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Hydrogen production from glucose by inhibiting hydrogenotrophic methanogens carbon-18 long-chain fatty acids.

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HYDROGEN PRODUCTION FROM GLUCOSE BY INHIBITING HYDROGENOTROPHIC METHANOGENS USING C_{18} LONG CHAIN FATTY ACIDS

by
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ABSTRACT

Dark fermentation is an attractive process for hydrogen (H_2) production from organic substrates. Rapid conversion of H_2 to other products, particularly methane, is a major hindrance to H_2 accumulation and recovery from the process. Long chain fatty acids (LCFAs) namely oleic ($C_{18:0}$) acid (OA) and linoleic ($C_{18:1}$) acid are inhibitors of aceticlastic methanogens and are suspected inhibitors of hydrogenotrophic methanogens. However, the effect of such inhibition on increasing H_2 recovery from organic substrates has not been examined. Hence, in this study, C_{18} LCFAs were used to increase the quantity of H_2 from glucose degradation.

Batch experiments were conducted at 23 ± 2°C to examine the effect of LCFA concentration (0 to 2,000 mg l^{-1}) and the initial pH (pH 5, 6 and 7.8) on the fermentative H_2 production. Glucose was re-injected on day 4 or day 5 to examine the combined effect of LCFA, volatile fatty acids (VFAs) and intermittent sparging on H_2 production.

H_2 production was a function of LCFA concentration and the initial pH. The maximum H_2 yield recorded was approximately 2.7 mol $H_2 \cdot mol^{-1}$ glucose in cultures receiving LA at an initial pH of 6. Glucose degradation was inhibited in cultures receiving LCFA. Inhibition of glucose degradation was enhanced at lower initial pH values. Overall, the data demonstrated that LA and OA can be used to enhance H_2 accumulation and recovery from organic substrates.

This work is dedicated to my parents.

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NOMENCLATURE

ADP	adenosine di-phosphate
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 Context

Energy is vital commodity to global prosperity, yet our dependence on fossil fuels as a primary energy source contributes to global climate change, environmental degradation, and health problems (Bockris, 2002). Hydrogen (H_2) is an ideal and clean energy source for the future because of its high conversion, recyclability and nonpolluting nature. H_2 has a high-energy yield of 122 KJ g^{-1}, which is 2.75 times greater than that compared to hydrocarbon fuels (Onodera *et al.*, 1999; Lay *et al.*, 1999; Mizuno *et al.*, 2000), and it is hailed as the fuel of the future. Three major energy consuming sectors which include transportation, industry, and heating and cooling buildings stand to benefit from this new technology. The most profound impact of H_2 as a fuel is expected in the transportation sector.

H_2 can be produced by a number of processes, including electrolysis of water, thermochemically, thermocatalytic reformation of H_2-rich compounds, and biological processes. Currently, H_2 is produced almost exclusively by water electrolysis or by steam reformation of methane (Levin *et al.*, 2004). Water electrolysis is economically feasible when cheap electricity is available (Lay *et al.*, 1999). Although in the thermocatalytic method, water is the only material input and H_2 and oxygen the only material outputs, energy is required to achieve high temperatures (Funk, 2001). However, this method of H_2 production cannot be considered as an alternative nonpolluting energy source since, traditional nonrenewable fossil fuels are used to produce the H_2 (Van Ginkel and Sung, 2001).

Biological systems provide a wide range of approaches to generate H_2, which include direct biophotolysis, indirect biophotolysis, photo-fermentations, and dark-fermentation (Das and Veziroglu, 2001; Hallenbeck and Benemann, 2002; Nandi and Sengupta, 1998). Photosynthetic H_2 producing systems are light dependent, have low light conversion efficiencies (expressed as solar energy conversion efficiency) and require elaborate, large area photobioreactors (Levin *et al.*, 2004; Hallenbeck and Benemann, 2002). Dark-fermentation bio-H_2 systems do not require light and therefore have an advantage of being able to produce H_2. Both light-dependent (direct photolysis, indirect photolysis, and photo-fermentation) and dark-fermentation bio-H_2 systems are under intense study to find methods to improve both the rates of H_2 production and increase the H_2 yield. An added benefit of bio-H_2 systems is that they could be used to produce H_2 by utilizing organic waste that would otherwise be incinerated or disposed of in landfill (Nandi and Sengupta, 1998; Zajic *et al.*, 1978; Harper and Pohland, 1986). H_2 fermentation could be economical at less than stoichiometric yields, using the same bioprocess as methane fermentation. According to Benemann (1996), the most promising method to produce H_2 is the production of H_2 from low cost substrates using dark fermentation methods.

H_2 production by fermentation has been studied using a large group of pure cultures of isolated strains, such as *Clostridia* and *Enterobacter* (Heyndrickx *et al.*, 1991; Fumiaki *et al.*, 1993; Rachman *et al.*, 1997). However, only recently studies of H_2 production by mixed cultures have attracted research attention. Furthermore, in a continuous process producing H_2 from a waste, such as a wastewater treatment reactor,

the bacterial culture would be a mixed culture composition since, the wastewater itself contains a mixed culture.

Research on H_2 gas production using anaerobic mixed cultures has progressed over the past 5 to 10 years. However, a major problem facing researchers is the removal of H_2 by hydrogenotrophic methanogens and sulfur reducing bacteria (SRB). Inhibiting hydrogenotrophic methanogens would block the reduction of carbon dioxide (CO_2) to methane (CH_4), leaving only acidogens to produce H_2, carbon dioxide, and volatile acids (Kumar and Das, 2000). Avoiding the presence of organisms utilizing H_2, particularly hydrogenotrophic methanogens, was achieved in laboratory studies by operating a bioreactor at low pH and/or (for continuous studies) short retention times. Methanogens are affected more by lower pH and are slower growing than fermentative organisms (Hawkes *et al.*, 2002). An enrichment procedure, known as heat-shocking, which inhibits or kills nonsporeforming bacteria (hydrogen-consuming methanogens) and enriches sporeforming bacteria (hydrogen-producing acidogens) was recently reported by Van Ginkel and Sung (2001). Major issues in applying the above methods for fermentative H_2 production would be the cost and hence the process economics.

Other researchers have described the use of inhibitors such as oxygen, 2-bromoethanesulfonate (BES), chloroform and acetylene (Sparling and Daniels, 1987; Patel *et al.*, 1984; Sprott *et al.*, 1982). Again, inhibition of methanogenesis is essential for increasing H_2 production and for full scale industrial application, the method employed must also be cost effective.

Several reports have described the use of long chain fatty acids (LCFAs) to inhibit to aceticlastic and hydrogenotrophic methanogens (Hanaki *et al.,* 1981; Koster and

Cramer, 1987; Angelidaki and Ahring, 1992; Rinzema *et al.,* 1994; Lalman and Bagley, 2000; Alves *et al.,* 2001). A major LCFA source is effluents from food-processing industries. Typically, vegetable oils consist of varying quantities of LCFAs. For example, safflower and cottonseed oils consist of approximately 75% (w/w) and 45% (w/w) linoleic acid (LA), while peanut and olive oils contain approximately 60% oleic acid (OA) (Sonntag, 1982). Vegetable oils rich in oleic or linoleic acids such as soyabean and corn could be a suitable source of methanogenic inhibitor for generating bio-H_2. The effect of C_{18} LCFAs inhibition of hydrogenotrophic methanogens has not been examined as a method to divert the reducing equivalence for H_2 production.

1.2 Objectives

In view of the inhibitory effects of C_{18} LCFAs on acetogens and methanogens, the following objectives were formulated to assess the potential of LA and OA to enhance bio-H_2 production potential during the dark fermentation of glucose.

1. Assess the degradation of LA and OA for producing bio-H_2.
2. Examine the effect of varying concentrations LA and OA on H_2 production from glucose.
3. Assess the effect of high VFA concentrations and intermittent sparging on H_2 production from glucose in the presence of varying LCFAs concentrations.
4. Determine the effect of varying the initial pH on H_2 production from glucose in the presence LA and OA.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview

Anaerobic digestion processes are widely applied to treat a variety of wastes. The increasing popularity of the process is mainly due to the fact that it couples the removal of organic compounds with the production of energy in the form of CH_4 (Koster and Cramer, 1987). In conventional anaerobic treatment processes, organic pollutants are first converted to acetate and H_2, both of which are eventually converted into methane. Only limited studies have been conducted to explore the feasibility of recovering the intermediate H_2, instead of CH_4. The following sections summarize biohydrogen production from the anaerobic fermentation process.

2.2 Fermentative hydrogen production

Anaerobic digestion is a complex multi-stage process of organic compound degradation to CH_4 and CO_2 by the action of numerous anaerobic microflora. Figure 2.1 (Gujer and Zehnder, 1983) summarizes the microbial process into four phases:

1. Hydrolysis
2. Acidogenesis
3. Acetogenesis
4. Methanogenesis

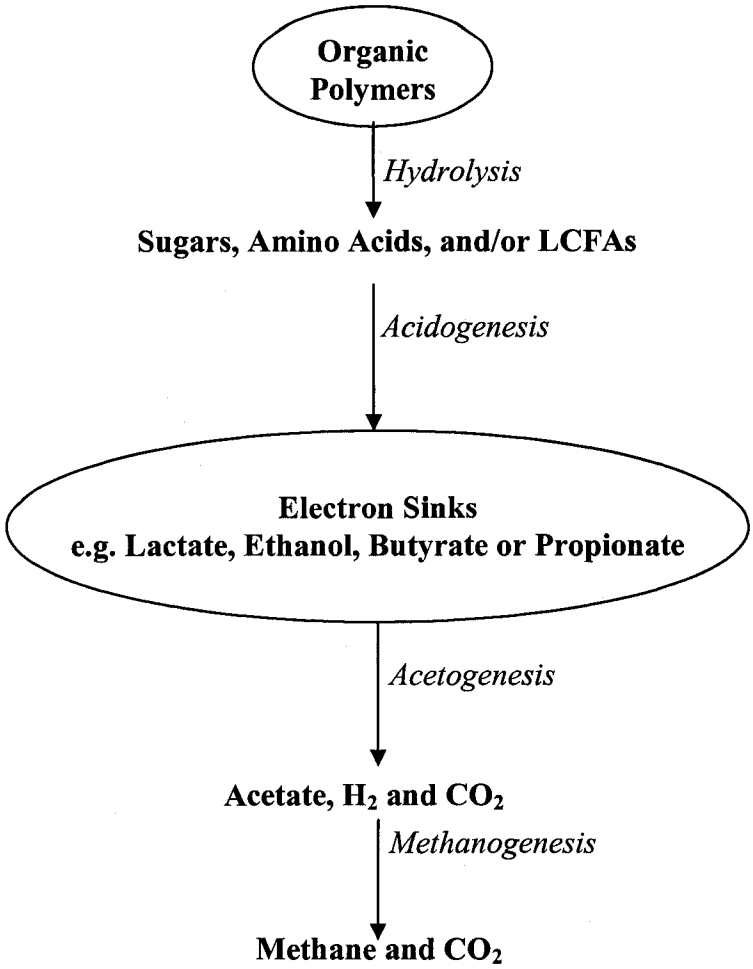

Figure 2.1: Main degradation pathways in anaerobic digestion.

In the first hydrolysis phase, complex insoluble matter is broken down into intermediate, readily fermentable organic monomers such as glucose, by the use of enzymes excreted by hydrolytic organisms (Veeken *et al.,* 2000). The second phase, acidogenesis, involves the fermentation of hydrolysis products to organic acids, alcohols, hydrogen and carbon dioxide (Veeken *et al.,* 2000). This phase is carried out by a large group of fast growing fermentative organisms, such as *Enterobacter aerogenes* and *Escherichia coli* (Malina and Pohland, 1992). In phase three, some of the acidogenesis products are converted to acetic acid, hydrogen and carbon dioxide. These processes are

executed by acetogenic, H_2-producing organisms such as *Clostridium thermoaceticum*. The final step, methanogenesis, involves conversion of acetic acid to methane and carbon dioxide by aceticlastic methanogens and reduction of CO_2 with H_2 to form CH_4 by hydrogenotrophic methanogens (Veeken *et al.*, 2000).

Figure 2.2 summarizes several common routes to some major end products from the microbial fermentations of pyruvic acid. Different microorganisms mediate the conversion of a variety of substrates via several pathways. Hence, it is important to screen a wide variety of organisms that will maximize the yield of a desired product, while minimizing the formation of other by-products (Shuler and Kargi, 2000).

Anaerobic microorganisms can produce H_2 using a variety of carbohydrates. These microorganisms transfer electrons released during metabolism to hydrogen ions to form molecular H_2 through the activity of hydrogenases (Das and Veziroglu, 2001). During the breakdown of glucose, H_2 evolution may occur by three different metabolic pathways (Tanisho *et al.*, 1998).

In the first pathway, organisms evolve H_2 as the result of anaerobic oxidation of pyruvate, an intermediate metabolite of carbohydrates and some other compounds. In this reaction the degradation of pyruvate to acetyl-CoA with H_2 evolution is catalyzed by pyruvate-ferredoxin oxidoreductase (Equations 2.1, 2.2, 2.3 and 2.4) (Luong *et al.*, 1983). The latter reactions are common in many *Clostridium* species and several other anaerobes: *T. brockii*, *Peptococcus anaerobus*, *Eubacterium limosum*, *M. elsdenii*, *Sarcina maxima*, *Sar. Ventriculi, Rum. Albus, Veillonella alcalescens*, certain sulfate-reducing bacteria and spirochetes (Mortenson and Chen, 1974).

$$\text{Pyruvate} + \text{Fd} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + CO_2 + \text{Fd}\,H_2 \qquad (2.1)$$

$$\text{Acetyl-CoA} \longrightarrow \text{Acetyl-phosphate} \qquad (2.2)$$

$$\text{Acetyl-phosphate} \xrightarrow{+ADP} \text{Acetate} + \text{ATP} \qquad (2.3)$$

$$\text{Fd}\,H_2 \longrightarrow \text{Fd} + H_2 \qquad (2.4)$$

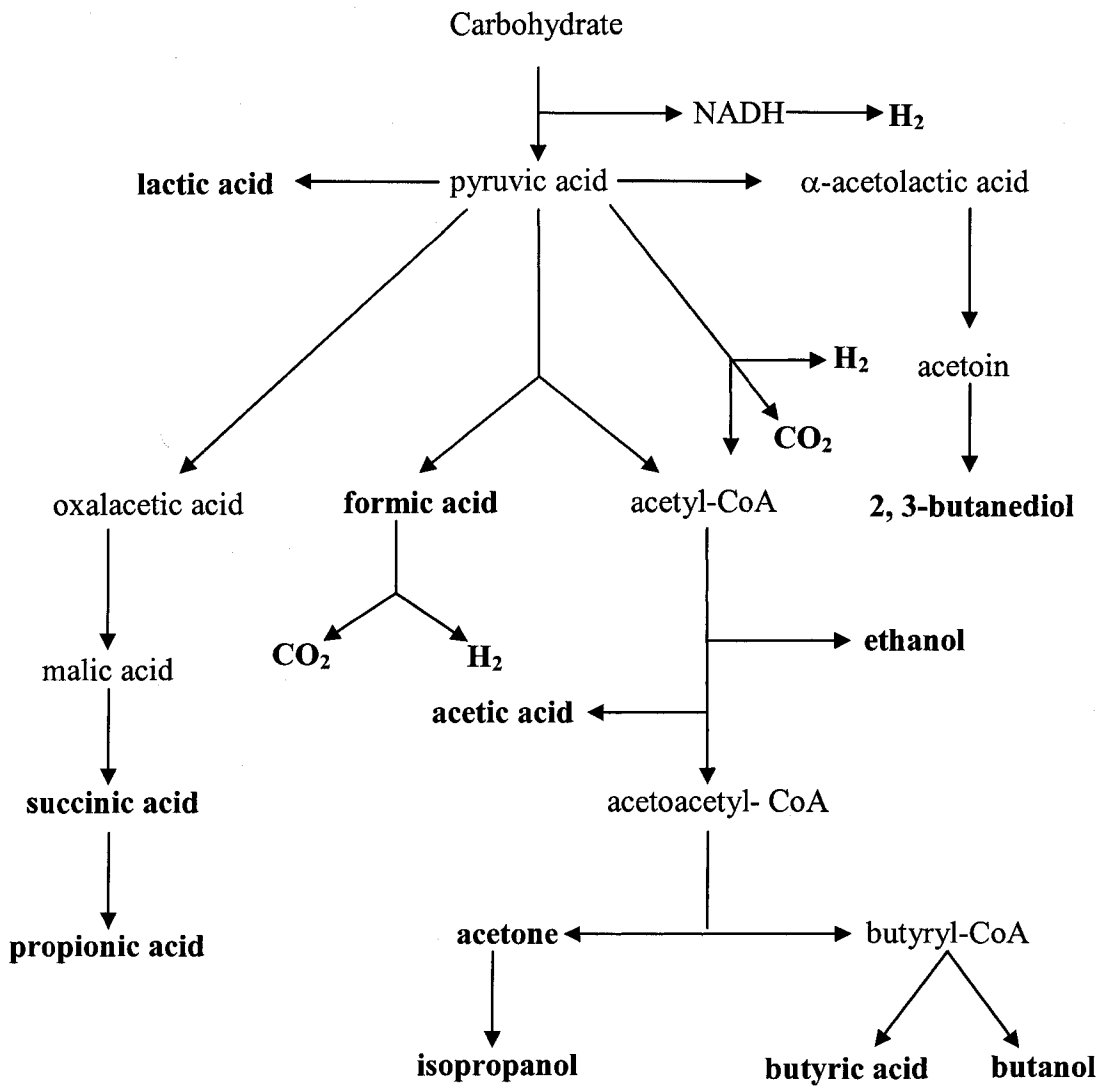

Figure 2.2: Major end products of the bacterial fermentations of carbohydrate from pyruvic acid. The end products are shown in boldface type. (Stanier, 1986)

In the second pathway, formate formed through pyruvate decomposition (Equation 2.5) undergoes further decomposition to release H_2 (Equation 2.6, Das and

Veziroglu, 2001). Formate degradation with the evolution of H_2 in microorganisms is catalyzed by pyruvate H_2 lyase. Members of the enterobacteriaceae and other facultative anaerobes produce H_2 from formate (Gottschalk and Andreesen, 1979).

$$\text{Pyruvic acid} + \text{HCoA} \longrightarrow \text{Acetyl-CoA} + \text{Formic acid} \quad (2.5)$$

$$\text{Formic acid} \longrightarrow H_2 + CO_2 \quad (2.6)$$

In the third pathway commonly referred to as NADH (nicotinamide adenine dinucleotide, reduced form) pathway, H_2 is evolved by the reoxidation of NADH catalyzed by NADH ferredoxin oxidoreductase (Equation 2.7, Tanisho *et al.,* 1998).

$$NADH + H^+ \longrightarrow H_2 + NAD^+ \quad (2.7)$$

Thus, any reaction that increases the amount of NADH within the cell has the potential to increase the H_2 yield.

2.3 Product formation and distribution

Carbohydrates are the preferred substrate for H_2-producing fermentations. The fermentation of carbohydrate predominately gives rise to acetic acid, butyric acid and H_2 gas (Classen *et al.,* 1999). Glucose, isomers of hexoses, or polymers in the form of starch or cellulose, yield different amounts of H_2 per mole of glucose depending on the fermentation pathway and end product(s) (Levin *et al.*, 2004). The type of product formed and distribution of volatile fatty acids (VFAs) have been used as indicators for monitoring H_2 production. VFAs typically found in the anaerobic systems are acetic, propionic, butyric, n–butyric and iso-butyric acids (Rittmann and McCarty, 2001). Acetic acid is the most common product formed during fermentation of carbohydrates and proteins. Propionic acid is mostly formed by fermentation of carbohydrates, while butyric acid is generated in the fermentation of proteins, lipids and carbohydrates.

When acetic acid is the end-product, a theoretical maximum of 4 mol $H_2 \cdot mol^{-1}$ glucose is obtained (Equation 2.8, Table 2.1). A theoretical maximum of 2 mol $H_2 \cdot mol^{-1}$ glucose is possible if butyrate is the only VFA produced (Equation 2.9, Table 2.1).

Table 2.1: Products of acidification of glucose (adapted from IWA, 2002)

Product	Reaction	Equation
Acetic acid	$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + CO_2$	2.8
Butyric acid	$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$	2.9
Propionic acid	$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O$	2.10
Lactic acid	$C_6H_{12}O_6 \longrightarrow 2CH_3CHOHCOOH$	2.11
Propionic + acetic acids	$3C_6H_{12}O_6 \longrightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2H_2O + 2CO_2$	2.12
Ethanol	$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$	2.13

Thus, the greatest theoretical H_2 yield is associated with acetate as the fermentation end-product, followed by butyrate. However since acetate alone is not produced in practice, high H_2 yields are associated with a fermentation mixture dominated by acetate and butyrate, and low H_2 yields are associated with propionate and reduced end-products like alcohols, lactic acid, etc (Equations 2.10 to 2.13, Table 2.1) (Levin *et al.*, 2004). Equation 2.10 has not been observed in cultured samples, because a H_2 source is required (IWA, 2002). Propionate formation is more common from Equation 2.12 (IWA, 2002).

Furthermore, degradation of products of glucose acidification is shown in Table 2.2. At low H_2 concentration ethanol and lactic acid can be degraded to acetic acid and H_2 (Equations 2.15 and 2.18). However, if lactic acid is converted into propionic acid, H_2 is consumed in the process (Equations 2.14).

Table 2.2: Fermentation reactions of byproducts from glucose acidification products (Bagley and Brodkorb, 1999)

Substrate	Product	Reaction	Equation
Lactic acid	Propionic acid	$CH_3CHOHCOOH + H_2 \longrightarrow CH_3CH_2COOH + H_2O$	2.14
Lactic acid	Acetic acid	$CH_3CHOHCOOH + H_2O \longrightarrow CH_3COOH + 2H_2 + CO_2$	2.15
Butyric acid	Acetic acid	$CH_3CH_2CH_2COOH + 2H_2O \longrightarrow 2CH_3COOH + 2H_2$	2.16
Propionic acid	Acetic acid	$CH_3CH_2COOH + 2H_2O \longrightarrow CH_3COOH + 3H_2 + CO_2$	2.17
Ethanol	Acetic acid	$CH_3CH_2COOH + H_2O \longrightarrow CH_3COOH + 2H_2$	2.18

Reduced fermentation end-products such as ethanol, butanol, and lactate, contain additional electrons not present in the corresponding acids, so alcohol production gives correspondingly lower H_2 yields (Hawkes *et al.*, 2002). To maximize the H_2 yield, the metabolism pathway must be directed away from producing alcohols (ethanol, butanol) and reduced acids (lactate) and towards short chain VFAs. *C. pasteurianum* is a classic H_2 and VFA producer, but its metabolism can be directed away from H_2 production and towards solvent production using high glucose concentrations (12.5% w/v) or CO (which inhibits Fe-hydrogenase) or by limiting iron concentrations (Dabrock *et al.*, 1992). The H_2 partial pressure (pH_2) is an extremely important factor in controlling the type of end product. H_2 synthesis pathways are sensitive to the H_2 partial pressure and hence, subject to end-product inhibition. As the H_2 concentrations increase, H_2 synthesis decreases due to a shift in the metabolic pathways and favour the production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine (Levin *et al.*, 2004).

2.4 Methanogenic Inhibition

H_2 can be produced sustainably by dark fermentation reactions using carbohydrate-rich substrates. Enterobacter (Fabiano and Perego, 2002), Bacillus (Kalia *et al.*, 1994) and Clostridium (Taguchi *et al.,* 1995) sp. have been used in pure cultures for producing H_2. For a technologically and economically feasible process, a stable mixed culture easily obtainable from natural sources, which is able to operate on non-sterile feedstocks, is required. The presence of organisms utilizing H_2, particularly methanogens has to be avoided in a sustainable production process.

2.4.1 Chemical inhibitors

Oxygen, 2-bromoethanesulfonate (BES), chloroform and acetylene are the four major inhibitors used by researchers in laboratory studies to inhibit methanogens (Sparling *et al.,* 1997). Air (oxygen) reduces the viability of most methanogens, but only to an extent similar to other anaerobes (Patel *et al.,* 1984). BES, an analog of the methanogens' coenzyme M (DiMarco *et al.,* 1990), is regarded as a methanogen specific inhibitor (Sparling and Daniels, 1987). However, BES resistant mutants (Santoro and Konisky, 1987) and reversal of BES inhibition of methanogenesis in specific species have been reported (Smith, 1983). Acetylene and chloroform, in low concentrations (0.5%), are potent inhibitors of methanogenic bacteria in pure cultures (Bomar *et al.,* 1985; Sprott *et al.,* 1982) and natural samples, but there are reports of acetylene inhibition of other anaerobes (Brouzes and Knowles, 1971).

Sparling *et al.* (1997) reported H_2 production from a model lignocellulosic waste in inhibited solid substrate anaerobic digesters. They compared the effectiveness of air, acetylene and BES as methanogen inhibitors in small batch reactors for producing H_2

using paper (25% w/w) as a model solid waste and either a mixed culture derived from anaerobic digesters or a pure culture of *Clostridium thermocellum* as inocula. Sparling *et al.* (1997) indicate that acetylene at 1% v/v in the headspace was as effective as BES in inhibiting methanogenic activity. The net H_2 produced and H_2 production rate were similar in a consortium of cultures exposed to either acetylene or BES Acetylene had no effect on the rate and amount of H_2 produced from a pure culture of *Clostridium thermocellum* grown under the same conditions. On the other hand, pre-incubation in air led to an initial inhibition of methane and transient production of net H_2 followed by methanogenic recovery.

Though several methods have been used in the laboratory to inhibit methanogenesis, large-scale production of H_2 is likely uneconomical and not environmentally friendly (Brosseau and Zajic, 1982).

2.4.2 Effect of heat-shocking and pH

Heat-shock treatment is a method used to remove nonsporeforming bacteria, such as methanogens, from inoculum that consumes H_2 (Lay, 2001). In this method, the mixed inoculum culture is heated to about 104°C for 2 hr to eliminate nonsporeformers. Logan *et al.* (2002) reported that heating ordinary soil was sufficient to remove H_2 consuming methanogens and leave the spore forming H_2-producing bacteria unaffected. The heat treatment allowed producing a 60% pure H_2 gas stream (40% CO_2) using carbohydrates such as glucose, sucrose and starch. However, a low pH was simultaneously used to limit methanogen growth in batch tests, so the effects of heat treatment and pH on methane production were not separated.

Oh *et al.* (2003) studied the effectiveness of heat treatment and pH control to limit methanogenesis in a mixed culture for enhancing H_2 gas production. In their study the combined effects of low pH and the heat treatment procedure on biological H_2 production were conducted in batch, continuous-gas-release and respirometric tests. Oh *et al.* (2003) reported that H_2 concentrations between 57 to 72% were produced in all tests and that heat treatment (HT) of the inoculum (pH 6.2 or 7.5) produced greater H_2 yields than low pH (6.2) conditions with a non-heat treatment (NHT) inoculum. The conversion efficiencies of glucose to H_2 (based on theoretical yield of 4 mol $H_2 \cdot mol^{-1}$ glucose) reported were as follows: 24.2% (HT, pH = 6.2), 18.5% (HT, pH = 7.5), 14.9% (NHT, pH = 6.2), and 12.1% (NHT, pH 7.5). The main products of glucose (3 g-COD l^{-1}) utilization (≥99%) in batch tests reported were acetate (3.4 to 24.1%), butyrate (6.4 to 29.4%), propionate (0.3 to 12.8%), ethanol (15.4 to 28.8%) and hydrogen (4.0 to 8.1%), with lesser amounts of acetone, propanol and butanol (COD basis). The major problem in these experiments was the H_2 concentrations in all batch cultures reached a maximum of 57 to 72% after 30 hr but thereafter rapidly declined to non-detectable levels within 80 hr. However, in the latter studies H_2 was not converted to methane. Additional experiments conducted by Oh *et al.* (2003) indicated a loss of H_2 to form acetate by homoacetogens.

2.4.3 Effect of VFAs

The toxicity of VFAs to methanogens is pH dependent since only the nonionized form exhibits microbial toxicity. VFAs exhibit microbial toxicity at pH below 7. At pH 6, 5% of the acetate present is protonated, or in the nonionized form, and is toxic to microorganisms. The proportion of ionized to nonionized form of a VFA is dependent upon the pH of the solution. The concentration of the nonionized form of an individual

VFA in solution can be estimated using the Henderson-Hasselbalch equation (equation 2.16) as follows:

$$\text{nonionized VFA} = \frac{mM(TotalVFA)}{\left[\frac{10^{(-pk_a)}}{10^{(-pH)}}+1\right]} \qquad (2.16)$$

where mM (Total VFA) = concentration of a VFA in solution (millimolar), pK_a (24°C) = equilibrium constant (Frutun, 1982).

Protonated (nonionized) VFAs can easily penetrate the lipid cell membrane. When they enter the cell where the pH is 7, these fatty acids are ionized and release hydrogen ions which cause a decrease in the intercellular pH (McCarty and McKinney, 1961). Moreover, VFAs have pK_a values in the range of 4.7 to 4.9 and an accumulation of these acids results in a pH decrease (Wilcox *et al.*, 1995).

2.4.4 Effect of LCFAs

LCFAs are inhibitory to a variety of anaerobic microorganisms. Nieman (1954) illustrated that unsaturated fatty acids exerted antibacterial effects on gram-positive bacteria and yeast, while they did not have any effect on gram negative bacteria. Furthermore, he concluded that the inhibitory effects of unsaturated fatty acids increased as the number of double bonds increases. Hwu *et al.* (1998) showed that LCFA adsorption is a prerequisite for biodegradation. However, this adsorption to the surface of anaerobic sludge could limit the transport of soluble substrates by reducing the permeability of the cell wall, eventually leading to a decrease in the substrate conversion rate (Sayed *et al.*, 1988; Rinzema *et al.*, 1993; Demeyer and Hendrrickx, 1967).

The impact of LCFAs on acidogens, acetogens, aceticlastic methanogens and hydrogenotrophic methanogens have been described in several reports. Aceticlastic

methanogens and hydrogenotrophic methanogens are affected by the presence of linoleic, oleic, stearic, lauric, capric and caprylic acid (Koster and Cramer, 1987; Lalman and Bagley, 2000; Lalman and Bagley, 2001). Hanaki *et al.* (1981) concluded that LCFA mixtures inhibited aceticlastic methanogens and hydrogenotrophic methanogens at 37°C. Similarly, other researchers have shown that LCFAs are inhibitory to aceticlastic methanogens and hydrogenotrophic methanogens (Lalman and Bagley, 2002; Angelidaki and Ahring, 1992; Rinzema *et al.,* 1994; Alves *et al.,* 2001). Demeyer and Hendrickx (1967) observed that linolenic (C18:3) acid also affected H_2 consumption.

Lalman and Bagley (2000) examined the impact of LA on aceticlastic and hydrogenotrophic methanogens at 21°C. They reported a threshold concentration of 30 mg l^{-1} LA was inhibitory to aceticlastic methanogens; however, concentrations greater than 30 mg l^{-1} was slightly inhibitory to hydrogenotrophic methanogens. Lalman and Bagley (2001) also reported the effects of OA and stearic acid (SA) on aceticlastic and hydrogenotrophic methanogens. SA was not inhibitory to aceticlastic methanogens even at 100 mg l^{-1} whereas a threshold concentration of 30 mg l^{-1} OA was enough to inhibit acetic acid consumption and affect hydrogenotrophic methanogens. Lalman and Bagley (2002) investigated the effect of LA, OA and SA on H_2 degradation at 21°C using culture unacclimated to LCFAs. They observed low hydrogen consumption rates in cultures receiving LA and OA compared to cultures receiving SA.

2.5 Environmental factors affecting H_2 production

H_2 production by bacteria is highly dependent on the process conditions such as temperature, pH, and gas partial pressure (Levin *et al.*, 2004). H_2 production by different microorganisms is intimately linked with their respective energy metabolism (Nandi and

Sengupta, 1998). Kumar *et al.* (2000) suggested that H_2 production associated the microbial growth. Their experimental results showed that the specific growth rate is linearly correlated with the specific H_2 production rate. Thus, optimizing environmental conditions to increase bacterial metabolism and growth rate could increase the H_2 yield.

2.5.1 Effect of pH

Each microbial group involved in anaerobic degradation has a specific operating pH region for optimal growth. For acidogens, the optimal pH is approximately 6, whereas for acetogens and methanogens the optimum is 7 (Moosbrugger *et al.*, 1993). Dabrock *et al.* (1992) and Lay (2000) indicated that pH control is crucial for optimizing H_2 production, due to the effects of pH on the hydrogenase activity and/or on the metabolism pathways. In order to obtain dominant microorganisms for producing H_2 production and preventing H_2 removal by methanogenesis, the pH has to be controlled at an optimum value. Very low pH can inhibit H_2 production, and at pH values lower than 6.3 and higher than 7.8, methanogenesis decreases or stops (Chen *et al.,* 2002). A range of pHs was reported to optimize carbohydrate fermentation by mixed bacterial cultures. An optimal pH value of 9 was reported for H_2 production using batch fermentation of sucrose (Lee *et al.*, 2002), while for the continuous fermentation of sucrose (Ren *et al.*, 2001) and starch (Lay, 2000) the pH ranges were 4.0 to 4.5 and 4.7 to 5.7, respectively.

Zheng and Yu (2004) performed batch experiments to examine the role of pH in the biological production of H_2 and VFAs from glucose using mixed anaerobic cultures. They demonstrated that the H_2 yield fluctuated between 1.3 and 1.57 mol $H_2 \cdot$ mol^{-1} glucose when the reactor was operated at pH values of 4.0 and 5.0. However, a further increase in pH led to a considerable decrease in the H_2 yield, especially at pH 7.5 and 8.0.

Acetate, propionate, butyrate and ethanol were the major products of acidogenesis. Zheng and Yu (2004) also observed that butyrate production was favorable at pH 4.0 to 5.0, whereas the production of acetate was more pronounced at pH 6.0 to 8.0. They illustrated that the optimum pH for specific H_2 production was 5.5. Zoetemeyer *et al.* (1982), using glucose at pH 5.7, showed an optimum H_2 evolution rate and acetate/butyrate with negligible propionate production at a 4 hr hydraulic retention time (HRT). While at pH 6.4 the optimal HRT for H_2 production was 7.1 hr. Fang and Liu (2002) investigated the optimum pH for H_2 production from glucose using a 6 hr HRT over a pH range from 4.0 to 7.0 and observed an optimum H_2 yield at pH 5.5. Van Ginkel *et al.* (2001), using a heat-shocked anaerobic culture and sucrose as a substrate, demonstrated that the H_2 production rate increased and the potential decreased with a decrease in the sucrose concentration from 45 to 15 g-COD l^{-1}. The data indicated that substrate deprivation was more important than initial pH on H_2 production/clostridial germination. A substrate concentration of 15 g-COD l^{-1}, a pH value of 5.5 could be an optimum for clostridial growth. Khanal *et al.* (2004) investigated the effects of pH and intermediate products on biological H_2 production. They observed the initial pH had a profound effect on both H_2 production potential and the H_2 production rate. The data demonstrated that the lowest initial pH of 4.5 provided the highest specific H_2 production potentials of 1.8 mol $H_2 \cdot mol^{-1}$ glucose and 1.05 mol $H_2 \cdot mol^{-1}$ glucose for sucrose and starch, respectively.

In most of the experiments conducted to determine the optimum pH favouring VFAs and H_2 production, HCl and NaOH have been used to control the pH in the reactor (Lee *et al.*, 2002; Ren *et al.*, 2001; Chen *et al.*, 2002). This pH control method could be expensive in large scale reactors. An alternative solution, proposed by Yokoi and

coworkers is to use Enterobacter *aerogenes* strain H-39, which is able to grow anaerobically and produce H_2 at a low pH value of 4 (Yokoi *et al.*, 1995).

Many studies indicate that methanogenic activity can be blocked by adjusting the pH to one that is weakly acidic and by shortening the solids retention time (SRT) even further than the minimum for methanogenic growth. On the contrary, Kim *et al.* (2004) reported that initially at pH 4.3 ± 0.2 in a semi-continuous reactor, the H_2 yield was relatively low due to the methanogenic activity. The methanogenic activity originating from the H_2 utilizing methanogens was inhibited by nitrate addition. Similarly, Lee *et al.* (2002) reported that a maximum specific production H_2 yield of 1.79 mol $H_2 \cdot mol^{-1}$ sucrose at an initial pH 9. This high yield could be because of the high substrate concentration (10,000 mg l^{-1}) used in batch studies. The substrate concentration regulated the quantity of VFAs produced and at lower pH could become toxic to the microbial population, especially in the nonionized forms.

2.5.2 Effect of temperature

H_2 production systems are mainly operated at mesophilic (30 to 35°C) or thermophilic (50 to 60°C) temperatures (Lin and Chang, 2004). Rittmann and McCarty (2001) indicated that at optimal temperature range (i.e., up to 50-60°C for thermophilic microorganisms), the reaction rates approximately doubled for each 10°C raise in temperature. Thus, the advantage of operating in the thermophilic range is that the reaction rates are larger allowing for higher loading rates. Higher reaction temperatures facilitate smaller reactors with low retention times. Zhang *et al.* (2002) concluded that more H_2 can be produced from starch under thermophilic condition (55°C) than mesophilic condition (37°C). Fang and Yu (2001) reported higher H_2 yields for lactose

acidification by granular methanogenic sludge at 55°C and at pH 5.5. The main disadvantage of thermophilic H_2 production is the energy input required and hence, the cost. Thermophilic systems are known to be sensitive to temperature changes (Rittmann and McCarty, 2001). H_2 gas solubility is another factor that depends on temperature. An increase in temperature causes a decrease in gas solubility (greater Henry's constant). Hence, a thermophilic system would allow smaller dissolved H_2 concentrations than a mesophilic system, which favours higher H_2 yield.

Most of the studies on H_2 production were conducted under mesophilic conditions (Lay, 2001). Zoetemeyer *et al.* (1982) reported that product distribution was affected by temperature and a system operating on glucose fed with 6 hr HRT. Butyrate concentrations fell sharply above 30°C, while acetate and propionate concentrations increased.

2.5.3 Effect of H_2 partial pressure

H_2 partial pressure in the liquid phase is a key factor affecting H_2 production. Producing H_2 is a process by which bacteria oxidizes reduced ferredoxin and H_2-carrying coenzymes. These reactions are thermodynamically less favorable as the H_2 concentration in the liquid increases (Hawkes *et al.*, 2002). Anaerobic degradation reactions in the liquid are controlled by free energy changes, which depend on H_2 partial pressure (Guwy *et al.*, 1997). Thermodynamically unfavorable conditions can develop for organic acids and alcohols degradation, resulting in formation and accumulation of reduced organic acids such as propionic and butyric acid in the case of high H_2 partial pressure (Phelps *et al.*, 1985). Many reactions that produce H_2 are not thermodynamically favourable at standard conditions (H_2 partial pressure of 1 atmosphere). For instance,

butyric and propionic acids can only be fermented when the H_2 partial pressure is less than 32.04 and 40.34 Pa respectively. Ethanol and lactic acid, however, are readily fermented at H_2 partial pressures 2 to 3 orders of magnitude higher (Fennell *et al.*, 1997).

Mizuno *et al.* (2000) have demonstrated that lowering dissolved H_2 by sparging with N_2 caused a 68% increase in H_2 yield from a reactor operating on an enriched mixed microflora with 10 g l^{-1} glucose-mineral salts at a pH 6.0 and an 8.5 hr HRT. Tanisho *et al.* (1998) reported that when argon or H_2 was sparged into a culture liquid in order to remove CO_2 from culture liquid, the amount of residual NADH increased, causing the H_2 yield to increase. Heat treatment of the inoculum increased H_2 yields by preventing the loss of H_2 to methanogenesis; however, H_2 is still lost to acetic acid or formic acid generation from H_2 and CO_2 (Equations 2.17 and 2.18).

$$4H_2 + 2CO_2 \longrightarrow CH_3COOH + 2H_2O \quad (2.17)$$

$$H_2 + HCO_3^- \longrightarrow HCOO^- + H_2O \quad (2.18)$$

To reduce H_2 losses via acetogenesis, Park *et al.* (2005) reduced the CO_2 concentrations in the headspace substantially using a chemical scavenger (KOH). Park *et al.* (2005) also indicated that the reduction in CO_2 increased the H_2 yield from 1.4 to 2.0 mol $H_2 \cdot mol^{-1}$ glucose.

Logan *et al.* (2002) investigated biological production of H_2 from different substrates using a heat shocked mixed culture. An intermittent pressure release method (Owen method) and continuous gas release method using a bubble measurement device (respirometric method) was used to assess gas sparging on H_2 production. The respirometric method was shown to result in a 43% increase in H_2 yield compared to the Owen method. The lower glucose conversion to H_2 using the Owen method could have

been due to the repression of hydrogenase activity from the high H_2 partial pressures in the gas-tight bottles.

Dissolved H_2 concentrations may be related to substrate concentration. Batch tests conducted with microcrystalline cellulose levels over 25 g l^{-1} significantly inhibited H_2 production, with a maximum H_2 yield occurring at cellulose to initial sludge inoculum concentration of 8 g cellulose·g^{-1} VSS (Lay, 2001). Dissolved H_2 concentrations may also be related to the degree of agitation. Lay (2000) also indicated that an increase in agitation speed of a continuous laboratory-scale reactor mixed by a magnetic stirrer (from 100 to 700 rev/min) more than doubled the daily rate of H_2 production using starch as a substrate.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental plan

The experimental plan was developed in order to achieve the objectives. The experiments were divided into four different stages (Table 3.1). The first stage of experiments incorporated control studies to examine H_2 production from LCFAs degradation over an 8 day period. The second stage of experiments assessed the effect of varying concentrations of LA and OA on H_2 production and accumulation from microbial fermentation of 5,000 mg l^{-1} glucose. The third stage of experiments was formulated to examine the effects of high VFA and intermittent sparging in the presence of LCFA. Finally, the fourth stage of experiments examined the effect of LA and OA at varying initial pH on H_2 production potential during glucose fermentation..

3.2 Reagents and analytical equipments

Linoleic acid ($C_{18:2}$) (99%) and oleic acid ($C_{18:1}$) (>99%) were purchased from TCI, USA. The Dionex ion chromatograph (IC) (DX-500) was calibrated with acetic acid (99.8%), propionic acid (99.8%), and n-butyric acid (99.9%) (Fisher Scientific, Toronto, ON). Methane (99.99%) (Altech, USA), CO_2 (99.999%) and H_2 (99.99%) gases (Praxair, ON) were used to calibrate the gas chromatograph (GC). Carrier gas used was nitrogen (99.999%, Praxair, ON). Glucose (ACP Chemicals, Montreal, Quebec) degradation was monitored with a Dionex ion chromatograph (DX-600). Ethanol (95%), iso-propanol (99.9%), propanol (99%), iso-butanol (99%) and butanol (99.4%) (BDH Chemicals, Toronto, ON) were analyzed using the Dionex 600 IC configured with an electrochemical detector.

Table 3.1: Experimental plan and details

Exp. Stage	Experiment	Substrate Concentration (mg/L)	Initial pH	LCFA Concentration (mg/L)
1	LCFA Control Study	0	7.7 - 7.8	LA 0, 500, 1000, 1500 & 2000
				0A 0, 500, 1000, 1500 & 2000
2	Hydrogen Recovery Study	5000	7.7 -7.8	LA 0 , 500, 1000, 1500 & 2000
				0A 0, 500, 1000, 1500 & 2000
3	High VFA and Intermittent Sparging	5000	-	LA 0 , 500, 1000, 1500 & 2000
				0A 0, 500, 1000, 1500 & 2000
4	Initial pH Study	5000	7.8, 6.0 & 5.0	LA 0 & 2000
				OA 0 & 2000

3.3 Batch reactors

3.3.1 Inoculum source

Anaerobic digester sludge obtained from the wastewater treatment plant of an ethanol production plant in Chatham, Ontario, was used as the seed culture for two batch reactors A and B. An 8-l semi-continuous reactor (Reactor A) with a 4-l liquid volume was maintained at room temperature (23 ± 2°C) with approximately 20,000 mg l^{-1} volatile suspended solids (VSS). The basal medium at pH ranging from 7.6 to 7.8 (Table 3.2) was used to dilute the inoculum from Reactor A to 6,000 mg l^{-1} VSS into a second 4-l semi-continuous reactor (Reactor B). Reactor B was also maintained at room temperature (23 ± 2°C) with a 3-l liquid volume. Reactor A served as an inoculum source to reactor B, while inocula for the serum bottles (160 ml) were removed from Reactor B.

Table 3.2: Basal medium characteristics (Lalman and Bagley, 2000)

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

3.3.2 Operation of inoculum reactors

Reactors A and B were operated in batch mode and acclimatized to 5,000 mg l^{-1} of glucose, which was fed in amorphous form once in every 5 to 6 days time when acetate and gas production measurements indicated that all glucose and byproducts were consumed. To maintain anaerobic conditions the reactors were purged with nitrogen after each feeding. Operational stability for both reactors was monitored using gas production, pH, alkalinity (as $CaCO_3$) and VFA measurements. Prior to inoculation of the 160 ml serum bottles, pH, total suspended solids (TSS) and volatile suspended solids (VSS) were measured to characterize the culture in Reactor B.

3.3.3 Serum bottle preparation

All batch experiments were conducted in 160 ml serum bottles, which were prepared in an anaerobic glove box (COY Laboratory Products Inc., Michigan, USA). The glove box atmosphere was 77.5% N_2, 20% CO_2 and 2.5% H_2 atmosphere (Praxair, ON, Canada). All experiments were conducted with 50 ml liquid volume. The serum bottles were prepared by diluting the inoculum from reactor B to 2,000 mg l^{-1} VSS with basal medium. The volume of glucose and LCFA to be added was considered while diluting the inoculum. The bottles were then sealed with 20 mm Teflon® - lined silicone rubber septa and aluminum crimps. All bottles received 20 ml overpressure of 77.5%/20%/2.5% $N_2/CO_2/H_2$ mixture to avoid the formation of negative pressure during sampling. The required amount of LCFA and glucose were injected using 50,000 mg l^{-1} LCFA and 100,000 mg l^{-1} glucose stock solutions. The bottles were then maintained at 200 rpm on an orbital shaker (Lab Line Instruments) at room temperature (23 ± 2°C).

3.3.4 Sample removal, preparation and storage

Sampling procedures and materials were the same during all experiments. Samples were analyzed for glucose, VFAs and gas composition. The liquid samples were withdrawn using a 2.5 ml Hamilton Gastight® (VWR, Canada) syringe, while gas samples were withdrawn using a 50 μl Hamilton Gastight® (VWR, Canada) syringe. The gas samples withdrawn were manually injected on the GC (Varian, 3800). During each experiment, 0.5 ml samples were periodically withdrawn for glucose, VFAs and alcohol measurement. Samples withdrawn were transferred to a 7.5 ml culture tube, which contained 4.5 ml of Milli-Q® (Millipore, Nepean, ON) grade water. The samples were then centrifuged at 1750 g for 5 minutes. The centrate was filtered through a 25 mm

diameter Easy pressure syringe filter holder (PAL Sciences, MI, USA) fitted with a 25 mm diameter 0.45 μm polypropylene membrane (GE Osmonics, MN). The filtrate from the first filter was filtered again using a 1 ml polypropylene cartridge with a 20 μ PE frit (Spe-ed Accessories, PA) filled with Chelex® 100 to 200 mesh, sodium resin (Bio-Rad Laboratories, CA). The first 1 ml of the filtered sample from the second filtration step was discarded and the next 3 ml was transferred into a 5 ml polypropylene IC vial (Dionex, Oakville, ON) and stored at 4°C prior to analysis.

3.4 Experimental details

A 4l semi-continuous fed batch reactor (Reactor B) maintained at room temperature (23 ± 2°C) was used to provide inoculum (2,000 mg l^{-1} VSS) for the serum bottles. The performance of this reactor was determined by monitoring the glucose degradation, formation and removal of VFAs, total gas production, pH and alkalinity.

The first stage experiments were conducted over an 8 day period in 160 ml serum bottles at an initial pH of 7.8. The batch studies were conducted with varying LCFAs concentrations ranging from 0 to 2,000 mg l^{-1} (Table 3.1). One set of controls prepared for this study consisted of adding only culture to the serum bottles. Headspace samples were removed and analyzed for H_2 and CH_4 and liquid samples were removed and analyzed for VFAs.

In the second stage of experiments, glucose and a LCFA were injected into the culture to assess the effects of LCFA on H_2 production during glucose degradation at an initial pH of approximately 7.8 and at 23 ± 2°C. Two sets of controls were prepared for each subsequent experiment. In one control, no substrate was added while in another, only glucose was added. In the second experiment, glucose samples were removed over

the first 24 hr at the following intervals: every 2 hr for 8 hr.; every 4 hr. for the next 8 hr. and then after another 8 hr. Twelve hours after the first glucose injection, headspace samples and liquid samples were removed and analyzed for expected byproducts (VFAs, H_2, CH_4 and CO_2). Samples were also removed every 24 hr over a 4 day period for VFAs, H_2, CH_4 and CO_2 analysis. The third set of experiments used the same serum bottles from the second stage of experiments. On day 4, the bottles were opened and the headspace was sparged with N_2 gas for 1 to 2 minutes and resealed. The serum bottles were each injected with 20 ml of CO_2. Next, glucose was injected again and liquid and headspace samples were removed for VFAs, H_2, CH_4 and CO_2 analysis every 24 hr over the next 4 day period. Samples were not removed for glucose analysis.

The effect of initial pH was examined in the fourth set of experiments. The initial glucose (5,000 mg l^{-1}) and LCFA (2,000 mg l^{-1}) concentrations were constant and the initial pH conditions were 5.0, 6.0 and 7.8 (Table 3.1). These experiments were similar to the second stage of experiments, except the pH was adjusted to the initial value before glucose was added and when the serum bottle headspace was sparged with N_2. The pH was adjusted to the initial value using 1 M HCl or 1 M NaOH. The serum bottles were sparged with N_2 only on day 4. pH was monitored every 24 hr along with other parameters.

All the experiments were conducted at 23 ± 2°C. All controls and samples containing LCFAs were prepared in triplicate.

3.5 Analytical methods

3.5.1 VFAs measurement

VFAs were measured using a DX-500 IC (Dionex, Oakville, ON) equipped with CD 20 conductivity detector and GP 40 gradient pump. An IonPac® 24-cm x 4-mm diameter AS11-HC analytical column and an IonPac® AG11-HC guard column were used for the analysis (all from Dionex). A 25 μl sample loop was used for injecting a constant volume onto the analytical column. The VFA analysis method was previously described by Lalman and Bagley (2000). The method used three different eluents. Eluent A was Milli-Q® grade water (18 MΩ) (Millipore, Nepean, ON), eluent B was 5 mM NaOH and eluent C was 50 mM NaOH. The total eluent flow rate was 2 ml·min^{-1} and the individual flow rates are as shown in the Figure 3.1. The regenerant used was 12.6 mM H_2SO_4 solution with a flow rate of approximately 4.0 ml min^{-1} at a pressure of 5 psi. Both the eluents and the regenerant were prepared using Milli-Q® grade water.

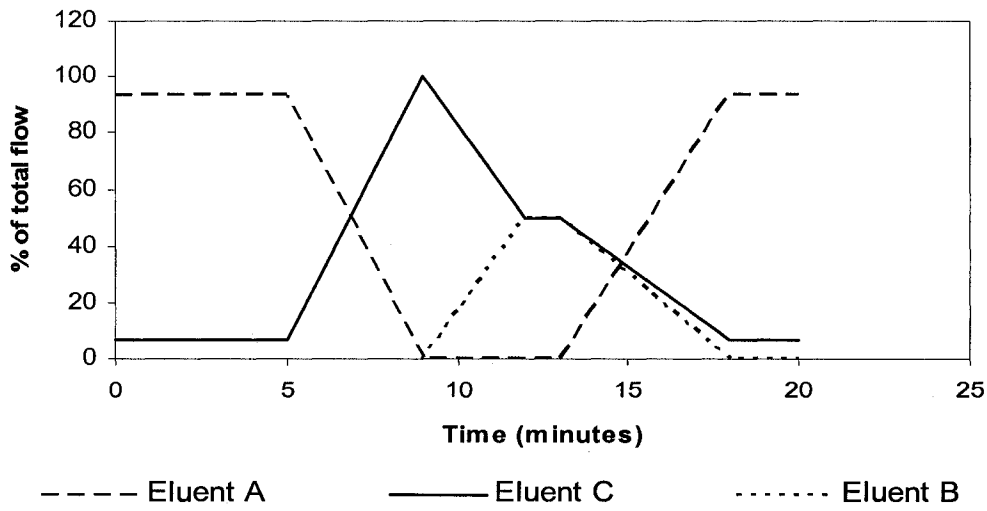

Figure 3.1: Individual eluent flow rate for VFA analysis on the Ion Chromotograph.

For the calibration graph, twelve standards were prepared and analyzed in triplicate at concentrations of 0 (Milli-Q® water), 0.5, 1, 2, 3, 4, 5, 10, 30, 50, 70, 100 and 200 mg l^{-1} for acetate, propionate and butyrate. The detection limit for the VFAs was 0.5 mg l^{-1}. An example calibration curve is presented in Appendix-A. During analysis, either a 50 or a 100 mg l^{-1} standard was analyzed after every 10 to15 samples. When analyzing the standards, a difference of less than ±5% from the standard value was considered acceptable.

3.5.2 Glucose and alcohol measurement

Dissolved glucose and alcohol concentrations in the reactors were measured using a DX-600 Ion Chromatograph (Dionex, CA, USA) equipped with a GP 50 multigradient pump, an AS 40 automated sampler and an ED 50 electrochemical detector. A CarboPac™ 25-cm x 4-mm diameter MA1 analytical column and a CarboPac™ 5-cm x 4-mm diameter MA1 guard column were used to achieve separation of the analytes (all from Dionex). A 25 μl sample loop was used to maintain a constant volume injection onto the analytical column. The method used 480 mM NaOH eluent at a flow rate of 0.4 ml min^{-1}. Glucose was detected at approximately 25 minutes, and ethanol, propanol i-propanol, butanol and i-butanol were detected at 7.38, 7.68, 8.98, 10.46 and 11.53 minutes, respectively

For the calibration graph, standards were prepared and analyzed in triplicate. The detection limit based on the lowest standard concentration was 1 mg l^{-1} for glucose and 5 mg l^{-1} for each alcohol. A sample calibration curve for glucose is presented in Appendix-B and the curves for the alcohols are given in Appendix-C. During the analysis, a blank and either a 50 or a 100 mg l^{-1} standard were analyzed after every 10 to 15 samples.

Sample carry over was a concern and none was detected. Eventually, the blank was removed from the sample queue. When analyzing the standards, a difference of less than ±5% from the standard value was considered acceptable.

3.5.3 Headspace gas measurement

To analyze the headspace in the reactors and serum bottles, a Varian 3800 gas chromatograph equipped with a thermal conductivity detector (TCD) and a 2-m x 1.0-mm diameter (ID) (OD = 1.6-mm) packed Shincarbon ST (Restek) column was used. The analysis was isothermal at 200°C with nitrogen as the carrier gas at 21 ml min^{-1} and the detector and injector temperatures set at 200°C and 100°C, respectively. H_2, CH_4 and CO_2 were detected at 0.43, 1.01 and 1.35 minutes, respectively.

Calibration standards for the gas chromatograph were prepared in serum bottles (160 ml) that had been purged with nitrogen (99.998%) for 2 to 3 minutes. The bottles were sealed with Teflon® lined septa and capped with aluminum crimp seals. Known quantities of H_2, CH_4 and CO_2 were injected into the capped bottles. During each headspace analysis, a standard was prepared within the calibration range and analyzed for the different gases. An example calibration curve for the gases is presented in Appendix-D. The detection limits for H_2, CH_4 and CO_2 were 0.000287 kPa, 0.000119 kPa and 0.000228 kPa, repectively.

3.5.4 LCFA delivery strategy

LCFA are hydrophobic compounds and the hydrophobicity is a function of the number of methylenic groups. Thus, the dispersion of these compounds will be affected by their dissolution rates in solution. Short chain fatty acids (SCFAs) below C8 are

relatively miscible in water, however as the number of carbon atoms increases, the solubility decreases.

Wodzinski *et al.,* (1972) reported that microorganisms utilized only dissolved hydrocarbon molecules in the aqueous phase. In order to increase LCFAs aqueous solubility, a delivery method was used to increase the amount of substrate available to the microorganisms.

Previous studies assessed the dispersion characteristics of LCFAs in aqueous solution (Lalman and Bagley, 2000; Sikkema *et al.* 1995). Diethyl ether, dimethyl formamide, dimethyl sulfoxide, acetone, and ethanol were used as dispersing agents. These agents assist in increasing the substrate surface area and thus, dispersing the LCFAs into solution. Although, these dispersing agents are quite effective, a major problem associated with these compounds is their toxicity to microorganisms (Lalman and Bagley, 2000; Sikkema *et al.* 1995).

In this study, a delivery method developed by Angelidaki and Ahring (1992) was adopted to avoid solvent toxicity. The LCFAs were melted *au bain-marie* and dissolved in hot (50°C) vigorously stirred NaOH solution. The quantities of sodium hydroxide used (expressed as g of NaOH per g of LCFA) are provided in Table 3.3.

Table 3.3: Quantity of NaOH used for LCFA stock solution preparation

LCFA	NaOH ($g \cdot g^{-1}$ of LCFA)
Oleic	0.142
Linoleic	0.143

3.5.5 pH and solids measurement

The pH of each batch reactor was measured at the beginning and at the end of each experiment using a VWR SR40C, Symphony pH meter.

Liquid samples from batch reactors were analyzed for total suspended solids (TSS) and volatile suspended solids (VSS) at the beginning of each experiment. Also, liquid samples TSS and VSS measurements were performed at the end of each experiment. The analyses were performed using 5 ml samples, following *Standard Methods* (APHA, AWWA, and WEF, 1998). All solids measurement was conducted using 1 μm pore size glass fiber filter papers (VWR, Canada).

CHAPTER 4: SOURCE CULTURE CHARACTERIZATION

4.1 Experimental results

Semi-continuous fed batch reactors (reactor A and reactor B) were maintained at approximately 20,000 mg l^{-1} and 6,000 mg l^{-1} VSS respectively at room temperature (23 ± 2°C). The average alkalinity in the reactors was 3,500 ± 500 mg l^{-1} (as $CaCO_3$) and the pH range was 7.6 ± 0.3. Reactor B was monitored frequently for glucose degradation, VFA and gas productions to assess reactor performance.

4.1.1 Glucose degradation

The glucose degradation profile for cultures from reactor B receiving 5,000 mg l^{-1} glucose is shown in Figure 4.1. Undetectable level of glucose was attained within 8 hr.

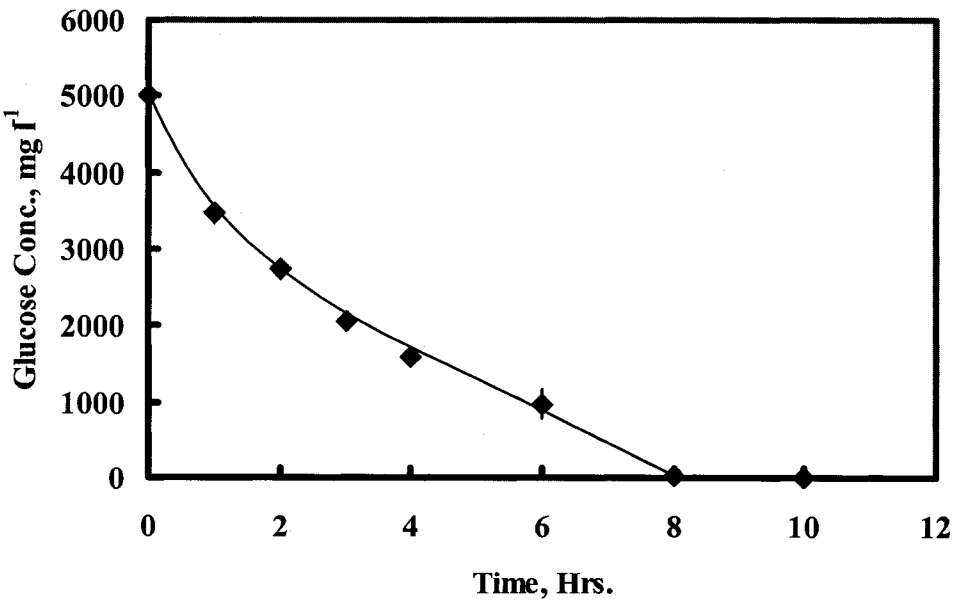

Figure 4.1: Glucose degradation profile for cultures from reactor B. Mean and SD of triplicate cultures are shown.

4.1.2 VFA production and removal

VFA profiles for cultures from reactor B receiving 5,000 mg l^{-1} glucose are shown in Figure 4.2. A maximum concentration of acetate was achieved within day 2 and more than 90% removal was observed within day 6. For propionate and butyrate, maximum concentrations were achieved within day 1. Complete butyrate removal was achieved within day 4, while propionate removal took 6 days.

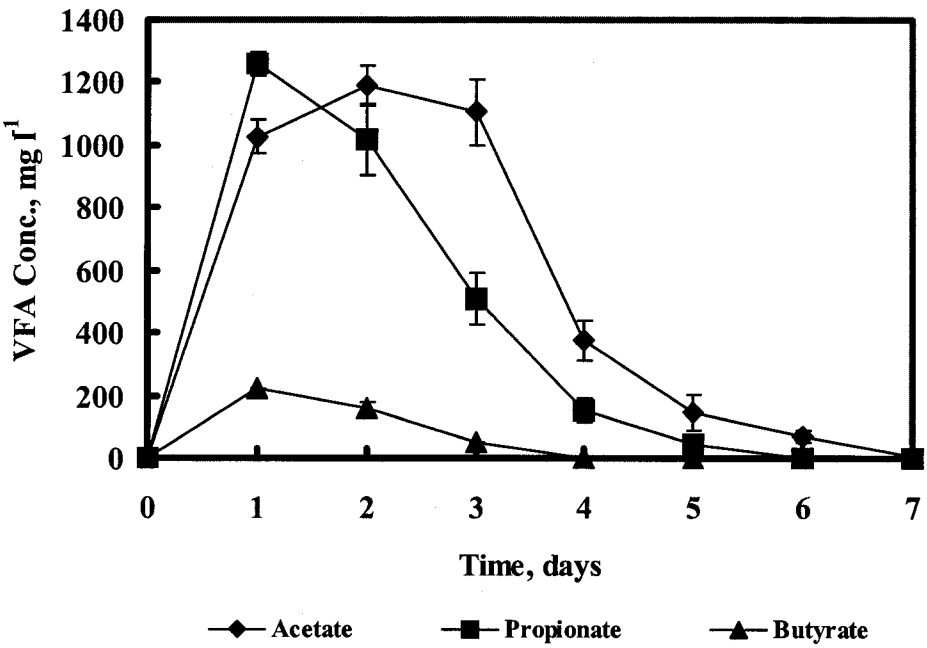

Figure 4.2: VFA profiles for cultures from reactor B. Values shown are mean ± SD from triplicate cultures.

4.1.3 Gas production

Gas production profile reactor B is shown in Figure 4.3. Complete conversion of glucose was achieved within 6 to 7 days.

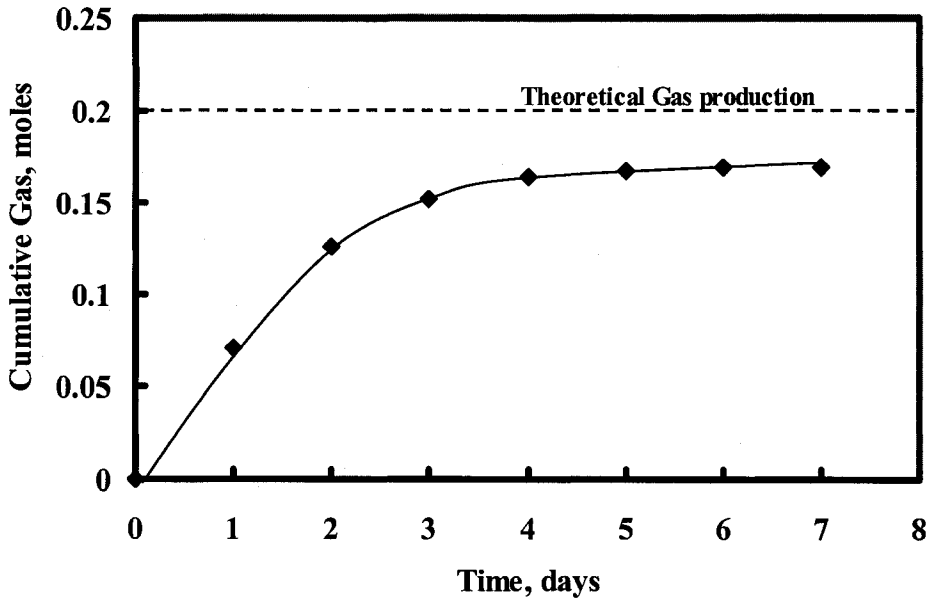

Figure 4.3: Gas production profile for reactor B.

CHAPTER 5: EFFECTS OF LINOLEIC ($C_{18:2}$) AND OLEIC ($C_{18:1}$) ACID ON HYDROGEN PRODUCTION FROM GLUCOSE

5.1 Experimental results

5.1.1 LA and OA degradation

In control experiments performed with LA and OA, the H_2 levels observed in the headspace were below the instrument detection limit. Acetate, a LCFA degradation by-product, accumulated in all experiments (data not shown).

5.1.2 Hydrogen and methane production

Hydrogen production profiles for the cultures receiving glucose at 5,000 mg l^{-1} plus LA and OA are shown in Figures 5.1 and 5.2, respectively. No detectable quantities of H_2 were observed in the controls.

No H_2 was detected in cultures inoculated with 500 mg l^{-1} LA. However, in cultures receiving 1,000 mg l^{-1} LA, maximum H_2 levels were observed at 28 hr, where as the maximum H_2 levels in cultures receiving ≥ 1,500 mg l^{-1} LA were observed at 54 hr after the first glucose injection. An initial lag-phase of 12 hr was observed in cultures receiving ≥ 1,000 mg l^{-1} LA. The maximum H_2 production of approximately 1 to 1.1 mole $H_2 \cdot mol^{-1}$ glucose was observed after the first glucose injection in cultures inoculated with ≥ 1,500 mg l^{-1} LA. Hydrogen yields obtained from cultures inoculated with LA and OA are shown in Table 5.1. Statistical comparison between data set for each LCFA concentration was performed using the Tukey's paired comparison procedure (Box *et al.*, 1978). The H_2 levels decreased in all cultures inoculated with LA after attaining a maximum.

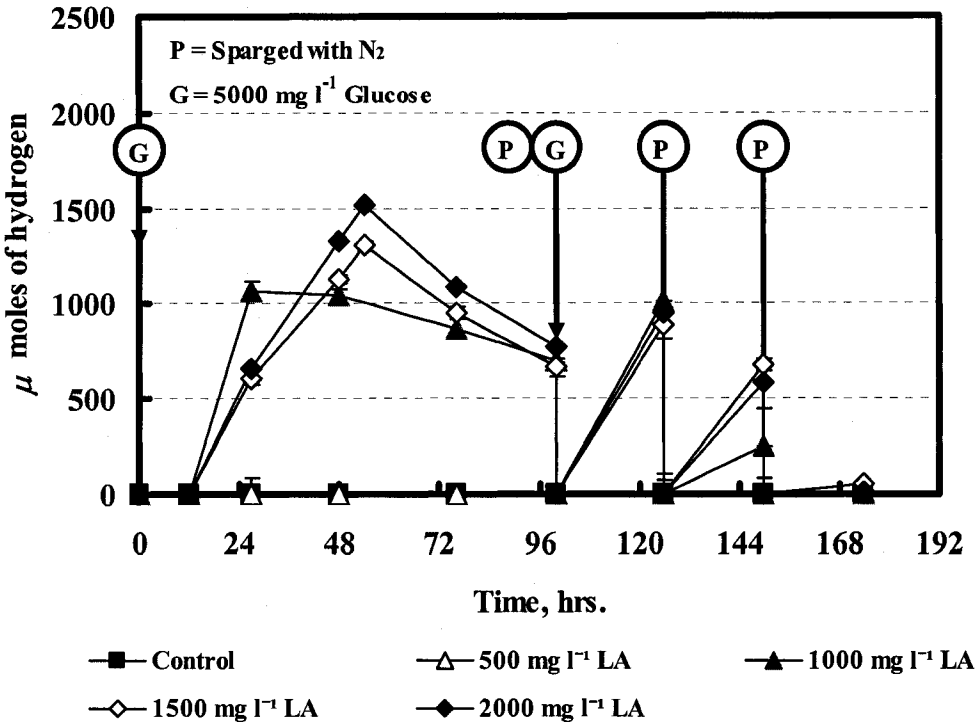

Figure 5.1: Hydrogen production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 100 hr) and LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

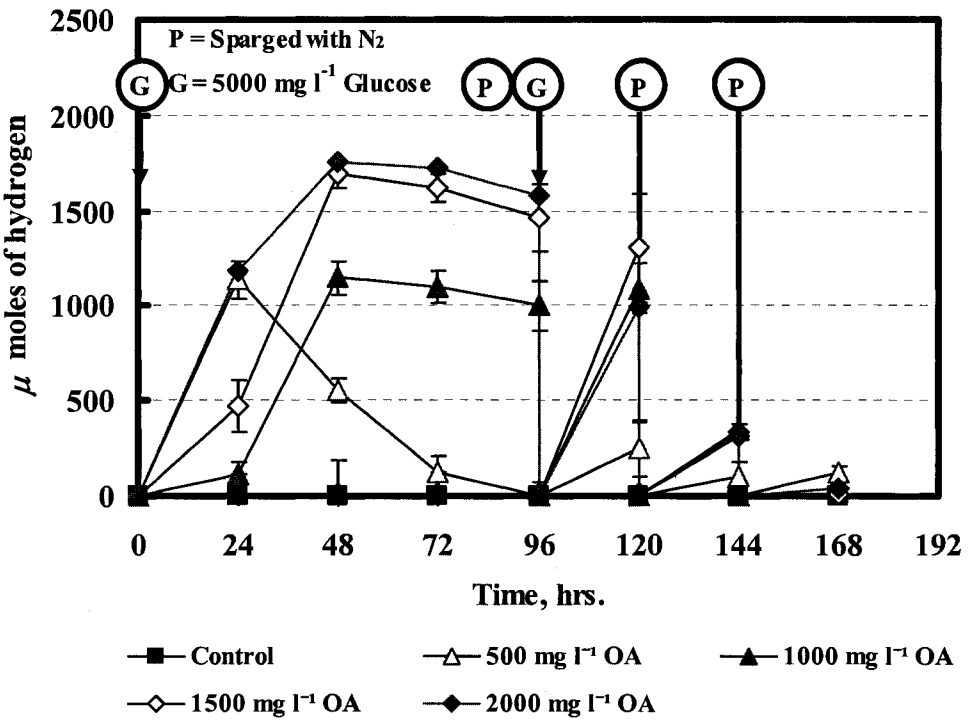

Figure 5.2: Hydrogen production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) and OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

Increases in H_2 production were observed in cultures receiving ≥ 1,000 mg l^{-1} LA after re-injecting glucose at 100 hr (Table 5.1). More than 90% of the maximum H_2 was produced within 48 hr following the glucose re-injection. The maximum amount of H_2 produced, after glucose re-injection was approximately 1.1 to 1.2 mol H_2·mol glucose^{-1} (Table 5.1) in cultures inoculated with ≥ 1,500 mg l^{-1} LA. H_2 production in cultures inoculated with 1000 and 1500 mg l^{-1} LA was enhanced after glucose re-injection due to the combined effect of LCFAs, VFAs and intermittent sparging.

Table 5.1: Hydrogen yields obtained from cultures receiving 5,000 mg l^{-1} glucose plus LA or OA

LCFA Concentration (mg l^{-1})	Hydrogen yields (mol·mol^{-1} glucose)			
	LA		OA	
	1st glucose injection	2nd glucose injection	1st glucose injection	2nd glucose injection
500	ND[a]	ND[a]	0.82 ± 0.08[a]	0.27 ± 0.27[a]
1000	0.77 ± 0.06[b]	0.91 ± 0.08[b]	0.82 ± 0.05[a]	1.06 ± 0.10[b]
1500	0.94 ± 0.04[c]	1.17 ± 0.06[c]	1.22 ± 0.06[b]	1.17 ± 0.09[b]
2000	1.09 ± 0.02[d]	1.11 ± 0.06[c]	1.27 ± 0.05[b]	0.98 ± 0.20[b]

Notes: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a, b, c, and d are used to indicate the means that are statistically different within the same column. The notations cannot be used for comparing data within the same row or between rows.
2. ND = Not detectable

In cultures inoculated with 500 mg l^{-1} OA, H_2 levels peaked at 24 hr, while a 24 hr lag was observed in cultures receiving ≥ 1,000 mg l^{-1} OA (Figure 5.2). The maximum amount of H_2 produced, after the first glucose injection was approximately 1.2 to 1.3 mol H_2·mol^{-1} glucose in cultures inoculated with ≥ 1,500 mg l^{-1} OA (Table 5.1). The H_2 levels decreased in all cultures inoculated with OA after reaching a maximum. In cultures inoculated with 500 mg l^{-1} OA after the first glucose injection, the H_2 concentration reached undetectable levels at 96 hr.

Following the second glucose injection, the H_2 production decreased in cultures receiving 500 and 2,000 mg l^{-1} OA (Table 5.1). However, cultures inoculated with 1,000 mg l^{-1} of OA showed an increase in H_2 production and cultures inoculated with 1,500 mg l^{-1} OA had similar H_2 production as after the first glucose injection. More than 90% of the maximum H_2 was produced within 48 hr following the glucose re-injection. The maximum H_2 production observed after glucose re-injection was approximately 1 to 1.17 mol $H_2 \cdot mol^{-1}$ glucose in cultures receiving $\geq$ 1,000 mg l^{-1} OA.

Figures 5.3 and 5.4 illustrate the methane production profiles for cultures receiving 5,000 mg l^{-1} glucose plus LA and OA, respectively. The degree of inhibition of methane production increased as the LA or OA concentration increased. Following the first glucose injection, a maximum methane production was observed in the control cultures. The methane production in the cultures receiving 1,000 and 1,500 mg l^{-1} LA were similar. A lag phase of 12 hr was observed in cultures receiving $\geq$ 1,000 mg l^{-1} LA. Cultures receiving $\geq$ 1,000 mg l^{-1} OA had negligible or no increase in methane production. Furthermore, cultures receiving 500 mg l^{-1} LA and OA produced approximately 50% and 22% less methane than the control cultures. A significant decrease in methane production was observed in the control and cultures receiving either LA or OA after the second glucose injection. No detectable levels of methane were observed in the cultures inoculated with LA and cultures inoculated with $\geq$ 1,000 mg l^{-1} OA after glucose re-injection.

5.1.3 VFAs production and removal

Acetic, propionic and butyric acids were the three major VFAs observed during fermentation of 5,000 mg l^{-1} glucose (Figures 5.5 to 5.10).

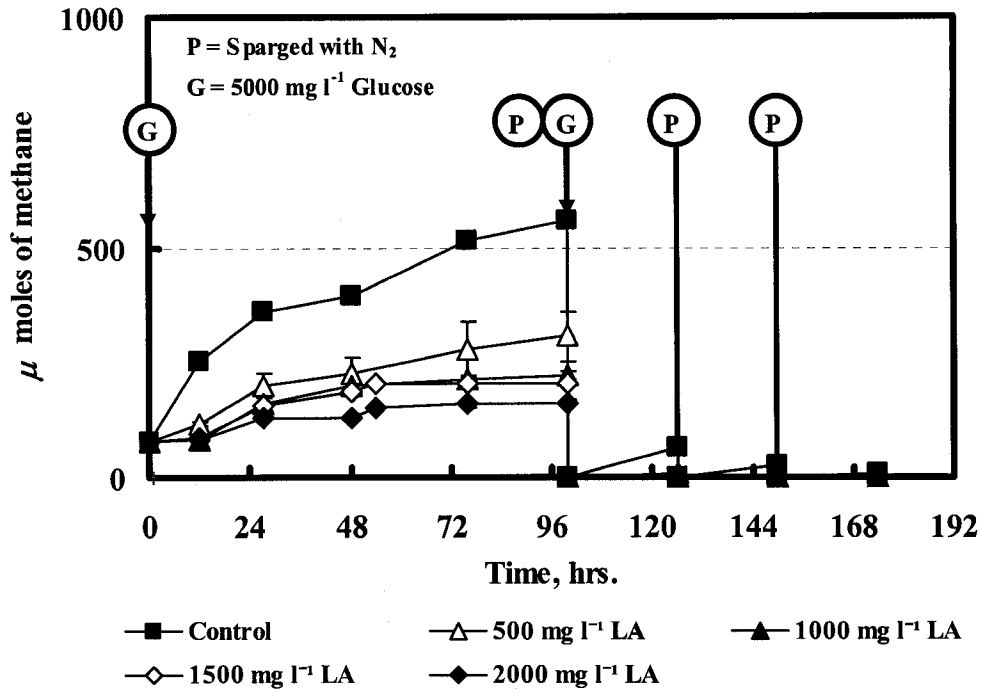

Figure 5.3: Methane production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 100 hr) and LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

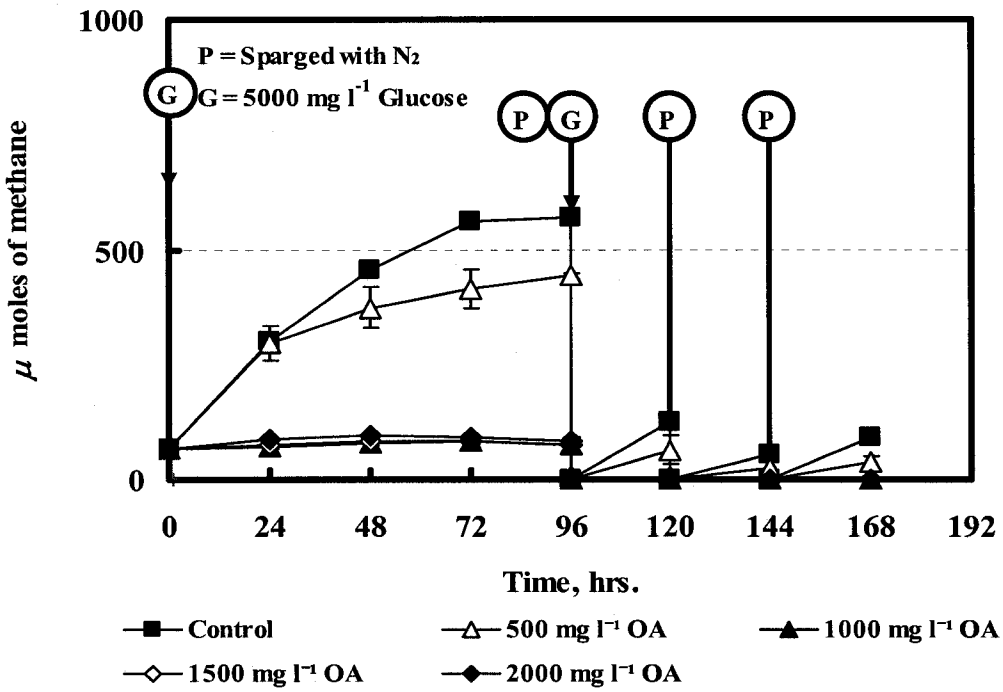

Figure 5.4: Methane production profiles for cultures receiving 5,000 mg l^{-1} glucose (0 hr and 96 hr) and OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

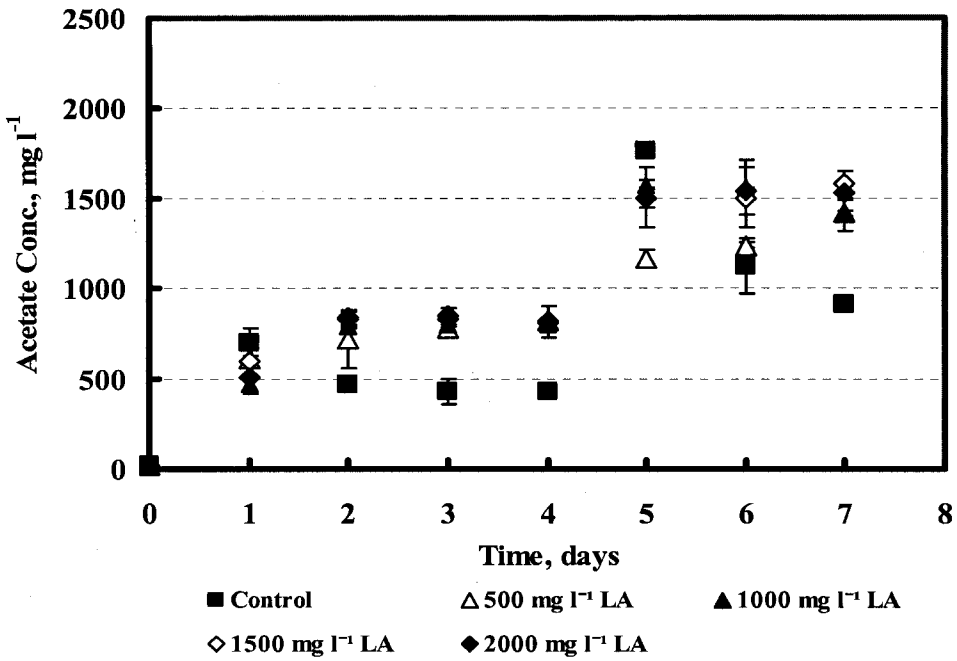

Figure 5.5: Acetic acid production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 100 hr) and LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

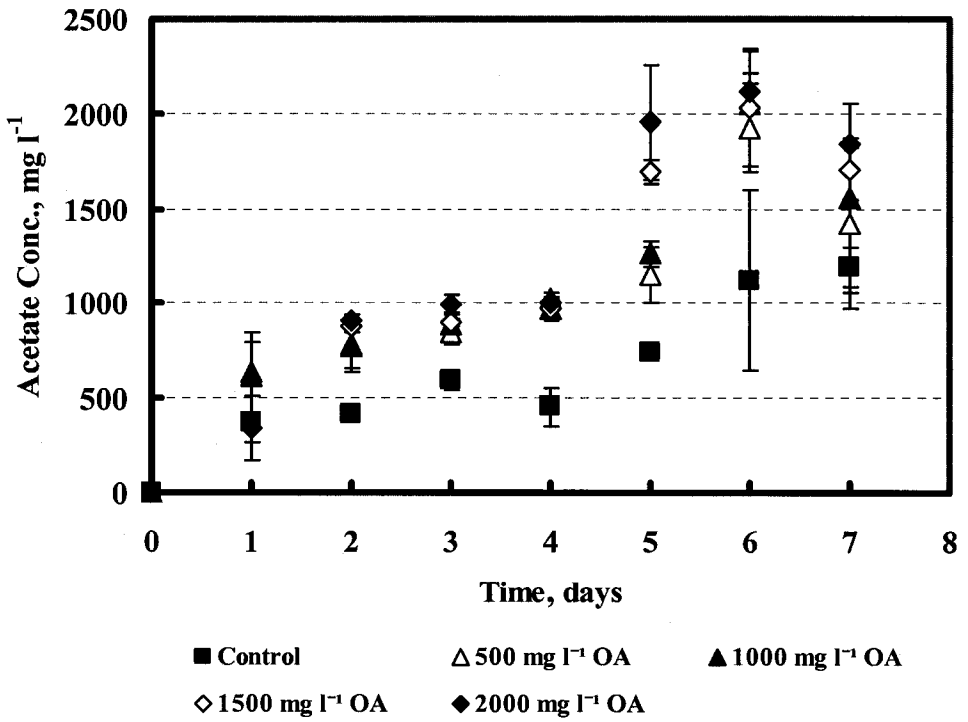

Figure 5.6: Acetic acid production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) and OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

Figures 5.5 and 5.6 illustrate the acetate production profiles for cultures receiving 5,000 mg l^{-1} glucose plus LA and OA, respectively. Following the first glucose injection, acetate concentrations accumulated in all cultures. On day 4, the acetate concentrations in cultures inoculated with LA or OA ranged from approximately 750 mg l^{-1} to 1,000 mg l^{-1}, while acetate concentration in control cultures was approximately 500 mg l^{-1}. Acetate concentrations continued to accumulate after glucose re-injection on day 4. On day 7, the acetate concentrations in the cultures inoculated with either LA or OA ranged from approximately 1500 to 1700 mg l^{-1}. The percentage conversions of glucose to acetate (on carbon basis) were as follows: 8.7 to 12% (in control cultures), 14 to 16.5% (in cultures inoculated with LA) and 14.3 to 20.5% (in cultures inoculated with OA).

Propionate production profiles in controls and cultures receiving LA and OA are shown in Figures 5.7 and 5.8, respectively. Following the first glucose injection, propionate concentrations accumulated in all the conditions examined. Propionate concentrations continued to accumulate after glucose re-injection. Relative to the controls, less propionate was detected in cultures inoculated with either LA or OA after injecting glucose on day 0 and day 4. In general, accumulated propionate levels seem to decrease with increase in LA or OA concentrations. Glucose to propionate conversions (percentage carbon basis) observed were; 24.6 to 30.4% (in control and cultures inoculated with 500 mg l^{-1} LA), 19.2% (in cultures inoculated with 500 mg l^{-1} OA), 9.8 to 14.3% (in cultures inoculated with $\geq$ 1000 mg l^{-1} LA) and 10.7 to 16.3% (in cultures inoculated with $\geq$ 1000 mg l^{-1} OA).

Butyric acid production profiles for cultures receiving 5,000 mg l^{-1} glucose plus LA and OA are shown in Figures 5.9 and 5.10, respectively. Butyrate production was

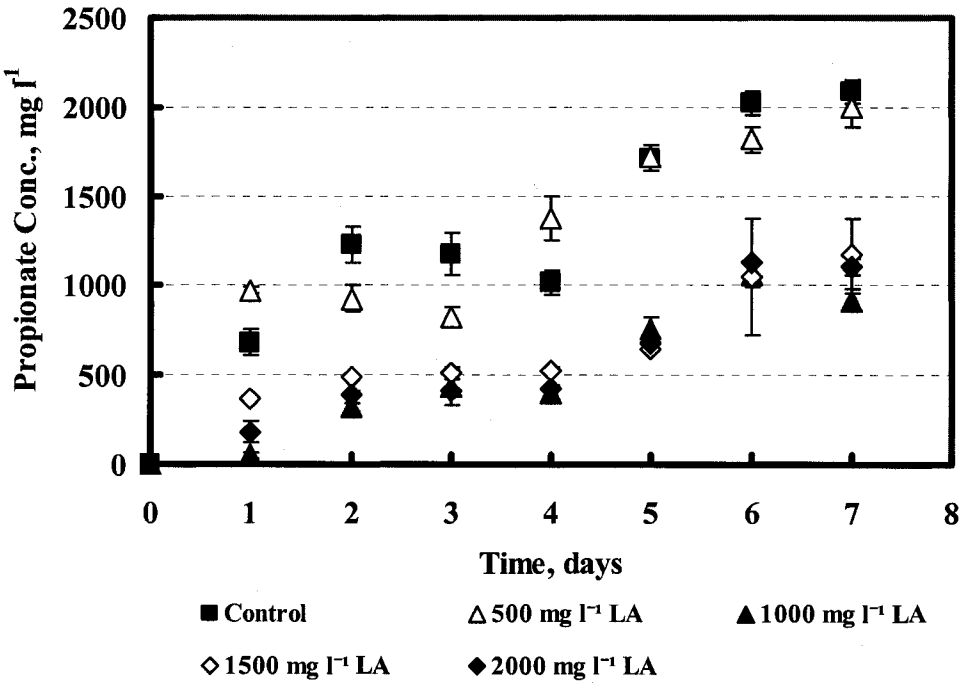

Figure 5.7: Propionic acid production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 100 hr) and LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

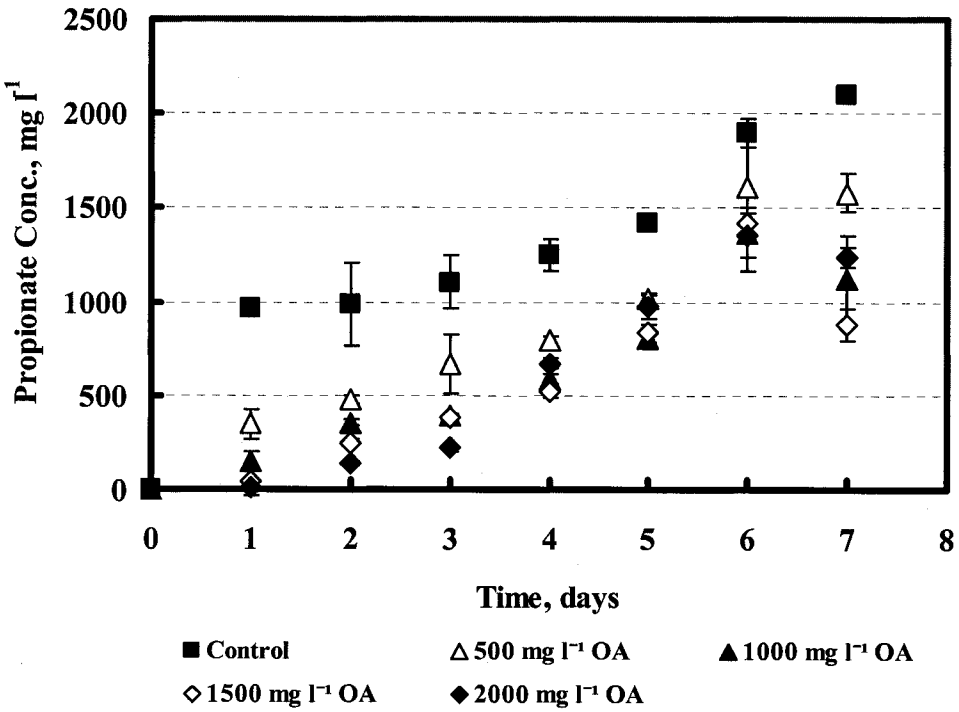

Figure 5.8: Propionic acid production profiles the cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) and OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

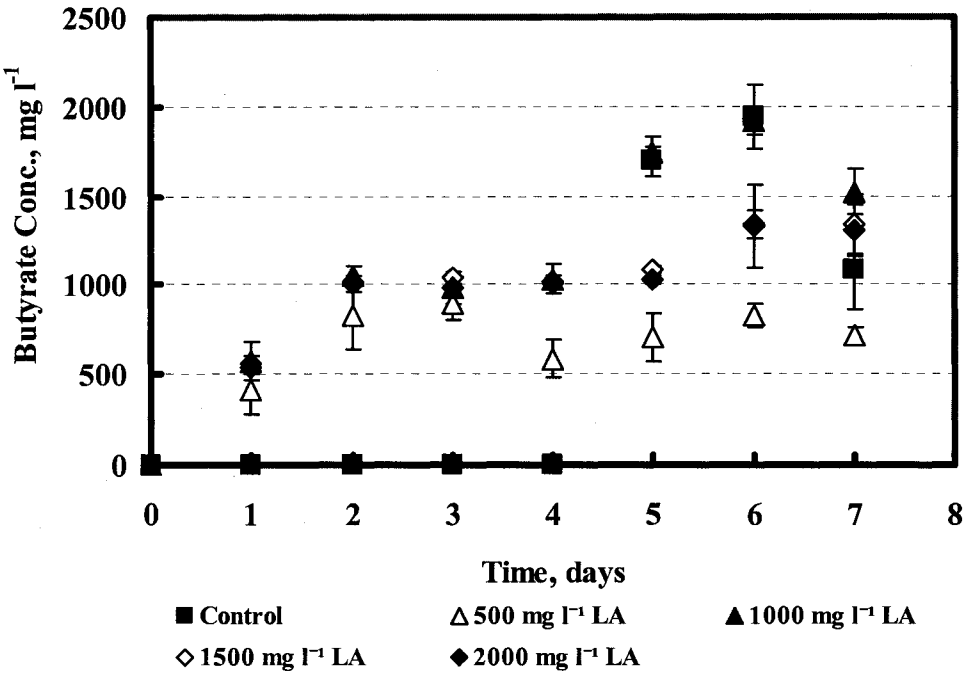

Figure 5.9: Butyric acid production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 100 hr) and LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

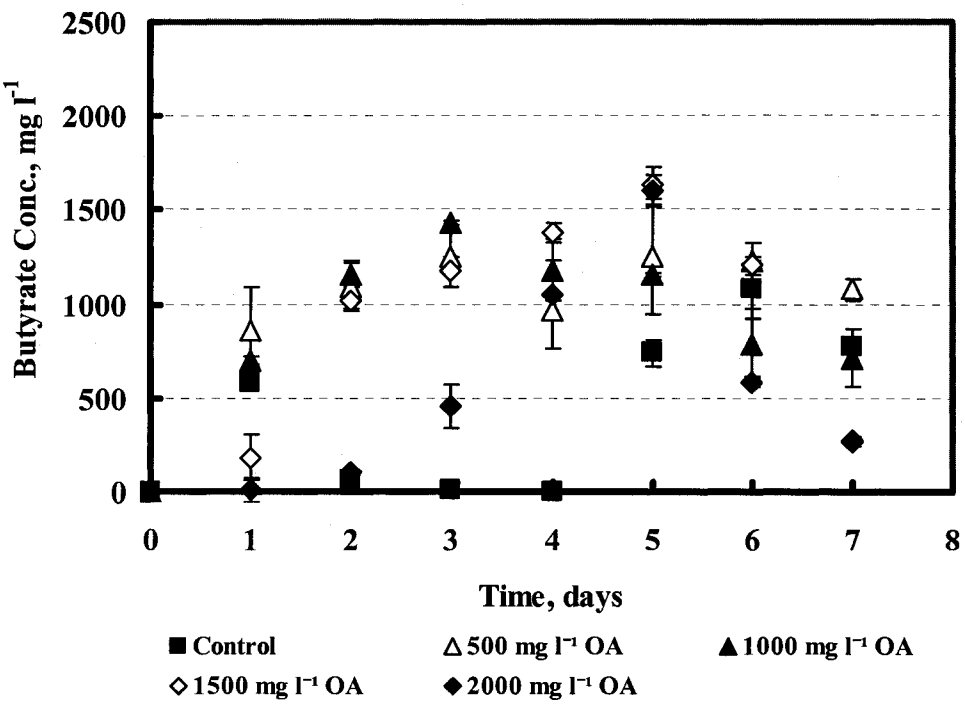

Figure 5.10: Butyric acid production profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) and OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

observed under all conditions examined. No clear butyrate trend was observed. The butyric acid concentrations ranged from approximately 500 to 1,800 mg l^{-1} for most of the conditions examined for the entire duration of the experiments. The conversion efficiencies from glucose to butyrate (on percentage carbon basis) were; 0 to 14.6% (in control cultures), 9.7 to 28.2% (in cultures inoculated with LA) and 9.6 to 37.6% (in cultures inoculated with OA).

5.1.4 Glucose degradation

Glucose degradation profiles for cultures receiving 5,000 mg l^{-1} glucose plus LA and OA are shown in Figures 5.11 and 5.12. The degradation profiles for cultures inoculated with LA and OA were similar. Undetectable levels of glucose were achieved in control cultures within 12 to 16 hr after inoculation. Glucose degradation was inhibited with increase in LCFA concentration. Initial glucose degradation rates for cultures receiving LA and OA plus 5,000 mg l^{-1} glucose are shown in Table 5.2. Statistical comparison between each data set was performed using the Tukey's paired comparison procedure (Box *et al.,* 1978). Glucose degradation rates for cultures inoculated with $\geq$ 500 mg l^{-1} LA or OA were statistically different from the control cultures. In the presence of 500 mg l^{-1} LA and OA, glucose removal was achieved within 24 to 28 hr. Notice approximately 70% of the acidogenic activity was lost in cultures fed with 500 mg l^{-1} LA when compared to the control cultures. In the cultures receiving $\geq$ 1,000 mg l^{-1} LA a residual glucose (on carbon basis) of approximately 13%, 37% and 49%, respectively was observed at 28 hr after glucose was injected. Similarly, residual glucose (on carbon basis) were observed (approximately 11 to 40%) in the cultures receiving $\geq$ 1,000 mg l^{-1}

OA at 24 hr. In all conditions examined, no detectable levels of glucose were observed after 48 hr.

Table 5.2: Initial degradation rates ($\mu g \cdot mgVSS^{-1} \cdot min^{-1}$) for cultures receiving 5,000 mg l^{-1} glucose plus LA or OA

LCFA Concentration (mg l^{-1})	Initial degradation rates ($\mu g \cdot mgVSS^{-1} \cdot min^{-1}$)	
	LA	OA
0	7.99 ± 0.93^{a}	7.18 ± 0.40^{a}
500	2.30 ± 0.49^{b}	2.84 ± 0.16^{b}
1000	1.65 ± 0.55^{b}	2.04 ± 0.09^{c}
1500	0.98 ± 0.05^{b}	1.17 ± 0.13^{d}
2000	0.95 ± 0.01^{b}	0.98 ± 0.03^{d}

Notes: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a, b, c, d, and e are used to indicate the means that are statistically different within the same column. The notations cannot be used for comparing data within the same row or between rows.
2. Sample calculation of initial degradation rate is shown in Appendix E

5.2 Discussion of results

Methanogenic inhibition is essential to attain high H_2 yields during dark fermentation. However, there are other microorganisms such as homoacetogens that may still consume H_2 and reduce the yields. The inhibitory effect of LCFAs on hydrogenotrophic methanogens has been reported in previous studies (Demeyer and Hendrickx, 1967; Lalman and Bagley, 2000 and 2001), where the primary focus was the degradation of LCFAs. However, the impact of such an inhibition on H_2 accumulation from degradation of carbon sources has not been examined.

The present study shows that the presence and inhibitory effects of LA and OA in mixed anaerobic cultures can facilitate H_2 accumulation. Increasing amounts of H_2 were observed in cultures inoculated with elevated amounts of LA or OA. Maximum H_2 accumulation of approximately 1.1 to 1.3 mol $H_2 \cdot mol^{-1}$ glucose was observed in cultures inoculated with LA and OA. The conversion efficiencies of glucose to H_2 (based on a

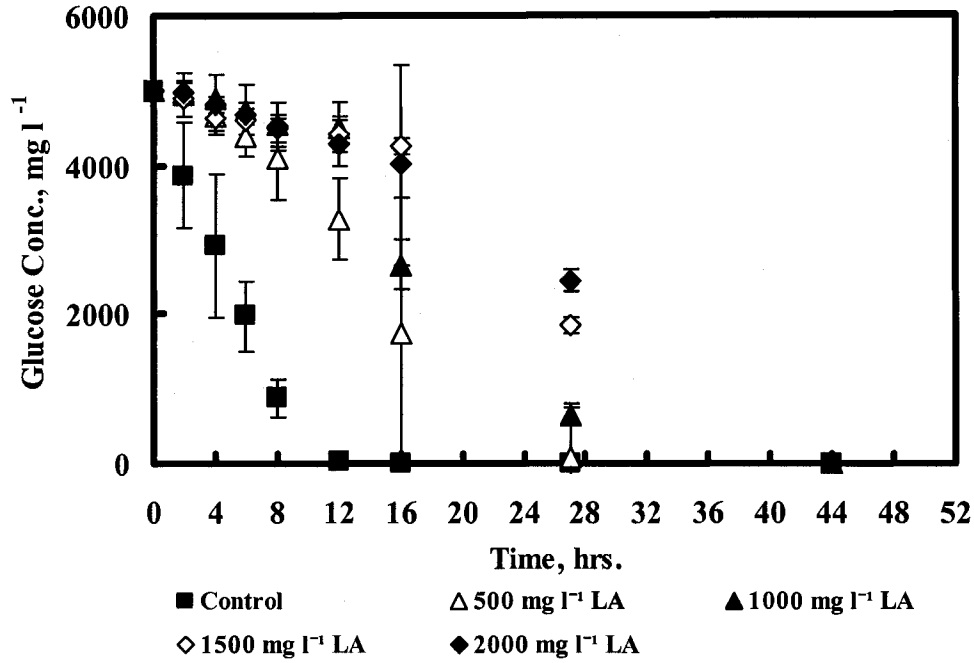

Figure 5.11: Glucose degradation profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr) and LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

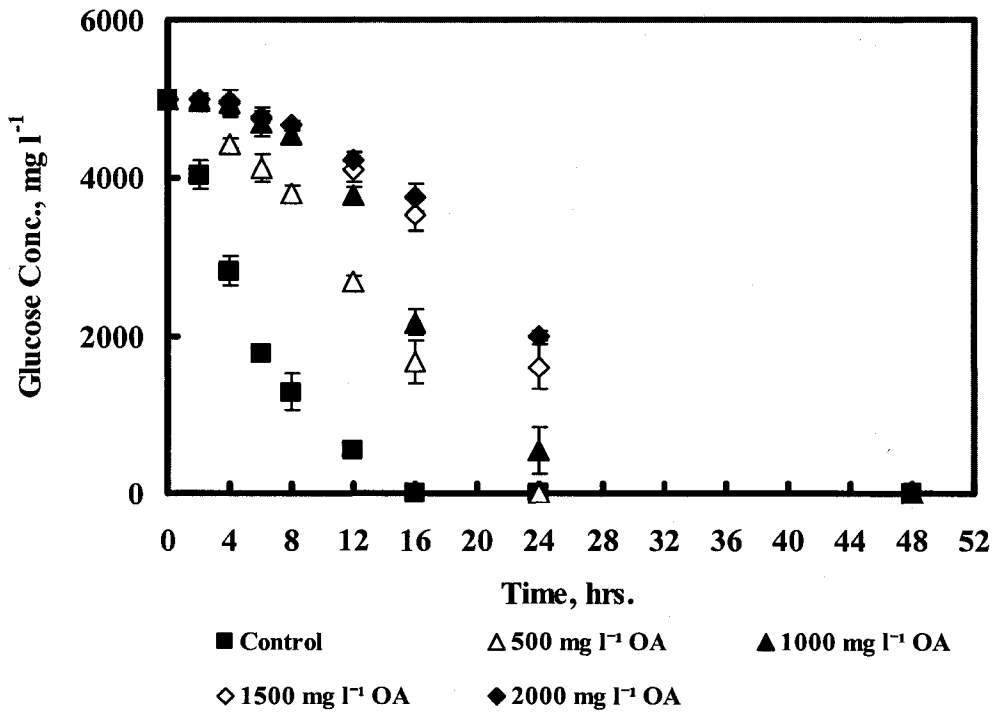

Figure 5.12: Glucose degradation profiles for cultures receiving 5,000 mg l^{-1} glucose (at 0 hr) and OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

theoretical yield of 4 mol $H_2 \cdot mol^{-1}$ glucose) in cultures receiving LA were approximately 19 to 30%, while in the cultures receiving OA the conversion efficiencies were approximately 7 to 32%. H_2 yields obtained by various researchers under different conditions using glucose and mixed cultures are shown in Table 5.3. Notice the H_2 yields in this study are comparable to those reported for batch cultures (Logan *et al.*, 2002; Oh *et al.*, 2003; Zheng and Yu, 2004).

The H_2 yields in cultures receiving OA were greater than cultures receiving LA, after the first glucose injection. However, H_2 could be consumed by homoacetogens to produce acetate (Equation 2.17) (Oh *et al.*, 2003) and through formate-utilizing organisms to formate (Equation 2.18) (Bleicher and Winter, 1994). These organisms are known to be more dominant when they are not competing with the methanogens (Schink, 1997). Therefore, it is unclear from this study that whether LA or OA is more inhibitory to hydrogenotrophic methanogens than the other.

Table 5.3: Hydrogen yields for different microbial systems and environmental conditions

Culture	Condition	pH	Temperature (°C)	H_2 yield (mol·mol^{-1} glucose)	Source
Heat treated mixed culture	Batch	6.0	26	0.92	Logan *et al.* (2002)
Heat treated mixed culture	Batch	6.2	24	0.97	Oh *et al.* (2003)
Heat treated mixed culture	Batch	6.5	30	1.10	Zheng and Yu (2004)

Increases in H_2 yields were observed in the cultures receiving LA after the second glucose addition. This is likely because of the combined effects of high VFAs concentration at lower pH, intermittent sparging with nitrogen and the presence of LA. The fraction of undissociated VFA increases when the pH decreases due to VFA

production by acetogens. Undissociated VFAs are toxic to methanogens (Andrews, 1969; Duarte and Anderson, 1982). Sung *et al.* (2002) reported that operating a bioreactor with a relatively high total VFA concentration ensures additional inhibition of H_2 consuming bacteria, such as methanogens. Previous studies have reported gas sparging to be an effective method to increase the H_2 yield by reducing H_2 partial pressure in the liquid phase. Hussy *et al.* (2005) observed an improved H_2 yield from 1.0 to 1.9 mol $H_2 \cdot mol^{-1}$ glucose using nitrogen sparging in a continuous reactor maintained with mixed culture at pH 5.2 and 32°C. Contrary to the results obtained for cultures inoculated with LA, the H_2 yields decreased after glucose re-injection in cultures receiving $\geq$ 1,500 mg l^{-1} OA. Likely, in this case H_2 producing organisms were inhibited along with methanogens because of high VFAs and LCFA concentrations.

The addition of LA or OA to the cultures inhibited aceticlastic and hydrogenotrophic methanogens. Between the two LCFAs tested, OA levels $\geq$ 1,000 mg l^{-1} inhibited methane production to a greater extent than the same amount of LA. The results suggest that a threshold condition may have been reached for OA inhibition at concentrations greater than 1,000 mg l^{-1}. The methane production decreased significantly after the second glucose injection; negligible or undetectable methane productions were observed in the cultures receiving either LA or OA. This could be a combined effect of high VFAs concentration and lower pH. Aguilar *et al.* (1995) reported that the degradation of soluble carbohydrates leads to the formation of products such as acetate, propionate and butyrate and accumulation of these intermediate products, especially in the undissociated form, leads to the inhibition of several microbial species with consequent decrease in methane production.

Acetate, propionate and butyrate were the main fermentation byproducts observed in these experiments. In the cultures receiving LA, the acetate, propionate and butyrate concentrations accumulated, especially at higher LA concentrations. Similarly, VFAs accumulation was observed in the cultures receiving OA. Aceticlastic methanogens and propionate degraders are inhibited by 30 to 50 mg l^{-1} LA and 150 to 250 mg l^{-1} OA (Kim *et al.* (2004). Mykhaylovin *et al.* (2005) observed inhibition of propionate degradation at 50 mg l^{-1} LA or OA concentration.

VFAs formation and distribution have been used as indicators for monitoring H_2 production. High H_2 yields are associated with a mixture of acetate and butyrate fermentation products, and low H_2 yields are associated with propionate. The difference in the hydrogen production potential in the cultures receiving LA and that receiving OA can be correlated to the product formation. Less propionate and high butyrate formations were observed in the cultures receiving OA in comparison with that receiving LA. Also, decreasing amounts of propionate formation were observed in the cultures receiving increasing amounts of LA or OA concentrations. Based on the VFA distributions profiles, it appears that the butyrate-type fermentation was dominant in the present study, especially, in the cultures receiving LA and OA. Zheng and Yu (2004) in their study examined the effect of pH variation on hydrogen production from glucose by enriched anaerobic cultures at 30°C and showed high H_2 yields (approximately 1.3 and 1.47 mol $H_2 \cdot mol^{-1}$ glucose) when the reactor was operated at pH 4.0 to 5. They reported propionate formation between approximately 3.5 to 11%. However, a further increase in pH led to a considerable decrease in H_2 yield, especially when the pH was at 7.5 and at 8.0.

Glucose degradation was inhibited in the cultures inoculated with either LA or OA. Increase in the inhibition of glucose degradation was observed with increasing LA or OA levels. These findings corroborate evidence reported by several studies (Lalman and Bagley, 2002, Lalman *et al.*, 2003; Alosta *et al.*, 2004).

CHAPTER 6: EFFECTS OF LA AND OA ON HYDROGEN PRODUCTION FROM GLUCOSE AT DIFFERENT INITIAL pH

6.1 Experimental results

This section discusses the effect of varying initial pH in the presence of LCFA on hydrogen production using 5,000 mg l^{-1} glucose. During the course of the experiment, the parameters monitored included H_2, CH_4, VFAs, alcohol production and pH along with glucose degradation.

6.1.1 Hydrogen and methane production

Hydrogen production profiles for cultures with varying initial pH receiving 5,000 mg l^{-1} glucose plus LA and OA are shown in Figures 6.1 and 6.2. No detectable quantity of H_2 was observed in cultures with an initial pH of 7.8 and without LCFA. Following the first glucose injection, no detectable level of H_2 was observed in cultures with an initial pH of 6 and without LCFA. However, H_2 accumulated in cultures receiving LCFA and with initial pH values of 7.8, 6 and 5. Hydrogen yields obtained from cultures inoculated with LA and OA are shown in Table 5.1. Statistical comparison between each data set was performed using the Tukey's paired comparison procedure (Box *et al.,* 1978). Cultures receiving LA with an initial pH of 6 had the greatest H_2 yield of 2.7 mol $H_2 \cdot mol^{-1}$ glucose (Table 6.1). A lag-phase of 12 hr was observed in cultures receiving LCFA at initial pH values of 5, 6 and 7.8. Significant increases in H_2 yields was observed after re-injecting 5,000 mg l^{-1} glucose in cultures at initial pH values of 5 and 6 without LCFA (Table 6.1), whereas, H_2 yields in cultures receiving LCFA with an initial pH of 5 and in cultures receiving LA with an initial pH of 6 decreased significantly. H_2 yields remained consistent with yields obtained from the first glucose injection in cultures receiving

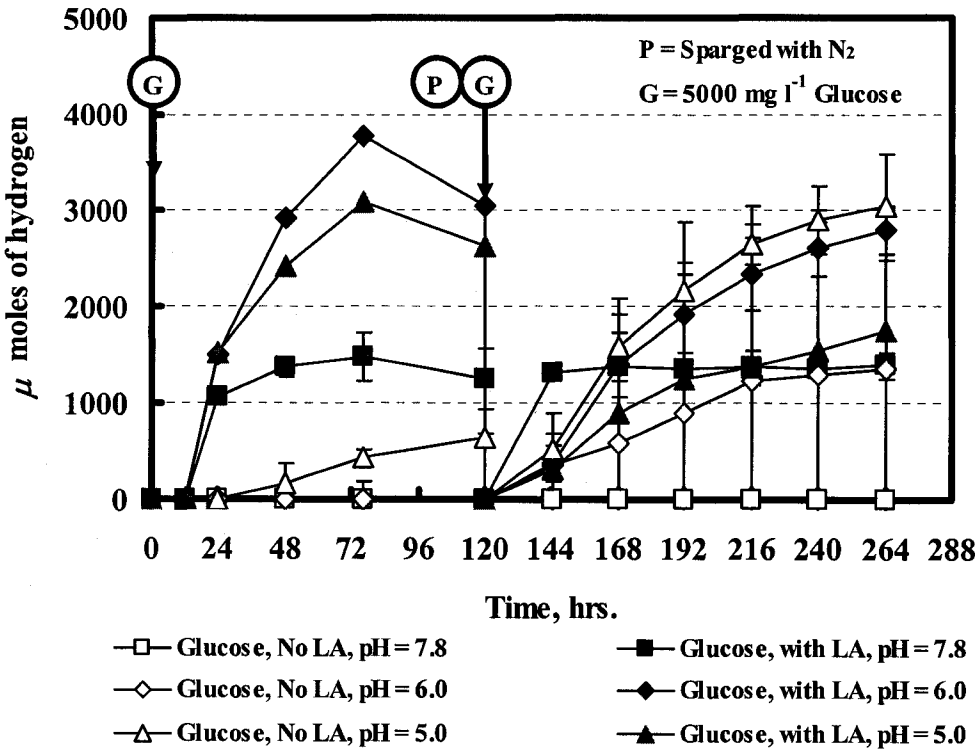

Figure 6.1: Hydrogen production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

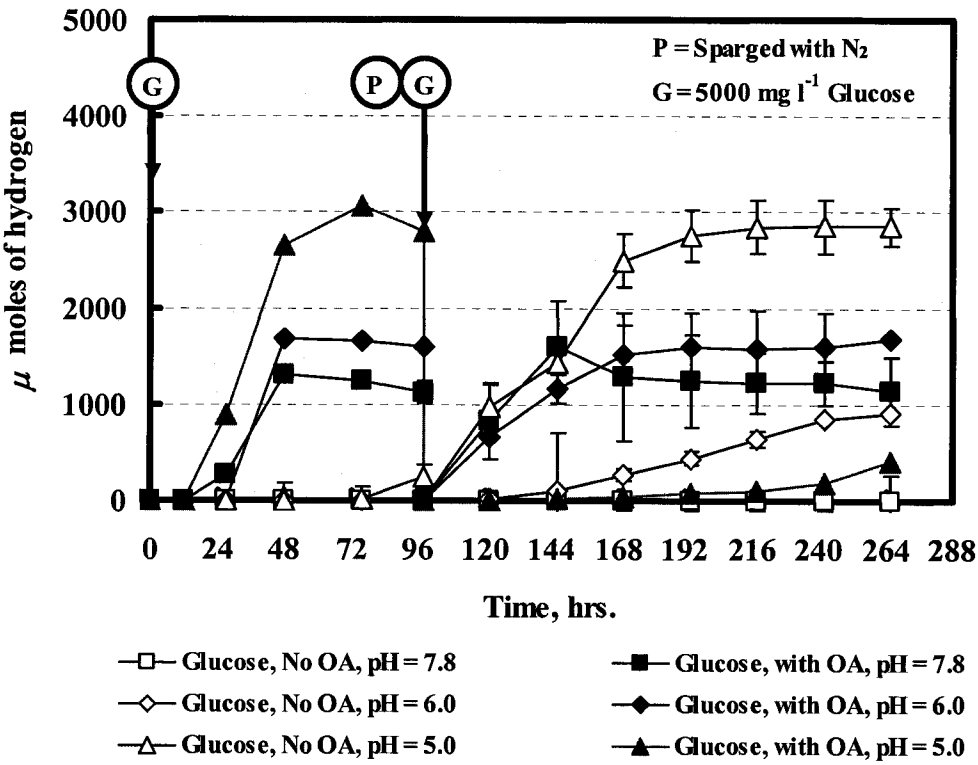

Figure 6.2: Hydrogen production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} of OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

Table 6.1: Hydrogen yields for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose plus LA or OA

Initial pH	Hydrogen yields (mol·mol^{-1} glucose)			
	LA		OA	
	1st glucose injection	2nd glucose injection	1st glucose injection	2nd glucose injection
5.0 (No LCFA)	0.46 ± 0.23^{a}	2.18 ± 0.10^{a}	0.18 ± 0.08^{a}	2.06 ± 0.26^{a}
6.0 (No LCFA)	ND^{b}	$0.98 \pm 0.17^{a,b}$	ND^{b}	$0.67 \pm 0.23^{b,c}$
7.8 (No LCFA)	ND^{b}	ND^{b}	ND^{b}	ND^{b}
5.0 (with LCFA)	2.21 ± 0.13^{c}	$1.25 \pm 1.20^{a,b}$	2.20 ± 0.05^{c}	$0.41 \pm 0.36^{b,c}$
6.0 (with LCFA)	2.70 ± 0.06^{d}	2.00 ± 0.39^{a}	1.20 ± 0.05^{d}	1.20 ± 0.14^{c}
7.8 (with LCFA)	1.07 ± 0.04^{e}	$1.0 \pm 0.03^{a,b}$	0.93 ± 0.10^{e}	1.15 ± 0.50^{c}

Notes: 1. Average and standard deviation for triplicate cultures are shown. The superscript notations a, b, c, d, e, and f are used to indicate the means that are statistically different within the same column. The notations cannot be used for comparing data within the same row or between rows.
2. ND = Not detected

LCFA with an initial pH of 7.8 and in cultures receiving OA with an initial pH of 6.

Figures 6.3 and 6.4 show methane production profiles for cultures with varying initial pH and fed with 5,000 mg l^{-1} glucose plus LA and OA, respectively. Maximum methane production after the first glucose injection was observed in cultures with an initial pH of 7.8 and without LCFA. The degree of methane inhibition increased with lower initial pH values and in the presence of LCFA. Cultures receiving LCFA and with initial pH values of 5, 6 and 7.8 had undetectable or negligible amounts of methane. After glucose re-injection, significant increase in methane production was observed in cultures with an initial pH of 6 and without LCFA. No detectable or negligible levels of methane were observed in cultures receiving LCFA with initial pH values of 5, 6 and 7.8.

6.1.2 VFAs production and removal

Acetate, propionate and butyrate were the major VFAs detected during glucose fermentation in cultures at varying initial pH and in the presence of LCFA (Figures 6.5 to

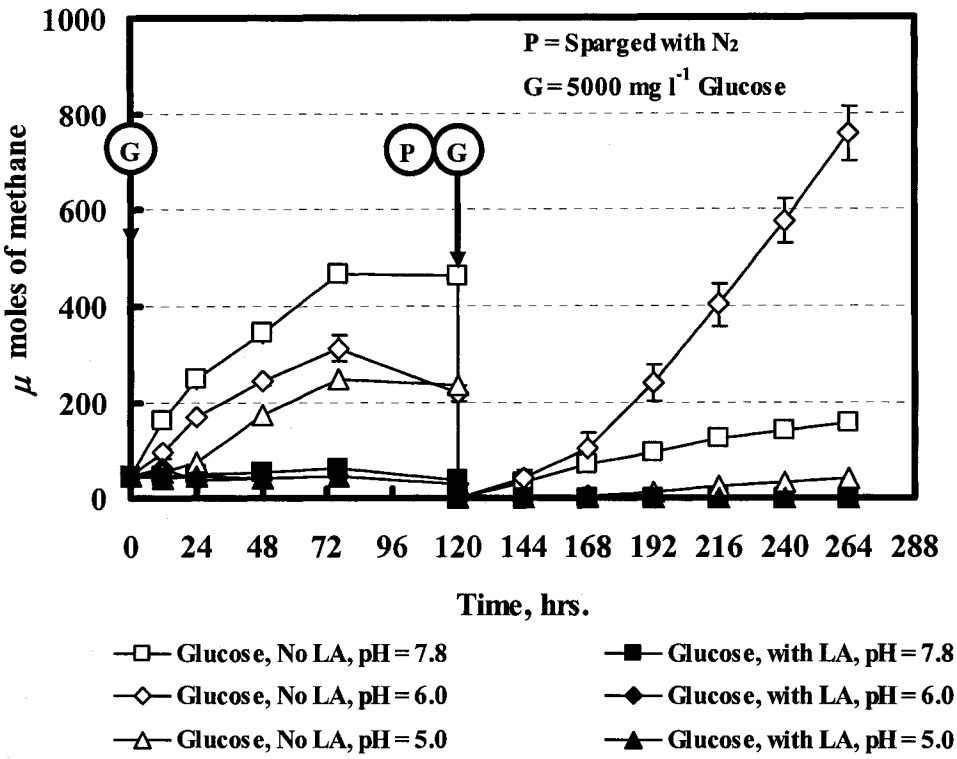

Figure 6.3: Methane production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

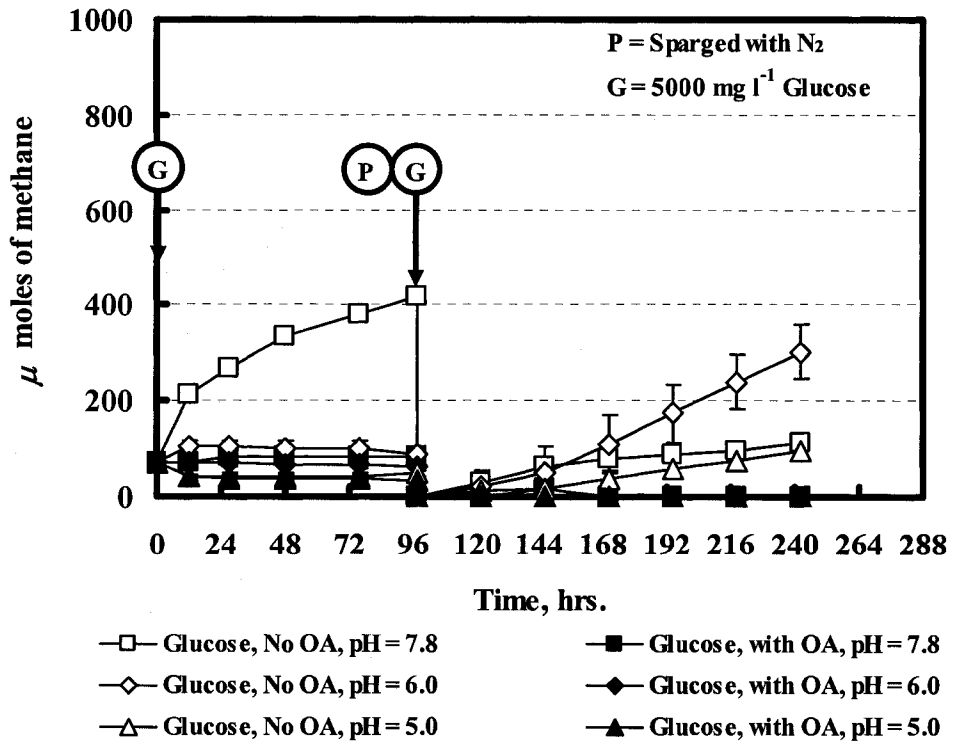

Figure 6.4: Methane production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

6.10). Acetate production was observed under all conditions examined (Figure 6.5 and 6.6). Following the first glucose injection, maximum acetate concentrations were observed in cultures with an initial pH of 7.8 and inoculated with LCFA. Acetate concentrations were higher in cultures fed with LCFA, than in cultures without LCFA. Cultures with higher initial pH had higher acetate concentration in comparison to those at lower initial pH. Increase in acetate concentrations were observed after glucose re-injection under all conditions examined. After re-injecting glucose, the acetate concentration in cultures with LCFA was comparatively less than cultures without LCFA, except in case of cultures with an initial pH value of 7.8 and with LCFA. The cultures with the latter conditions had maximum acetate concentration after re-injecting glucose. The glucose to acetate percentage conversions (on carbon basis) observed for cultures with an initial pH value of 7.6 and with LCFA ranged between 8 to 17%. In comparison, cultures at initial pH 5.0 and 6.0 and with LCFA the contained 5 to 14% acetate (on carbon basis).

Propionic acid was observed in all conditions examined (Figures 6.7 and 6.8). Higher propionate concentrations were observed in cultures without LCFA compared to cultures with LCFA. Further, higher propionate concentrations were observed in cultures at elevated initial pH than in cultures at lower initial pH. Increase in propionate concentrations were observed after glucose re-injection in all conditions examined. A similar propionate trend was observed after glucose re-injection compared to the first glucose injection. Glucose to propionate conversion percentages (on carbon basis) were 5 to 17% for cultures at an initial pH 7.8 and with LCFA and 1 to 9% for cultures at initial pH 5.0 and 6.0 and with LCFA.

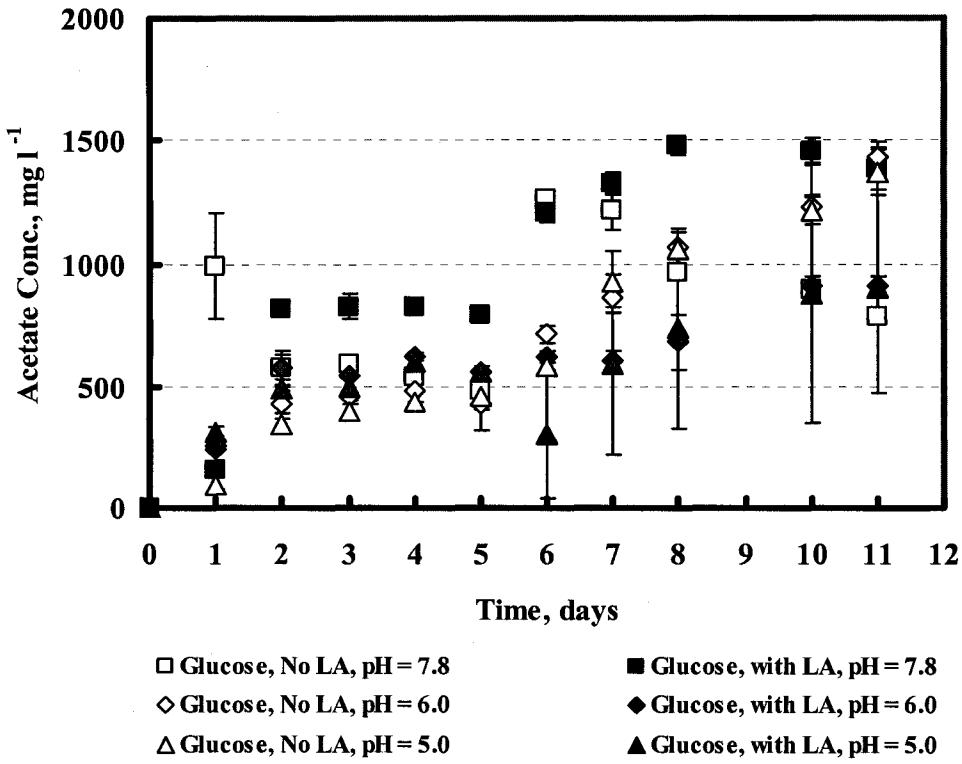

Figure 6.5: Acetic acid production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

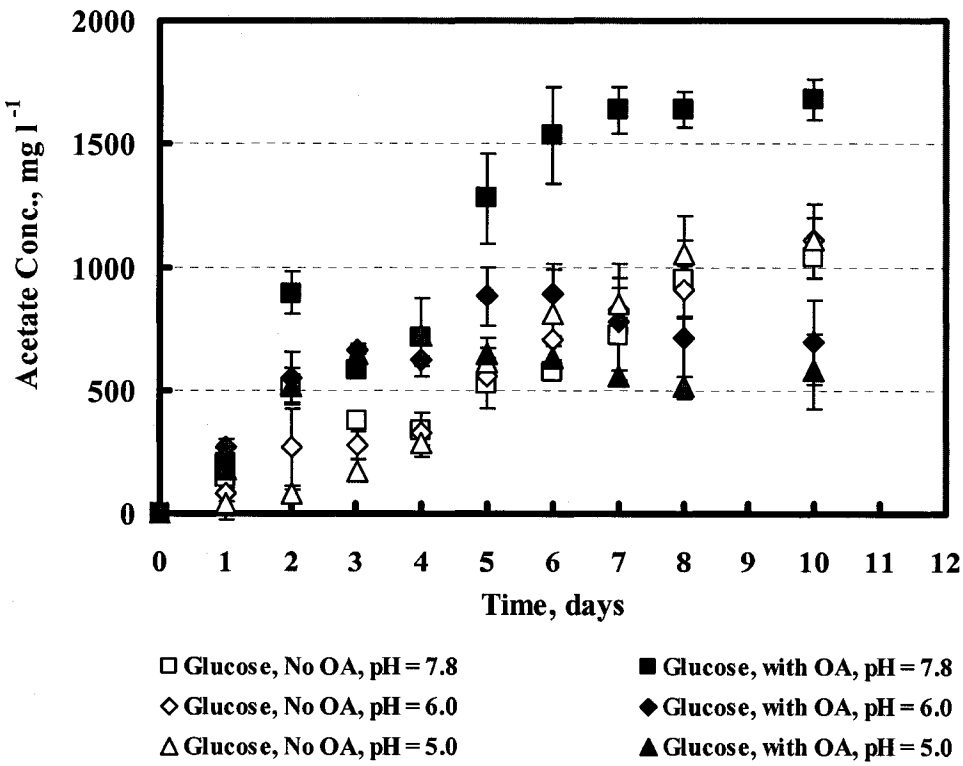

Figure 6.6: Acetic acid production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

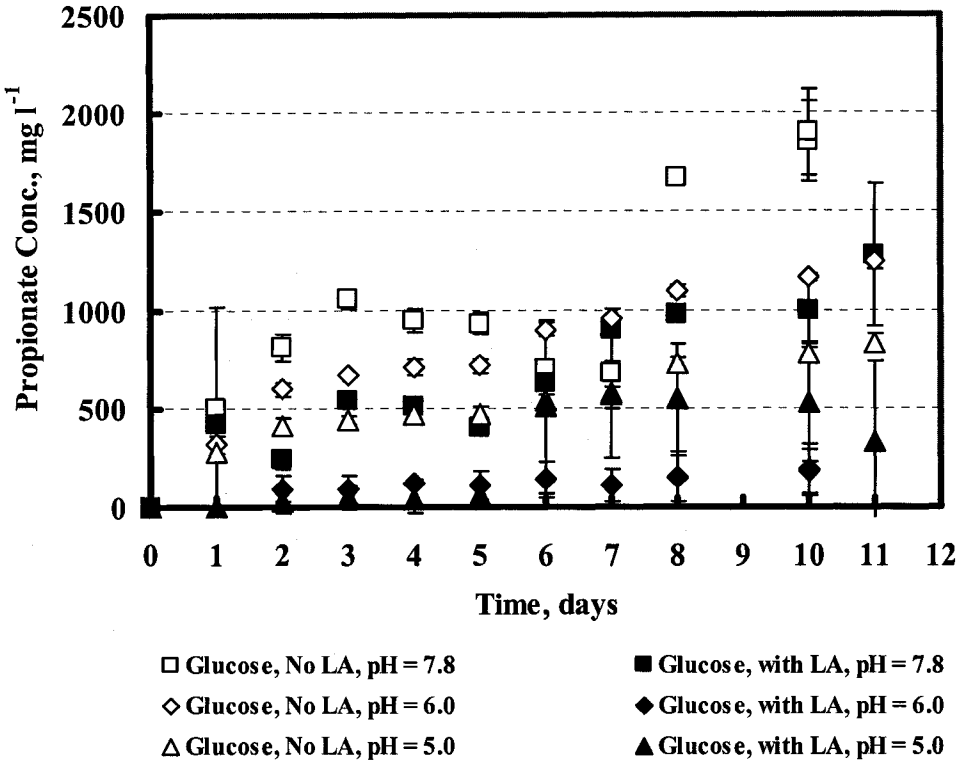

Figure 6.7: Propionic acid production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

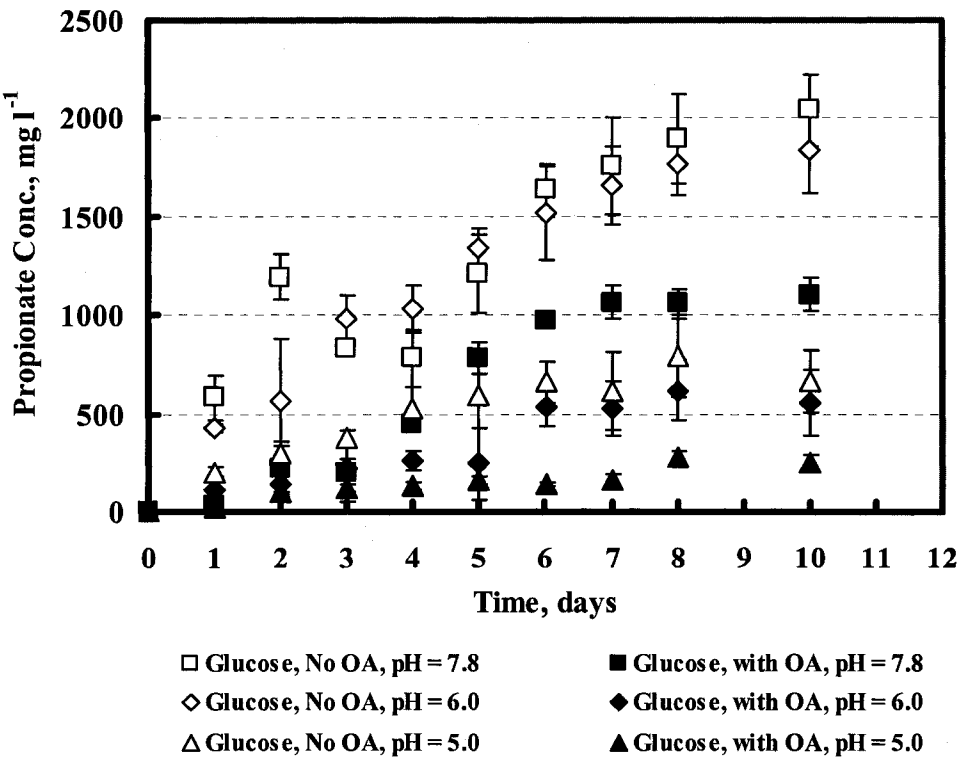

Figure 6.8: Propionic acid production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

Butyric acid production profiles for cultures at varying initial pH and fed with 5,000 mg l^{-1} glucose plus LCFA are shown in Figures 6.9 and 6.10. The butyrate formation trend was opposite to the propionate formation. Higher butyrate levels were observed in cultures with LCFA than in cultures without LCFA. Further, higher butyrate concentrations were observed in cultures at lower initial pH than in cultures at higher initial pH. Conversion efficiencies from glucose to butyric acid (on carbon basis) ranged between 1 to 10% for cultures at an initial pH 7.8 and with LCFA and 13 to 33% for cultures at initial pH 5.0 and 6.0 and with LCFA.

6.1.3 Alcohol production

The alcohols observed in cultures with varying initial pH and fed with 5,000 mg l^{-1} glucose plus LCFA were ethanol, propanol, i-propanol and butanol (Figures 6.11 to 6.17). No detectable levels of propanol were observed in cultures receiving OA.

Ethanol and i-propanol were the dominant alcohols observed in these studies. In general, elevated ethanol concentrations were observed in cultures fed with LCFA and at higher initial pH. Similar observations were found for propanol profiles for cultures receiving LA. However, i-propanol and butanol production trends were opposite to the ethanol profiles. Greater i-propanol or butanol concentrations were observed in cultures without LCFA and at lower initial pH. The glucose to ethanol conversion efficiencies (on carbon basis) were 2 to 18% for cultures at an initial pH of 7.8, 5 to 14% for cultures at an initial pH of 6.0 and 4 to 11% for cultures at an initial pH of 5.0). Similarly, conversion efficiencies (on carbon basis) from glucose to i-propanol were 0 to 5% for cultures at an initial pH of 7.8, 5 to 21% for cultures at an initial pH of 6.0 and 5 to 22% for cultures at an initial pH of 5.0.

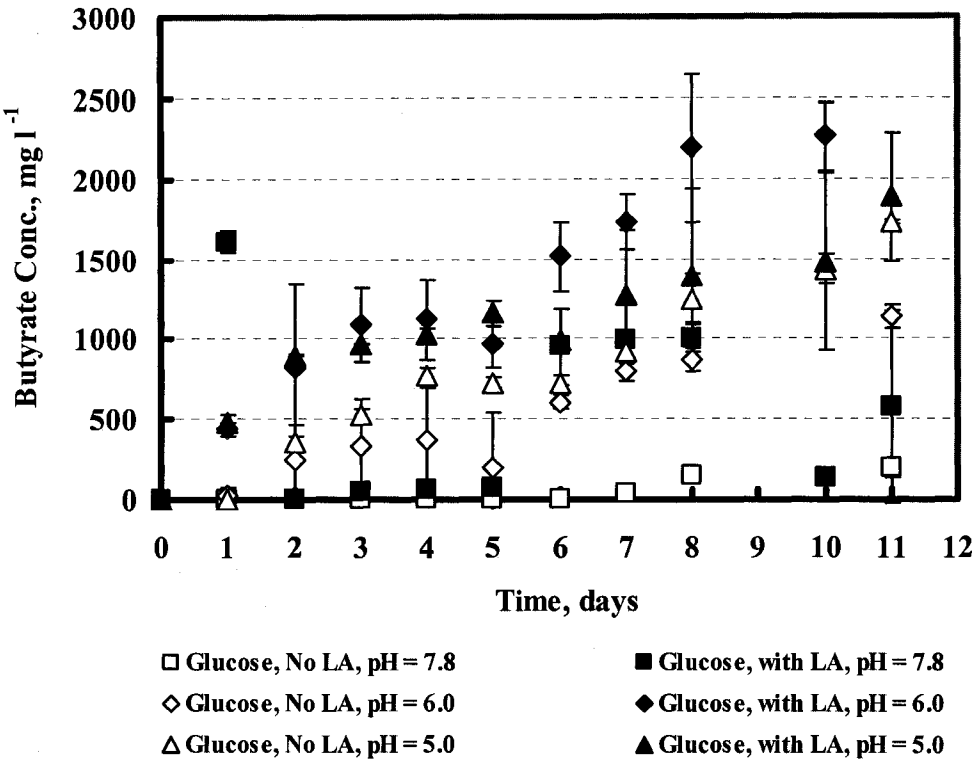

Figure 6.9: Butyric acid production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

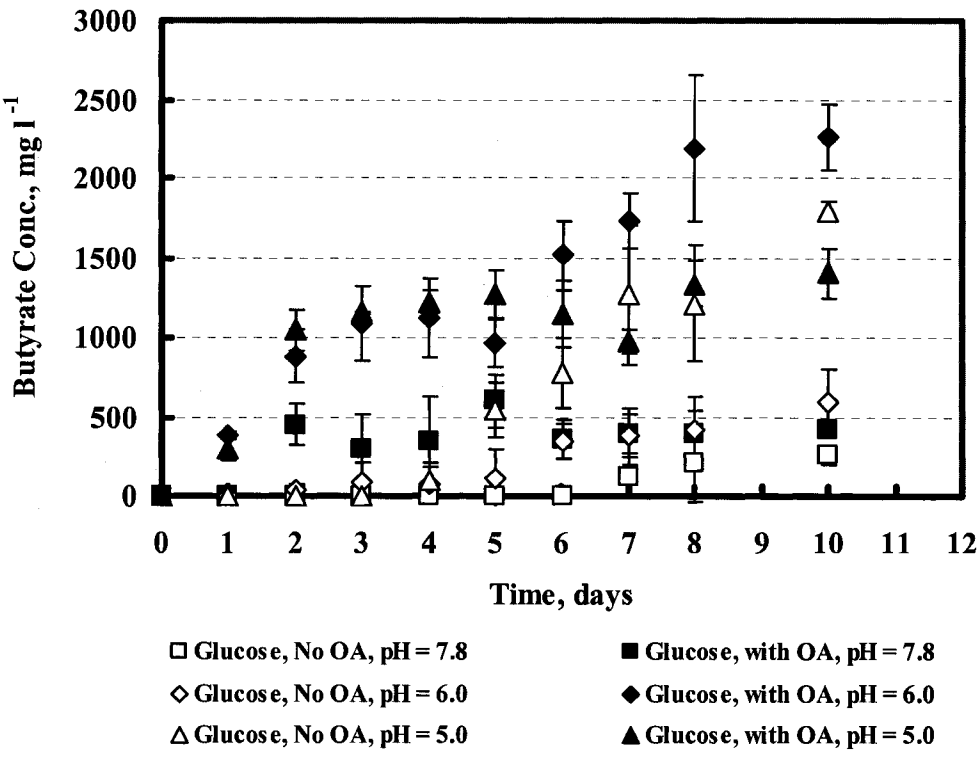

Figure 6.10: Butyric acid production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

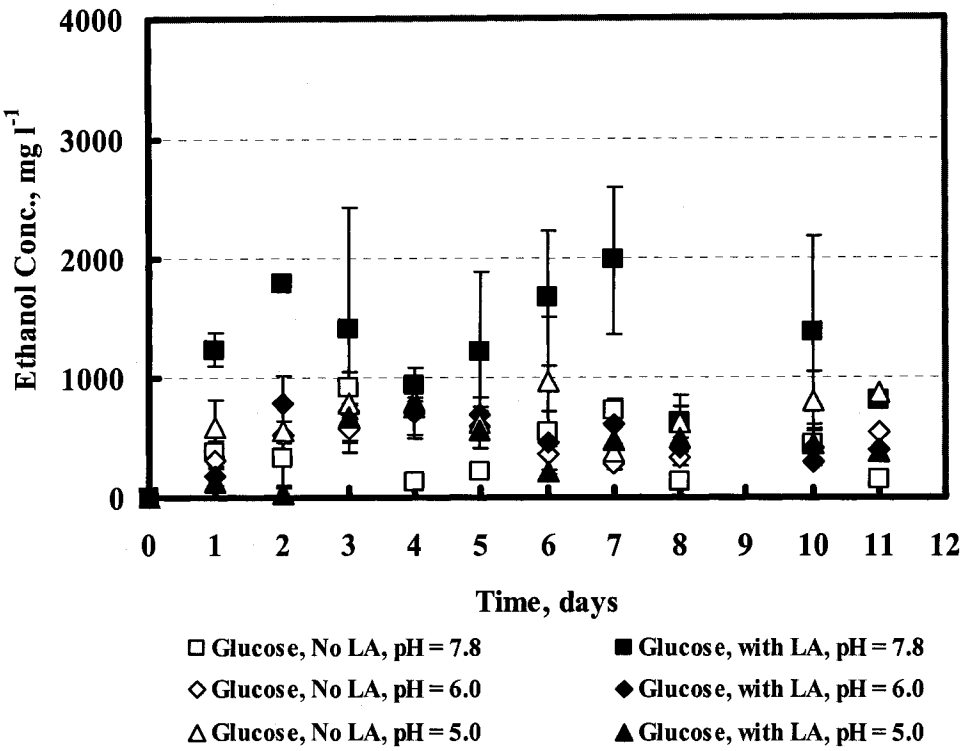

Figure 6.11: Ethanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

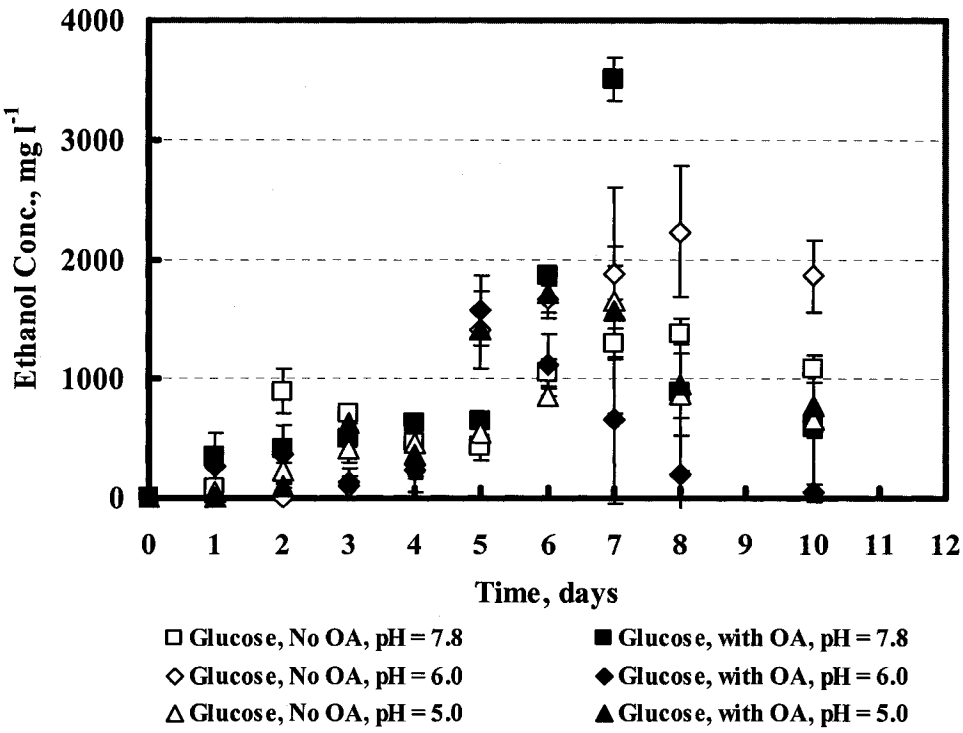

Figure 6.12: Ethanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

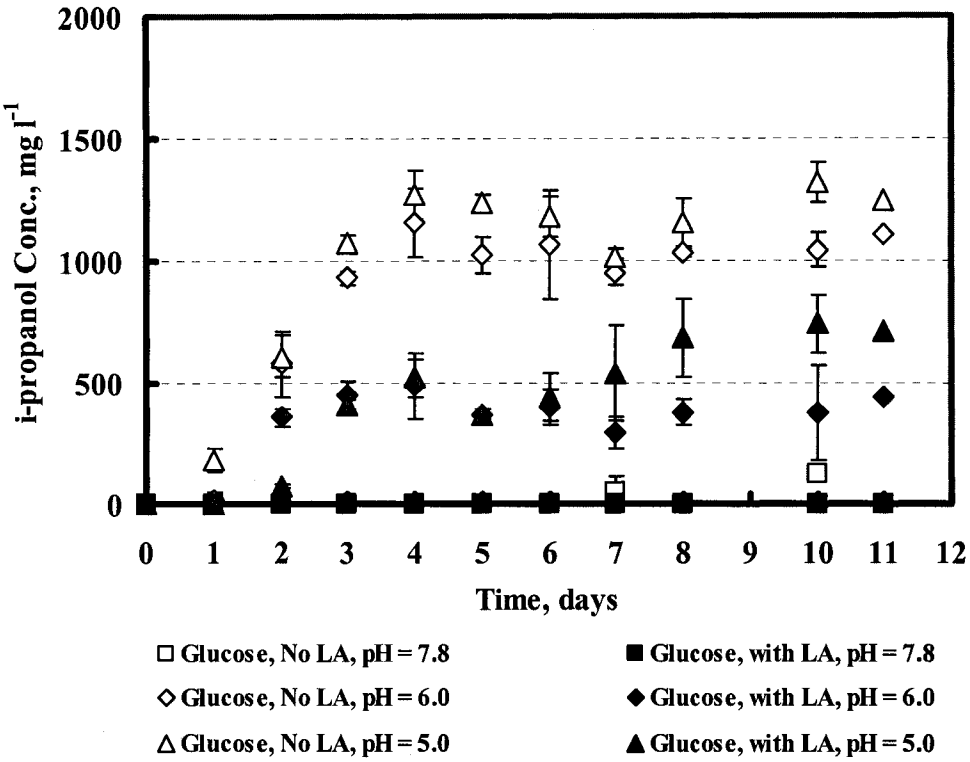

Figure 6.13: i-propanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

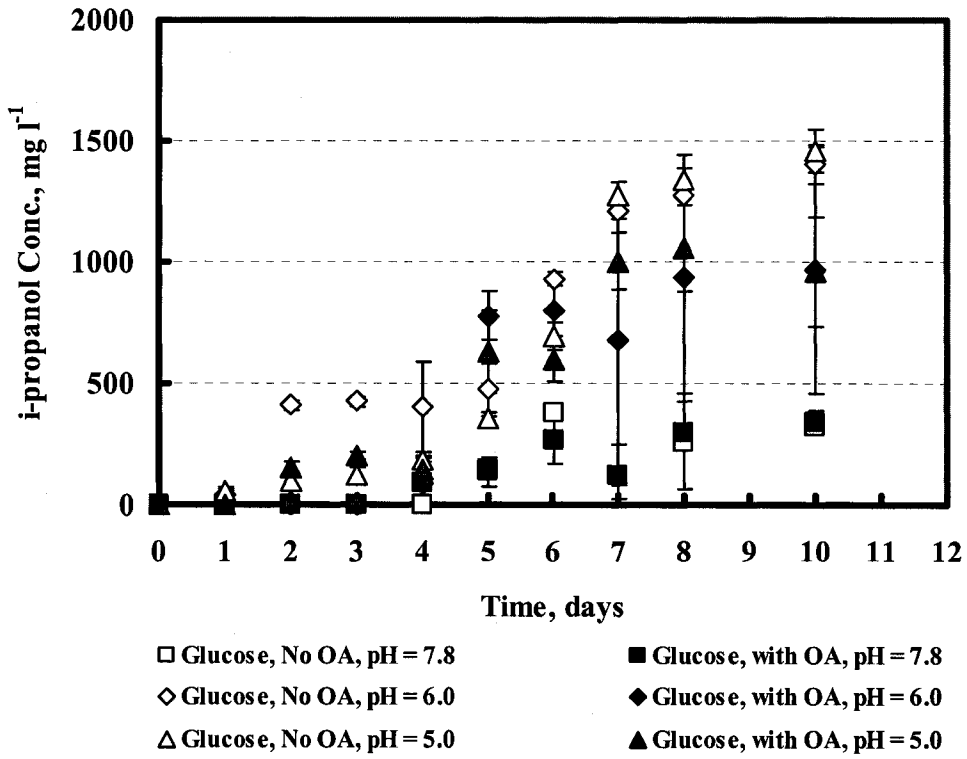

Figure 6.14: i-propanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

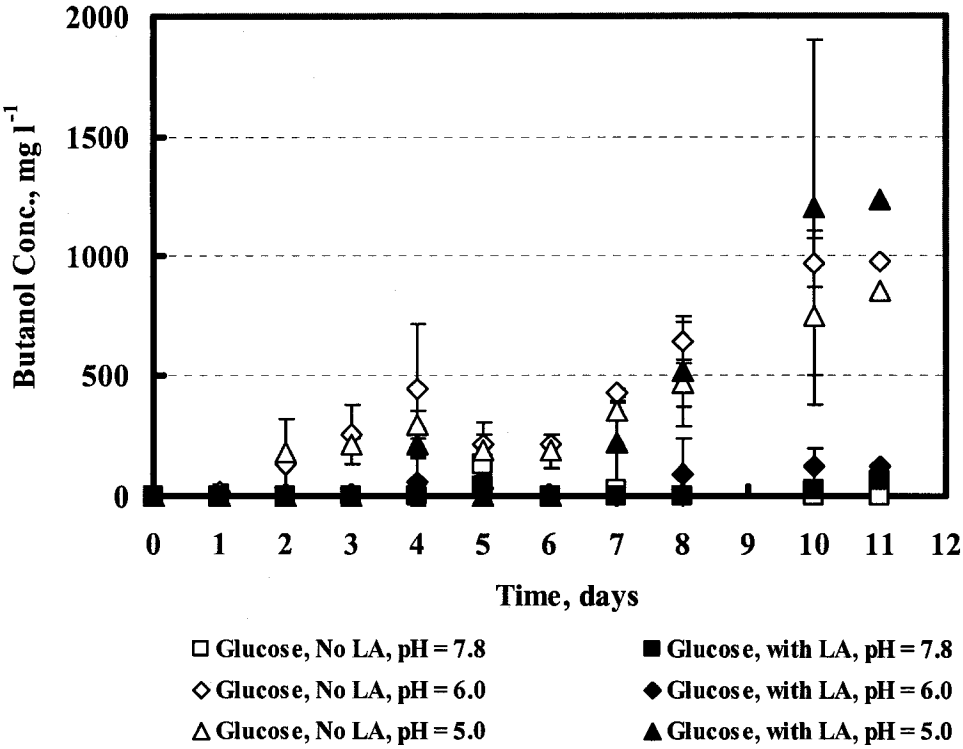

Figure 6.15: Butanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

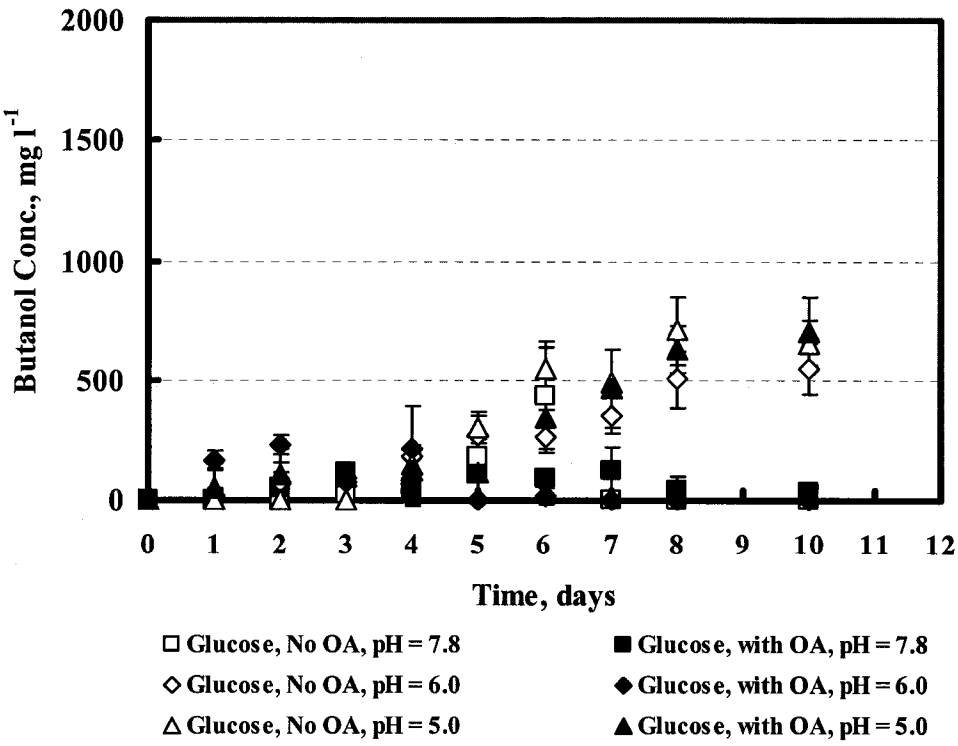

Figure 6.16: Butanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

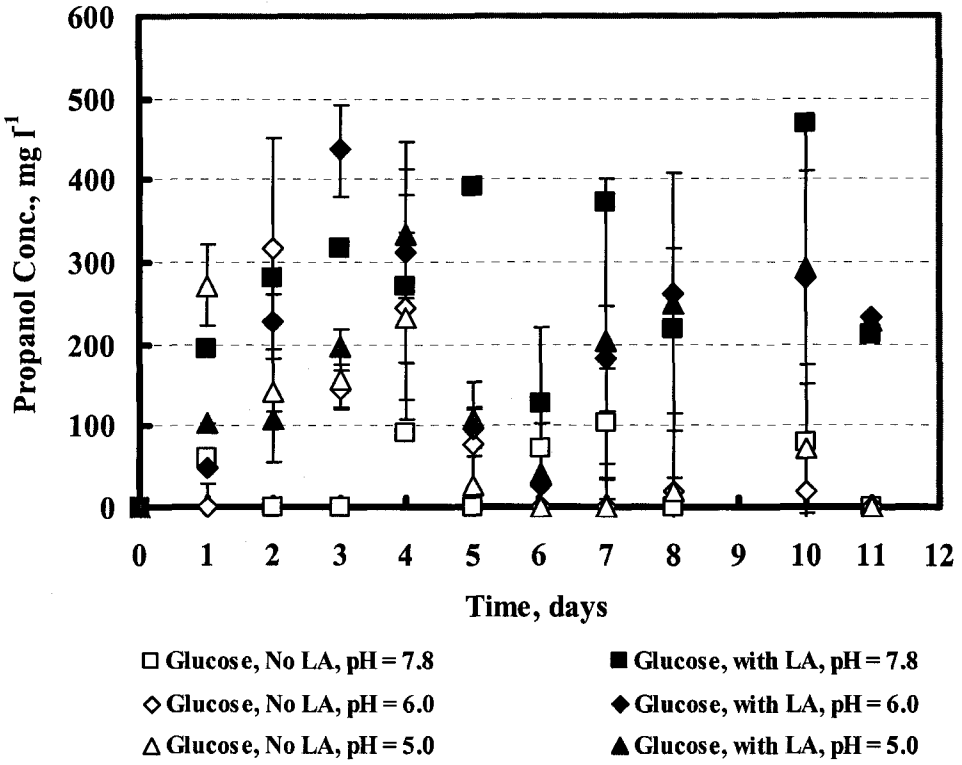

Figure 6.17: Propanol production profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

6.1.4 Glucose degradation

Figure 6.18 and 6.19 show glucose degradation profiles for cultures with varying initial pH and fed with 5,000 mg l^{-1} glucose plus LA and OA (at 0 h), respectively. Glucose degradation was inhibited in cultures inoculated with LCFA and with lower initial pH. Undetectable glucose levels were achieved in the controls after approximately 16 hr. The initial glucose degradation rates for cultures receiving LCFA were low. Glucose degradation was inhibited with increase in LCFA concentration. The initial glucose degradation rates for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose plus LCFA are shown in Table 6.2. Statistical comparison between each data set was performed using the Tukey's paired comparison procedure (Box *et al.*, 1978). Glucose was degraded to undetectable levels between 48 to 72 hr in cultures

inoculated with LCFA at an initial pH of 7.6 and 6, while cultures without LCFA at an initial pH of 7.6 and 6 took between 24 to 48 hr. Cultures with an initial pH of 5 and with LA were observed with 20% residual glucose, even after 96 hr.

Table 6.2: Initial degradation rates ($\mu g \cdot mgVSS^{-1} \cdot min^{-1}$) for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose plus LA or OA.

LCFA	0 mg l^{-1}			2,000 mg l^{-1}		
	pH=7.8	pH=6.0	pH=5.0	pH=7.8	pH=6.0	pH=5.0
LA	6.06±0.03[a]	1.58±0.10[b]	1.14±0.09[c]	1.62±0.01[d,b]	0.99±0.07[e,c]	0.82±0.04[f]
OA	5.66±0.82[a]	2.24±0.05[b]	2.92±1.03[c]	2.01±0.19[b]	0.93±0.05[b]	1.24±0.05[b]

Notes: 1. Average and standard deviation for triplicate are shown. The superscript notations a, b, c, d, e, and f are used to indicate the means that are statistically different within the same rows. The notations cannot be used for comparing data within the same column or between columns.
2. Sample calculation of initial degradation rate is shown in Appendix E

6.1.5 Carbon mass balance

Carbon mass balances for cultures at varying initial pH and fed with glucose plus LA and OA are shown in Figures 6.20 and 6.21. The carbon balance is based on the mass of carbon from glucose plus the carbon mass from all the products detected in the culture (an example calculation is shown in Appendix E). The percentage of carbon in the cultures under different conditions ranged from approximately 20 to 125%. The percentage of carbon observed up to day 5 varied between 50 to 125% for cultures inoculated with LA. In cultures with an initial pH of 7.8 and without LA the percent carbon was approximately 50%. A possible cause of this low carbon accounting is a large fraction of the methane may have been lost during headspace sampling. The percent carbon in the mass balance was low for cultures inoculated with OA after the first glucose injection.

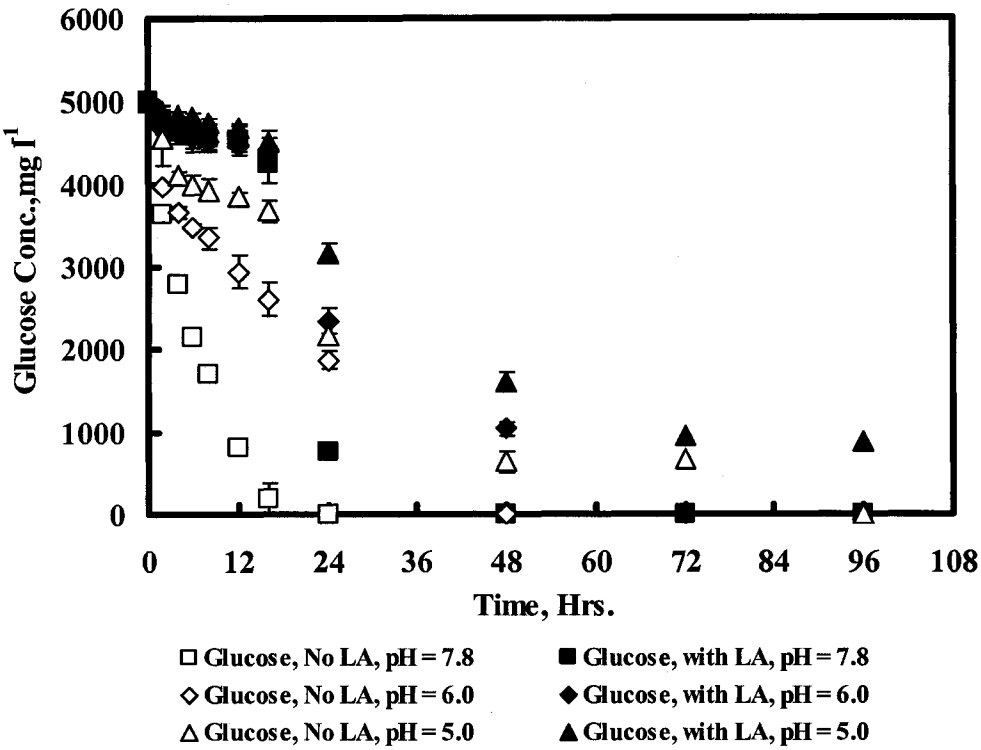

Figure 6.18: Glucose degradation profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

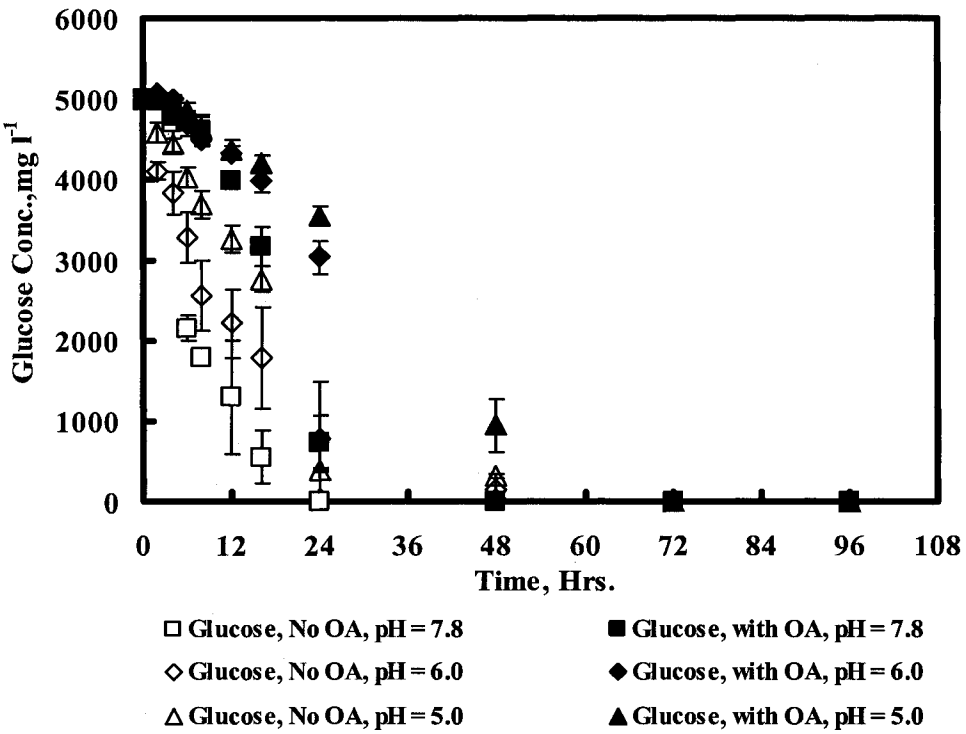

Figure 6.19: Glucose degradation profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

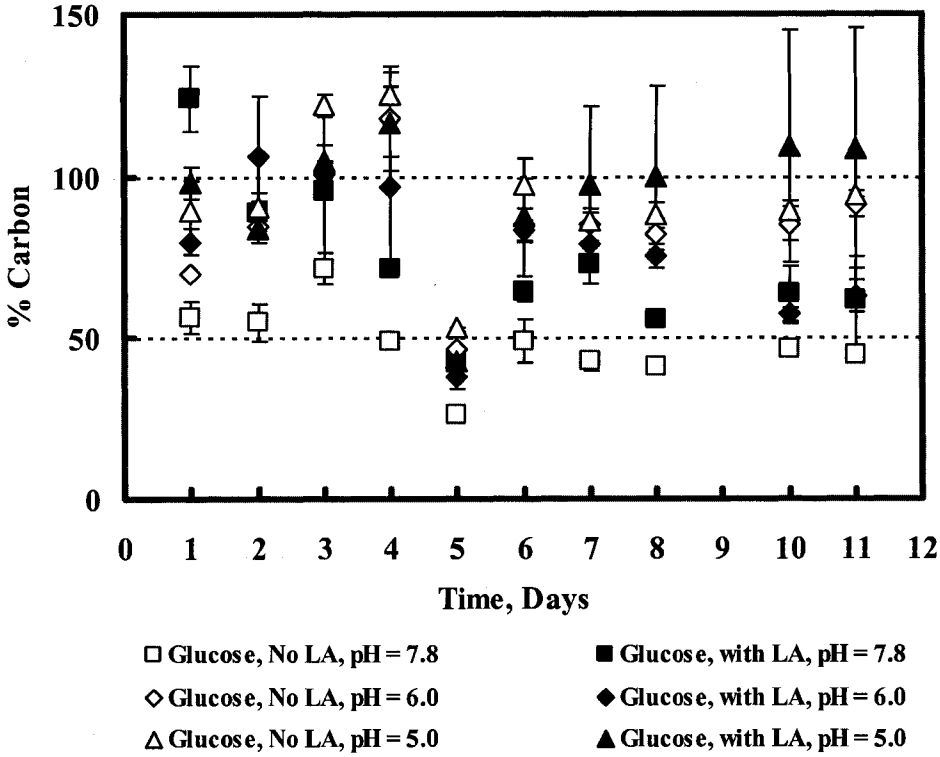

Figure 6.20: Carbon mass balance profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2000 mg l^{-1} LA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

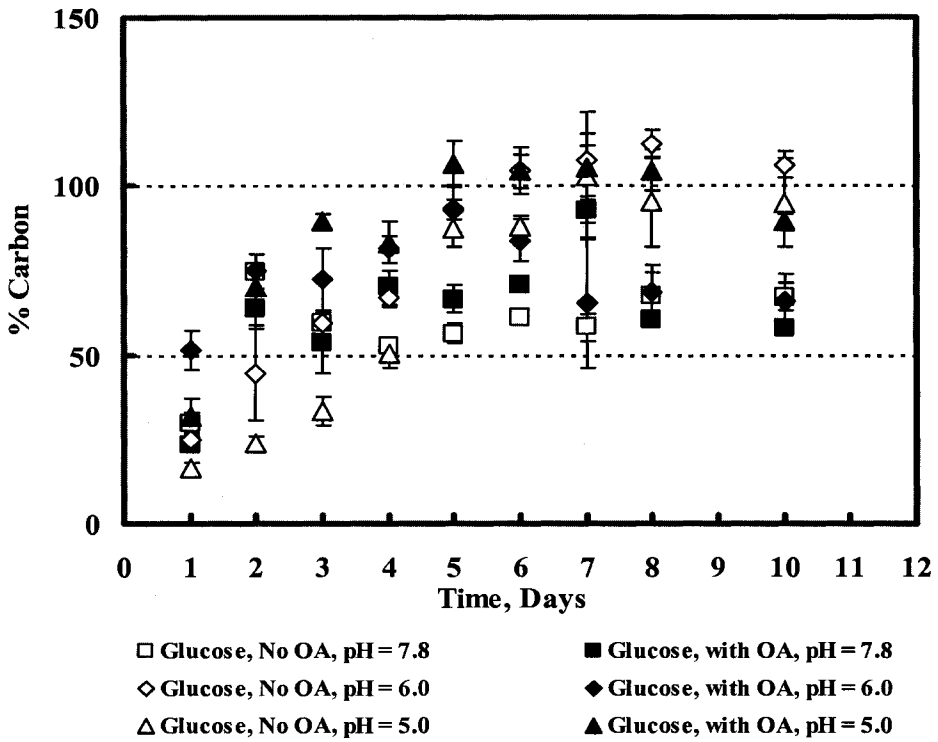

Figure 6.21: Carbon mass balance profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2000 mg l^{-1} OA (at 0 hr). Values shown are mean ± SD from triplicate cultures.

6.1.6 pH variation

Figures 6.22 and 6.23 show pH variation profiles for cultures with varying initial pH conditions and fed with 5,000 mg l^{-1} glucose plus LCFA. The pH was adjusted on day 5 to the initial value. The fluctuation of the pHs for cultures with an initial pH of 7.8, with and without LCFA, were similar. However, in the case of cultures at initial pH values of 5 and 6, the pH change for cultures without LCFA decreased compared to those receiving LCFA. The pH decrease for cultures with an initial pH of 7.8 was similar after the first and second glucose injection. In comparison, a significant decrease in pH was observed after the first glucose injection compared to after the second injection in cultures with an initial pH of 5 and 6.

6.2 Discussion of results

In an anaerobic process, high H_2 production rates are due to exponential growth of H_2 producing organisms (Kim and Zeikus, 1985). pH affects the growth rate and changes in pH may cause drastic shifts in the relative numbers of different species in a heterogeneous population (Minton and Clarke, 1989). In the present study, the presence of LCFA and a lower initial pH demonstrated higher H_2 production during glucose fermentation. The maximum H_2 yields observed in cultures inoculated with LA and with an initial pH of 5 and 6 were approximately 2.21 and 2.7 mol $H_2 \cdot mol^{-1}$ glucose, respectively. Similarly, the maximum H_2 yields for cultures inoculated with OA and at initial pH conditions of 5 and 6 were approximately 2.2 and 1.2 mol $H_2 \cdot mol^{-1}$glucose, respectively.

Several studies have reported high H_2 yields at lower initial pH (Zheng and Yu, 2004; Khanal *et al.*, 2004; Fan *et al.*, 2004; Van Ginkel *et al.*, 2001). In batch

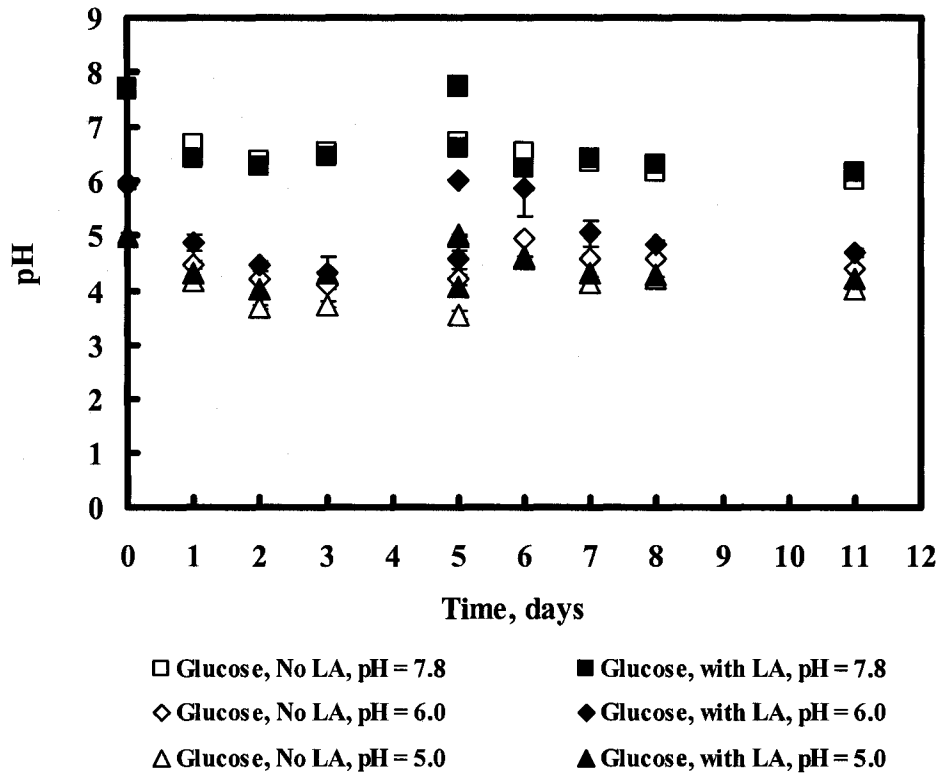

Figure 6.22: pH variation profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 120 hr) plus 0 and 2,000 mg l^{-1} LA (at 0 hr). pH was re-adjusted to initial pH on fifth day. Values shown are mean ± SD from triplicate cultures.

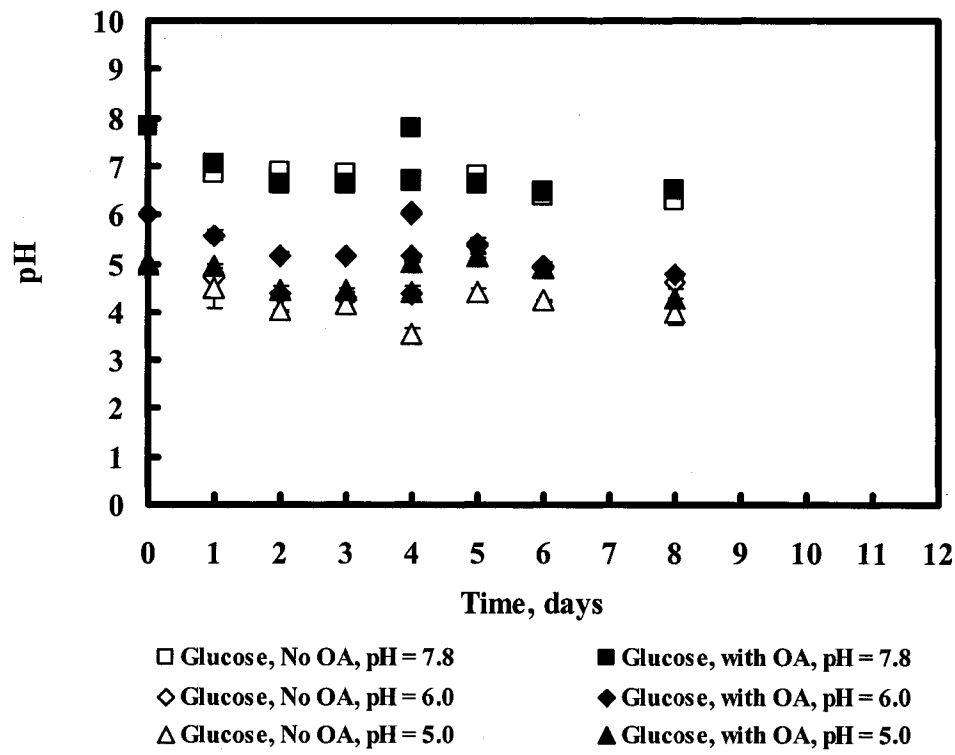

Figure 6.23: pH variation profiles for cultures at varying initial pH conditions and fed with 5,000 mg l^{-1} glucose (at 0 hr and 96 hr) plus 0 and 2,000 mg l^{-1} OA (at 0 hr). pH was re-adjusted to initial pH on fourth day. Values shown are mean ± SD from triplicate cultures.

experiments, a maximum H_2 yield between 1.30 to 1.57 mol $H_2 \cdot mol^{-1}$ glucose was reported by Zheng and Yu (2004) using a heat treated mixed culture at pH 4.0 to 5.0. Likewise, Khanal *et al.*, (2004) reported 1.8 mol $H_2 \cdot mol^{-1}$ glucose using heat shocked mixed cultures at 37°C and at an initial pH of 4.5. In continuous acclimated culture at 35°C and at a pH of 5.5 Chen *et al.* (2001) measured 3.47 mol $H_2 \cdot mol^{-1}$ sucrose. Van Niel *et al.* (2002) reported 3.3 mol $H_2 \cdot mol^{-1}$ glucose using *Thermotoga elfi* at 65°C and a pH of 7.4 in the batch cultures.

Higher hydrogen accumulation was observed after the second glucose injection in the cultures with an initial pH of 5 and 6 without LA or OA. This observation suggests greater inhibition of methanogens due to the presence of VFAs at low pH. Sung *et al.* (2002) reported that a reactor operating with relatively high total VFA concentrations ensured additional inhibition of H_2 consuming bacteria. However, in cultures inoculated with either LA or OA with an initial pH of 5 and 6, the H_2 yields either decreased or remained the same as after the first glucose injection. This suggests that the combined effect of high VFAs concentration at low pH along with LA or OA inhibited hydrogenase activity along with the methanogens.

Acetate, propionate and butyrate were the major VFAs observed during glucose degradation in cultures at different initial pH. Butyrate and acetate were the two most abundant species in the cultures receiving LA or OA at an initial pH values of 5 and 6. Increase of the initial pH from 5 to 7.8 resulted in the increase in acetate concentration. In comparison, reducing the initial pH from 7.8 to 5.0 resulted in elevated butyrate concentration. The H_2 yield obtained and the product distributions from different conditions examined imply that the butyrate-type fermentation dominated at the lower

initial pH conditions. Similar results reported by Zheng and Yu (2004) showed that production of butyrate was favored at pH 4.0 to 5.0; whereas production of acetate was favored at pH 6.0 to 8.0. They concluded that the butyrate-type fermentation is favored at lower initial pH. Similar observations have been reported in other studies (Fang and Liu, 2002; Khanal *et al.,* 2004; Kim *et al.,* 2004).

Ethanol and i-propanol were the dominant alcohols observed in all the conditions examined. Higher concentrations of ethanol were observed in cultures inoculated with either LA or OA compared to the controls. i-propanol was dominant in cultures without LA or OA. The ethanol-acetate pathway is another route through which H_2 is produced (Hwang *et al.,* 2004). Acetate and ethanol can be simultaneously produced by this route (Moat, 1979). Other alcohols observed in small amounts included butanol and propanol. Dabrock *et al.* (1992) reported the effect of pH, growth rate, and carbon source on product formation by Clostridium *pasteurianum*. They noticed 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. The metabolic pathways for butyric acid fermentation from glucose and simultaneous production of i-propanol and butanol have been reviewed by Papoutsakis (1984, 1985). Kim and Zeikus (1985) reported that the specific H_2 production rate decreased as the culture became solventogenic. Their data showed that at a pH of 5.8, the specific activity of hydrogenase remained constant during growth and only acids were produced, while at pH 4.5, the shift from acidogenesis to solventogenesis was accompanied by a corresponding decrease in the hydrogenase activity.

Glucose was removed relatively easily in most of the cultures within 3 days after glucose injection. Cultures inoculated with LA or OA had lower initial degradation rates compared to the controls. Furthermore, cultures set at higher initial pH values had faster initial degradation rates compared to those at lower initial pH conditions. Similarly, Zheng and Yu (2004) reported that 5,000 mg l^{-1} of glucose at pH 8.0 was degraded rapidly and depleted within 12 hours. However, only 40% of glucose was degraded in the reactors at pH 4.0 within 24 hr and it took more than 100 hours to deplete glucose completely. In another study, the glucose degradation increased from 90.3 ± 1.0% at pH 4 to 99.3 ± 0.9% at pH 5.5 (Fang and Liu, 2002).

The carbon mass balance for serum bottles based on the conversion to %carbon per bottle varied from 10 to 70% of the theoretical amount of carbon. The deficit in the carbon balance is expected for two reasons. Firstly, accountability of all the CO_2 produced by fermentation and the amount added to the head space gas caused an error in the carbon mass balance. In particular, because CO_2 is aqueous soluble, a fraction could be removed from the headspace and converted into aqueous carbonate species. Secondly, there could be other unidentified glucose fermentation products which are not taken into account. The carbon mass balances in some case exceeded 100% which indicates that CO_2 could have diffused from liquid phase and into the gas phase or LCFA degradation contributed to acetate formation.

CHAPTER 7: CONCLUSIONS

The motivation for this study was to investigate the fermentative H_2 production potential of mixed culture in the presence of LCFA as a methanogenic inhibitor and using carbohydrate (glucose) as a substrate. Two LCFAs, linoleic acid (LA) and oleic acid (OA) were selected as potential inhibitors based on previous studies. In this study, LCFA concentration and initial pH were selected as target factors to increase the H_2 production potential. Effect of high VFAs and intermittent sparging on H_2 production was assessed. Batch experiments were conducted in order to evaluate the influence and optimum of two factors on the culture generating H_2. The following conclusions are derived from this study:

1. No detectable amount of H_2 was produced during anaerobic degradation of LCFA or glucose.
2. The H_2 yield varied from 0.77 to 1.27 mol $H_2 \cdot mol^{-1}$ glucose in cultures inoculated with either LA or OA. High H_2 accumulation was observed with higher LA or OA concentration.
3. Glucose degradation was affected by the presence of LA or OA. Higher initial degradation rates were observed with decrease in LA or OA concentration. Glucose degradation was inhibited with lower initial pH. Higher initial degradation rates were observed with high initial pH.
4. At lower initial pH, higher total hydrogen production was observed. The maximum yield of 2.7 mol $H_2 \cdot mol^{-1}$ glucose was achieved in cultures receiving LA with initial pH 6.

5. Acetate, propionate and butyrate were the main VFAs observed in the cultures. Increase in hydrogen production potential accompanied with increase in acetate and butyrate formation. Butyrate type fermentation was observed in cultures with high hydrogen yields.
6. Ethanol and i-propanol were the major alcohols observed during glucose fermentation in cultures maintained at different initial pH. Propanol and butanol were other alcohols detected.

CHAPTER 8: ENGINEERING SIGNIFICANCE AND FUTURE RECOMMENDATIONS

The recapturing of energy in the form of H_2 is a promising alternative strategy. The use of LCFAs could turn out to be a very inexpensive approach for inhibiting hydrogenotrophic methanogens, in comparison with conventional techniques such as heat treatment. The experimental data from this study, such as H_2 yields and LCFAs concentrations could be useful to establish design data for a continuous fermentative H_2 production system.

Future recommendations to gain a better knowledge and improve understanding of inhibiting hydrogenotrophic methanogens are as follows:

- Further study is required to examine the effects of maintaining a constant optimum pH.
- Determine the effect of thermophilic operating conditions on H_2 production by dark fermentation using glucose in the presence of LCFA.
- The glucose to H_2 conversion efficiency is required to be further investigated in a continuous fermentative H_2 production system to assess the feasibility of LCFA inhibition on hydrogenotrophic methanogens.
- Assess the feasibility of a two-stage reactor system to produce H_2 using dark fermentation in the first stage and phototrophic fermentation H_2 production in the second stage reactor system.

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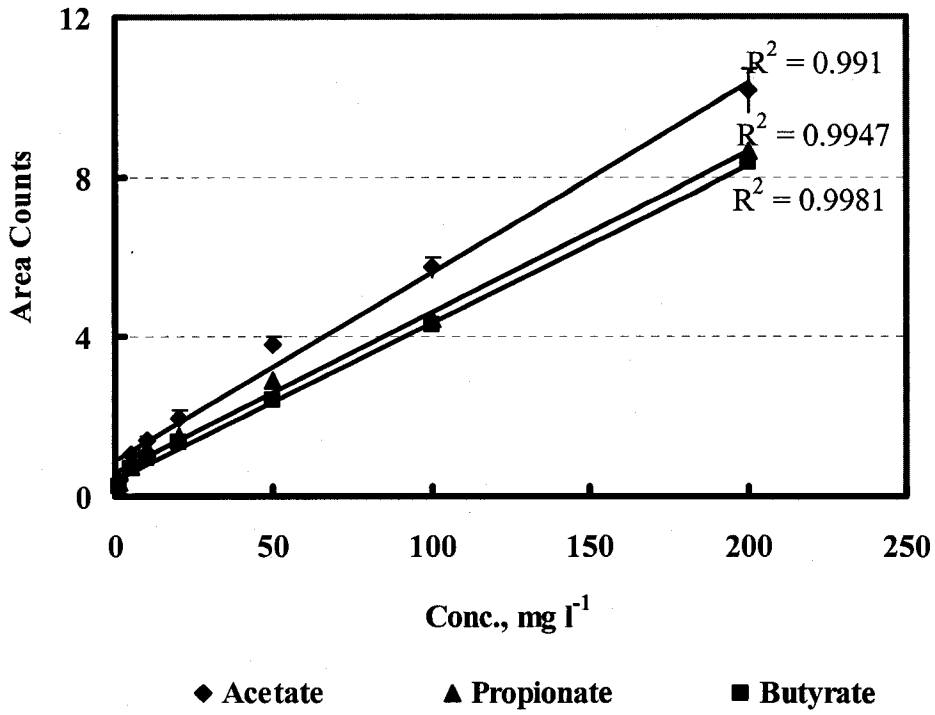
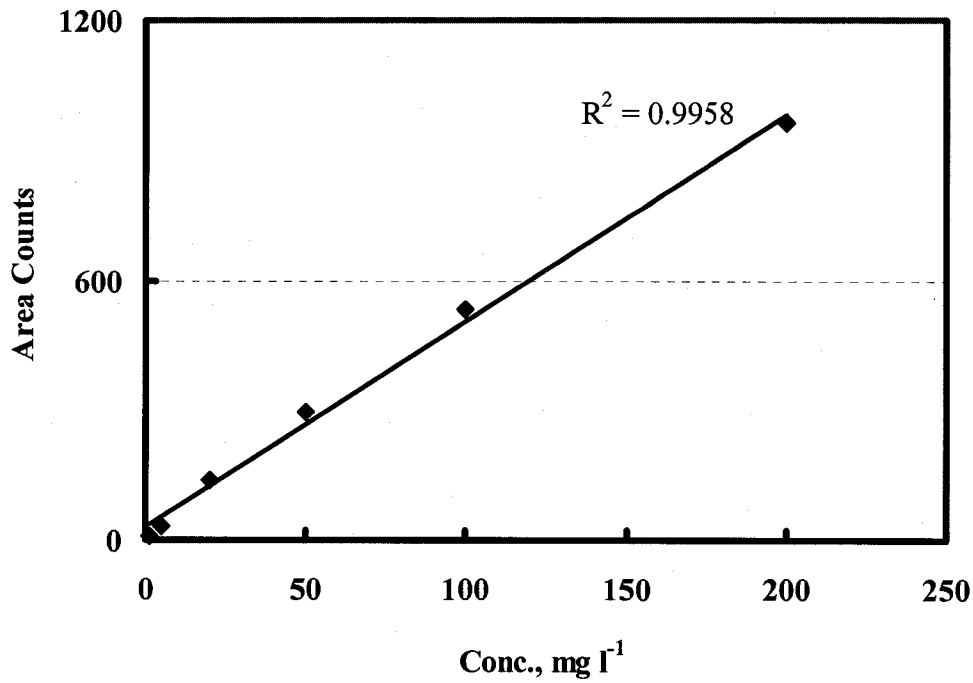
Appendices

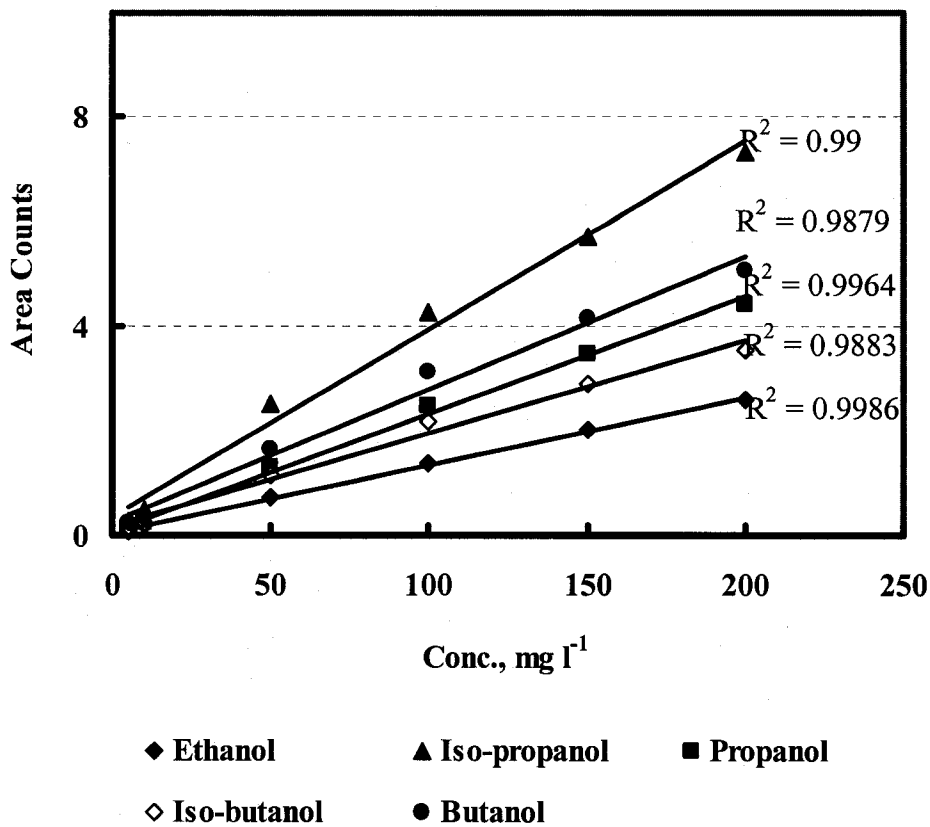
Appendix A: Volatile fatty acids calibration curves



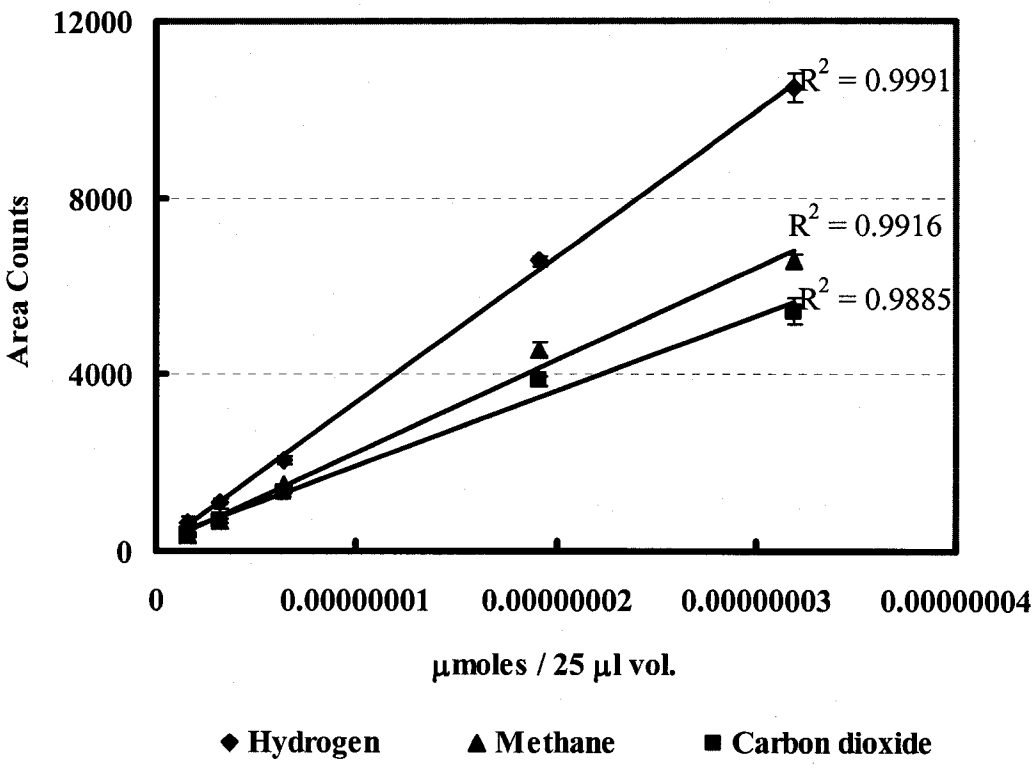
Appendix B: Glucose calibration curve



Appendix C: Alcohol calibration curves



Appendix D: Hydrogen, methane and carbon dioxide calibration curves



Appendix E: Example calculations for statistical comparisons, hydrogen yields, initial glucose degradation rates and carbon mass balance

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

- t is the batch number or the number of cases, t = 5 (0, 500, 1000, 1500, and 2000 mg/L).
- n_i is the sampling number = 3 (sampling is done in triplicates).
- $df_w = n_i - 1$
- α is the confidence level, the default value is used (0.95)
- q_α: upper percentage points of the studentized range, values are pre-calculated and can be obtained from any statistical textbook.
- Sw^2

Hydrogen yields were calculated based on mole of H_2/mole of glucose. Example: hydrogen recovery profilesnfor the cultures receiving 5000 mg l^{-1} glucose and LA (see Figure 5.1, cultures receiving 2000 mg l^{-1} LA).

Maximum hydrogen production (in the first phase) = 1513 μmoles.

Net amount of glucose (in the first phase) = 1388.9 μmoles.

Therefore, Hydrogen yield $= \frac{1513}{1388.9} = 1.09$ mo H_2/mol glucose

Degradation rates were calculated using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
Example: glucose degradation profiles for the cultures receiving 5000 mg l^{-1} glucose (see Figure 5.6, Control).

$$C\ mgl^{-1} = 5896\left(1 - e^{-0.1531t}\right) \Rightarrow v = \frac{-dC}{dt} = 902.7e^{-0.485t}$$

At t = 0 $\Rightarrow$ initial degradation rate = 902.7 mg $l^{-1}hr^{-1}$ = 15.05mg l^{-1} min^{-1}

Biomass concentration = 2000 mgVSS l^{-1} $\Rightarrow$

intial degradation rate = 7.52 μg mgVSS^{-1} min^{-1}

Mass balance calculations were done using the following formula:

$$\Sigma Substrate_{=0} = \Sigma Product_t + \Sigma Substrate_t$$

Example: cultures receiving 5000 mg l^{-1} glucose and LA/OA (see Figures 6.11 and 6.22) at t = 0; theoretical amount 100 mg C (from glucose) = 100 mg C (assuming there is no carbon contributed from LA/OA degradation during the short span of the experiments) at 1 day; C mass = 5.28 mg C (from Ethanol) + 0.52 mg C (from formate) + 60.8 mg C (from residual glucose) + 5.7 mg C (from Acetate) + 11.3 mg C (from Butyrate) + 0.55 mg C (from CH_4) + 8.5 mg C (from CO_2) = 92.65 mg C

Therefore, percentage carbon = $\frac{C_{Actualmass}}{C_{Theoreticalmass}} * 100 = 92.65\ \%$.

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