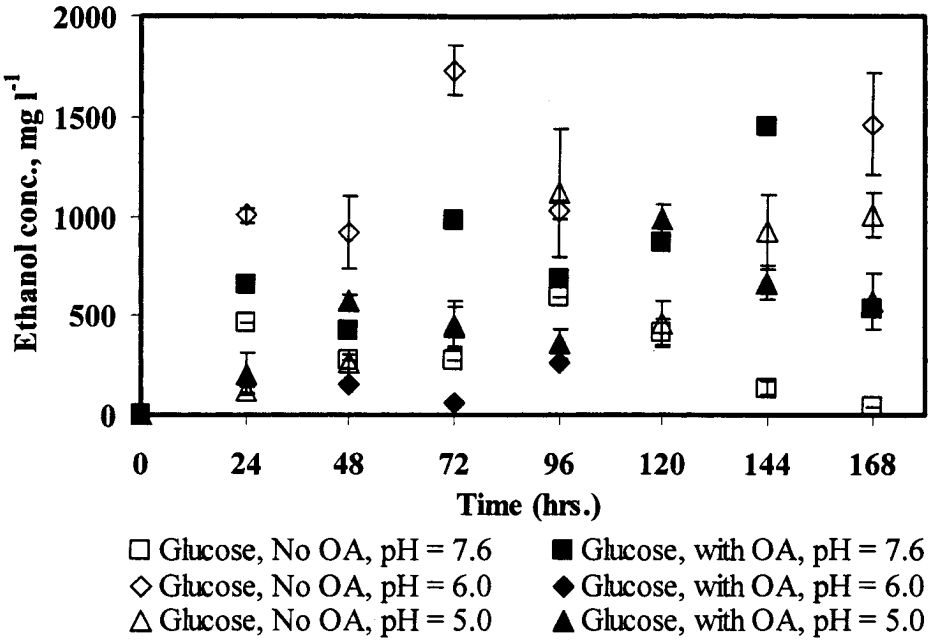

Figure 7.14: Ethanol production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

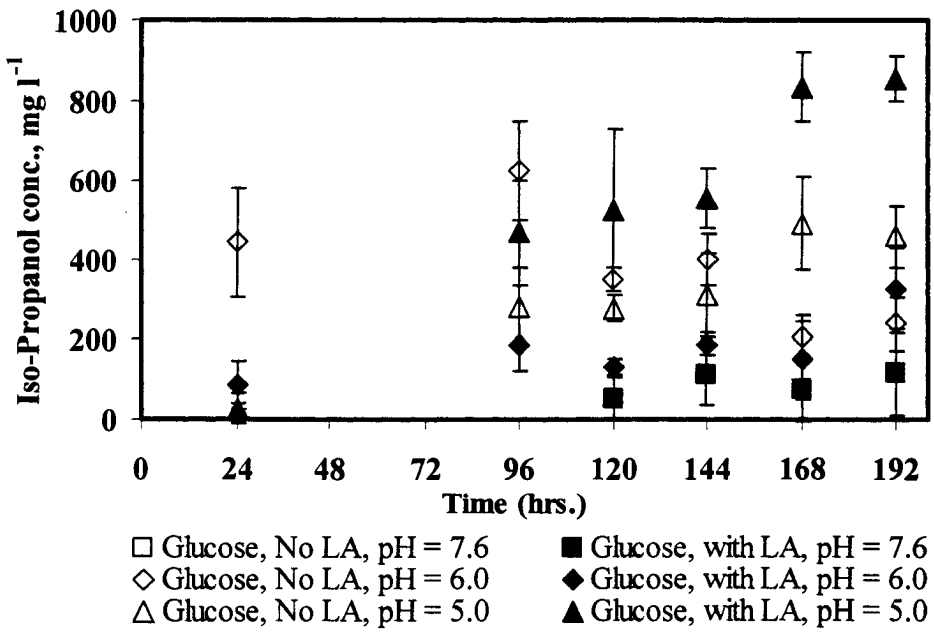

Figure 7.15: Iso-propanol production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

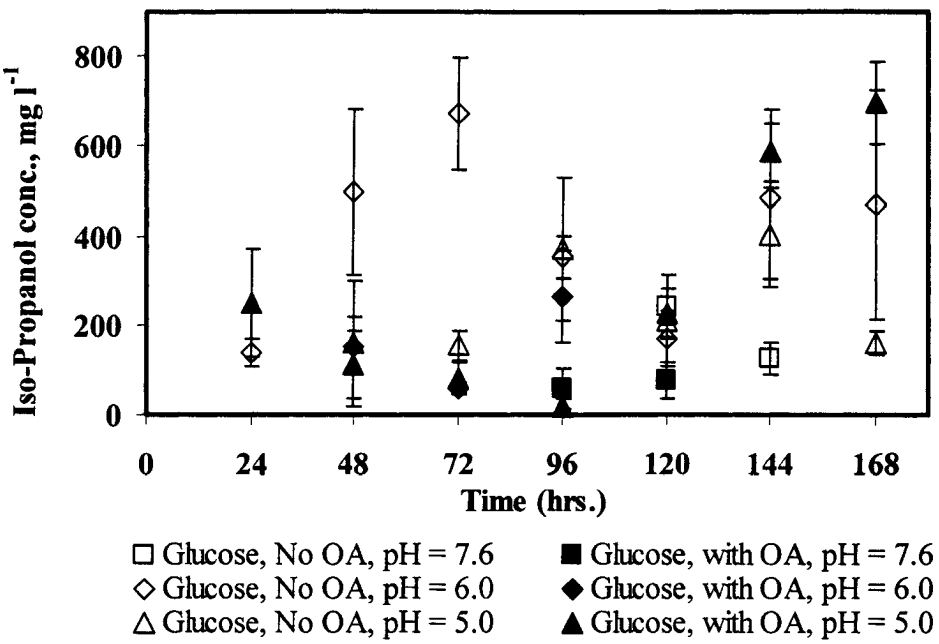

Figure 7.16: Iso-propanol production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

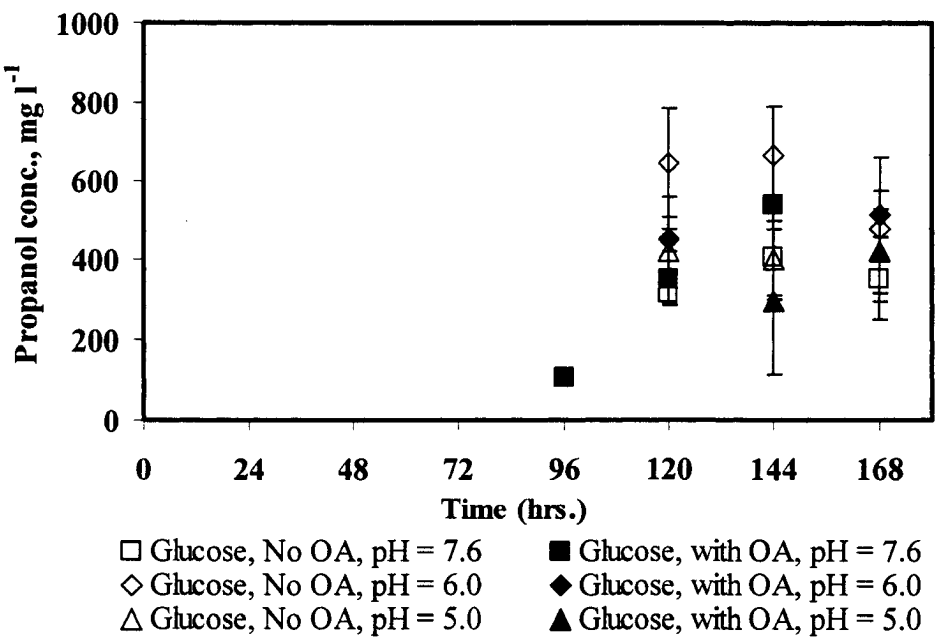

Figure 7.17: Propanol production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

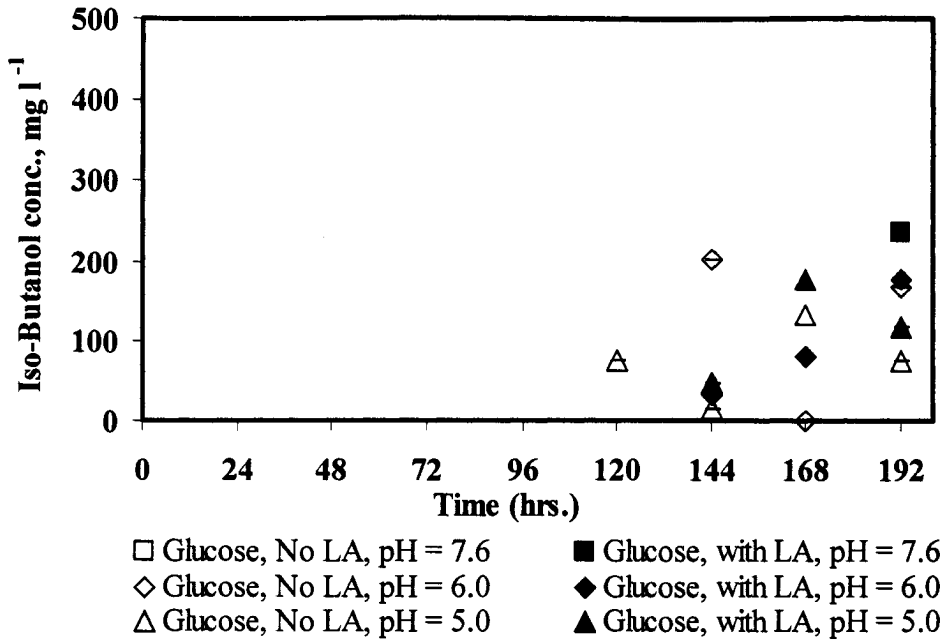

Figure 7.18: Iso-butanol production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

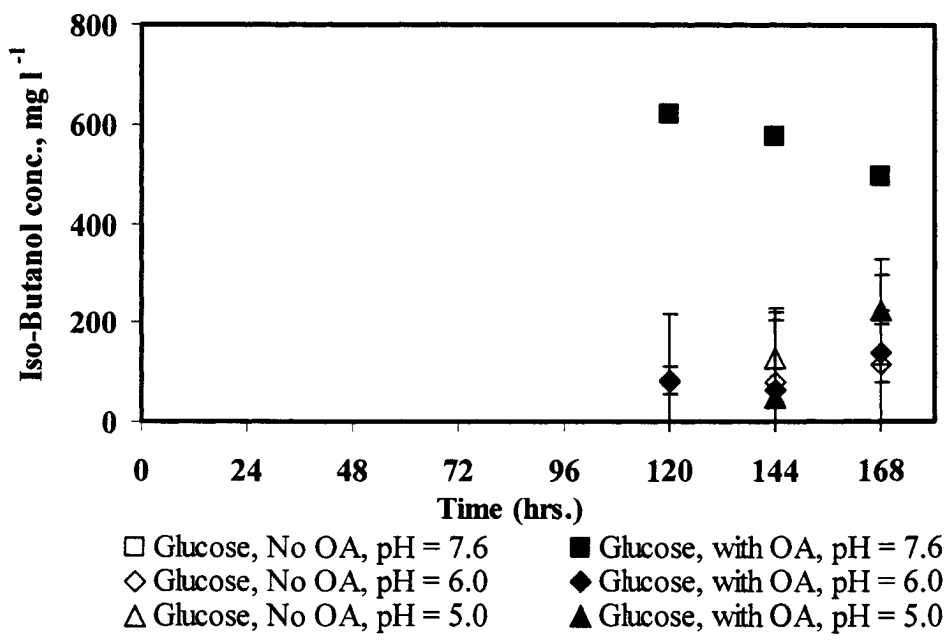

Figure 7.19: Iso-butanol production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

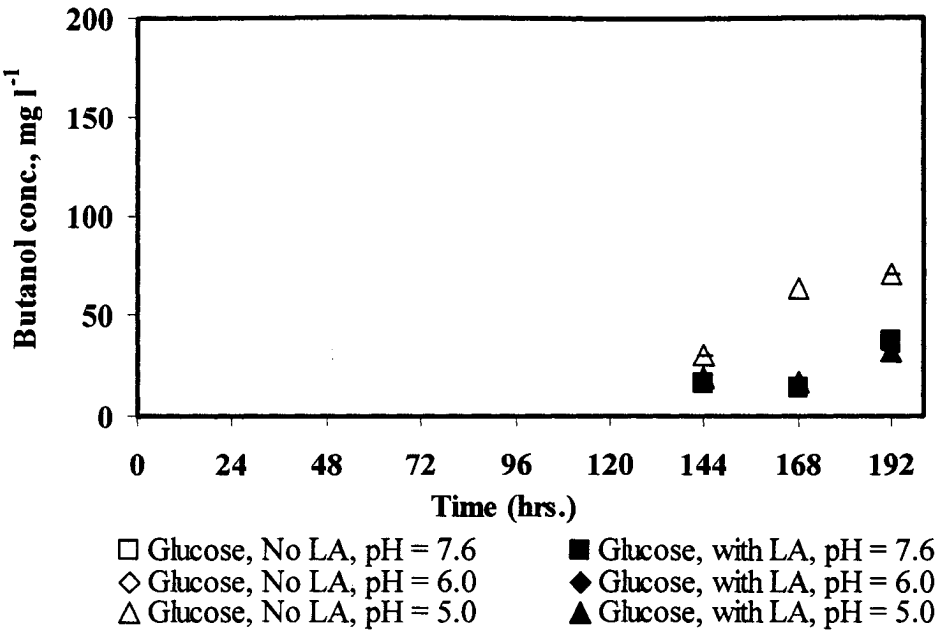

Figure 7.20: Butanol production in cultures receiving 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

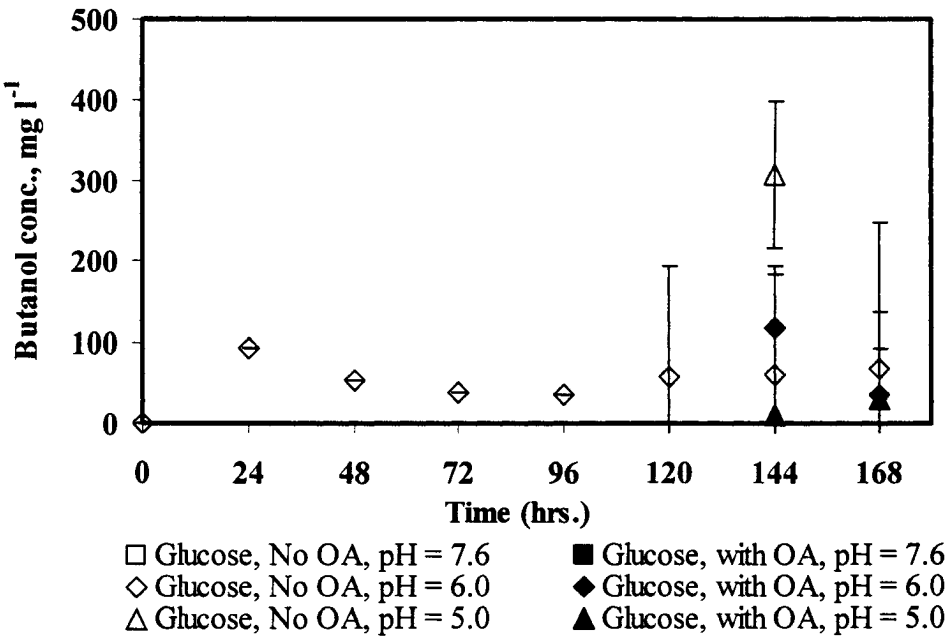

Figure 7.21: Butanol production in cultures receiving 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

Ethanol was the major alcohol observed; at concentrations between 1,000 to 1,500 mg l^{-1} (alcohol data for LA batch at 48 and 72 hours were lost because of malfunction of the analytical equipment). Iso-propanol was observed between 200 to 600 mg l^{-1} in cultures after injecting glucose. Propanol, iso-butanol, and butanol were also detected in the cultures after re-injecting glucose. Alcohols were either undetectable or negligible in the controls whereas, greater amounts were observed in cultures with initial pH values of 6.0 and 5.0. These findings indicate that lower initial pH shifts the metabolic pathway to produce neutral solvents such as ethanol and butanol, which are more reduced than the corresponding fatty acids.

7.1.5 Carbon Mass Balance

A carbon mass balance in cultures receiving 5,000 mg l^{-1} glucose and LA or OA at different initial pH is shown in Figures 7.22 and 7.23.

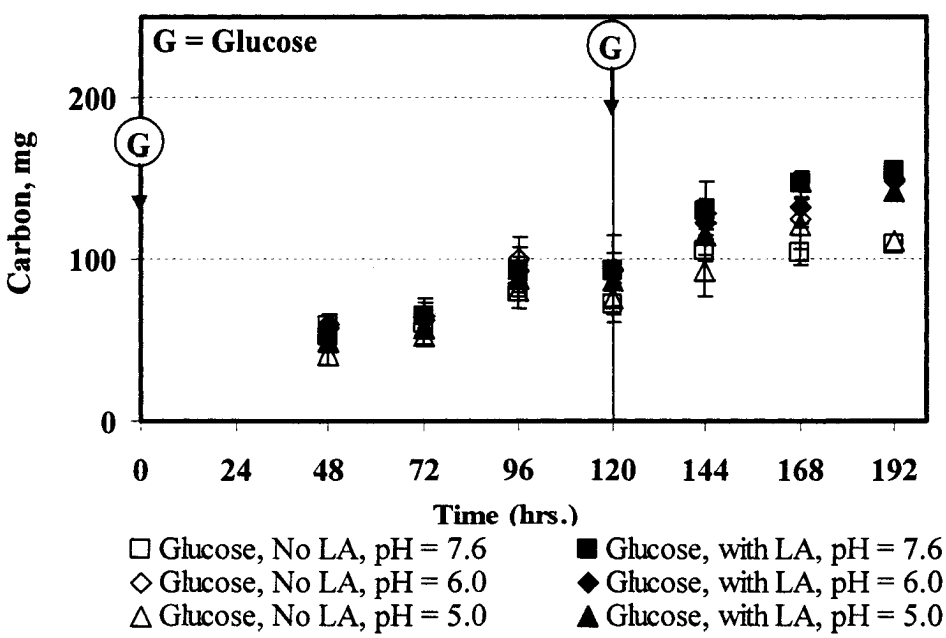

Figure 7.22: Mass balance in cultures receiving 0 or 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).
---------- Theoretical carbon balance

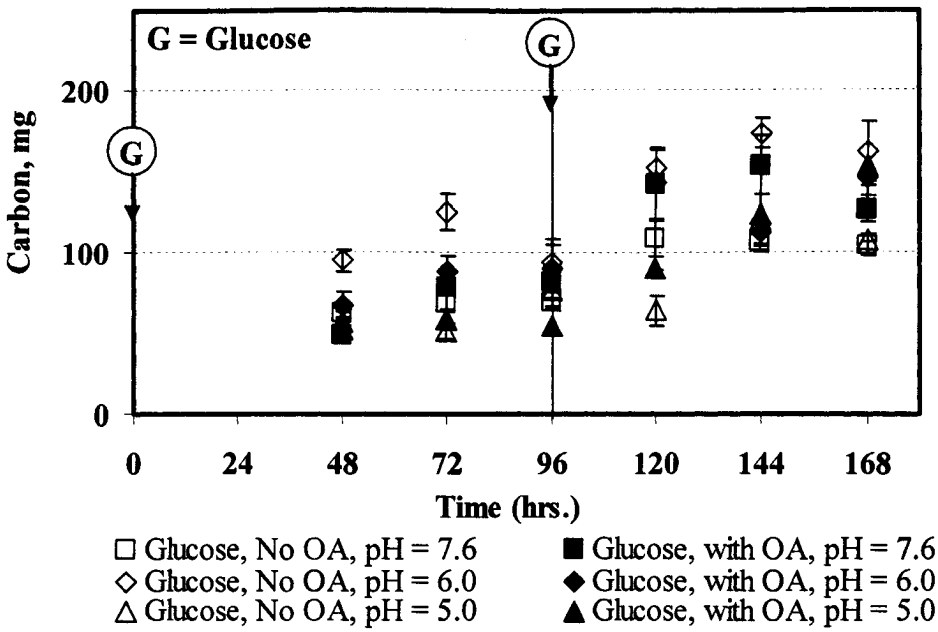

Figure 7.23: Mass balance in cultures receiving 0 or 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).
---------- Theoretical carbon balance

Approximately 80 to 90% of theoretical carbon (added as glucose) recovery was observed in all cultures after the first glucose addition. After re-injecting glucose, carbon the mass balance was approximately 60 to 80%. This deficiency in carbon is likely due to the production of biomass and other solvents that were not considered in the carbon mass balance calculation.

7.1.6 pH Profile

Initial pH in the cultures was adjusted at 7.6, 6.0, and 5.0 and was not controlled after glucose addition. A pH change was observed in the cultures (shown in Figures 7.24 and 7.25) during glucose degradation, as a result of the formation of short chain fatty acids.

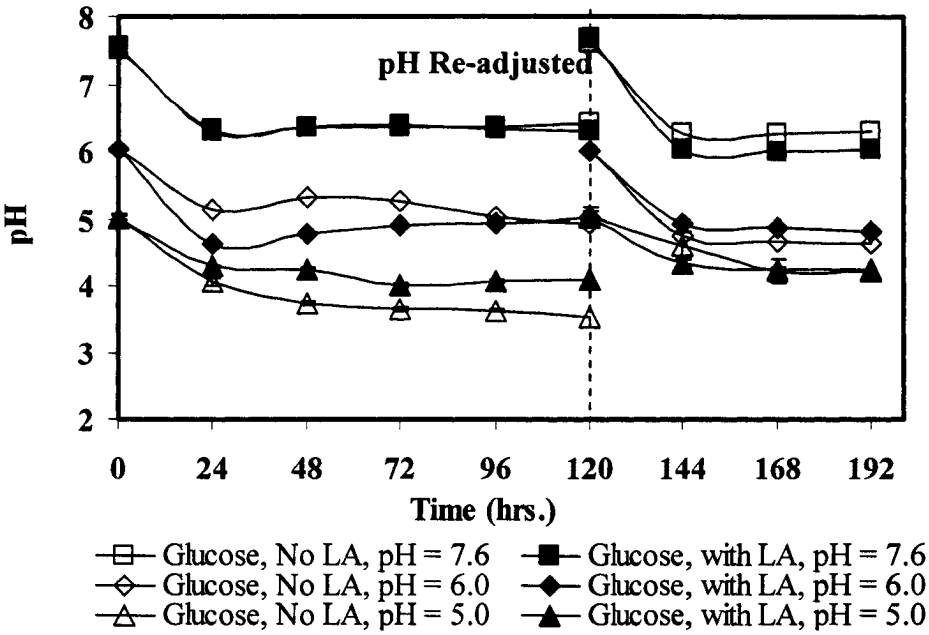

Figure 7.24: pH profile in cultures receiving 0 or 2,000 mg l^{-1} LA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 120 hr with pH adjustment, average with SD for triplicate samples are shown).

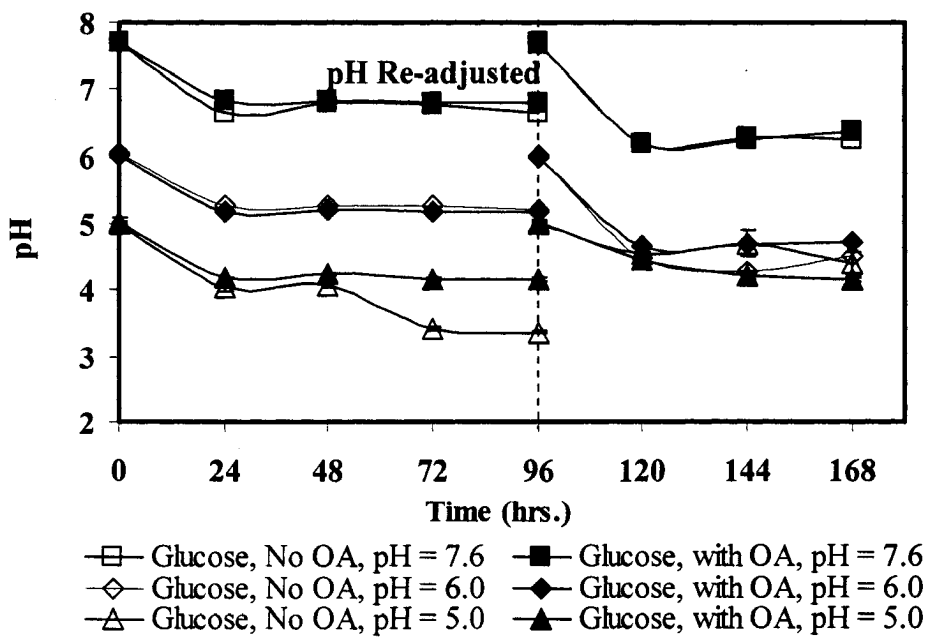

Figure 7.25: pH profile in cultures receiving 0 or 2,000 mg l^{-1} OA and 5,000 mg l^{-1} glucose at different initial pH conditions (glucose added at 0 hr and 96 hr with pH adjustment, average with SD for triplicate samples are shown).

A similar pattern in pH change was observed in cultures after readjusting the pH and the second glucose addition.

7.2 Discussion of Results

The presence of LCFA and a lower initial pH effectively aided in improving the hydrogen yield during glucose degradation. The maximum yield, 2.37 moles H_2.mole^{-1} glucose (59% glucose to hydrogen conversion efficiency based on a theoretical maximum of 4 moles H_2.mole^{-1} glucose), observed in the cultures inoculated with LA at 37°C and at an initial pH of 5.0 was substantially greater than the yields reported for other mixed cultures. In batch tests, Khanal *et al.* (2004) measured 1.8 moles H_2.mole^{-1} glucose (45% conversion efficiency) using heat shocked mixed cultures at 37°C and an initial pH of 4.5. Zheng and Yu (2004) observed 1.3 to 1.57 moles H_2.mole^{-1} glucose using a heat treated granular sludge in batch tests at 30°C and at a pH of 4.0 to 5.0. In a continuous cultures tests, using mixed cultures from the hydrogen producing reactors at 36°C, a pH of 5.5, and a 6 hour HRT, Fang and Liu (2002) observed 2.1 moles H_2.mole^{-1} glucose (53% conversion efficiency). In continuous cultures using an acclimated culture at 35°C and pH of 6.7, Chen *et al.* (2001) measured 3.47 moles H_2.mole^{-1} sucrose (43% conversion efficiency based on the theoretical maximum of 8 moles H_2.mole^{-1} sucrose). Higher hydrogen yields have also been found using pure cultures. Van Niel *et al.* (2002) measured 3.3 moles H_2·mole^{-1} glucose using *Thermotoga elfi* at 65°C and a pH of 7.4 in the batch cultures. Hydrogen yield could be increased using higher temperatures or pure cultures in the presence of LCFA.

Acetate and butyrate were the major VFAs produced during glucose degradation in the cultures at different initial pH. A weakly acidic pH is a fundamental operating

parameter for biological hydrogen production. Reducing the initial pH from 7.6 to 5.0 in the cultures resulted in an increase of butyrate and a decrease of the acetate concentrations. The cultures inoculated with LCFA and an initial pH of 7.6 contained acetate (20 to 28% on carbon basis) and butyrate (8 to 10%). In comparison, cultures at initial pH of 6.0 and 5.0 contained acetate (12 to 20%) and butyrate (15 to 25%). These findings indicate that reduced initial pH favored butyrate production. Similar results were observed by Fang and Liu (2002), and Zheng and Yu (2004). Fang and Liu (2002) observed that an increase in pH from 4.0 to 7.0 resulted in a decrease in the butyrate concentration and an increase in the acetate concentration during glucose degradation using mixed cultures at 36°C.

Dabrock *et al.* (1992) reported the effect of pH, growth rate, and carbon source on product formation by *Clostridium pasteurianum*. They observed the formation of reduced products such as ethanol and butanol with other short chain fatty acids from glucose degradation at different pH and growth rates. Ethanol was the major alcohol (5% to 15% on carbon basis), observed at different initial pH conditions during glucose degradation at 37°C. The maximum ethanol production was observed in cultures with an initial pH of 6.0. Fang and Liu (2002) reported ethanol (4.6% to 10.1%) was the third most abundant byproduct in the effluent of a continuous hydrogen production system using mixed cultures at 36° and a pH ranging from 5.0 to 6.0. Moat (1979) reported an ethanol-acetate fermentation pathway where ethanol and acetate were produced simultaneously. Iso-propanol and butanol were also detected in cultures during glucose degradation. Papoutsakis (1984, 1985) discussed metabolic pathways for butyric acid fermentation

from glucose, where iso-propanol and butanol were produced in the presence of iso-propanol dehydrogenase and butanol dehydrogenase.

In this current study, glucose was degraded within 16 to 24 hours in cultures at an initial pH of 7.6 and 6.0 but it took more than 24 hours in cultures maintaining at an initial pH of 5.0. Similar work by Zheng and Yu (2004), showed glucose (5,000 mg l^{-1}) was degraded rapidly and depleted within 12 hours in cultures maintained at 30°C and at a pH of 8.0. Only 40% of glucose was degraded within 24 hours in cultures maintained at a pH of 4.0. The time for complete glucose removal was approximately 100 hours.

CHAPTER 8

CONCLUSIONS

To examine the fermentative hydrogen production potential of wastewater in the presence of LCFA, batch studies were conducted at different LCFA (LA or OA) concentrations and initial pH conditions. Glucose was used as the principal carbohydrate source. VFA production and glucose degradation along with the headspace gases were monitored over the duration of each study. To study the inhibitory effects of LCFAs and VFAs combination, glucose was re-injected into the cultures on day 1 or day 5. After re-injecting glucose, the headspace was purged every 24 hours in selected experiments with N_2 to maintain low hydrogen partial pressure in the headspace.

These experiments were conducted based on the hypothesis that LA or OA would inhibit hydrogen consumers. Hydrogen accumulated in cultures inoculated with LA or OA. The glucose to hydrogen conversion efficiency was greatly influenced by LA or OA concentrations and the initial pH condition in the cultures.

The following useful conclusions are obtained from the present study:

1. Hydrogen was undetectable during anaerobic degradation of only LCFA or glucose.
2. Approximately 1.0 to 1.5 moles $H_2.mole^{-1}$ glucose was observed in cultures inoculated with LA or OA. The hydrogen yields were greater after the second glucose addition. This could be due to synergistic effects of LCFAs and VFAs on hydrogen consumers.
3. Most of the hydrogen was produced within 24 hours of glucose addition in the cultures inoculated with LA or OA.

4. The maximum hydrogen yield was 2.37 moles H_2.mole^{-1} glucose (59% glucose to hydrogen conversion efficiency based on a theoretical maximum yield of 4.0 moles H_2.mole^{-1} glucose) in cultures inoculated with LA at an initial pH of 5.0. Lowering the initial pH in the cultures increased the glucose to hydrogen conversion efficiency.
5. Acetate, propionate and butyrate accumulation in the cultures inoculated with LA or OA indicate that LCFA inhibits aceticlastic and methanogenic activities. The hydrogen yield was higher in cultures with high acetate and butyrate accumulation and it was lower in the cultures where propionate was produced.
6. Initial pH of the cultures affected the glucose fermentation products. Butyrate production was favored at initial pH of 6.0 and 5.0, whereas, acetate production was favored at an initial pH of 7.6.
7. Ethanol was the major alcohol found from glucose degradation in cultures maintained at different initial pH. Iso-propanol, propanol, iso-butanol and butanol were also detected.

CHAPTER 9

ENGINEERING SIGNIFICANCE AND FUTURE RECOMMENDATIONS

In fermentative hydrogen production system using mixed cultures, inhibition of hydrogenotrophic activities is a major problem to attain elevated hydrogen yield. LCFA inhibition of hydrogenotroph will make this system more economical and sustainable. The hydrogen yields obtained in this research could be used to provide some design data for a continuous fermentative hydrogen production system. Also the glucose degradation data will be useful to understand the conversion of substrate to hydrogen in the presence of LA or OA. A solid retention time (SRT) for optimum hydrogen yield can be established using the data from this research.

Based on this study, several recommendations to improve and gain a better understanding for future research are as follows:

- A continuous reactor study is essential to assess the feasibility of LCFA inhibition on hydrogenotrophs in a large-scale fermentative hydrogen production system.
- A two-stage reactor system can be used to produce hydrogen using dark fermentation in the first stage and phototrophic hydrogen from the effluent in the second stage (Fang *et al.*, 2005). Separation of aceticlastic methanogens and hydrogenotrophic methanogens to optimize hydrogen yield is a major design issue to be resolved by engineering design of anaerobic systems.
- pH needs to be optimized for anaerobic hydrogen production form wastewater containing LCFAs. Further study is required to examine the effects of maintaining a constant pH.

- Temperature affects the inhibition of LA, OA and reaction rates reported by Lalman and Komjarova (2004). Additional work is required to asses the anaerobic hydrogen production potential from carbohydrates in the presence of LCFAs under thermophilic conditions.

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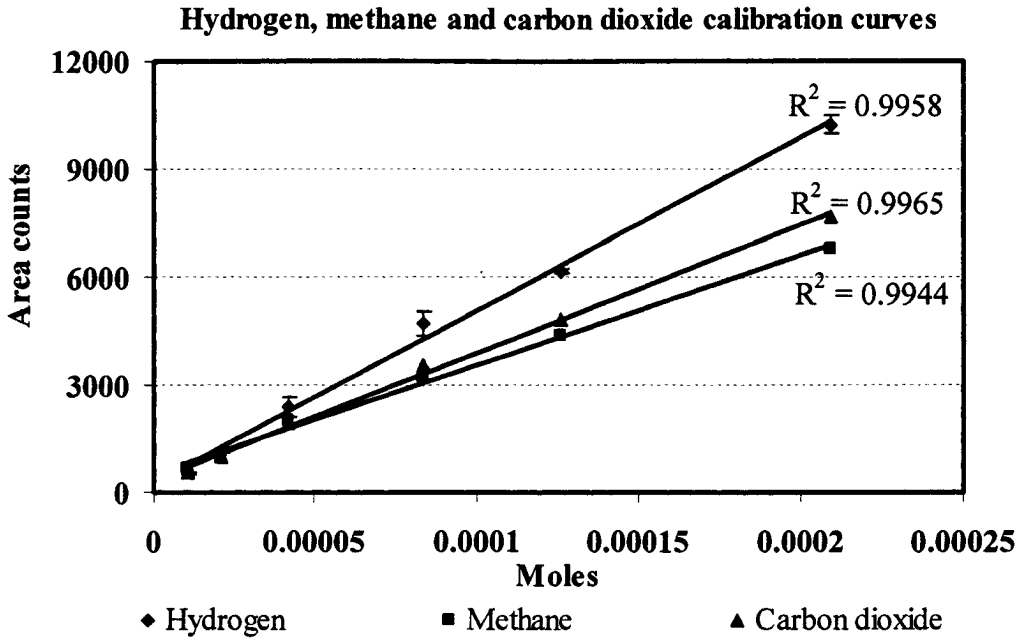
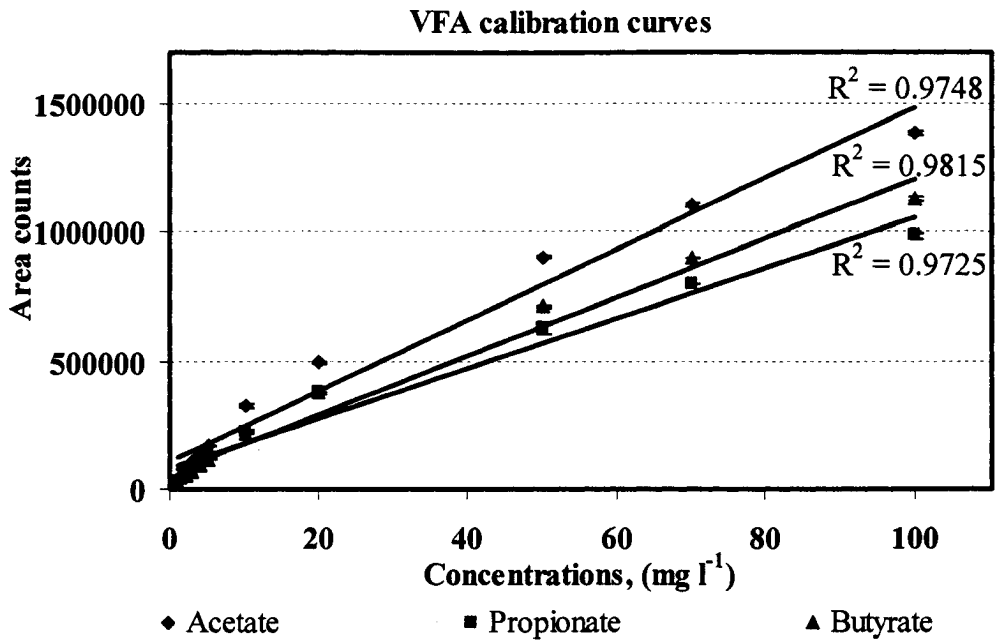
APPENDICES

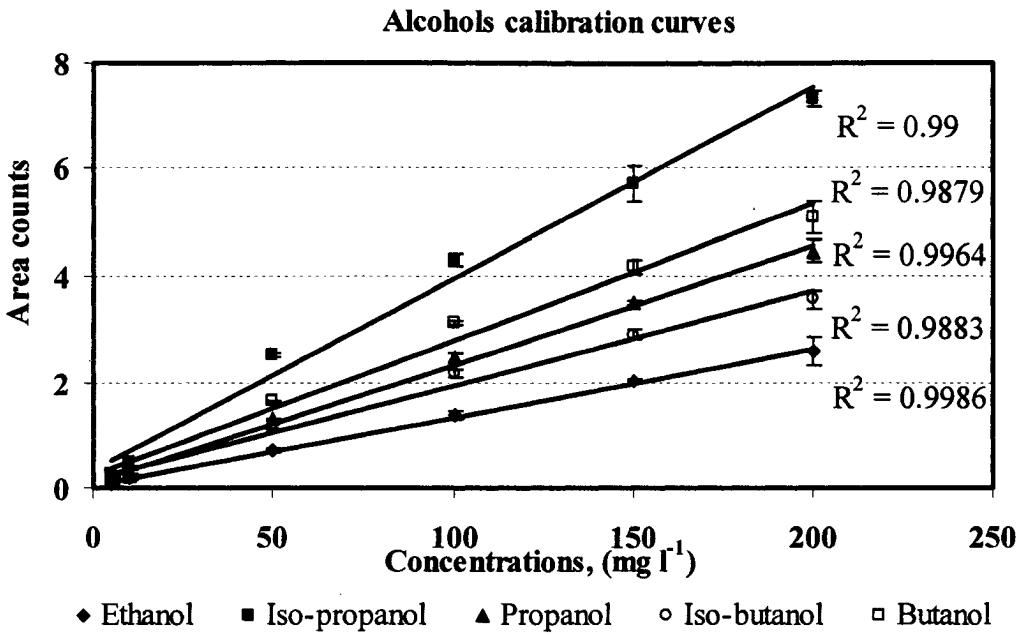
Appendix A: Hydrogen, Methane and Carbon Dioxide Calibration Curves



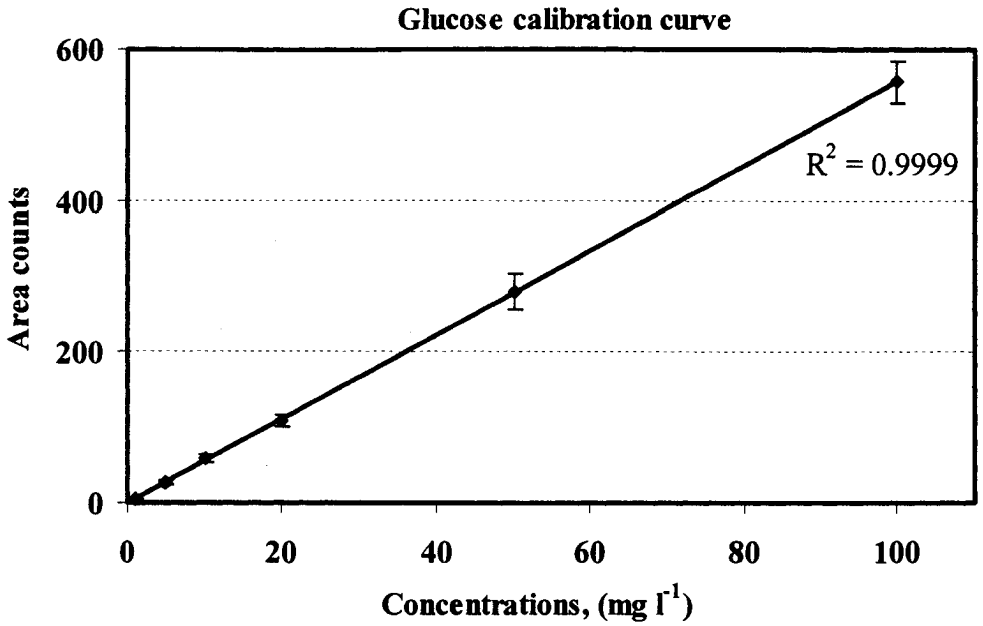
Appendix B: Volatile Fatty Acids Calibration Curves



Appendix C: Alcohol Calibration Curves



Appendix D: Glucose Calibration Curve



Appendix E: Example Calculations for Hydrogen Yields, Degradation Rates and Carbon Mass Balance

Hydrogen Yields

Sample calculation for hydrogen yield in the cultures receiving 5000 mg l^{-1} glucose in the presence of 2000 mg l^{-1} LA (Figure 5.1)

At 24 hour; Amount of hydrogen found in the batch reactor (160 ml) = 1537.27 μmoles

Amount of glucose injected = 1390 μmoles

So, Hydrogen yield = 1.11 mole H_2 mole glucose^{-1}

Degradation Rates

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

Sample calculation: glucose degradation profiles for the cultures receiving 5000 mg l^{-1} glucose. (Figure 5.6)

$$C\,mgl^{-1} = 5104 - 857.4t + 34.75t^2$$

$$-\frac{dC}{dt} = 857.4 + 69.50t$$

At, t = 0; initial degradation rate = 857.4 mg l^{-1} hr^{-1}

= 7.14 μg mg VSS^{-1} min^{-1}

(Biomass concentrations 2000 mg VSS l^{-1})

Carbon Mass Balance

Mass balance calculation was done by using following principle:

$$\Sigma Substrate_0 = \Sigma Products_t + \Sigma Substrate_t.$$

Sample calculation for the cultures receiving 5000 mg l^{-1} glucose and 0 mg l^{-1} LA at initial pH 7.6 (Figure 5.12)

At t = 0 hour,

$\Sigma Substrate_0$ = 100.00 mg C (theoretical amount of carbon from glucose)

At t = 96 hours,

$\Sigma Products_t$ = 19.97 mg C (from acetate) + 9.23 mg C (from propionate) + 11.03 mg C (from butyrate) + 6.05 mg C (from ethanol) + 14.49 mg C (from methane) + 20.99 mg C (from carbon dioxide)

= 81.37 mg C

$\Sigma Substrate_t$ = 0 mg C

Total amount of carbon at 96 hours = 81.37 mg C

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