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Fermentation of Glucose and Xylose to Hydrogen in the Presence of Long Chain Fatty Acids

by
Stephen Reaume

A Thesis
Submitted to the Faculty of Graduate Studies
through the Environmental Engineering Program
in Partial Fulfillment of the Requirements for
the Degree of Master of Applied Science at the
University of Windsor

Windsor, Ontario, Canada
2009

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Abstract

Hydrogen is a clean, efficient and versatile energy source which makes it a suitable alternative to fossil fuels. Mixed anaerobic cultures has the potential to produce hydrogen in a sustainable way in methanogenic bacteria can be inhibited.

Batch studies were performed to assess the fermentation of glucose and xylose individually and together to observe if the sugar mixture is effective in hydrogen fermentation. Experiments were performed using a variety of LCFAs in order to inhibit methanogens so hydrogen can be collected.

The highest amount of hydrogen produced took place in cultures fed LA plus xylose, glucose and the 50%/50% sugar mixture with yields of 2.13±0.05, 2.46±0.19 and 2.32±0.17 mol H_2/mol sugar, respectively. The maximum yields generated on a mol hydrogen per mass of sugar was 13.65, 14.20 and 14.08 mmol H_2/g sugar for the respective sugars fermented. The final results showed that the ratio of the two different sugars did not have a significant difference in the hydrogen yield.

DEDICATION

I dedicate this thesis to my loving family, John, Mary and Mark Reaume

Acknowledgements

I wish to truly thank my advisor, Dr. Jerald A. Lalman for his leadership, wisdom and guidance throughout this thesis. Without his advice, support and selfless contributions this study would not have been able to be conducted or completed. I would like to extend my gratitude to Dr. A. A. Asfour and Dr. P. Vacratsis for their valuable suggestions as my committee members.

Thanks to all my close friends who have helped me along in my studies, including special thanks to Srimanta Ray for his invaluable advice, Noori Saady and SubbaRao Chaganti for their help in the lab and Paul Huggard for all his help and together all their help throughout this thesis was instrumental.

I would like to thank Mr. Bill Middleton for his advice, valuable assistance and technical support during my entire thesis without which I would still be stuck in the lab trying to figure out the instruments.

Lastly I would like to thank NSERC, OGS and the Civil and Environmental Engineering Department for the financial support throughout this study.

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Nomenclature

ATP	Adenosine tri-phosphate
ADP	Adenosine di-phosphate
CoA	Coenzyme A
COD	Chemical oxygen demand
Fd	Ferrodoxin
GC	Gas chromatograph
HRT	Hydraulic retention time
IC	Ion chromatograph
LA	Linoleic acid
LAU	Lauric acid
LCFA	Long chain fatty acid
LC	Liquid chromatograph
MA	Myristic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OA	Oleic acid
PA	Palmitic acid
SA	Stearic acid
TCD	Thermal conductivity detector
TSS	Total suspended solids
VFA	Volatile fatty acid
VSS	Volatile suspended solids

Chapter 1
General Introduction

1.1 Hydrogen Production Methods

Energy security and environmental protection are two key issues which have arisen from the use of fossil fuels by developed and undeveloped nations. Over 80% of the current energy usage is derived from fossil fuels and with developing countries making a bid for these resources it is expected these fossil fuels will disappear at much faster rates than currently. Burning fossil fuels contributes to major climate changes, environmental destruction and health problems. Hydrogen is a reliable and clean alternative to fossil fuels. Hydrogen gas can be used as a transportation fuel, generating electricity and for heating purposes.

For many years engineers and scientists have been converting waste organic residues to methane using anaerobic fermentation. During this multi-reaction anaerobic fermentation process hydrogen is produced at several steps and is subsequently converted to methane by hydrogenotrophic methanogens. Other hydrogen consuming microorganisms includes homoacetogens and sulfate reducing bacteria (SRB) (Mara and Horan, 2003). Inhibiting microorganisms which consume hydrogen ultimately leads to hydrogen accumulation. Hydrogen is a preferred fuel when compared to methane for several reasons. One of which is that during combustion of hydrogen gas water is the only by-product. Industrial uses of hydrogen include the synthesis of ammonia, hydrogenation of oils and hydrogen is used in fuel cells to generate electricity. Hydrogen having such a low viscosity is theoretically more efficient in the transfer of energy as hydrogen gas in pipes as opposed to electricity in wires. Since the conversion of hydrogen to electricity is not 100% efficient there is a minimum distance in its distribution before energy is saved. This could mean that hydrogen, or more specifically electricity production could be at remote sites then transferred as hydrogen to cities for conversion into electricity (Bockris, 2002).

In the last few decades a number of international organizations and countries have been promoting research in order to assess the conversion of renewable agriculture residues or waste material into hydrogen. (http://imagine.gsfc.nasa.gov/docs/ask_

astro/answers/971113i.html, Retrieved on 2009-03-10.) Iceland is a country which has implemented an active hydrogen economy. With many geological active sites, Iceland is able to harness geothermal energy and subsequently transform it into hydrogen. The steam generated from those geothermal energy facilities is used by turbines to generate electricity which is eventually used to produce hydrogen. The hydrogen can then be transferred to cities where it is used in fuel cells to be transferred back into electricity which is utilized to heat buildings and for transportation purposes (Kiaviat, 2003). Since the electricity produced from the geothermal facilities is from remote areas, it is feasible to create hydrogen through electrolysis and transport that energy to the cities. This allows for greater storage in vehicles for long trips, since batteries cannot store as much energy compared to a tank of hydrogen. For example submarines that operate on batteries can move at a speed of only a few knots for 4–6 h. The time in which the submarine may remain submerged using fuel cells depends on the capacity for storing on board hydrogen and oxygen which could be at least 12 h. (Bockris, 2002).

Many current methods for producing large amounts of hydrogen for industrial uses utilize fossil fuels as their source of energy. The following four processes used to produce hydrogen from fossil fuels include (Rosen and Scott, 1998):

a) Steam reforming of natural gas. Steam and methane are mixed at high temperatures (700 – 1000^{o}C) in the presence of metal catalysts which react to form hydrogen and carbon monoxide (Reaction 1.1).

$$CH_4 + H_2O \rightarrow CO + 3\ H_2 \quad \Delta G = +191.7\ kJ/mol \qquad (1.1)$$

b) Thermal cracking of natural gas. Heating natural gas in the presence of a catalyst to produce carbon monoxide and hydrogen.

c) Partial oxidation of heavy hydrocarbons. Partial oxidation of hydrocarbons involves the heating of the hydrocarbon in a low oxygen environment to create a hydrogen rich gas.

d) Coal gasification. Is the heating and pressurizing of coal and water to create hydrogen and carbon monoxide according to the reaction (Reaction 1.2).

$$C + H_2O \rightarrow CO + H_2 \quad \Delta G = -40.4\ kJ/mol \qquad (1.2)$$

Hydrogen produced from natural gas accounts for approximately 80% of the total hydrogen production while production from fossil fuels accounts for over 90% of the commercial hydrogen production (Rosen and Scott, 1998). These industrials processes are energy intensive as they operate at very high temperatures and pressures. Other methods to produce hydrogen include the following (Hallenbeck and Benemann, 2002):

a) Electrolysis. Using an electrical current to drive a non-spontaneous chemical reaction. In the case of water electrolysis, the reaction is mediated in a strong ionic solution in order to split water into hydrogen and oxygen gases.

b) Photolysis. Using light energy to split water into hydrogen and oxygen by way of a biological process.

c) Thermo chemical and Thermal decomposition. Organic materials which are broken down into hydrogen gas and various other constituents under extreme chemical and environmental conditions.

These processes again require a large amount of energy in order to produce sufficient amounts of hydrogen gas. The energy required to produce hydrogen gas by electrolysis is 118.7 kJ/mol (United States Department of Energy, USDOE). Electrolysis of water is not economically feasible because the process used to convert hydrogen to electricity in a fuel cell is approximately 60% efficient which leaves 40% of the energy unused. (Bockris, 2002)

1.2 Biological Methods

The biological methods for the production of hydrogen can be classified into two categories. Light fermentation using photosynthesis and dark fermentation which is the breaking down of complex organic molecules in the absence of light. Photosynthetic production of hydrogen is carried out by photosynthetic bacteria where water is converted into hydrogen in the presence of light. Cyanobacteria are an example of a microorganism which can synthesize hydrogen in the presence of light. Light fermentation has

deficiencies such as low conversion efficiencies and is light dependant (Hallenbeck and Benemann, 2002). Dark fermentation of organic molecules on the other hand has a much greater potential for commercialization than that of light fermentation. The microorganisms that mediate these reactions have relatively high reproduction and growth rates which are required in order to produce the necessary amount of enzymes required for rapid hydrogen production (Tanisho et al., 1994).

In the dark fermentation process however hydrogen is consumed and transformed into methane. For the hydrogen to be recovered the methane producers must be somehow inhibited. Long chain fatty acids (LCFAs) (Lalman and Bagley, 2000) are a safe low cost method for accomplishing methane inhibition (Hwu and Lettinga, 1997). LCFAs are available from two major sources. They are present in wastewater effluents from many food processing industries and are produced from renewable agricultural sources. Other than chemical inhibition, several other factors play an important role in affecting the hydrogen gas yield. These include feedstock type such as glucose and xylose, culture source, temperature, pH, product concentration and hydrogen partial pressure. Engineering design conditions can also affect the yield of hydrogen gas. These include reactor size, continuous or batch reactor, hydraulic retention time (HRT), pressure regulation, and oxygen barriers. Feedstock type is important for fermentation to be economically feasible. Xylose is a five carbon sugar which is found along with glucose in woody and non-woody biomass (Huang and Logan, 2008). Glucose, a six carbon sugar is commonly found in starches and many food products and can be an expensive feedstock source. Many fermentation studies have reported using glucose; however, a glucose plus xylose mixture could much better serve as a source of electron donors for hydrogen production. Hence, xylose is an excellent sugar which can be compared with past studies using only glucose.

1.3 Objectives

The purpose of this study is to optimize the hydrogen production from a mixture of sugar feedstocks using LCFAs and reaction conditions set at 23^{o}C. Optimization studies will be performed using different LCFAs, pH values, individual sugar type (C5 or C6) plus sugar mixtures at various ratios.

The objectives are as follows:

1. Determine optimal LCFAs and pH conditions that maximize the hydrogen yield using glucose as the electron donor.
2. Acclimate the culture to the sugar xylose and optimize hydrogen yield by screening a wide range of long chain fatty acids (LCFAs).
3. Determine if the ratio of two different sugars has an effect on hydrogen yield by the use of a combine sugar mixture of both glucose and xylose at various ratios using the optimal conditions from objectives 1 and 2.

Chapter 2
Literature Review

Hydrogen has a wide variety of uses as an electron donor in fuel cells and in combustion processes. Hydrogen is also used as an electron donor for C1 compounds and various other unsaturated hydrocarbons. Hydrogen has the potential to replace a variety of hydrocarbons as a fuel source of the future. Hydrogen is clean and produces only water as a combustion by-product. Hydrogen gas has the potential to be more efficient to transport than electricity due to a very low viscosity (Bockris, 2002). Environmental engineers have developed successful techniques for treating waste using anaerobic fermentation. During anaerobic treatment complex organic substrates are converted into simple C1 compounds (CH_4 and CO_2). Under certain conditions, hydrogen is produced as a by-product. Minimizing CH_4 formation and increasing hydrogen production is possible by manipulating the microbial culture.

2.1 Anaerobic Fermentation

Anaerobic fermentation is a process whereby complex organic material is degraded in the absence of oxygen. In a reactor operating under optimal conditions, the electron equivalences are ultimately diverted to methane (CH_4), carbon dioxide (CO_2) and biomass. The four main stages of anaerobic digestion are as follows (Figure 2.1):

a) Hydrolysis
b) Acidogenesis
c) Acetogenesis
d) Methanogenesis

2.1.1 Hydrolysis

During hydrolysis, the first step in anaerobic fermentation, complex organic polymers are broken down into simpler oligomers and monomers. Hydrolysis is the rate limiting step and is dependant on a variety of factors such as pH, temperature and availability of hydrolytic enzymes (Jordan and Mullen, 2007).

2.1.2 Acidogenesis

Acidogenesis, the second step in the fermentation process is where the degraded compounds from hydrolysis (i.e. sugars, amino acids, LCFAs) are converted primarily into volatile acids, alcohols, hydrogen and carbon dioxide. The bacteria that mediate these reactions are typically fast growing, resistant to many toxins and inhibitors and as a result are the most abundant bacterial group in most biological reactors (Joubert and Britz, 1987)

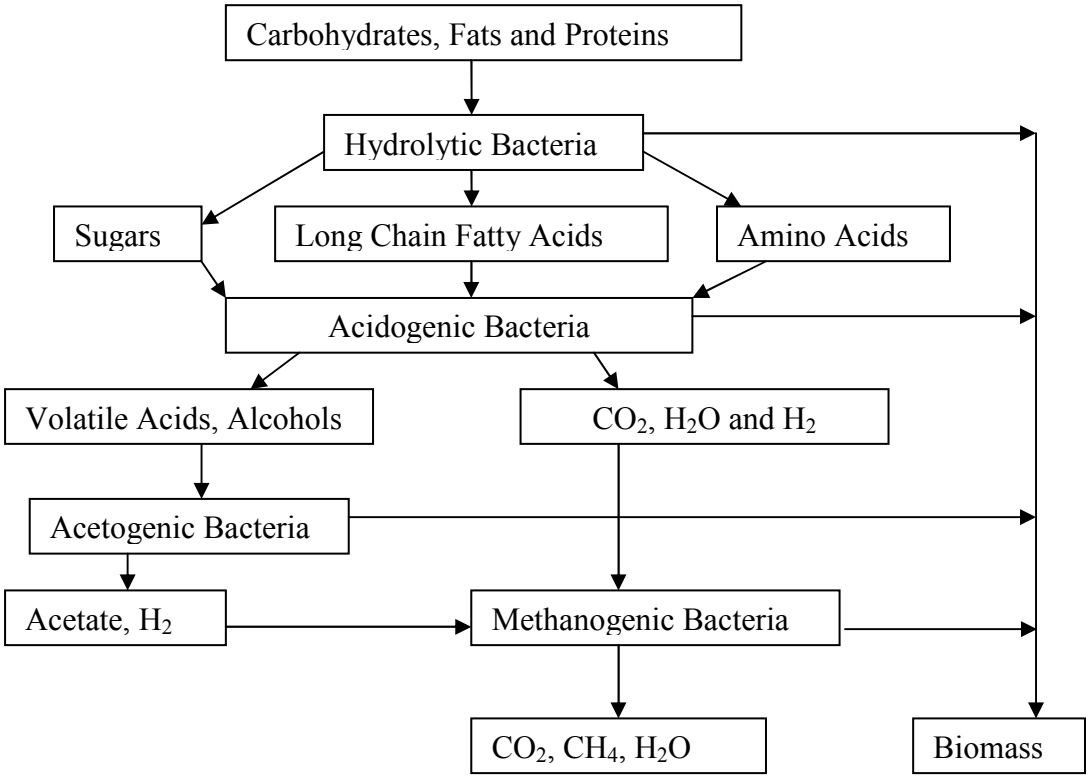


Figure 2.1 Typical anaerobic degradation pathway of organic substrates

2.1.3 Acetogenesis

During acetogenesis, the third reaction step, intermediary products are broken down into acetate, carbon dioxide and hydrogen. The organisms mediating these reactions are affected by end-product concentration and pH (Joubert and Britz, 1987). Hydrogen production can be increased at an optimum pH however it can also be hindered

under elevated hydrogen partial pressures. At elevated hydrogen partial pressure greater than 10^{-4} atm in solution (Mara and Horan, 2003) or under low pH conditions of pH 4.3 alcohols are the main by-product, (Kim et al., 2004) and are used to offset the unfavorable thermodynamic effects.

2.1.4 Methanogenesis

Methanogenesis is the final step in anaerobic fermentation. Bacteria mediating these reactions are the terminal organisms in the process. In this step acetate is converted into methane plus carbon dioxide by acetoclastic methanogens. In a parallel process hydrogen plus carbon dioxide are converted into methane by hydrogenotrophic methanogens (Gujer and Zehnder, 1983). These bacteria are slow growers and sensitive to changes in environmental conditions. Methane production by hydrogenotrophic bacteria is the main mechanism maintaining low hydrogen partial pressures in anaerobic communities. This is important as high hydrogen pressures can inhibit the acidogenic reactions.

A wide range of microorganisms facilitate the different steps of anaerobic degradation using a wide range of organic electron donors. This relationship is defined as a syntrophic relationship whereby the products of one organism are used by another. (Gujer and Zehnder, 1983) A general example of this is the relationship between soybean plants and the bacteria that live on their roots. The bacteria in their metabolic process fix nitrogen from the air into various nitrogen compounds which the plant then absorbs in order to grow. The microorganisms involved in fermentation of sugars exist in a large syntrophic relationship. An example involving hydrogen production and consumption is how methanogens are fed by the products of the acidogenic and acetogenic bacteria which are fed by the products of the hydrolytic bacteria. Though this process is not strictly dependant on syntrophy, syntrophy is a major advantage to the microorganisms involved. The process of syntrophy is shown in Figure 2.2 where the products of the organisms of one step of the fermentation process feed the organisms in the next step of the process.

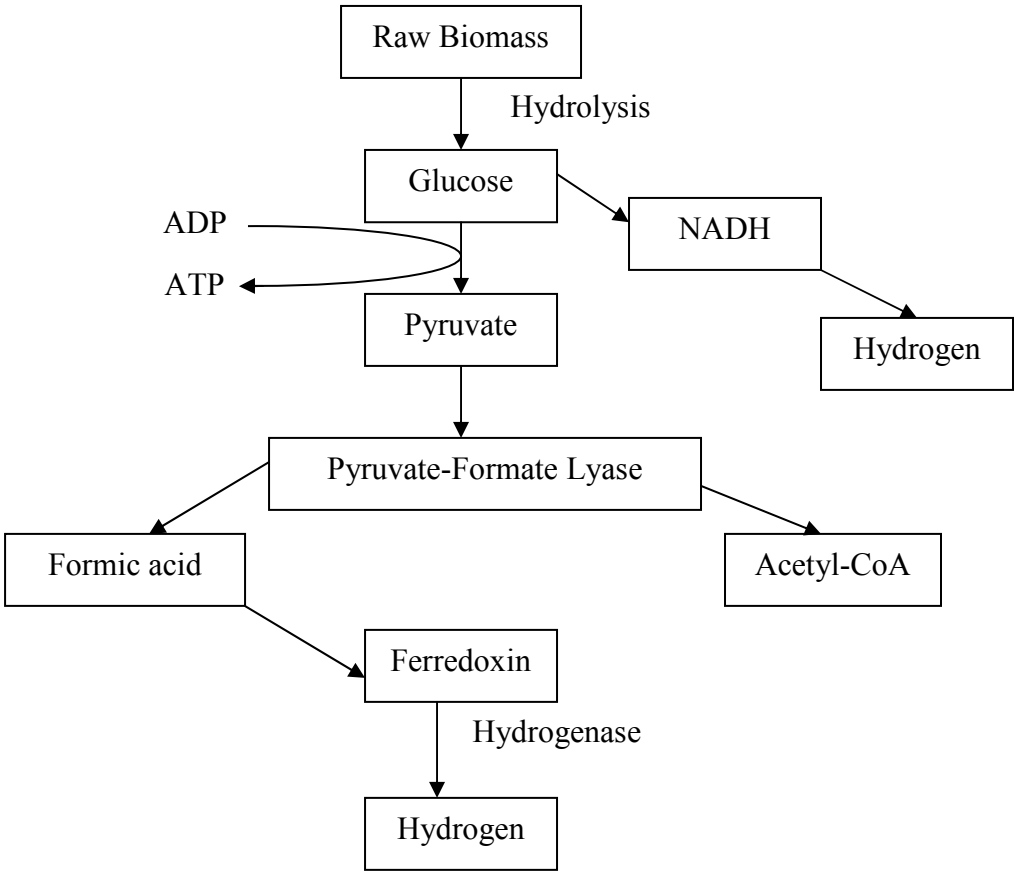


Figure 2.2 Biochemical decomposition of biomass into hydrogen (adapted from Nath and Das, 2004).

2.2 Anaerobic Hydrogen Production

Hydrogen production from the fermentation of organic compounds in an anaerobic environment is accomplished by diverting electron equivalences towards proton reduction. During heterotrophic bacteria metabolism these organisms are able to use various energy sources and oxidize many organic substrates providing energy for cell growth. Oxidation of organic compounds generates electrons which reduces cofactors such as NAD^+ to NADH the common electron carriers in many cells. There are a limited number of these cofactors in a system and are regenerated by the loss of electrons to a terminal electron acceptor such as carbon dioxide or protons. In aerobic systems, oxygen accepts the electrons and is reduced to water. In anaerobic systems other electron

acceptors such as protons accept these electrons and are reduced to molecular hydrogen (H_2) (Nandi and Sengupta, 1998). The biochemical pathway for converting reduced carbon compounds into hydrogen by way of fermentation is shown in Figure 2.2. Pyruvate is the main product from the breakdown of sugars. Figure 2.2 illustrates the main hydrogen producing pathway via pyruvate fermentation.

In aerobic environments oxygen is used to oxidize vital cofactors that have been reduced during the various breakdown pathways. In anaerobic environments there is no oxygen and such the excess protons are reduced into hydrogen in order to oxidize and recharge the cofactors such as NAD^+. The metabolism of pyruvate is responsible for most of the hydrogen produced during anaerobic fermentation. Pyruvate decomposition is catalyzed by the enzyme pyruvate formate lyase (PFL) to produce formate (Equation 2.1). Formate can be further degraded by enteric bacteria in order to produce hydrogen (Equation 2.2) (Hallenbeck and Benemann, 2002). The degradation of pyruvate into acetyl-CoA resulting in hydrogen production is catalyzed by the enzyme pyruvate ferrodoxin oxidoreductase (PFOR) (Equation 2.3). Molecular hydrogen is also produced by the oxidation of the reduced ferrodoxin (Fd) by the enzyme hydrogenase (Equation 2.4). The acetyl-CoA generated from pyruvate can then be converted into acetyl-phosphate, resulting in the formation of ATP and acetate (equation 2.5) (Nath and Das, 2004).

$$\text{Pyruvate} + \text{CoA} \xleftrightarrow[\text{lyase}]{\text{Pyruvate:formate}} \text{Acetyl-CoA} + \text{formate} \quad (2.1)$$

$$\text{Formate} + H^+ \leftrightarrow H_2 + CO_2 \quad (2.2)$$

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \xleftrightarrow[\text{oxidoreductase}]{\text{Pyruvate:ferrodoxin}} \text{Acetyl-CoA} + 2\text{Fd(red)} + CO_2 \quad (2.3)$$

$$2\text{Fd(red)} \xleftrightarrow{\text{Hydrogenase}} 2\text{Fd(ox)} + H_2 \quad (2.4)$$

$$\text{Acetyl-phosphate} + \text{ADP} \leftrightarrow \text{Acetate} + \text{ATP} \quad (2.5)$$

In the dark fermentation route, hydrogen is also generated through NADH oxidation (nicotinamide adenine dinucleotide, reduced form). Residual NADH from the metabolic reactions is reoxidized, forming molecular hydrogen (Equation 2.6) (Tanisho et al., 1998). Therefore, an increase in residual NADH could improve the hydrogen yield.

$$NADH + H^+ \leftrightarrow H_2 + NAD^+ \quad (2.6)$$

Not all pathways from pyruvate lead to hydrogen production (Table 2.1). The pathways are dependant upon many different environmental and engineering design conditions so an optimization of the correct pathway conditions is necessary to maximize the hydrogen yield.

Table 2.1: Products of acidification of glucose

Product	**Reaction**	**Eq.**
Acetic acid	$C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 4H_2 + 2CO_2$	(2.7)
Butyric acid	$C_6H_{12}O_6 \leftrightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$	(2.8)
Propionic acid	$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$	(2.9)
Lactic acid	$C_6H_{12}O_6 \leftrightarrow 2CH_3CHOHCOOH$	(2.10)
Propionic and Acetic acid	$3C_6H_{12}O_6 \leftrightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2H_2O + 2CO_2$	(2.11)

2.3 Thermodynamics

Microbial thermodynamics is the study of the relationship between metabolic reactions and the heat and other forms of energy that drive them to completion. The different thermodynamic variables include temperature, pressure, volume, concentration of reactants and products and free energy (ΔG). Free energy is the amount of energy available to do work in a biological system. Cell synthesis, the building up of molecules is a reaction that consumes energy and has a positive ΔG value, inhibiting its completion. Thus, the breakdown of carbohydrates which releases energy and has a negative ΔG

value is coupled with cell synthesis in order to satisfy the energy requirements. The energy produced by the breakdown of the carbohydrates can be used in cell synthesis. Reactions in microbial systems are usually coupled in order to accomplish a task.
Free energy is defined as Gibbs free energy and is represented by the following equation:

$$\Delta G = -n*F*\Delta E \quad (2.12)$$

ΔG = change in Gibbs free energy (J)
n = number of electrons transferred (mol)
F = number of Coulombs / Faraday (96485 C/mol)
ΔE = potential difference (V)

If ΔG is negative the reaction releases energy and proceeds spontaneously. If ΔG is positive the reaction requires energy and does not proceed spontaneously. Another factor important in microbial thermodynamics is the rate of the reaction. The rate of reaction depends on a few factors including temperature, concentration and the most important being activation energy (E_a). The activation energy is an energy barrier which controls the forward reaction rate. If the activation energy of a reaction is high, the rate of the reaction will be slow where if the activation energy is low the rate of reaction is increased. A technique to increase the rate of reaction is to lower the activation energy. Compounds that lower activation energy of a reaction are called catalysts. Catalysts are used in a reaction but are not consumed by it. The free energy of the reaction remains unchanged as well. Enzymes are proteins that act as catalysts to increase the rate of a reaction. They are present in all living microorganisms and are essential components in all living systems. Figure 2.3 illustrates the activation energy and free energy of a reaction with the effect of enzyme activity on the reaction. (Mara and Horan, 2003)

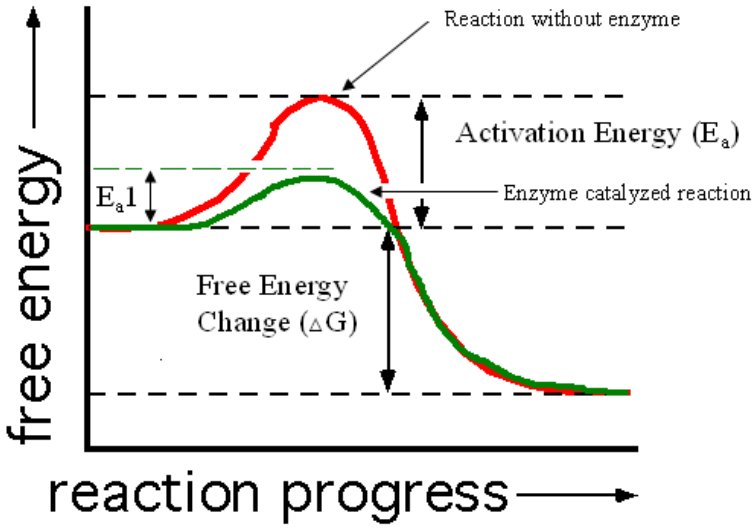


Figure 2.3 Effect of enzyme activity on activation energy of a reaction

Microbial reactions are divided into two types; oxidation and reduction reactions. These reactions describe the transfer of electrons from one substance to another. The substance oxidized is called the electron donor and the substance reduced is called the electron acceptor. Examples of electron donors are organic molecules such as glucose and xylose. Examples of electron acceptors are oxygen (free oxygen), nitrates, sulphates, carbonates (combined oxygen). Examples of various reduction and oxidation reactions are summarized in Table 2.2.

In microbial systems energy is used to build and repair biomass, since many of these reactions are thermodynamically unfavorable (Table 2.2), they require energy to proceed. This energy is harnessed from the breakdown of organic materials. In aerobic microorganisms which use oxygen as an electron acceptor, of all the available energy that is absorbed as substrates, 60% can be used to produce new biomass, with 40% wasted as heat and products (CO_2 and H_2O) (Mara and Horan, 2003). For anaerobic microorganisms which use other organic matter and CO_2 as an electron acceptor, of the available energy coming in as substrates only 10% is used to produce biomass with 90% released as waste heat and products (organic material, CH_4, CO_2) (Mara and Horan, 2003).

Table 2.2 Biological half-reactions and free energy associated with the reaction (Yang and Okos, 2007)

Half-Reaction	ΔG (aq) (kJ/eq)	Equation
Electron Donor (Oxidation)		
Carbohydrates $1/4CH_2O + 1/4H_2O \rightarrow 1/4CO_2 + H^+ + e^-$	-41.8	(2.13)
Fats and Oils $1/46C_8H_{16}O + 15/46H_2O \rightarrow 4/23CO_2 + H^+ + e^-$	-27.6	(2.14)
Protien $1/66C_{16}H_{24}O_5N_4 + 27/66H_2O \rightarrow 8/33CO_2 + 2/33NH_4^+ + 31/33H^+ + e^-$	-32.2	(2.15)
Acetate $1/8CH_3COO^- + 3/8H_2O \rightarrow 1/8CO_2 + 1/8HCO_3^- + H^+ + e^-$	-27.6	(2.16)
Ethanol $1/12CH_3CH_2OH + 1/4H_2O \rightarrow 1/6CO_2 + H^+ + e^-$	-31.8	(2.17)
Electron Acceptor (Reduction)		
Oxygen $1/4O_2 + H^+ + e^- \rightarrow 1/2H_2O$	-78.2	(2.18)
Nitrate $1/5\ NO_3^- + H^+ + e^- \rightarrow 1/10\ N_2 + 3/5H_2O$	-71.6	(2.19)
Carbon Dioxide $1/8CO_2 + H^+ + e^- \rightarrow 1/8CH_4 + 1/4H_2O$	+24.3	(2.20)
Sulphate $1/8SO_4^{2-} + 19/16H^+ + e^- \rightarrow 1/16H_2S + 1/16HS^- + 1/2H_2O$	+21.3	(2.21)
Cell Mass $1/20NH_4^+ + 1/20HCO_3^- + 1/5CO_2 + H^+ + e^- \rightarrow 1/20C_5H_7O_2N + 9/20H_2O$	+31.4	(2.22)

2.4 Xylose Production from Lignocellulose

Xylose is a 5-carbon sugar found in the hemi-cellulose composition of lignocellulosic biomass. Lignocellulosic biomass is the non-starch fibrous part of the plant its composition makes up the structure of the plant. Biomass is abundant and is an ideal feedstock source for alternative fuel production due to the fact it is present in many renewable agricultural resources (Huang and Logan, 2008; Wu et al., 2008). Lignocellulosic biomass contains a mixture of cellulose, hemi-cellulose and lignin. Cellulose is the major component of biomass comprising 30-50% of the total dry matter. Hemi-cellulose makes up approximately 20-40% of the total dry matter of biomass depending on the feedstock type. The main component of hemi-cellulose is the 5-carbon

sugar xylose. Xylose when compared to the total dry mass of the feedstock can make up approximately 19-23% depending on the type of feedstock used. Hemi-cellulose is an ideal feedstock due to the ease of breakdown compared to cellulose (Lee et al., 2007). When a sugar polymer is broken down the process is called hydrolysis. Hydrolysis takes place when a water molecule is introduced into an ether, ester or amide bond and cleaves the molecule in two monomers. Hemi-cellulose is more readily hydrolyzed compared to cellulose because of its branched and amorphous nature (Lee et al., 2007). The breakdown of hemi-cellulose is carried out by acid hydrolysis and steam explosion in order to first separate the hemi-cellulose from the plant material and then break it down into the sugar xylose (Verenium, 2008). The cellulose component is not as easily broken down due to the β–1,4 glycosidic bonds between the glucose sugars. Specific enzymes are needed to attack these bonds and hydrolyze them to separate and extract the sugars.

There are five advantages to using cellulosic biomass as the raw material for biofuels: use of non-food crops, relatively low feedstock cost, use of marginal lands for feedstock growth, beneficial net energy balance, and less fertilizer and water usage (Verenium, 2008). The United States Department of Energy (USDOE) and United States Department of Agriculture (USDA) have estimated that more than 1.3 billion dry tons of biomass (368 million dry tons of biomass from forestlands and 998 million dry tons from agricultural lands) can be produced per year in the U.S. (Perlack et al., 2005).

2.4.1 Xylose versus Glucose Degradation

Xylose is a five carbon aldose sugar that is present in woody and non-woody biomass with a chemical formula of $C_5H_{10}O_5$. The xylose degradation pathway is denoted as the "Xylose Reductase-Xylitol Dehydrogenase" or XR-XDH pathway. In the first step D-xylose is reduced into xylitol by the cofactors NADH or NADPH. Xylitol is further oxidized into D-xylulose, exclusively with the NAD^+ cofactor. As there are a variety of cofactors needed in this pathway, which may not always be available for usage, an overproduction of xylitol may result. In the following step the D-xylulose is converted into D-xylulose-5-P where it then enters the pentose phosphate pathway (Figure 2.4). (Kruger and von Schaewen, 2003)

In comparison the breakdown of glucose is much less complex. In the first step a phosphate molecule is added to glucose converting it into glucose-6-phosphate and then it is further converted into fructose-6-phosphate through an isomerization reaction. A second phosphate molecule is added to form fructose-1,6-bisphosphate. Next glyceraldehyde-3-phosphate is produced and ultimately converted into pyruvate.

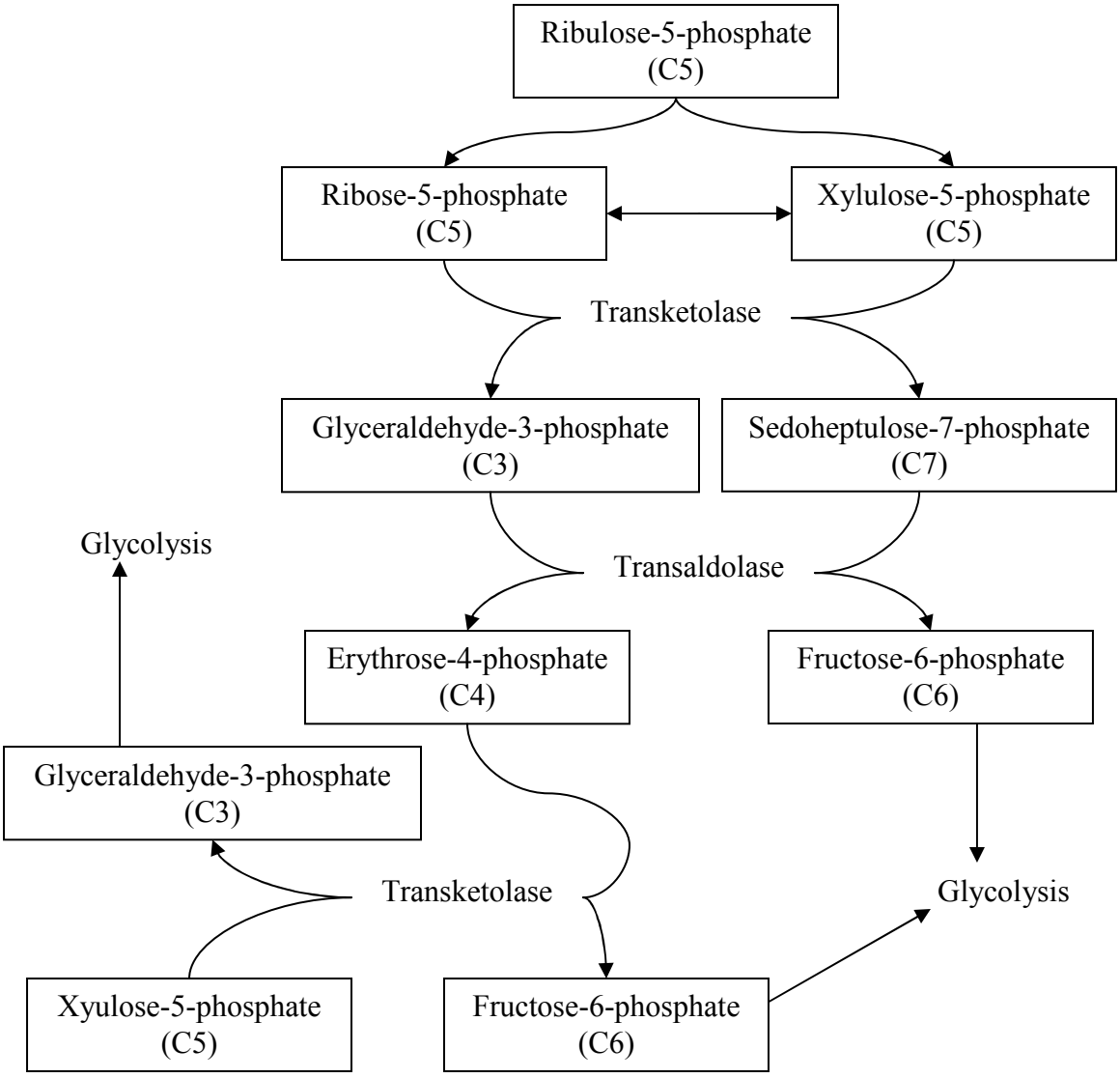


Figure 2.4 Breakdown pathway of the xylose-5-phosphate formed from the XR-XDH breakdown pathway (adapted from Kruger and von Schaewen, 2003)

The two most common hydrogen producing routes for glucose and xylose are the acetate and butyrate pathways. Note reactions 2.23 through 2.26 which show that the yields of hydrogen gas are different per mol of each sugar. The glucose degradation reactions produce 4.0 and 2.0 moles of hydrogen per mol glucose according to the acetate

and butyrate pathway, respectively. In case of xylose, the hydrogen yields are 3.33 and 1.67 mol of hydrogen per mol xylose for the acetate and butyrate pathways, respectively.

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_3(CH_2)_2COO^- + 2\ HCO_3^- + 2\ H_2 + 3\ H^+ \quad (2.23)$$

$$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + 4\ H_2 + 4\ H^+ \quad (2.24)$$

$$C_5H_{10}O_5 + 1.67H_2O \rightarrow 0.83CH_3(CH_2)_2COO^- + 1.67HCO_3^- + 1.67H_2 + 2.5H^+ \quad (2.24)$$

$$C_5H_{10}O_5 + 3.33H_2O \rightarrow 1.67\ CH_3COO^- + 1.67HCO_3^- + 3.33\ H_2 + 3.33H^+ \quad (2.26)$$

Since both the xylose and glucose pathways converge at the final stages of glycolysis, the same end products are formed. The difference between the sugar substrates is one fewer oxygen atom, one fewer carbon atom and 2 fewer hydrogen atoms per molecule of xylose when compared to glucose therefore the different yields of hydrogen are produced.

2.5 Factors Affecting Hydrogen Production

Hydrogen yields are affected by factors which include substrate type, environmental conditions, and microbial and engineering design factors. Substrates rich in electrons and that are easily degradable will generate greater hydrogen yields compared to those which are poor electron donors. Temperature and pH are factors affecting enzymatic activities which in turn control the hydrogen yield. Under elevated temperature conditions the hydrogen yield is greater than that obtained from low temperatures. For example, hydrogen yields for cultures maintained at 50^{o}C are greater than those obtained for cultures maintained at 20^{o}C (Yu et al., 2002). The operating pH also affects the hydrogen yield. Under low pH conditions, the production of reduced products is more favorable such as butyrate than the more oxidized products such as acetate.

Figure 2.5 shows different pathways for various acidogenic reactions. From the reactions expressed in Equation 2.23 through 2.26 the optimal theoretical yield of hydrogen is 4.0 mol/mol glucose or 3.33 mol/mol xylose. To obtain the maximum amount of hydrogen production the pathway must lead to the production of highly oxidized by-products. The hydrogen yield if acetate is the only oxidized carbon by-product is twice the amount produced if butyrate is the only by-product. For each of

these reactions if propionate, lactate, and ethanol are the only byproducts the hydrogen yield is zero. Under acetogenesis, butyrate, propionate, lactate and ethanol is further degraded into hydrogen and acetate (Table 2.3).

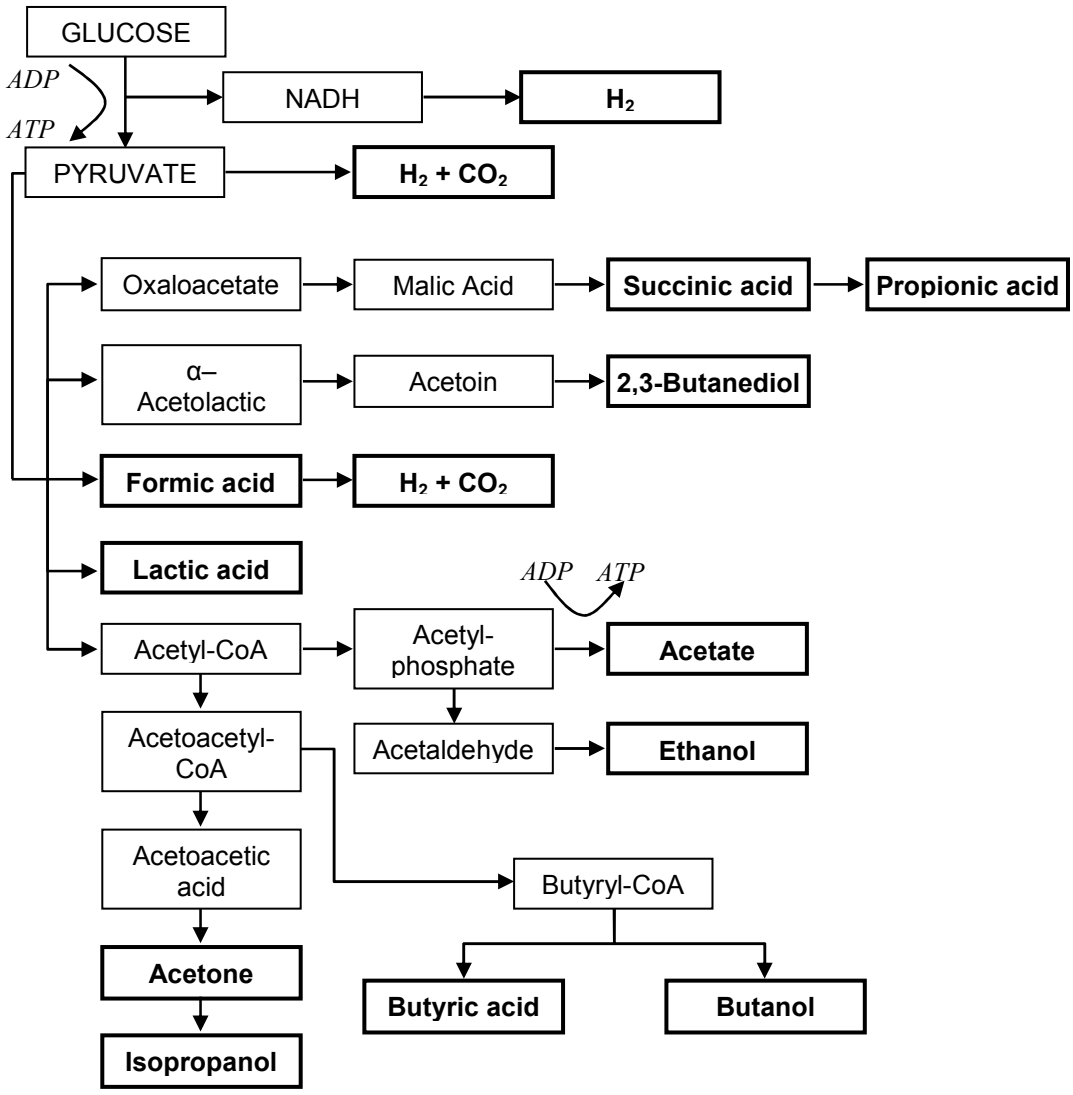


Figure 2.5 Pathways to the end products of the fermentation of sugars. (End products indicated by bold text) (Adapted from Gaudy & Gaudy, 1980)

Table 2.3 Acetogenic reactions

Substrate	Reaction	ΔG (kJ/mol)	Equation
Lactic acid	$CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H_2$	+21.4	(2.26)
Butyric acid	$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$	+43.6	(2.27)
Propionic acid	$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2$	+73.6	(2.28)
Ethanol	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	+1.9	(2.29)

The reactions shown in Table 2.3 are all thermodynamically unfavorable under standard conditions. They do however proceed only under very low hydrogen partial pressures. The reactions are dependant on pH, temperature, nutrient availability and most importantly hydrogen partial pressure. The reactions of butyric and propionic acid only occur if the hydrogen partial pressure is below 32.04 Pa and ethanol and lactate at about 65 Pa (Fennel et al., 1997). The effect of hydrogen partial pressure is shown in Figure 2.6. As hydrogen partial pressure rises, hydrogen production becomes unfavorable and hydrogen consumption becomes more favorable. Figure 2.6 illustrates that at a partial pressure of approximately 10^{-4}, the hydrogen producing reaction has a positive Gibbs free energy value. The optimal operating pressure for the two reactions is where the two curves meet at approximately 10^{-5}.

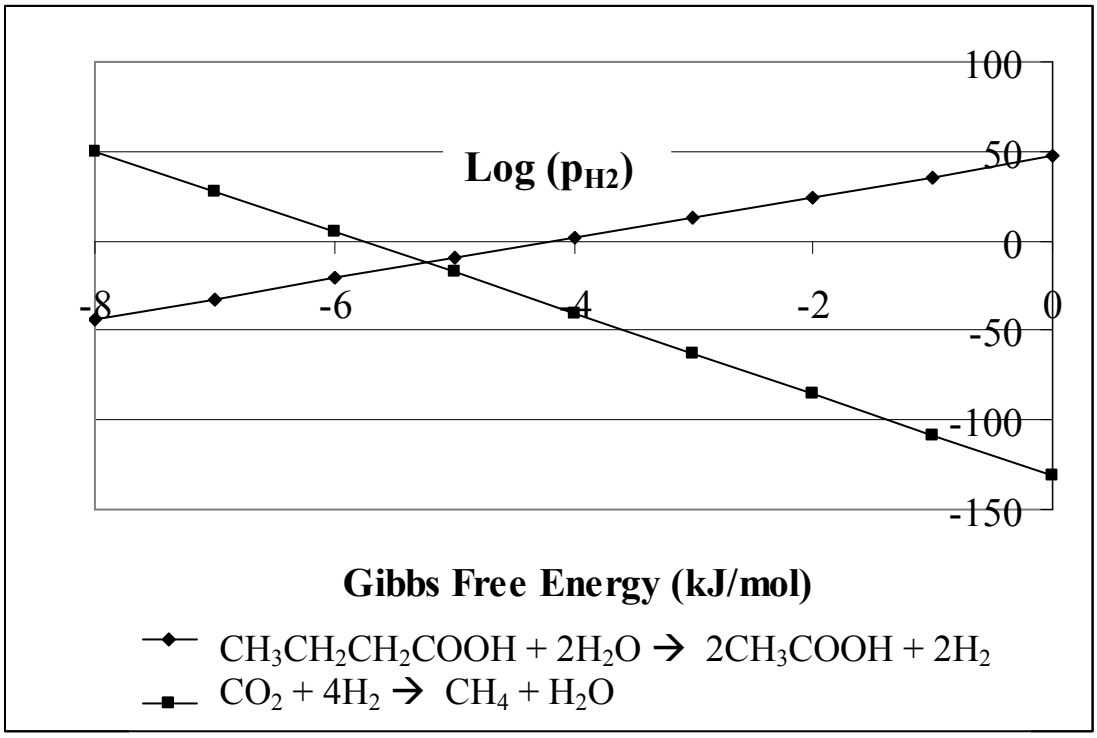


Figure 2.6 ΔG vs. hydrogen partial pressure (P_{H2}) plot of a hydrogen producing reaction and a hydrogen consuming reaction

2.5.1 Nutrients

Bacteria require an electron donor, a carbon source and nutrients. Nutrients such as nitrogen which is used for DNA and protein synthesis and phosphorus is another important nutrient which is used for energy storage and in DNA synthesis. Certain heavy metals at low levels are also important for the metabolism in bacteria cells. Metals such as magnesium, sodium, zinc, and iron are important cofactors for enzyme function (Lin and Lay, 2005). Other metals though have been shown to have toxic effects. Zinc, sodium, iron, and magnesium are toxic if added in very high concentrations (Li and Fang, 2007). Some metals have inhibitory effects on enzymes and can destroy protein pumps in the cell membrane which can prevent the bacteria from getting food sources. These metals include cadmium, chromium, copper, nickel, and lead (Fang et al., 2004). An important component of operating a successful biological reactor is to provide the proper amount of necessary nutrients. Too much macro and micronutrients can have a negative effect on the biological processes. That is why the reactors must be properly maintained

so that the bacteria have ideal operating conditions in order to produce the maximum amount of hydrogen gas.

Both macro and micro nutrient levels beyond toxic levels are a problem in wastewater effluents as the effect they impose on microbial communities is detrimental. In some cases, a macronutrient such as phosphorus can cause overgrowth of the culture. This leads to overgrowth of the bacterial culture making the reactor solution too thick leading to inefficient operation of the reactor.

2.5.2 pH

The pH is another important factor affecting the production of hydrogen. It has effects on the hydrolytic and catabolic bacterial pathways (Lay, 2001). Low pH is inhibitory to methanogenic activity in anaerobic biological processes (Li and Fang, 2007). Also, the activity of hydrogenase, an iron-containing enzyme, is inhibited by low pH (Dabrock et al., 2002). Therefore, controlling the pH is necessary due its effects on hydrogenase enzymes and enzymes mediating many metabolic pathways (Chen et al., 2002). The pH is a major factor affecting the control of methanogenic pathways in which hydrogen is consumed (Lay et al., 2001). The pH can also have a large effect on the amount of hydrogen produced by a culture. In dark fermentation process, there are many pathways which sugar degradation can proceed and these pathways can impact the yield of hydrogen. Many reports suggest many optimal pH values, due to the fact that many experiments were conducted in batch reactors without pH buffer or control system. In these cases, only the initial pH was reported and since the main product of sugar degradation was acids, the pH decrease observed is shown to affect the pathway. Many of the optimal pH values reported was in the range of 5.2 to 5.6 (Li and Fang, 2007). Another reason that so many optimal pH values exist is that these studies used mixed cultures instead of pure cultures. It is difficult to produce the exact same mixed culture because there are a variety of microorganisms in a given culture. Even undisturbed cultures will show different results over a period of time (Mara and Horan, 2003). At pH values lower than 4.5, solvent production is activated which produces no hydrogen gas, and pH values above 7.0, propionate production predominates which is a reaction which consumes hydrogen gas (Li and Fang, 2007). Evidence by Kim et al. (2004) has shown

butyrate predominates at pH 5.5; however, butanol production dominates at a pH value of 4.3. Hwang et al. (2004) reported ethanol as the main product at a pH value of 4.5. Table 2.4 shows the effect of different pH values on hydrogen yield from glucose fermentation suggesting an optimal lower pH of approximately 5.0.

Table 2.4: Typical hydrogen yields for mixed anaerobic microbial populations

Reactor Type	Culture treatment	Chemical Inhibitor	pH	Temp. (^{o}C)	H_2 yield (mol/mol glucose)	Source
Batch	No heat treatment	LA	7.6-8.0	37	1.7	Chowdhury *et al.* (2007)
Batch	No heat treatment	LA	5.0	37	2.4	Ray *et al.* (2008)
Continuous	No heat treatment	None	5.5	33	0.97	Mu *et al*. (2006)
Continuous	Heat treatment	None	5.5	41	1.67	Mu *et al*. (2006)
Continuous	Heat treatment	None	5.5	36	1.91	Fang *et al*. (2004)
Batch	Heat treatment	None	6.0	26	0.92	Logan *et al.* (2002)

During xylose fermentation to hydrogen, studies by Lin et al. (2006) showed an optimal pH for hydrogen production of 6.5 using a mixed anaerobic culture at 35°C in a batch reactor. Results from work reported by Wu et al. (2008) have shown an optimal pH of 6.5 for a mixed anaerobic culture in a CSTR operation at 50°C. This illustrates the difference in operating conditions when comparing glucose and xylose fermentation. The optimal pH of the cultures varies in the breakdown of the different sugars.

Fang and Liu (2002) investigated the degradation of glucose to acid products at different initial pH values and they showed that butyrate was the predominant product at a pH value of 5.0 followed by acetate at a pH value of 6.5 and propionate at a pH value of 7.0 and greater. As shown earlier in Table 2.1, the production pathway affects the amount of hydrogen that is produced where the two acid producing pathways that yield hydrogen are acetic acid and butyric acid.

The effect of pH on *Clostridium acetobutylicum,* a hydrogen producing bacteria was investigated by Gottwald and Gottschalk (1985). They studied the internal and

external pH of the cell on the switch from acid to solvent production. Their findings included the difference in external pH is close to the internal pH which suggests the bacteria maintain a small ΔpH. Further results of the study showed that the change from acid to solvent production occurs when the internal pH value of the cell falls below 5.7 corresponding to an external pH of approximately 4.5. These researchers also showed that if the internal pH value fell below 5.3 cell death began and acid or solvent production was not observed. The internal and external pH values did not have a large difference due to the undissociated acids being able to freely cross the cell membrane.

2.5.3 Temperature

The effect of temperature has a significant impact on the production of hydrogen in a biological reactor. Research has shown that in mesophilic and thermophilic reactor systems the rate of hydrogen production is higher than in psychropilic systems. For every increase of $10^{o}C$, the enzymatic activity within a range of $20^{o}C$ to $60^{o}C$ is expected to double (Li and Fang, 2007). There is a tradeoff in an increase in temperature and the increased rate of hydrogen production due to the many engineering and biological factors. At temperatures greater than approximately 35°C, energy input is required and this will add to the operating costs. The biological reason that temperature is a factor is that when a bacteria's cell membrane is warmer it behaves more fluid-like. The phospholipids become more fluid and flexible hence, there is easier diffusion of molecules by passive and active transport processes allowing more compounds to pass through (Cirne et al., 2007). Also, the rate of reaction is directly proportional to the temperature so a higher temperature means a faster rate thus a higher rate of hydrogen gas production. Zoetemeyer et al. (1982a) used glucose with an activated sludge inoculum to assess the hydrogen production capacity under thermophilic and mesophilic temperatures. The study reported an optimum temperature in the thermophilic range at 52°C but noted an optimum temperature of 37°C. Thermophilic temperatures should reduce dissolved hydrogen concentration (Hawkes et al., 2002); however, mesophilic conditions offer a more optimal and more stable product distribution and do not require additional energy input (Zoetemeyer et al., 1982b). Lin and Chang (2004) investigated the effect of temperature in the range of 15 -34°C and found that the optimal temperature

was somewhere between 30 - 34°C. Lin et al., (2008) report an optimal temperature for xylose fermentation to hydrogen of 40°C when using a batch reactor with a mixed anaerobic culture at a pH of 6.5.

Many studies have reported a range of hydrogen yield with temperature for a variety of cultures. The data indicates that warmer temperatures are best suited for increasing hydrogen yield (Table 2.5). The variety of results could be due to the fact that the experiments were conducted with mixed cultures instead of pure cultures making it difficult to recreate the exact same culture used in the studies and therefore difficult to replicate the results.

Table 2.5 Effects of temperature on hydrogen gas yield for mixed anaerobic cultures

Reactor Type	pH	Temperature (^{o}C)	H_2 yield (ml H_2/g hexose)	Source
CSTR	5.5	36	260	Fang et al. (2002)
Batch	5.5	50	285	Morimoto et al. (2005)
CSTR	5.5	23	140	Zheng et al. (2005)
CSTR	5.2	32	259	Hussy et al. (2005)
PBR	5.5	55	291	Yu et al. (2002)
CSTR	5.5	26	266	Fang et al. (2002)

2.5.4 Hydrogen Partial Pressure

Hydrogen itself can become inhibitory to its own production as it has been shown that a hydrogen partial pressure of 2.0 kPa prevented growth and butyrate consumption as compared to pressures as low as 0.1 kPa (Ahring and Westermann, 1988). The breakdown of VFAs is thermodynamically unfavorable (Table 2.3) and for these reactions to proceed, the hydrogen partial pressure must remain relatively low. The reason that the hydrogen partial pressure must remain low is that these are equilibrium reactions and thus, if there is a low concentration of products and a high concentration of reactants the system will shift towards the products side of the reaction. As shown in Table 2.6, the hydrogen producing reactions have positive free energy values suggesting the reactions require energy to proceed and the equilibrium favors the reactants side of

Table 2.6: Gibb's free energy changes under standard conditions for typical hydrogen-releasing and hydrogen-consuming reactions (Schink, 1997)

Reaction	ΔG (kJ / mol)	Equation #
Hydrogen-releasing reactions		
$CH_3CH_2CH_2COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + H^+ + 2H_2$	+43.6	(2.30)
$CH_3CH_2COO^- + 2H_2O \leftrightarrow CH_3COO^- + CO_2 + 3H_2$	+73.6	(2.31)
$CH_3COO^- + H^+ + 2H_2O \leftrightarrow 2CO_2 + 4H_2$	+94.9	(2.32)
$CH_3CH(CH_3)CH_2COO^- + CO_2 + 2H_2O \leftrightarrow 3CH_3COO^- + 2H^+ + H_2$	+25.2	(2.33)
$CH_3CH_2OH + H_2O \leftrightarrow CH_3COO^- + H^+ + 2H_2$	+1.9	(2.34)
Hydrogen-consuming reactions		
$4H_2 + 2CO_2 \leftrightarrow CH_3COO^- + H^+ + 2H_2O$	-94.9	(2.35)
$4H_2 + CO_2 \leftrightarrow CH_4 + 2H_2O$	-131.0	(2.36)
$H_2 + HCO_3^- \leftrightarrow HCOO^- + H_2O$	-1.3	(2.37)
$H_2 + S \leftrightarrow H_2S$	-33.9	(2.38)
$4H_2 + SO_4^{2-} + H^+ \leftrightarrow HS^- + 4H_2O$	-151.0	(2.39)
$H_2C(NH_3^+)COO^- + H_2 \leftrightarrow CH_3COO^- + NH_4^+$	-78.0	(2.40)
Fumarate + $H_2 \leftrightarrow$ succinate	-86.0	(2.41)

the equation. Lowering the partial pressure of hydrogen will favor the production of hydrogen thereby shifting the equilibrium to favor the product side of the reaction.

The elevated hydrogen partial pressure is a problem that has to be dealt with in order to reduce the levels of hydrogen in solution. Hence, researchers have developed methods to maximize the yield of hydrogen. One approach is to release the dissolved hydrogen by sparging the culture with an inert. The second is to use a specialized membrane which is permeable only to hydrogen. Due to the relatively small size of the hydrogen molecule, it can pass through a small pore size where other products or reactants are retained in the gas phase (Li and Fang, 2007). A stirred tank reactor is the simplest and most widely used configuration to enhance the release of hydrogen from solution into the headspace.

2.5.5 Substrate Source and Concentration

Carbohydrates, such as sugars, cellulose and starch are commonly used as electron donors for hydrogen-producing fermentations as they are relatively inexpensive and readily available. An optimization of the substrate or food to biomass concentration or microorganism (F/M) ratio is needed to ensure proper operational efficiency of the process (Lay, 2001). Research by Van Ginkel et al. (2001) suggests that higher substrate concentrations can have an inhibitory effect due to the increased acid production which leads to lower pH values and increased hydrogen partial pressures. Lay's (2001) batch experiments demonstrated that the hydrogen-producing activity of sludge at 37°C and a pH value of 5.0 was significantly inhibited when the initial substrate levels (microcrystalline cellulose) exceeded 25.0 g/L, with a maximum hydrogen yield occurring at a cellulose to initial sludge concentration ratio of 8 g cellulose/g VSS. The type of substrate used for hydrogen gas production is important as Li and Fang (2007) reported values of different substrates used including glucose, sucrose, molasses and cellulose at 37°C at pH 5.5. Li and Fang, (2007) reported that glucose (286 ml H_2/g hexose) and sucrose (266 ml H_2/g hexose) had the highest yields with molasses (109 ml H_2/g hexose) and cellulose (50 ml H_2/g hexose) falling far behind the other three. The ability of the culture to utilize a specific substrate was also found to be very important, such that if a population of microorganisms cannot degrade the substrate then very little hydrogen will be produced.

2.6 Microbial Inhibition

During the fermentation of simple electron rich electron donors, hydrogen is produced at several intermediate steps. Hydrogen is produced by acidogens and acetogens; however, it is readily used by the hydrogenotrophic methanogens in the final step of fermentation producing methane. To recover the hydrogen, mechanisms have to be developed in order to inhibit the hydrogenotrophic methanogenic population. These are based upon the spore forming characteristics of the hydrogen producing bacteria. Hydrogenotrophic methanogens which are non-spore forming organisms are more sensitive to environmental conditions than acidogens and acetogens (Oh et al., 2003; Lay, 2001). Several methods which have been reported to inhibit hydrogenotrophic

methanogenic populations include heat treatment, chemical addition, aeration and electric treatment. (Li and Fang, 2007)

2.6.1 Heat Treatment

During heat treatment of mixed anaerobic communities viable hydrogen consuming bacteria are destroyed at high temperatures and subsequently, the spore forming hydrogen producing bacteria survive (Oh et al., 2003; Sung et al., 2002; Van Ginkel et al., 2001; Lay et al., 1999). In spore forming bacteria the original cell replicates its genetic material, which is surrounded by a tough coating. The outer cell then is destroyed releasing the spore which is now well protected against a variety of environmental conditions, including temperature extremes, radiation, and an absence of nutrients. There was no reported optimal value for heat treatment in terms of temperature or duration of heating. However, the most common treatment for an anaerobic mixed community inoculum is at 100°C for 15 min (Li and Fang, 2007). According to some studies, heat treatment promotes germination of spores which increases the levels of active hydrogen producers and subsequently, the hydrogen yield (Sung et al., 2002). Several studies have concluded that heat treatment is not 100% effective at inhibiting all of the hydrogen consuming organisms. Oh et al. (2003) found that some homoacetogenic bacteria may survive heat treatment and eventually use hydrogen for the production of acetate.

2.6.2 Chemical Addition and Aeration

Adjusting the pH is a use of chemical inhibition for controlling the hydrogen consuming population. Under low pH conditions, the methane production rate decreases after the pH value decreases to 6.3 or less and increases to values above 7.8 (Fang and Li, 2007). Methanogens are strictly anaerobic and very oxygen sensitive. Methanogenic activity is severely impaired by the introduction of oxygen gas. Ueno et al (1996) reported that in compost sludge after aeration produced 330–340 ml H_2/g hexose without producing methane. Other chemicals that are used to inhibit hydrogenotrophic methanogens include bromomethanesulfonate (BES), acetylene, and chloroform (DiMarco et al., 1990).

2.6.3 Electric Current

Roychowdhury (2000) found that hydrogen-producing bacteria could be separated from methane gas producing bacteria in a sludge sample by electric current. Using an application of low-voltage (3.0–4.5 V) electric current, cellulose containing landfill sludge and municipal sewage sludge were shown to accumulate hydrogen without the accumulation of methane.

2.6.4 Product Inhibition

At low pH levels, a metabolic switch causes a shift from acid production to solvent production. During fermentative hydrogen production, organic acids are produced which lower the pH in solution or the external pH of the cell. If the pH value falls below the pKa of the acids the acids remain undissociated. These undissociated organic acids are non-polar molecules and can freely move across the cell membrane. After the threshold level of undissociated acids is reached the solvent production route becomes dominant. The reason for this is that the undissociated acids move across the cell membrane and dissociate inside the cell which causes the cell to use energy to regulate its internal pH. In order to reduce this effect the cell ceases to produce acids and begins producing solvents which do not reduce the pH of the solution. (Gottwald and Gottschalk, 1985)

2.6.5 Long Chain Fatty Acids Inhibition

LCFA's are compounds with hydrophilic and hydrophobic groups. They are attached to a glycerol back bone by ester bonds to form glycerides. The glycerides can exist in the mono- di- and tri- forms. LCFAs are linear carbon compounds classified into saturated and unsaturated fatty acids. Saturated fatty acids are carbon chains with only single bonds between the carbons whereas unsaturated fatty acids are carbon chains with one or more double bonds between the carbons. The double bond causes branching of the fatty acid which makes it more of a liquid at room temperature whereas saturated fatty acids tend to be solid at room temperature.

LCFAs act by several mechanisms to inhibit a variety of microorganisms. They cause the death of bacteria by surrounding the cell in a lipid layer (Alves et al., 2001). This lipid layer causes the bacteria to float and is washed out in the effluent. LCFAs also impose mass transfer limitations across the cell membrane (Cirne et al., 2007). Without transfer across the cell membrane the cell cannot properly maintain its metabolic machinery and death ensues. A mechanism to prevent LCFA accumulation is to lower the concentration of LCFA's in the system so they cannot accumulate around the cell. This is achieved by the addition of calcium ions, which binds to the LCFA creating a precipitate. This prevents the LCFA from surrounding the cell and allows the cell to use the LCFA as needed for energy.

2.6.6 LCFA Degradation

LCFAs are degraded by a process called β-oxidation (Figure 2.7). Essentially the β carbon or second carbon from the carboxylic acid group is oxidized to a carboxylic acid group and an acetyl group is liberated with every turn of the cycle. The process involves dehydrogenation, hydration, followed by another dehydrogenation ending with thiolytic cleavage. Fatty acids are degraded by fatty acid-β-oxidizing organisms which fall into the group known as acetogenic and methanogenic organisms (Cirne et al., 2007). LCFA degradation by β-oxidation involves the following steps (Kioka and Kunau, 1985), (Bloor, 1943):

• LCFA molecules are oxidized by abstraction of two hydrogen atoms from the α and β carbons using the enzyme fatty acyl-CoA dehydrogenase.

• Hydrogen atoms are donated to FAD (flavin adenine dinucleotide).

• The resulting LCFA acyl-CoA complex is then transformed into a trans-unsaturated isomer.

• Water is then added across the trans double bond by the enzyme 2,3-enoyl-CoA hydratase forming a γ-alcohol LCFA acyl-CoA complex.

• The LCFA molecule becomes further oxidized by 3-hydroxyacyl- CoA dehydrogenase when nicotine adenine dinucleotide (NAD^+) is reduced to NADH.

• Acetate is released and 3-oxoacyl-CoA thiolase again activates the shortened LCFA molecule using ATP (adenine triphosphate).

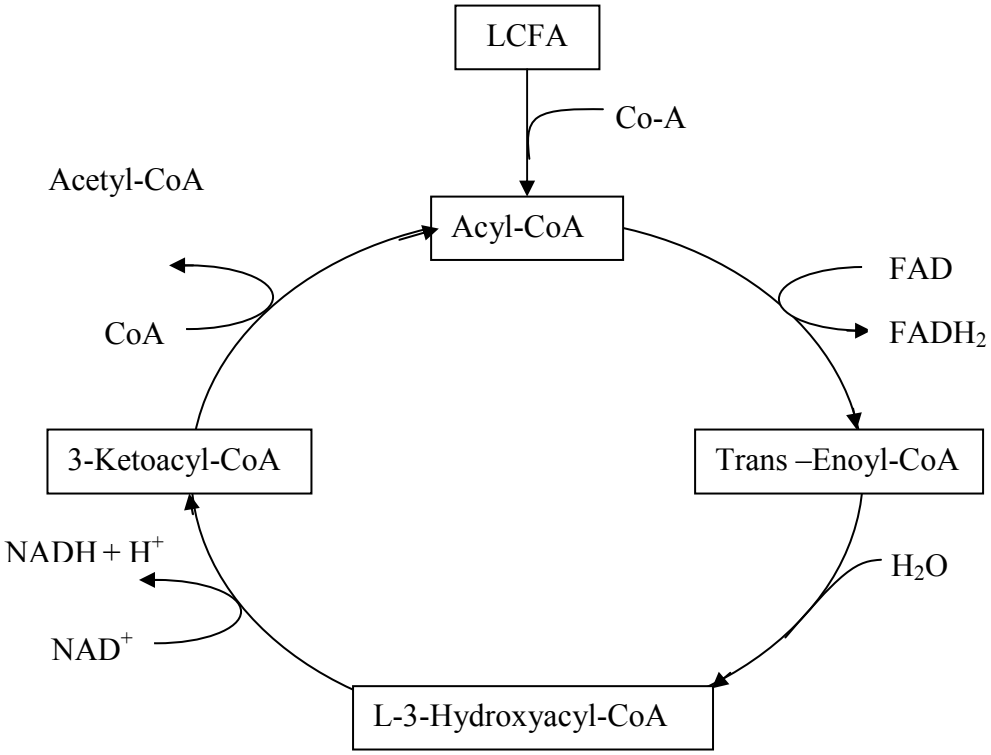


Figure 2.7 LCFA β-oxidation pathway (Kioka and Kunau, 1985)

The equation for LCFA degradation is summarized in the following equation:

$$CH_3(CH_2)_nCOOH + 2H_2O \rightarrow CH_3(CH_2)_{n-2}COOH + CH_3COOH + 2H_2 \quad (2.42)$$

Chapter 3

Materials and Methods

3.1 Experimental Plan

The experiments were divided into three stages in order to execute the research objectives. In the first stage, the experimental design examined the effect of OA, LA and different pH values on hydrogen production from glucose degradation. The experiments were performed in batch studies with 160 mL serum bottles (Table 3.1). The second phase of the study was carried out to examine xylose fermentation in the presence of multiple LCFAs and at two pH values (Table 3.2). The first series of experiments were designed to answer questions related to finding an optimal pH for glucose degradation in the presence of individual LCFAs. In the second phase studies, xylose degradation at pH 7.6 and 5.0 was examined in the presence of C18 to C12 LCFAs. In the third phase of the study, experiments were performed to examine hydrogen production from a mixture of glucose and xylose at different ratios in the presence of LCFA and at an optimal pH (Table 3.3).

Table 3.1 Experimental design for glucose fermentation to hydrogen in the presence of OA and LA at different pH conditions

Glucose (mg/L)	LCFA (mg/L)	pH value
5,000	0	7.6
5,000	1,000	7.6
5,000	2,000	7.6
5,000	0	6.0
5,000	1,000	6.0
5,000	2,000	6.0
5,000	0	5.0
5,000	1,000	5.0
5,000	2,000	5.0

Table 3.2 Experimental design for xylose fermentation to hydrogen in the presence of multiple LCFAs and at pH 7.6 and 5.6

Xylose (mg/L)	LCFA	LCFA (mg/L)
5,000	N/A	0
5,000	Linoleic Acid (LA)	2,000
5,000	Oleic Acid (OA)	2,000
5,000	Stearic Acid (SA)	2,000
5,000	Palmitic Acid (PA)	2,000
5,000	Myristic Acid (MA)	2,000
5,000	Lauric Acid (LAU)	2,000

Table 3.3 Experimental design for glucose and xylose mixture fermentation to hydrogen in the presence of optimal LCFA at pH 7.6 and 5.6

Ratio of Sugars X/G	Sugar (mg/L)	LCFA (mg/L)
25/75	5,000	0
25/75	5,000	2,000
50/50	5,000	0
50/50	5,000	2,000
75/25	5,000	0
75/25	5,000	2,000

For each condition examined, the experiment was conducted in triplicate. The temperature was set at 23°C±2°C, a VSS concentration of 2,000 mg/L and the total liquid volume was 50.0 mL. All experiments were conducted in 160 mL serum bottles. Samples were removed and analyzed for gases (CH_4, CO_2 and H_2), VFAs (acetate, propionate and butyrate), sugars (glucose and xylose) and alcohols (ethanol, i-propanol, n-propanol, i-butanol and n-butanol).

3.2 Inoculum Source

The inoculum was composed of a mixture in a ratio of 70% wastewater culture to 30% ethanol plant culture. The cultures were obtained from an ethanol producing facility (Chatham, ON) and the Chatham wastewater treatment facility (Chatham, ON). The culture (denoted as Reactor A) was maintained in an 8L serum bottle at a VSS concentration of 15,000 to 20,000 mg/L. A smaller 4L (denoted as Reactor B) kept at a VSS concentration of 8,000 to 10,000 mg/L. The contents from reactor B was diluted into reactor B with a basal material. The inoculum from reactor B served as a feed for all the experiments in the 160 mL serum bottles. The culture was fed on a weekly basis with

1,250 mg/L sugar, first glucose for four weeks then switching to xylose when glucose experiments were complete. For the xylose experiments, the culture was acclimated for a period of 25 weeks. The efficiency of the degradation reaction was determined by assessing the quantity of gas produced, the quantity of sugar degraded, the VFA concentration and the pH. When all sugar the VFAs were removed within approximately 5 days, the culture was used to conduct experiments in the 160 mL serum bottles.

3.3 Basal Medium

The basal medium used to dilute samples contained the following constituents (mg/L in deionized water): $NaHCO_3$, 6000; NH_4HCO_3, 70; KCl, 25; K_2HPO_4, 14; $(NH_4)_2SO_4$, 10; yeast extract, 10; $MgCl_2 \cdot 4H_2O$, 9; $FeCl_2 \cdot 4H_2O$, 2; resazurin, 1; EDTA, 1; $MnCl_2 \cdot 4H_2O$, 0.5; $CoCl_2 \cdot 6H_2O$, 0.15; Na_2SeO_3, 0.1; $(NH_4)_6MoO_7 \cdot 4H_2O$, 0.09; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $NiCl_2 \cdot 6H_2O$, 0.05; and $CuCl_2 \cdot 2H_2O$, 0.03.. (Weigant and Lettinga, 1985).

3.4 Analytical Methods

Liquid samples were analyzed for sugars (xylose and glucose), alcohols (ethanol, proponal and butanol) and VFAs (acetic acid, propionic acid and butyric acid) while gas samples were analyzed for H_2, CH_4 and CO_2. At fixed time intervals, a 0.5 ml liquid sample were removed from the serum bottles using a 0.5 mL Gastight® syringe and transferred to a 7.5 ml culture tube containing 4.5 ml of Milli-Q® (Millipore, Nepean, ON) grade water. The diluted samples were then centrifuged at 4000 rpm for 7 minutes. The centrate was filtered through a 25 mm diameter 0.45 µm polypropylene membrane (GE Osmonics, MN). The filtrate was filtered again using a 1 ml polypropylene cartridge fitted with a 20 µm PE frit (Spe-ed Accessories, PA) and filled with Chelex® 100 to 200 mesh, sodium resin (Bio-Rad Laboratories, CA). The filtered samples were stored in 5 ml polypropylene ion chromatography vials (Dionex, Oakville, ON) at 4˚C until further analysis by an ion chromatograph (IC).

Liquid samples were analyzed for VFAs (acetate, propionate and butyrate), alcohols (ethanol, proponal and butanol) and sugars (glucose and xylose) by a Dionex IC (DX-500) equipped with an AS40 automated sampler, LC10 liquid chromatograph, GP50

multi-gradient pump, 25 µl sample loop and a CD20 conductivity detector (Dionex, Oakville, ON). For VFA analysis the IC was configured with an IonPac® 24-cm x 4-mm diameter AS11-HC analytical column, IonPac® AG11-HC guard column and an ASRS-ULTRA® (4 mm) anion self-regenerating suppressor for VFA analysis. The IC was calibrated using acetic acid (99.8%), propionic acid (99.8%), and n-butyric acid (99.9%) (Fisher Scientific, Toronto, ON). The eluents were prepared with Milli-Q® grade water (18 MΩ) (Millipore, Nepean, ON). The three eluents used were: Milli-Q® grade water (Eluent A); 100 mM sodium hydroxide (NaOH) (Eluent B); and 1 mM NaOH (Eluent C). The eluent flows (as a percent of the total flow of 1 ml/min) were as follows: 0-15 min, 80% A, 20% C; 15-15.1 mins, A ramped up from 80 to 85%, B from 0 to 15%, C went to 0%; 15.1-25 mins, A ramp down from 85 to 65%, B up from 15 to 35%. The instrument detection limit for acetate, propionate and butyrate was 1 mg/L. Each concentration of the calibration standards was prepared in triplicate. The calibration standards were prepared using pure Milli-Q® water. VFA standards followed by blank consisting of Milli-Q® water were analyzed after every 12 to 15 samples to check the instrument calibration. The alcohols and sugars were analyzed on a MA1 column (Dionex, Oakville, ON). The IC was calibrated using ethanol (99.8%), isopropanol (99.8%), and propanol (99.9%) isobutanol (99.8%), and butanol (99.9%) (Fisher Scientific, Toronto, ON). The eluents were prepared with Milli-Q® grade water (18 MΩ) (Millipore, Nepean, ON). The eluent was 480 mM sodium hydroxide (NaOH) at a flow rate of 0.4 ml/min. The total analysis time was 33 min. The instrument detection limit for ethanol, n-propanol, i-propanol, i-butanol, and n-butanol was 1 mg/L. Each concentration of the calibration standards was prepared in triplicate. Sugars were calibrated using of glucose (99.9%) (ACP, Montreal, QC.) and xylose (99.9%) (TCI, Tokyo, Japan). The instrument detection limit for glucose and xylose were 1 mg/L.

To analyze for hydrogen, methane and carbon dioxide, headspace samples (25 µL) were removed and analyzed using a Varian 3800 gas chromatograph (GC) 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, USA) column. The operational temperatures of the injector, the oven and the detector were 100°C, 200°C, and 200°C, respectively. Nitrogen (99.999%, Praxair, ON) was used as the carrier gas at a flow rate of 21 ml/min.

Gas samples were injected manually using a 50 µl Hamilton Gastight® (VWR, Canada) syringe. The retention times of hydrogen, methane and carbon dioxide were at 0.49, 1.29 and 1.51 min, respectively with detection limits of 0.25 ml gas/160 ml bottle, 0.25 ml gas/160 ml bottle and 0.25 ml gas/160 ml bottle respectively. 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). Each concentration of the calibration standards was prepared in triplicate.

The volatile suspended solids (VSS) and total suspended solids (TSS) concentration in each reactor was determined in accordance with Standard Method of Analysis (APHA, AWWA, WEF, 1992). The TSS and VSS measurements were conducted at the beginning of each experiment. The analysis was conducted using 5 ml of liquid sample and filtered using 0.45 µm pore size glass fibers (VWR, Canada). The pH of each batch reactor was measured at the beginning, end and at time intervals throughout each experiment using a VWR SR40C, Symphony pH meter (Orion, Boston, MA.).

3.5 Chemicals

The electron donors were glucose and xylose and the methanogenic inhibitors included the following LCFAs: Linoleic (LA), Oleic (OA), Stearic (SA), Palmitic (PA), Myristic (MA), and Lauric (LAU) acids. The concentration of the LCFAs used in this study ranged from 0 to 3,000 mg/L with a sugar concentration offset at 5,000 mg/L. For experiments examining the use the sugars together the ratios of the two will be 75/25, 50/50 and 25/75 xylose to glucose, respectively.

The glucose experiments were conducted using LA (18:2) (>99%) and OA (18:1) (>99%) (TCI America). Experiments run with xylose were conducted in the presence of LA (18:2) (>99%), OA (18:1) (>99%), SA (18:0) (>99%), PA (16:0) (>99%), MA (14:0) (>99%), and LAU (12:0) (>99%) (TCI America, VW) to establish the effect of the inhibition using a different sugar.

3.6 LCFA Delivery, Culture Bottle Preparation and Reaction Time

Because of low solubilities, LCFAs do not disperse well in aqueous solutions (Sikkema et al., 1995). In order to overcome this problem, the LCFAs were saponified with NaOH (Angelidaki and Ahring, 1992). The quantities of sodium hydroxide used (expressed as g of NaOH per g of LCFA) are provided in Table 3.4. Two mL of the saponified stock LCFA solution (50,000 mg/L) was added to the culture bottles to provide an initial LCFA concentration of 2,000 mg/L.

Table 3.4: Quantity of NaOH used to saponify the LCFAs

LCFA	NaOH ($g.g^{-1}$ LCFA)
Linoleic Acid	0.142
Oleic Acid	0.143
Stearic Acid	0.141
Palmitic Acid	0.156
Myristic Acid	0.175
Lauric Acid	0.200

Varying amounts of culture from Reactor B and basal media were added to 160 ml serum bottles, depending on the condition examined. Each serum bottle received a total liquid volume of 50 ml minus the volume that was to be added for sugar and LCFA. The serum bottles were prepared under anaerobic conditions in a Coy® anaerobic chamber (COY Laboratory Products, Inc., Michigan) with a mixed gas composition of 70-75% N_2, 20-25% CO_2 and 1-5% H_2 (Praxair, ON, Canada). The sample bottles were sealed with Teflon®-lined silicone rubber septa and aluminum crimp caps. After sealing the bottles, 20 ml of the gas mixture from the anaerobic chamber was injected into each bottle to avoid negative pressure from forming in the bottle during headspace sampling. The bottles were inverted to ensure the formation of a liquid seal and agitated with an orbital shaker (Lab-Line Instruments) at 200 rpm and 23±2°C throughout the batch study. The cultures were left on the orbital shaker for 1 day prior to the initiation of the each experiment to remove the residual hydrogen from the headspace gas mixture and to initiate the LCFAs inhibitory reactions. Anaerobic conditions were maintained as indicated by the resazurin dye remaining colorless. Each experiment was conducted over

a 7 day period and samples (liquid and headspace) were withdrawn periodically to measure selected parameters.

3.7 Batch Reactor Operation and Culture Acclimation

The culture in Reactor B was maintained at a VSS concentration of 8,000 to 10,000 mg/L. The average gas production over a 4 week period with the theoretical quantity of gas is shown in Figure 3.1 and the production a degradation of volatile fatty acids (VFAs) over a ten day period is shown in Figure 3.2.

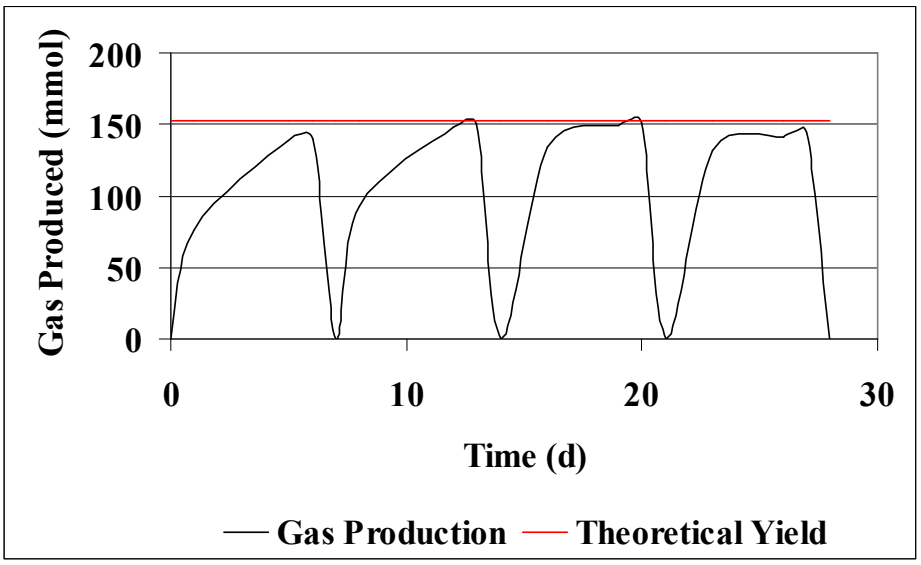


Figure 3.1 Gas production profile for Reactor B

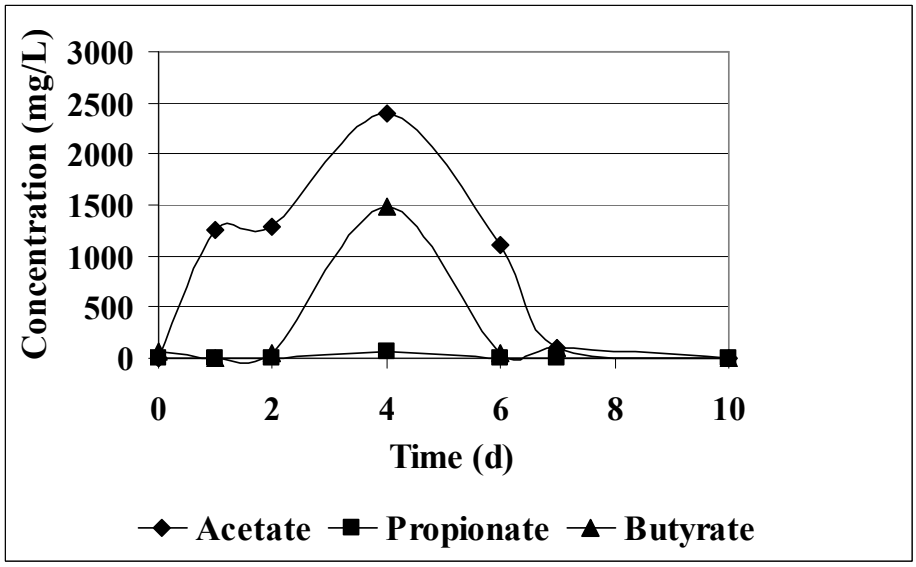


Figure 3.2 Profile for Reactor B VFA production and degradation

In Figure 3.1 the theoretical yield of gas is based on the amount of sugar added to the culture (5 g) from this mass a total production of CO_2 and CH_4 is calculated to be 153 mmol of gas. The gas production values are what were recorded each week of sampling. Figure 3.2 shows the production and breakdown of the VFAs produced during anaerobic fermentation over a ten day period. These results are used to determine if the culture was performing consistently.

The components from left to right in Figure 3.3 consists of a nitrogen gas tank, mother reactor (Reactor A), basal media bottle, tip bucket gas meter and reactor B (Figure 3.1). The reactors were operated in a semi-batch mode. After approximately 5 to 7 feeding periods, the culture was settled and approximately 1,000 mL of liquid was decanted using an inverted siphon. The headspace was purged with N_2 for 20 seconds and the rubber stopper on top of the serum bottle was used to seal the bottle. With the gas purge operating, the N_2 flow was diverted to the tipping bucket gas meter. Next, the basal medium was pressurized with approximately 1 psi N_2 gas and a know quantity of a mixture containing basal medium plus sugar was fed into Reactor B to ensure the sugar level was as a specified concentration. The procedure was repeated after feeding reactor B on 5 to 7 occasion.

Figure 3.3 Reactors A and B

The acclimation process was developed to ensure that the culture was able to consume sugar over a specific time period. Adjusting the feed from 100% glucose to 100% xylose was conducted over several weeks. The acclimation procedure was as follows:

1. Glucose was added at 1,250 mg/L on a weekly basis and gas production was monitored daily.
2. Varying ratios of xylose to glucose are added in sequence of 25%/75%, 50%/50% and 75%/25% totaling 1,250 mg/L.
3. The first ratio of 25%/75% xylose to glucose was added and gas production was monitored. When the weekly gas production reached the baseline levels established with glucose, the sugar mixture ratio was changed to 50%/50% and then 75%/25% xylose to glucose.
4. When the final ratio of 75%/25% xylose to glucose was reached and the gas production was stable, 100% xylose was added at 1,250 mg/L.

The culture bottles (mother and feed reactors) are feed 5 g of sugar once a week to a total concentration of 1,250 mg/L. Gas production is monitored using a tip scale gas meter. Every four weeks the ratio of sugars is changed from 100/0 to 75/25, 50/50, 25/75, and 0/100 glucose to xylose. Ensuring that the culture maintained proper gas production before the ration was changed.

Chapter 4

Fermentation of Glucose to Hydrogen in the Presence of Linoleic (LA) and Oleic acid (OA)

4.1 Experimental Results and Data Analysis

The experimental design for these studies considered the impact of two C18 LCFA on the hydrogen yield under three different pH conditions. To determine an optimum hydrogen yield in which the LCFA concentration and pH were the variables, a full factorial design (FFD) was used (Box et al., 2005). In this study, one center-point run was implemented in order to collect hydrogen yield data (mol H_2/mol glucose), while LCFA concentration and pH varied from 0 to 2,000 mg/L and from 5.0 to 7.6, respectively (Table 1). The experimental design under consideration was a full 3^2 experimental set, which required 9 experiments (Chatzisymeon et al., 2009).

Table 4.1: Experimental design matrix

Run	LA conc. (mg/l) (X1)	pH (X2)	LA conc. (mg/l)	pH	H_2 Yield (LA)	H_2 Yield (OA)
1	0	7.6	-1	1	0	0
2	2,000	7.6	1	1	0.91±0.28	0
3	1,000	5.0	0	-1	1.82±0.06	1.23±0.19
4	0	5.0	-1	-1	0.20±0.14	0.20±0.02
5	0	6.0	-1	0	0.09±0.04	0.09±0.12
6	1,000	6.0	0	0	1.44±0.10	0.32±0.11
7	1,000	7.6	0	1	0.90±0.18	0
8	2,000	5.0	1	-1	2.46±0.08	1.25±0.19
9	2,000	6.0	1	0	1.24±0.87	0.78±0.04

Full factorial designs are often not considered practical due to the large requirement of experiments to accurately predict the proper outcome (Box et al., 2005). However, in the case of 3 levels and 2 factors (9 runs (3^2)) the number of experiments is relatively small and the full design was considered.

4.1.1 Hydrogen and Methane Production

In comparison to the controls, methane production was inhibited under all the LCFA conditions examined. Varying amounts of hydrogen were produced in cultures receiving different LCFAs at the same concentration. The three LCFA concentrations examined were 1,000, 2,000 and 3,000 mg/L. In cultures fed with 1,000 and 2,000 mg/L LA, the hydrogen yield was 1.82 and 2.46 mol H_2/mol glucose, respectively (Figures 4.1 and 4.2 and Table 4.2). The optimal yield was detected in cultures fed with 2,000 mg/L. Methane inhibition was observed in all cultures fed with LA (Figure 4.1, 4.2 and 4.3). The data illustrates that very small quantities of methane was produced in cultures fed with 3,000 mg/L LA together with low quantities of hydrogen. The high LA level of 3,000 mg/L could have potentially affected the hydrogen producing microorganisms and hence, the low hydrogen yield. Increased hydrogen yields were observed with decreasing pH values.

Studies conducted with oleic acid (OA) also assessed its impact on glucose at 1,000, 2,000 and 3,000 mg/L. The results from these studies were similar to the experiments conducted with LA. In the presence of 2,000 mg/L of OA, the greatest amount of hydrogen was produced (Figure 4.4 and Table 4.2). The hydrogen yield data for cultures inoculated with OA (Table 4.2) indicate a strong dependence on pH for hydrogen yields.

The daily production of hydrogen was relatively consistent given their exposure to the different fatty acids (Figures 4.1 - 4.5). The maximum daily production of hydrogen was observed between day 0-2 followed by a consistent decrease in hydrogen production with values ultimately tapering off. After re-injection of glucose the highest daily hydrogen production was observed on day 6. The LCFAs type, LCFA concentration and pH affected the total amount of hydrogen produced. However, on day 2 a peak hydrogen level was observed.

Methane production data was very similar for cultures fed with LA or OA. However, note elevated methane levels were observed only in the control cultures. The greatest degree of methane inhibition was observed at pH 5 followed by pH 6 and with the least inhibition observed at pH 7.6.

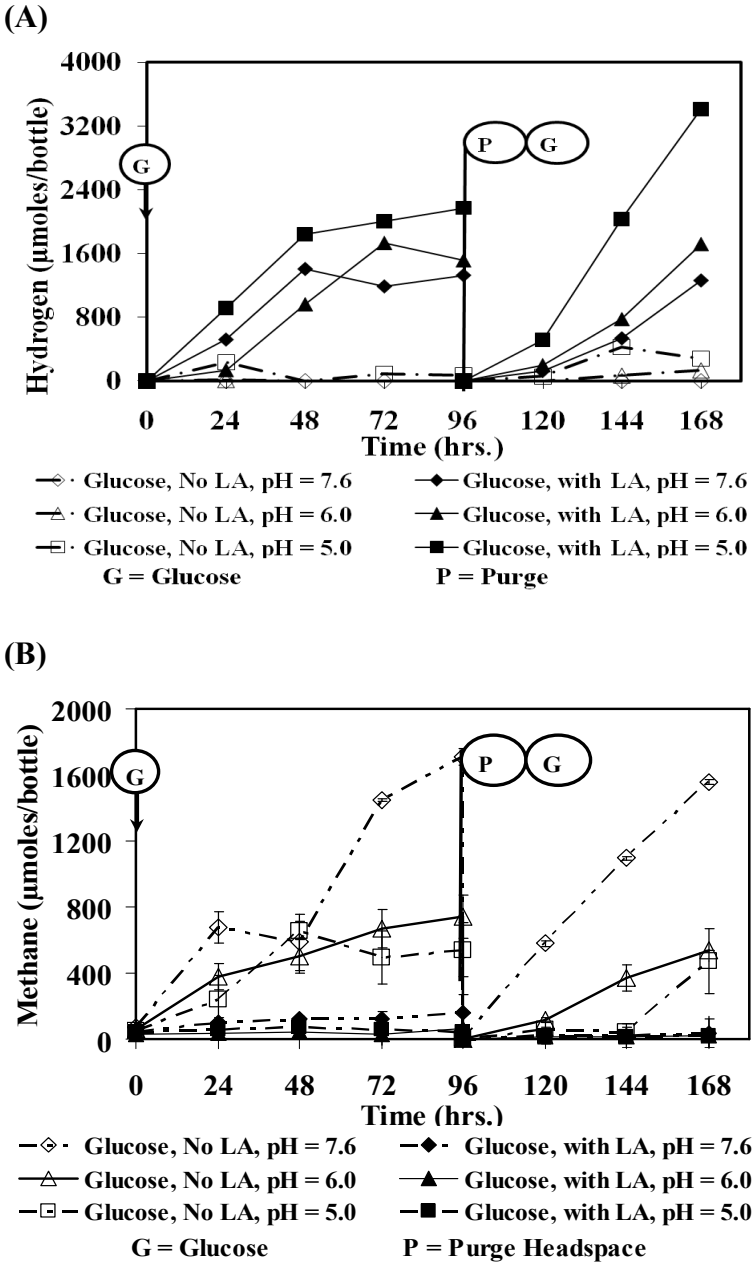


Figure 4.1 Effect of pH on gas production in cultures receiving 2,000 mg/L LA and 5,000 mg/L glucose. Glucose addition and pH adjustment at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen production, (B) Methane Production.

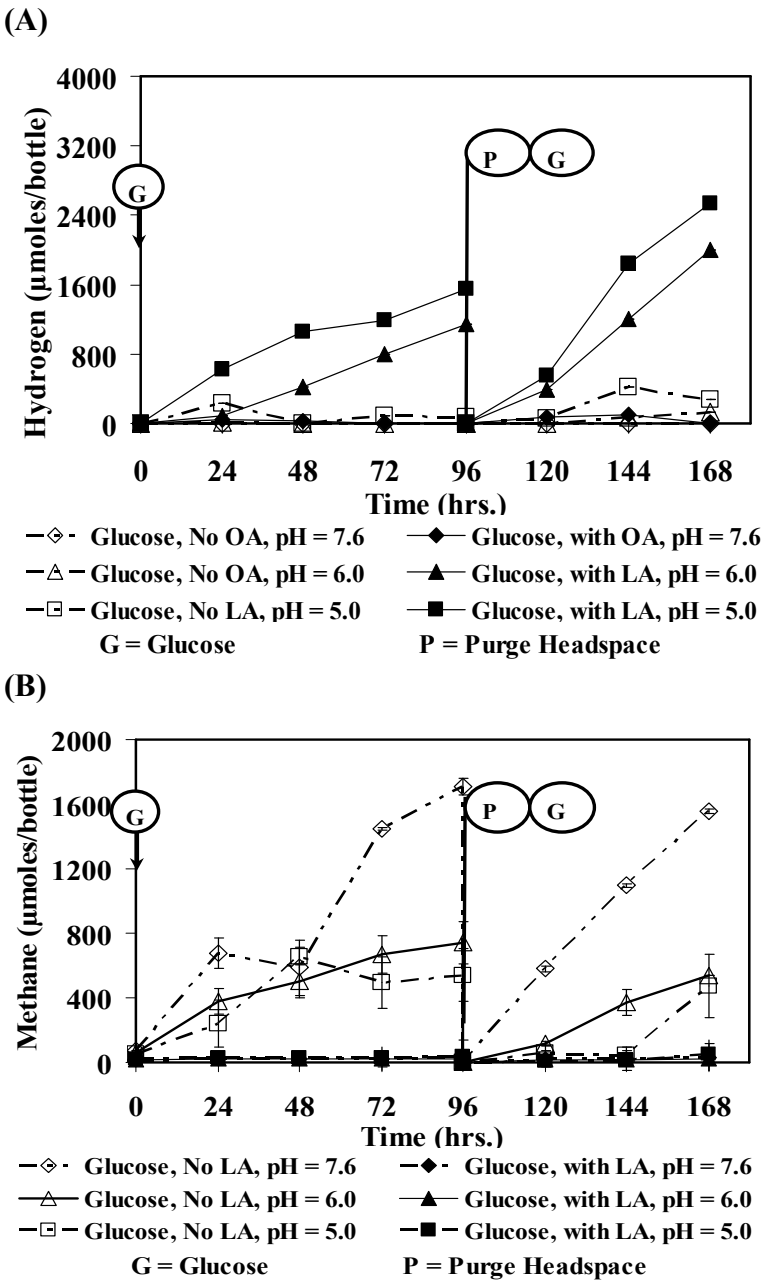


Figure 4.2 Effect of pH on gas production in cultures receiving 1,000 mg/L LA and 5,000 mg/L glucose. Glucose addition and pH adjustment at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen Production, (B) Methane Production.

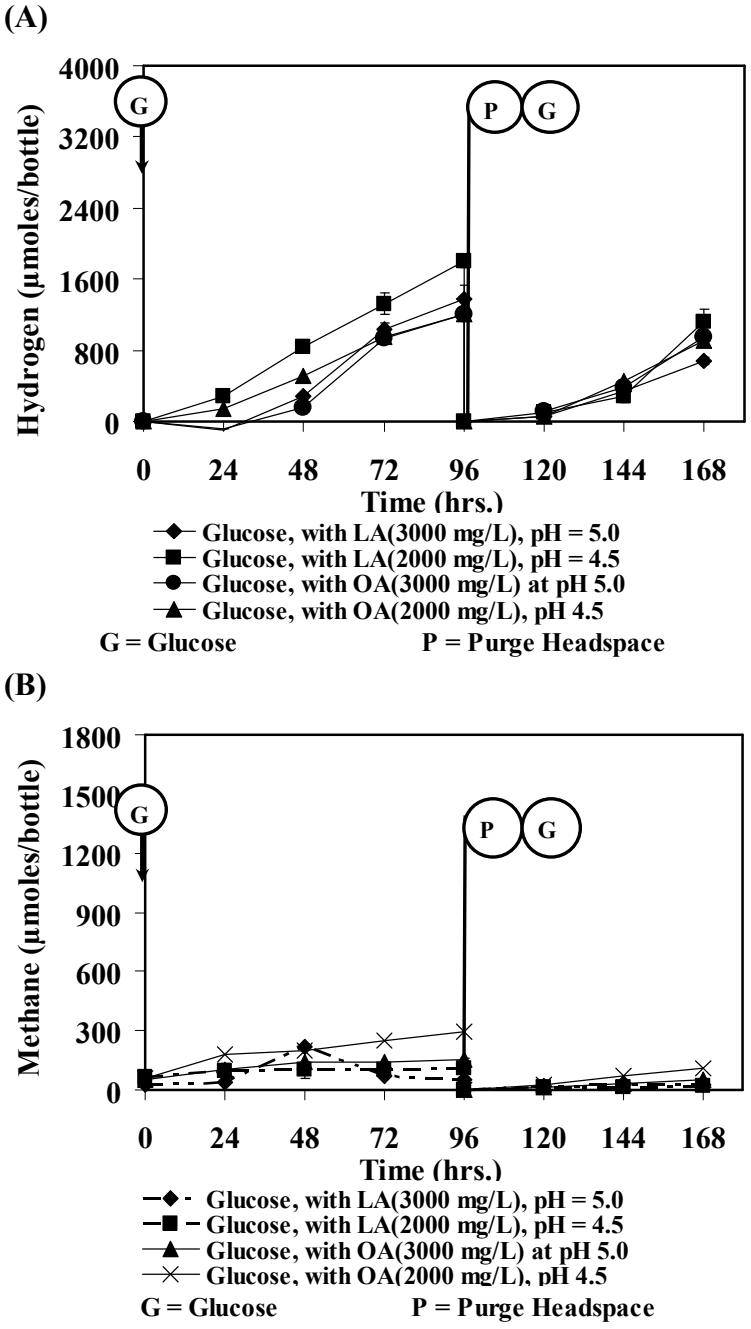


Figure 4.3 Gas production profiles for cultures receiving 3,000 mg/L LA or OA at pH 5 and culture receiving 2,000 mg/L LA or OA at pH 4.5, glucose addition (5,000 mg/L) at t = 0 and t = 96 hrs (each data point are for triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen Production, (B) Methane Production.

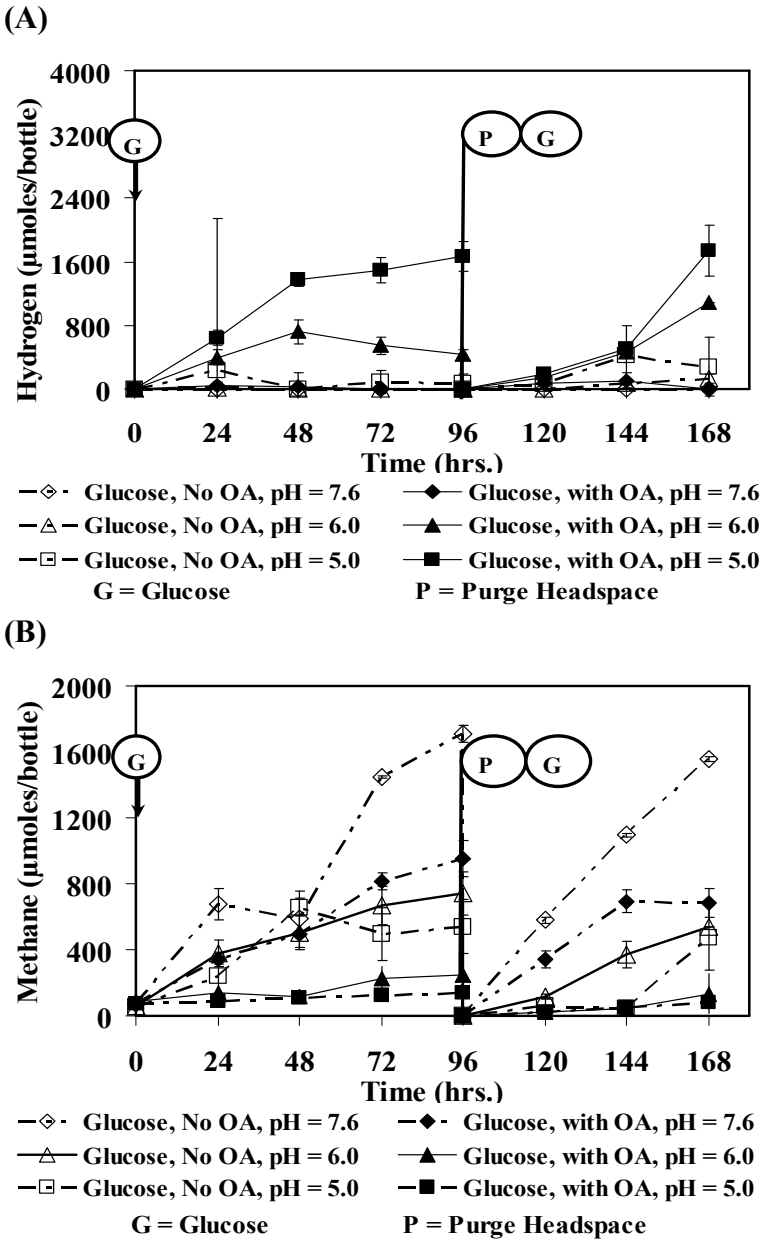


Figure 4.4 Effect of pH on gas production in cultures receiving 2,000 mg/L OA and 5,000 mg/L glucose. Glucose addition and pH adjustment at t = 0 and t = 96 hrs (each data point are for triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen Production, (B) Methane Production.

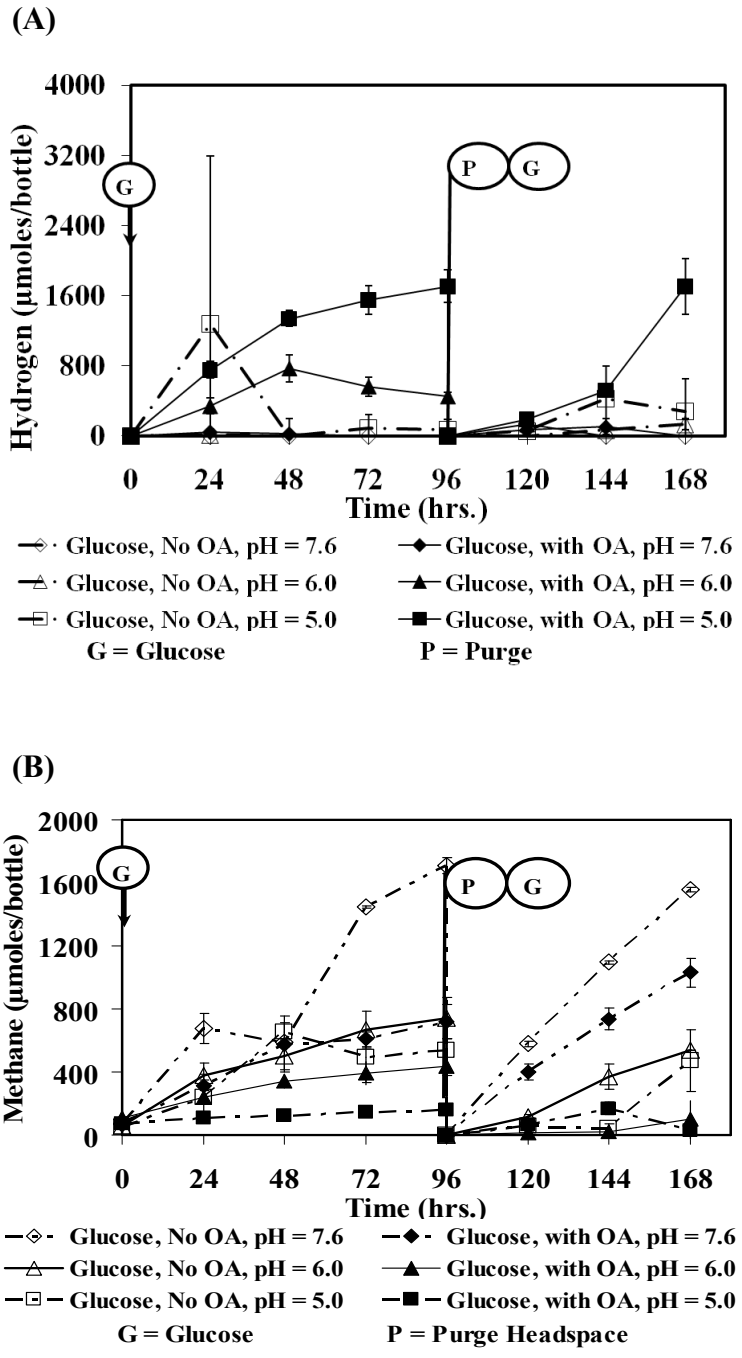


Figure 4.5 Effect of pH on gas production in cultures receiving 1,000 mg/L OA and 5,000 mg/L glucose. Glucose addition and pH adjustment at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen Production, (B) Methane Production.

Table 4.2: Hydrogen yield (mol H_2/mol glucose) in cultures fed with 5,000 mg/L glucose plus OA or LA (0, 1,000, 2,000, 3,000 mg/L) and maintained at pH 4.5, 5.0, 6.0 and 7.6

LCFA	Injection	0 mg/L			1,000 mg/L		
pH		7.6	6.0	5.0	7.6	6.0	5.0
LA	1st	0	0	0.05±0.01	0.71±0.04	0.82±0.02	1.12±0.26
	2nd	0	0.09±0.04	0.20±0.14	0.90±0.18	1.44±0.10	1.82±0.06
OA	1st	0	0	0.05±0.03	0	0.32±0.11	1.23±0.11
	2nd	0	0.09±0.12	0.20±0.02	0	0	1.23±0.19

Table 4.2 (continued)

LCFA	Injection	2,000 mg/L			2,000 mg/L	3,000 mg/L
pH		7.6	6.0	5.0	4.5	5.0
LA	1st	0.96±0.54	1.09±0.09	1.56±0.08	1.24±0.04	0.99±0.11
	2nd	0.91±0.28	1.24±0.87	2.46±0.08	0.77±0.13	0.49±0.06
OA	1st	0	0.31±0.06	1.20±0.05	0.87±0.08	0.86±0.07
	2nd	0	0.78±0.34	1.25±0.19	0.66±0.02	0.68±0.01

4.1.2 Statistical Optimization - Full Factorial Design (FFD)

The outcome from a FFD analysis shows the effect of multiple factors on a given outcome. An FFD surface analysis was performed for cultures fed with LA and OA. Two sets of nine experiments were conducted and analyzed using an analysis of variance (ANOVA) in order to evaluate the significance of the effects ($p<0.05$). The adequacy of the final model was verified by graphical and numerical analysis using Minitab 15 (Minitab Inc., State College PA).

For analysis of the hydrogen yield (H_2 yield) a surface model was developed for both LCFAs tested according to Equation 4.1.

$$H_2 \text{ yield} = a_0 + a_1*[\text{LCFA}] - a_2*(\text{pH}) - a_3*[\text{LCFA}]*(\text{pH}) \quad (4.1)$$

The coefficient a_0 is the constant of the equation and a_1 and a_2 are the main effects coefficients and a_3 is the two-way interaction coefficient. The effect of the two factors for both LA and OA subsets are shown in Figures 4.6A and 4.6B. The effect of pH 5 on the yield of hydrogen gas is larger in comparison to pH 6 and 7.6 for both LA and OA. The overall difference in the effect is similar for both LA and OA with a difference between high and low values of approximately 0.9 mol H_2/mol glucose (Figure 4.6). The effect of LCFA concentration is the same in both LA and OA with cultures fed with 0 and 2,000 mg/L having the highest and lowest yields, respectively.

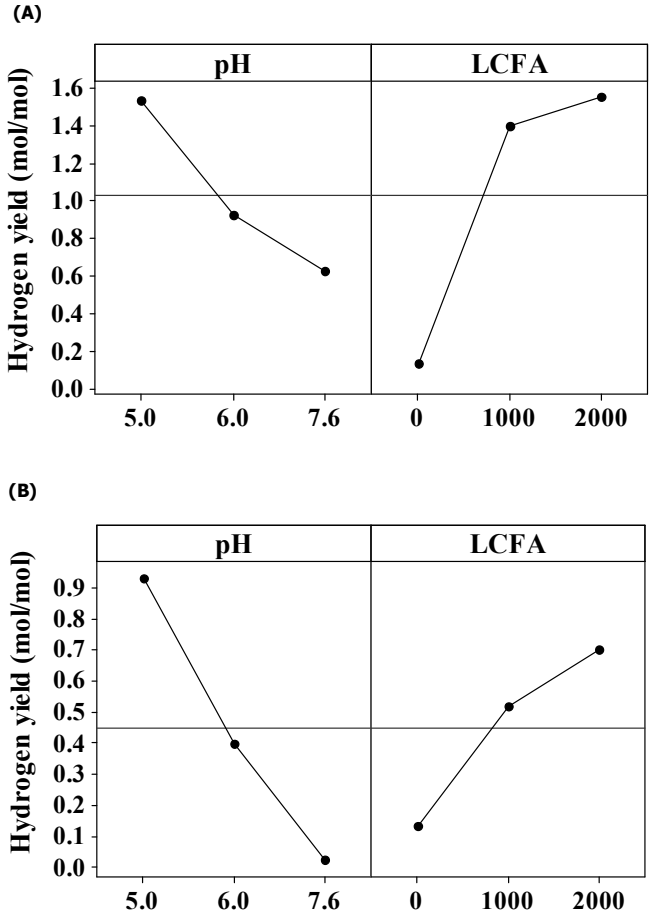


Figure 4.6 Factorial plots of hydrogen yield in a two factor, three level FFD, (A) LA main effects plot (B) OA main effects plot

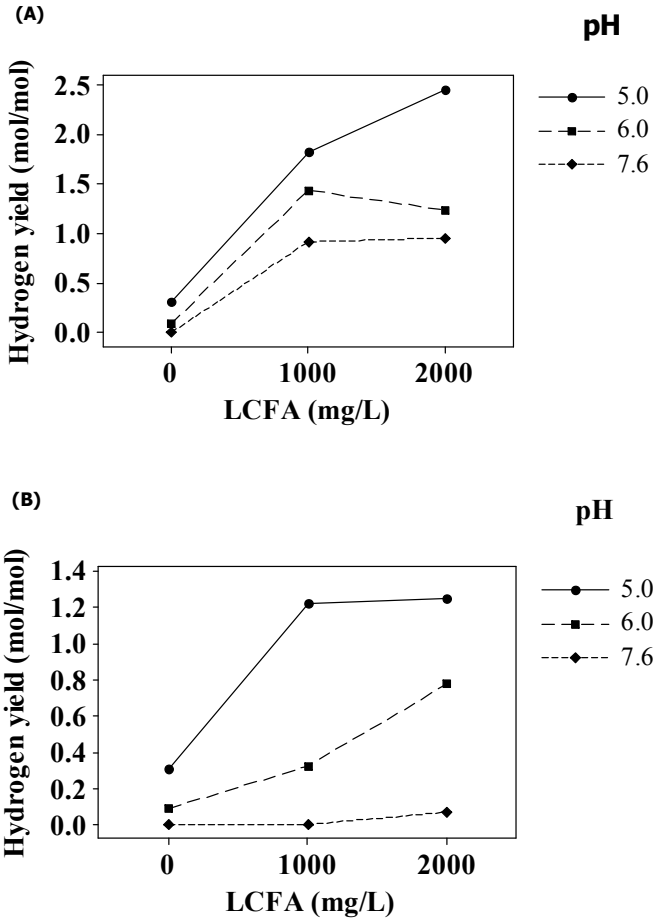


Figure 4.7 Factorial plots of hydrogen yield in a two factor, three level FFD, (A) LA two factor interaction plot (B) OA two factor interaction plot

The two factor interaction plot (Figures 4.7A and 4.7B) show the effect of combining pH and LCFA concentration simultaneously. The data clearly indicates that LA combined with pH of 5 has the greatest combined effect for studies conducted with both LA and OA. The contour lines of the response variable (H_2 yield) versus the two factors (pH and LCFA concentration) connect points of equal response (Figures 4.8A and 4.8B) for the LA and OA. The plots suggest that 2,000 mg/L LCFA together with a pH of 5 are the conditions which generate the highest hydrogen yield. A comparison of the plots in Figures 4.8A and 4.8B shows that the LA contour plot has a much higher hydrogen gas yield than the OA contour plot.

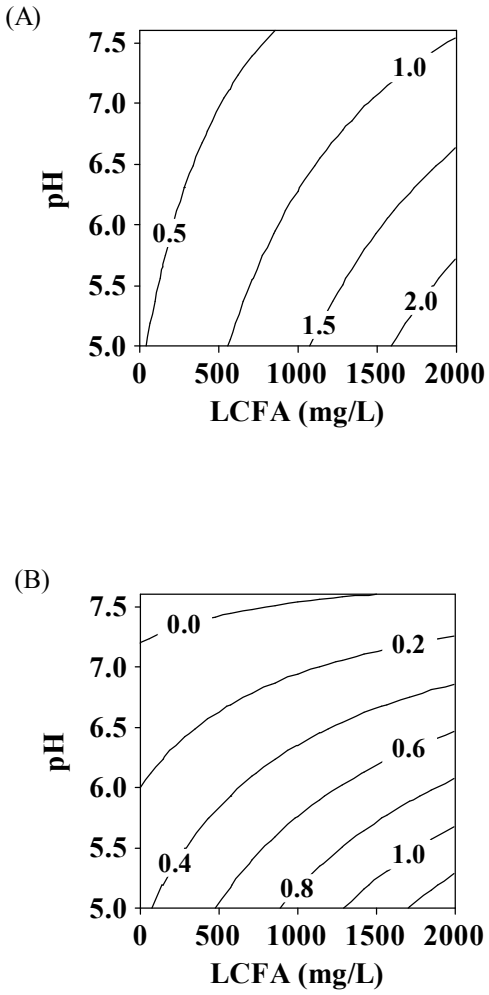


Figure 4.8 Effect of variables on response (H_2 yield). (A) Contour plot of response for pH versus LA concentration. (B) Contour plot of response for pH versus OA concentration.

An ANOVA (Table 4.3) was performed in order to evaluate the full linear response of the model presented in Table 4.1. The results reveal that the main effects of both LA and OA experiments are statistically significant ($p<0.05$); however, the two way interactions of the LA and OA ANOVA table are statistically insignificant ($p>0.05$) therefore the two-way interactions are removed from the surface model equation.

Table 4.3 ANOVA results of the experimental response with two factor interactions

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	F	P
LA						
Main Effects	2	4.1628	3.9589	1.9795	11.10	0.014
2-Way Interactions	1	0.3150	0.3150	0.3150	1.77	0.241
Residual Error	5	0.8914	0.8914	0.1783		
Total	8	5.3692				
OA						
Main Effects	2	1.6593	1.5992	0.79962	18.84	0.005
2-Way Interactions	1	0.2002	0.2002	0.20019	4.72	0.082
Residual Error	5	0.2122	0.2122	0.04243		
Total	8	2.0716				

[a] DF = degrees of Freedom, [b] Seq SS = sequential sum of squares, [c] Adj SS = adjusted sum of squares, [d] Adj MS = adjusted mean square

A regression analysis was performed on the two studies (LA and OA) in order to determine the coefficients of the surface equation (Equation 1) (Table 4.3).

Table 4.4 Response surface model regression coefficients for hydrogen yield

Term	Coefficient	Regression Coefficient	p-value
LA			
Constant	a_0	2.7902	0.001
[LCFA]	a_1	0.0007	0.011
pH	a_2	-0.3391	0.042
[LCFA]*pH	a_3	-0.000237	0.241
OA			
Constant	a_0	2.3651	0.002
[LCFA]	a_1	0.0003	0.025
pH	a_2	-0.3347	0.003
[LCFA]*pH	a_3	-0.0001004	0.082

Using the regression coefficients and p-values, two equations were formulated for studies conducted with both LA and OA. The equations can be used to calculate the theoretical H_2 yield in the range of pH 5.0 - 7.6 and LCFA concentration of 0 – 2,000 mg/L.

$$H_2 \text{ yield} = 2.7902 + 0.0007*[LA] - 0.3391*(pH) \quad (4.2)$$

$$H_2 \text{ yield} = 2.3651 + 0.0003*[OA] - 0.3347*(pH) \quad (4.3)$$

Validation of the surface model was performed by plotting the theoretical versus experimental hydrogen yields for culture receiving LA and OA (Figures 4.9A and 4.9B). The effectiveness of the design is based upon how close the values are to the line y = x. The R^2 value shows the linear relationship of the plotted values which show how effective the design equation is at predicting the hydrogen yield. The R^2 value indicates a reasonable high correlation between the theoretical and experimental hydrogen yields. The Anderson-darling plot is used to confirm normal distribution of residuals (Figure 4.10). The calculated Anderson-Darling statistics of 0.666 for LA and 0.205 for OA are less than the critical value of 0.751 for a 5% confidence level strongly suggests a normal distribution of the residuals (Montogomery, 2005). The p-values of 0.054 for LA and 0.813 for OA (>0.05) (Figure 4.10) indicates the model correlates reasonably well with the experimental results.

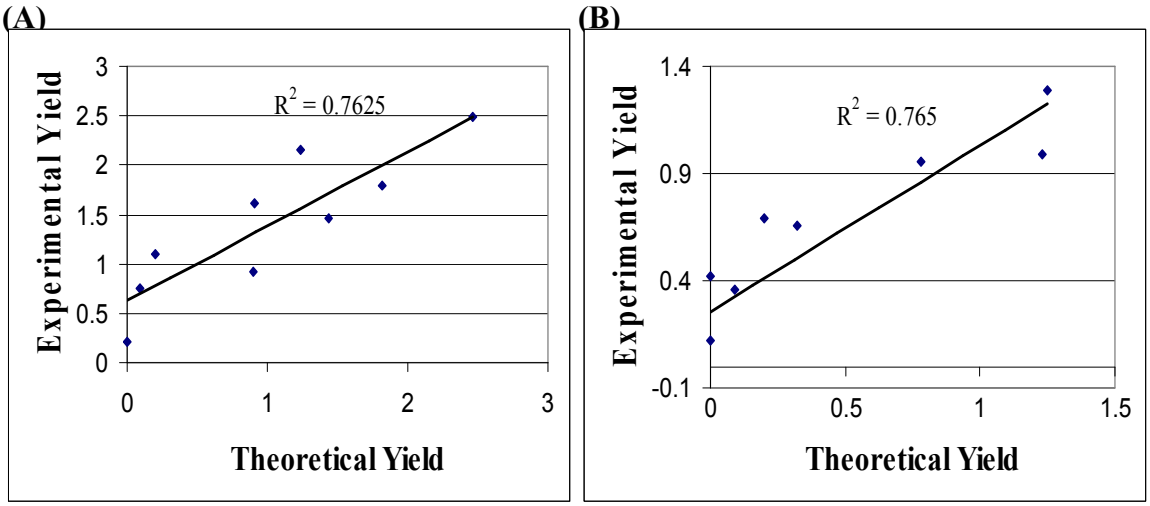
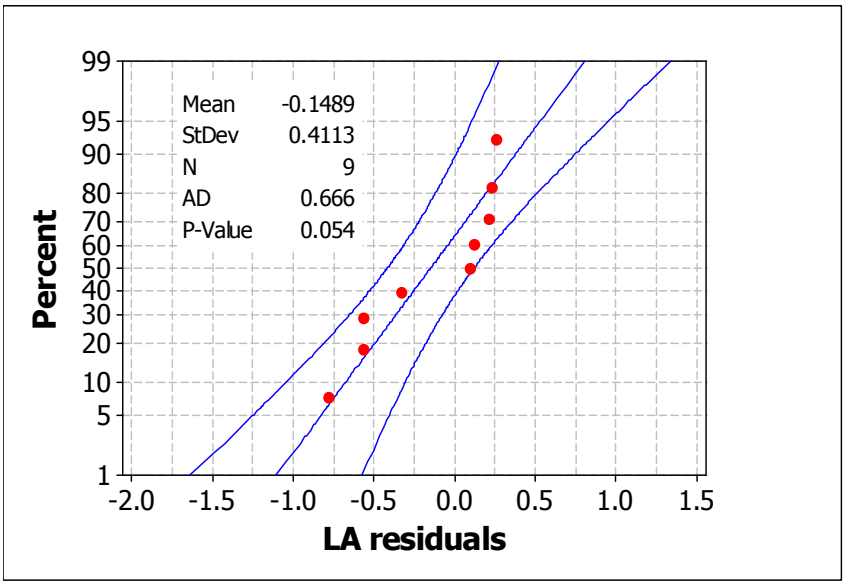


Figure 4.9 Validation of the response model. (A) Scatter plot for LA actual yield versus theoretical yield. (B) Scatter plot for OA actual yield versus theoretical yield.

(A)



(B)

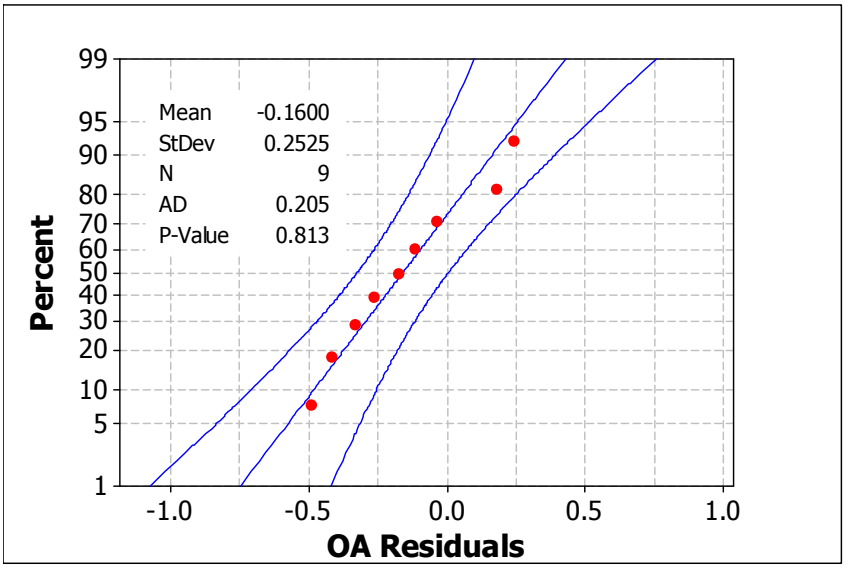


Figure 4.10 Anderson-Darling plots of residuals for surface model (A) Linoleic acid and (B) Oleic acid

4.1.3 Sugar Degradation

Glucose degradation after the first injection at t = 0 is shown in Figure 4.11. Notice glucose removal was slower in cultures inoculated with LCFAs. The initial rate of glucose degradation in both the presence and absence of LCFAs was affected the greatest with the rates matching up after approximately 4 hours and there were no measureable glucose levels after 24 hrs.

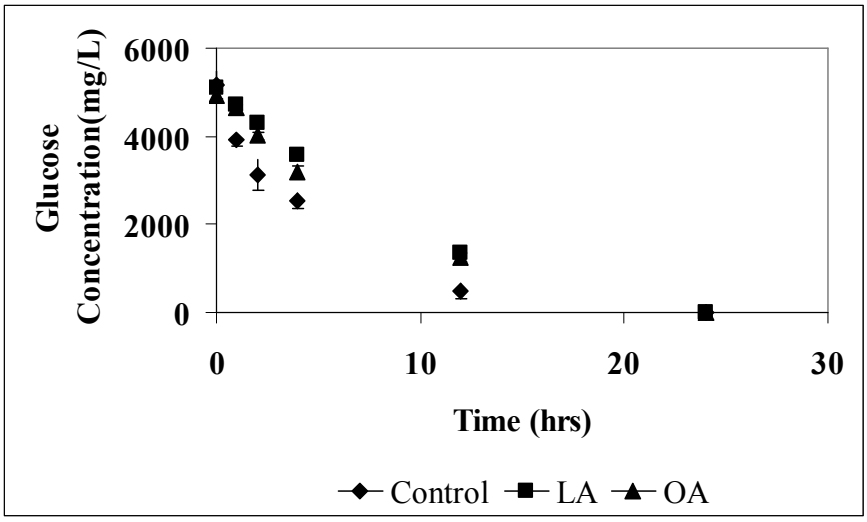


Figure 4.11 Glucose degradation of 5,000 mg/L glucose containing 0 and 2,000 mg/L LCFA at a pH of 7.6 (data for glucose added at t=0) (each data point are for triplicate samples; errors are indicated using the standard deviation).

The addition of the LCFAs had a significant effect on the initial degradation rate (Table 4.5). The samples inoculated with LA and OA show a much lower rate than the samples without LCFA inhibition. Tukey's analysis (Montogomery, 2005) was performed on the difference in the means between the cultures with and without LCFA. The analysis showed that the q_s values (225 for LA and 309 for OA) were greater than $q_{critical}$ values (3.2 for LA and 2.5 for OA) suggesting that the difference in the means is significant.

Table 4.5 Initial glucose degradation rates (µg/mg VSS/min) for cultures containing 5,000 mg/L glucose and LCFA at pH of 7.6 (data point are for triplicate samples; errors are indicated using the standard deviation).

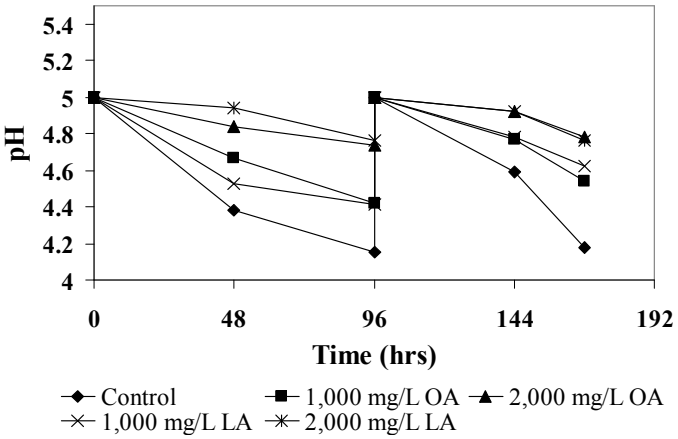
LCFA	0 mg/L	2,000 mg/L
LA	9.03±0.06	2.28±0.03
OA	9.03±0.06	2.85±0.02

4.1.4 Change in pH Value

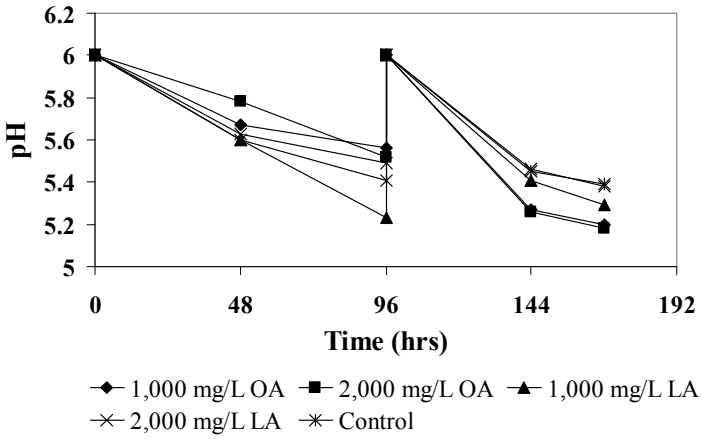
The initial pH value of the culture was adjusted to a value of 5.0, 6.0, and 7.6 on day 0 and re-adjusted at t = 96 hrs after re-injecting the sugar to the pre-determined levels (Figure 4.12). During the experiment, increasing acid formation caused a decrease in the

pH value of the sample. It is important to note that the pH change over the duration of the experiment differs under all three initial pH conditions. At initial pH values of 6.0 and 7.6, the pH value decreased quickly followed by a slower decrease until the readjustment. In cultures with a pH value of 5.0, a gradual pH decrease is shown. The pH 7.6 profile (Figure 4.12C) shows that for cultures receiving LA, the lowest pH attained was at t = 96 hrs and t = 168 hrs and was approximately 6.3 after the first glucose injection. After re-injecting glucose, the pH values decreased from 6.3 - 6.7. In cultures where the initial pH was set at a value of 6 (Figure 4.12B), the pH values at t = 96 hrs and t = 168 hrs were approximately 5.2 to 5.6, respectively. For cultures at an initial pH value of 5.0 (4.12A) the maximum pH decrease from t = 0 to t = 96 hrs and from t = 96 hrs to t = 164 hrs was approximately 0.8 pH units.

(A)



(B)



(C)

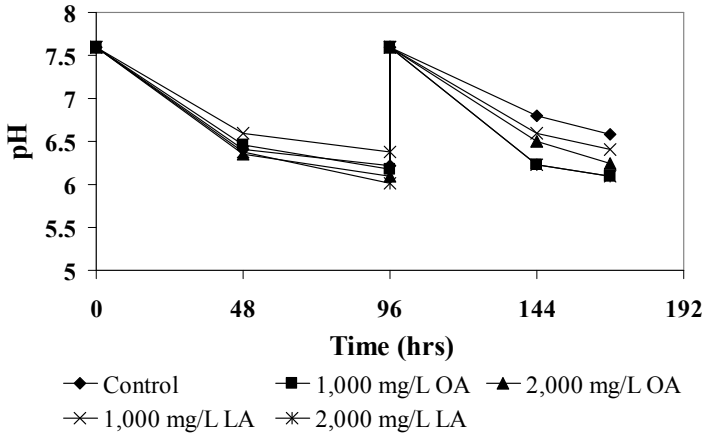


Figure 4.12 The pH profile of the experiments conducted at different initial pH values with the pH set at t =0 hrs and re-adjusted at t = 96 hrs. (A) Samples run at initial pH 5, (B) samples run at initial pH 6, and (C) samples run at initial pH 7.6.

4.2 Discussion

An anaerobic microbial community operating under stable thermodynamic conditions does not produce an excess amount of hydrogen. In a stable system hydrogen is produced and subsequently consumed to produce methane. Under conditions in which hydrogen begins to accumulate, the community will develop a mechanism to produce reduced volatile fatty acids such as butyrate and lactate. When the pH decreases to a threshold level, the system will begin to produce reduced products such as alcohols including ethanol, propanol and butanol (Li and Fang, 2007; Gaudy and Gaudy, 1980). These mechanisms are utilized to decrease the hydrogen partial pressure and cause the system to achieve thermodynamic stability.

LCFAs are known to inhibit the consumption of hydrogen and the production of methane in anaerobic systems (Lalman and Bagley, 2000; Hwu and Lettinga, 1997). Data from experiments conducted using glucose as an electron donor and C18 LCFAs as methanogenic inhibitors indicate there is little or no methane gas produced and hydrogen accumulation is observed (Chowdhury et al., 2007).

The purpose of this study on glucose fermentation was to assess optimal conditions under which a maximum hydrogen yield is attained. The conditions which were examined included pH, LCFA types and LCFA concentrations. The results show that an optimum hydrogen yield was observed at a pH value of 5.0 and 2,000 mg/L LA.

The maximum hydrogen yield of 2.46 mol H_2/mol glucose for cultures fed with 2,000 mg/L and maintained at an initial pH of 5.0 and 23°C was comparable to data reported by Ray et al., (2008). These authors used the same conditions as those reported herein for cultures fed with glucose plus LA. The optimal conditions for cultures fed with OA were 2,000 mg/L at a pH 5.0 and a temperature of 23°C. The yield reached a maximum value of 1.23 mol H_2/mol glucose under these conditions.

When the LA levels increased from 1,000 mg/L to 2,000 mg/L, the hydrogen yield increased and subsequently, decreased with a concentration of 3,000 mg/L LA (Table 4.1). In the case of cultures fed with OA, a similar trend was observed; however, the yield were less than cultures fed with LA (Table 4.2). The increase in hydrogen yield in cultures receiving 1,000 and 2,000 mg/L LCFA is likely due to the inhibition of hydrogen consuming microorganisms by both LA and OA. After reaching a threshold concentration, LA inhibited the hydrogen producers and a subsequent decrease in yield was observed. The greater degree of inhibition caused by LA in comparison to OA is confirmed by inspecting the methane production data. In the presence of LA, lower levels of methane were produced. The impact of increasing LA levels and decreasing pH values on hydrogen production was confirmed in works reported by Chowdhury et al. (2007) and Ray et al. (2008), respectively.

The effects of pH on hydrogen production have been documented in many reports. Fang and Liu (2002) reported a yield of 2.12 mol H_2/mol glucose for a heat treated mixed culture operating at a pH of 5.5 and maintained at 36°C. Other researchers have reported hydrogen gas yields of approximately 2.4 mol H_2/mol glucose (Ray et al., 2008) at pH 5.0 using LA as inhibitor and maintained at 37°C. Logan et al. (2002) reported a yield of 0.92 mol/mol glucose for a system operating at a pH value of 6.0 using heat treated cultures which were maintained at 26°C. Evidence form Chowdhury et al. (2007) has shown a yield of 1.71 mol/mol glucose at a pH of 7.6 and a temperature of 37°C.

Under all the conditions examined, methane was produced. However, in general the quantity produced was greater in cultures maintained at higher initial pH values. Notice the quantity of methane produced in cultures without any inhibitor was larger than those fed a LCFA inhibitor. The inhibitory effect of LA and OA on acetoclastic and

hydrogenotrophic methanogens have been widely documented by (Lalman and Bagley, 2002; Lalman and Bagley, 2000; Chowdhury et al., 2007; Ray et al., 2008; and Cirne et al., 2007). Alosta et al. (2004) and Lalman and Bagley (2002) studied the effects of different C18 LCFAs on acetogens, acetoclastic methanogens and hygenotrophic methanogens. Their work showed that these microorganisms were inhibited by the presence of the LCFAs. A study performed by Alosta et al. (2004) found that at LA significantly inhibited substrate degradation at 300 mg/L and higher.

The conditions of lower pH values of 4.5 and LCFA concentrations of 3,000 mg/L are shown in Figures 4.5A and 4.5B. The data suggests that the optimal conditions for producing hydrogen is at pH 5.0 and LA at 2,000 mg/L. The maximum yield at pH 4.5 and LA at 2,000 mg/L was 1.25 mol H_2/mol glucose and for the cultures operating at pH 5.0 and fed LA at 3,000 mg/L, the yield was 0.99 mol H_2/mol glucose. Note these yields are less than the 2.46 mol H_2/mol glucose value which was observed with cultures at an initial pH of 5.0 and fed 2,000 mg/L LA.

A low pH value has been shown to produce solvents such as ethanol and butanol which consumes hydrogen in the production of these products and the hydrogen is not released to the headspace for collection. The culture at 3,000 mg/L was set at pH 5.0 so solvent production would not have taken over however the increased LCFA had a lower hydrogen yield than the 2,000 mg/L culture. The effects of LCFA has been documented in many reports, Alosta et al. (2004) reports that acetogens are inhibited at concentrations of 300 mg/L LA, Hwu and Lettinga (1997) showed an 80% reduction in microorganism activity at 1,000 mg/L OA. This illustrates a poisoning effect of the high LA of OA concentration. LCFAs are slightly toxic to all bacteria, having a high concentration not only inhibited the methanogens but may have inhibited the acidogenic and acetogenic bacteria as well causing an overall reduction in hydrogen gas production.

The conversion efficiency of glucose to hydrogen based on a theoretical yield of 4 mol/mol glucose was approximately 62% These results are greater than yields reported by Fang and Liu (2002) who observed a yield of 2.1 mol H_2/mol glucose (53% conversion). Other researchers have reported lower yields Khanal et al. (2004) of 1.8 mol H_2/mol glucose at 37°C and pH 4.5.

Two response surface models were developed using a full factorial design (FFD) to describe the hydrogen yield using pH and LCFA concentration. The range of pH was chosen based on the data reported by Fang and Liu (2002) and Kim et al. (2004). The LCFA range is based on evidence that LCFAs are toxic at certain levels which can inhibit the entire culture not only the methanogens (Cirne et al., 2007; Lalman and Bagley, 2002). LCFA and pH values were tested at 3,000 mg/L and pH 4.5 in order to establish the hydrogen yield.

The optimal conditions based on the two FFDs presented are at a pH value of 5.0, LCFA concentration of 2,000 mg/L and LA**.** From the results shown by Figure 4.7, LA has more of an effect on hydrogen yield than OA as the effect of the pH is drastically reduced. In Figure 4.7B, the pH lines are distinctly diverse indicating more of a pH effect on hydrogen yield.

Optimizing conditions for a mixed anaerobic culture can be an expensive endeavor as chemical addition requires a carefully designed process. The concentration of the LCFA must be monitored and replenished and pH must also be tested and adjusted accordingly. The FFD model allows a cost benefit analysis to be preformed in order to establish acceptable operating ranges for the LCFA concentration and pH.

Glucose was removed within 24 hours in cultures with no inhibitor and in cultures fed the LCFA inhibitor, the degradation rate was less. The initial degradation rates were 9.03±0.06 μg/mg VSS/min without inhibitor and varied from 2.28±0.03 μg/mg VSS/min to 2.85±0.02 μg/mg VSS/min with LCFA inhibition (Table 4.5). The slower glucose degradation rate in the presence of LCFAs is evidence of the toxicity imposed by these inhibitors on acidogens. Chowdhury et al. (2007) reports a reduction in the initial degradation rates with LA when compared to absence of an inhibitor. Zheng and Yu (2004) reported degradation of glucose within 12 hrs at pH of 8 whereas at pH 4 only 40% of the glucose was degraded after 24 hrs. This clearly demonstrates the effect of pH on sugar degradation.

4.3 Conclusions

Glucose fermentation was studied over a wide range of conditions to find an optimal pH, LCFA and LCFA concentration. The conditions examined were pH 4.5 -

7.6, LCFA type (LA and OA) and concentration (0 – 3,000 mg/L). A factorial design analysis was use to model the experimental results and to develop an equation which would describe the effects of pH and LCFA concentration on the hydrogen yield. From the results of the study, it was concluded that the optimal conditions were pH 5.0 and 2,000 mg/LA L for a maximum hydrogen yield of 2.46±0.08 mol H_2/mol glucose.

Chapter 5

Fermentation of Xylose to Hydrogen in the Presence of Long Chain Fatty Acids (LCFAs)

5.1 Introduction

Xylose is a C5 carbon sugar which is found in low value biomass from woody and non-woody sources. Production cost is a key variable which is controlled by using low cost lignincellusoic feedstock material containing a mixture of both C5 and C6 sugars plus lignin. Using C6 based sugars and starches which are expensive and sourced from food supplies could cause a rise in the production costs. In corn stover, switchgrass and wheat straw, the xylose composition can vary from approximately 20 to 25% of the dry matter while the C6 sugar levels can reach up to 40% (Lee et al., 2007). Developing fermentation routes to use xylose will decrease the disposal of underutilized biomass and assist in increasing the efficiency of converting agricultural residues into useful biofuels. Developing a broad chemical platform which can be used to produce biofuels offers process flexibility. Using xylose to meet increasing demands of biofuels will allow more C6 sugar based agricultural products to be available for food consumption.

The conversion of C6 sugars into a variety of chemicals using fermentation routes is well documented. However, in comparison to C6 sugars, the quantity of data relating to use of C5 sugars is relatively small. In this study, LCFA inhibitors were used to inhibit methane production and ultimately divert electron fluxes into hydrogen production. Diverting electron fluxes in microbial systems can be achieved by adding chemicals as well as controlling environmental (pH) and physical parameters (heat). Lin et al. (2006) studied the effects of pH values with a range of 5.0 to 8.0 on xylose fermentation into hydrogen. These authors showed that the hydrogen yield values ranged from 0.5 to 1.3 mol H_2/mol xylose. Lin et al. (2008) also studied the effects of different temperatures ranging from 30°C to 55°C on hydrogen yield and reported an optimal temperature of 40°C. The electron fluxes for hydrogen production can be also controlled by engineering design parameter such as the hydraulic retention time (HRT). Wu et al. (2008) studied the effects of different bioreactors on hydrogen yield. They show a higher HRT of 2 h provided the highest yield of hydrogen (0.8 mol H_2/mol xylose).

A large quantity of data relating to the conversion of C6 sugars into hydrogen is available. However, data for studies examining C5 sugars and C6 plus C5 mixtures is lacking. Also, reports describing the effects of LCFAs on the conversion of C5 or C5/C6 sugars into hydrogen are non-existent. The purpose of this work outlined in this chapter was two-fold. In Chapter 4, glucose degradation in the presence of varying concentrations of linoleic acid (LA) and oleic acid (OA) was examined. In the first phase of the work outlined in Chapter 5, the optimum conditions (pH 5.0 and 2,000 mg/l LA) for glucose degradation was used as reference conditions for assessing the effects of a series of LCFAs (C12 to C18) on the degradation of xylose to hydrogen at initial pH values of 5.6 and 7.6 and at 23°C±1°C. The pH value of 5.6 was used as opposed to that of 5.0 due to the fact that hydrogen yields from xylose fermentation were lower at pH 5.0.

Data showing the change in the initial pH for work conducted in Chapter 4 demonstrated that the pH value of a culture fed 2,000 mg/L LA decreased by a value of 0.6. To prevent the pH of the culture from decreasing below a value of 5.0, an initial pH value of 5.6 was selected. The LCFAs under consideration included linoleic acid (LA) (C18:2), oleic acid (OA) (C18:1), stearic acid (SA) (C18:0), palmitic acid (PA) (C16:0), myristic acid (MA) (C14:0) and lauric acid (LAU) (C12:0).

The wide range of LCFAs are which were selected was based on the β-oxidation degradation by-products of C18 LCFAs. A C18 LCFA is successively degraded to a C16 acid and ultimately to acetate. One objective of this work was to establish the inhibitory effect of shorter chain LCFAs on hydrogen production.

In the second phase of the work in this chapter, the effects of optimal LCFA from both glucose and xylose fermentation, was used to assess the degradation of various glucose plus xylose mixtures to hydrogen at 23°C±1°C and initial pH values of 5.6 and 7.6. In these experiments the products which were analyzed are shown in Table 5.1.

Table 5.1 Chemical parameters analyzed for different experiments

Conditions			H_2	CH_4	VFA	Alcohol
LCFA	**pH**	**Sugar**				
LA, OA, LAU, SA, PA and MA	5.6 and 7.6	100% Xylose	Yes	Yes	No	No
LA	5.6 and 7.6	25%/75%, 50%/50% and 75%/25% xylose to glucose ratios	Yes	Yes	No	No
LA	5.6	100% Xylose , 50%/50% xylose to glucose and 100% glucose	Yes	Yes	Yes	Yes

5.2 Experimental Design and Analytical Methods

These studies were conducted at 23^{o}C±1°C and at initial pH values of 5.6 and 7.6. The experimental design is shown in Tables 3.2 and 3.3 and all analytical procedures are described in Chapter 3.

5.3 Results

The experiments were conducted at pH 5.6 and 7.6. The change in pH to 5.6 instead of 5.0 was based on the pH profile data in Chapter 4. In cultures with an initial pH adjusted to 5.0, the final pH was approximately 4.4.

5.3.1 Xylose Degradation in the Presence of LA, OA, SA, PA, MA, LAU

5.3.1.1 Hydrogen and Methane

A hydrogen yield of 2.13 mol H_2/mol xylose was observed at pH 5.6. In comparison, lower yields were observed in cultures with an initial pH adjusted to 7.6. The quantity of methane produced was relatively small from t = 0 hrs to t = 96 hrs and negligible amounts were observed after xylose was re-injected (t = 96 hrs to t = 168 hrs). The levels of hydrogen produced (1.65 mol H_2/mol xylose) in cultures fed with OA and

at an initial pH of 5.6 were similar to those receiving LA at the same pH. Methanogenic inhibition was also observed in the OA fed cultures under low pH conditions. The hydrogen production data indicates a strong dependence on pH for cultures fed OA and LA. In the presence of LAU, the cultures produced significant amounts of hydrogen (1.78 mol H_2/mol xylose). The hydrogen production profiles for LAU were different than those for cultures fed with LA or OA. As seen from Figure 5.3A, the pH has less of an effect when LAU is used as an inhibitor since there is a small difference in hydrogen production between the two pH values (852 μmol of hydrogen difference between pH 5.6 and pH 7.6). When compared to the LA and OA differences of 1692 μmol of hydrogen and 1692 μmol of hydrogen, respectively, were observed for pH 5.6 and 7.6 (Figure 5.1A and 5.2A). At pH 5.6 and 7.6, the hydrogen production trends were similar for LA, OA and LAU when xylose was injected at t=0 and t = 96 hrs. The methane production profiles for LAU show complete inhibition with negligible methane production occurring after the re-injection xylose at t = 96 hrs.

The methanogenic inhibition in cultures receiving SA, PA or MA (Figures 5.4B, 5.5B and 5.6B) was significantly less than in cultures fed LA, OA and LAU. Large quantities of methane were produced in cultures receiving SA (0.75 mol CH_4/mol xylose) at pH 7.6 in comparison to cultures fed PA or MA (from 0.36 mol CH_4/mol xylose to 0.58 mol CH_4/mol xylose). The methane production trend based on mass was as follows: SA > MA > PA. A small amount of hydrogen was produced in cultures operating at an initial pH of 5.6 and fed with MA at t = 168 hrs. The PA fed cultures showed consistent methane production. In cultures operating at initial pH values of 5.6 and 7.6 the methane production was 0.36 and 0.37 mol CH_4/mol xylose, respectively. Whereas in SA and MA fed cultures, the production at pH 7.6 was 0.75 and 0.58 mol CH_4/mol xylose, respectively. At pH 5.6, 0.26 and 0.21 mol CH_4/mol xylose of methane was produced in cultures fed with SA and MA, respectively.

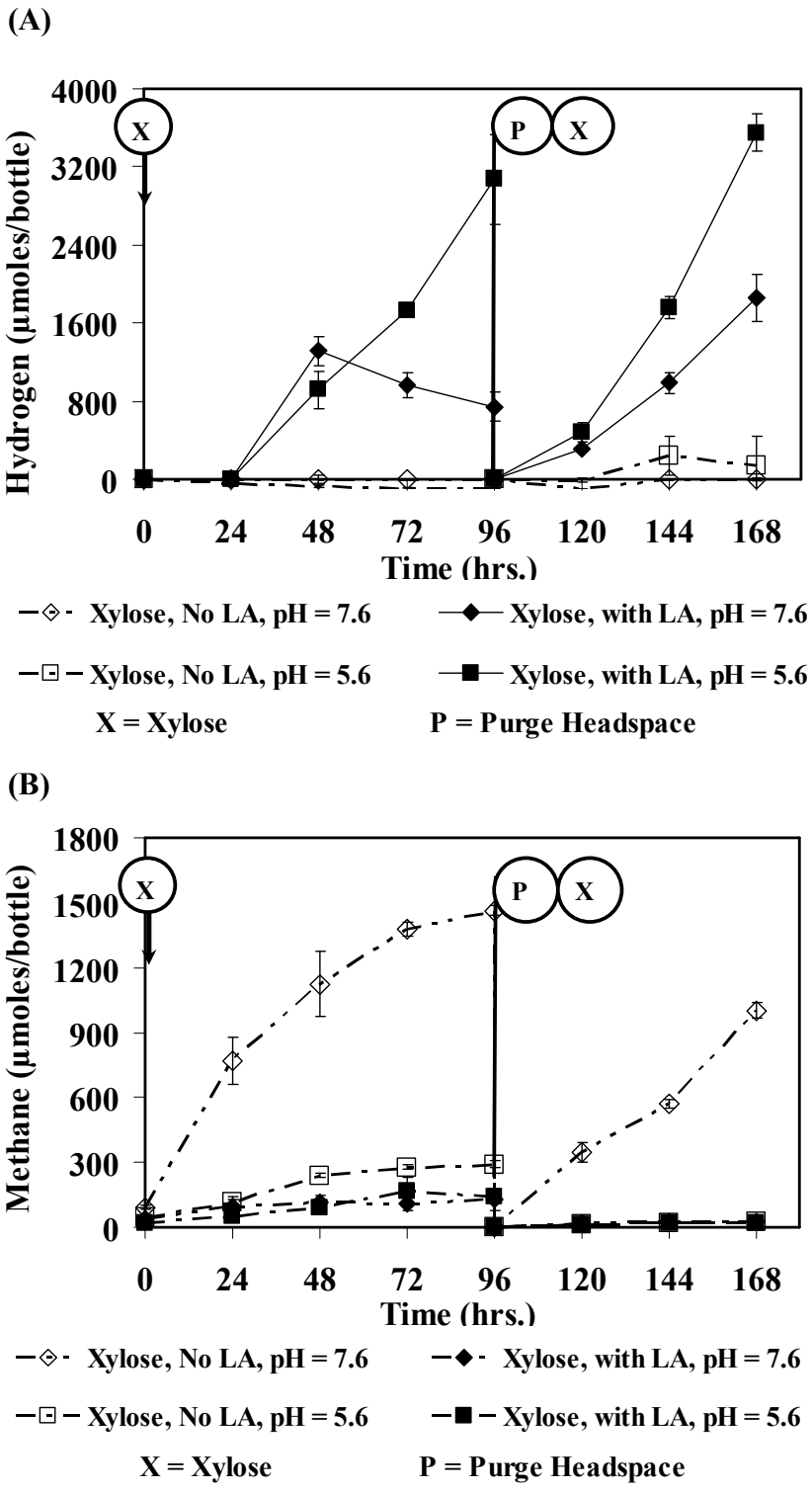


Figure 5.1 Gas production profiles for xylose (5,000 mg/L) degradation under different pH conditions in the presence of LA at 2,000 mg/L. Xylose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated by the standard deviation). (A) Hydrogen production and (B) Methane production.

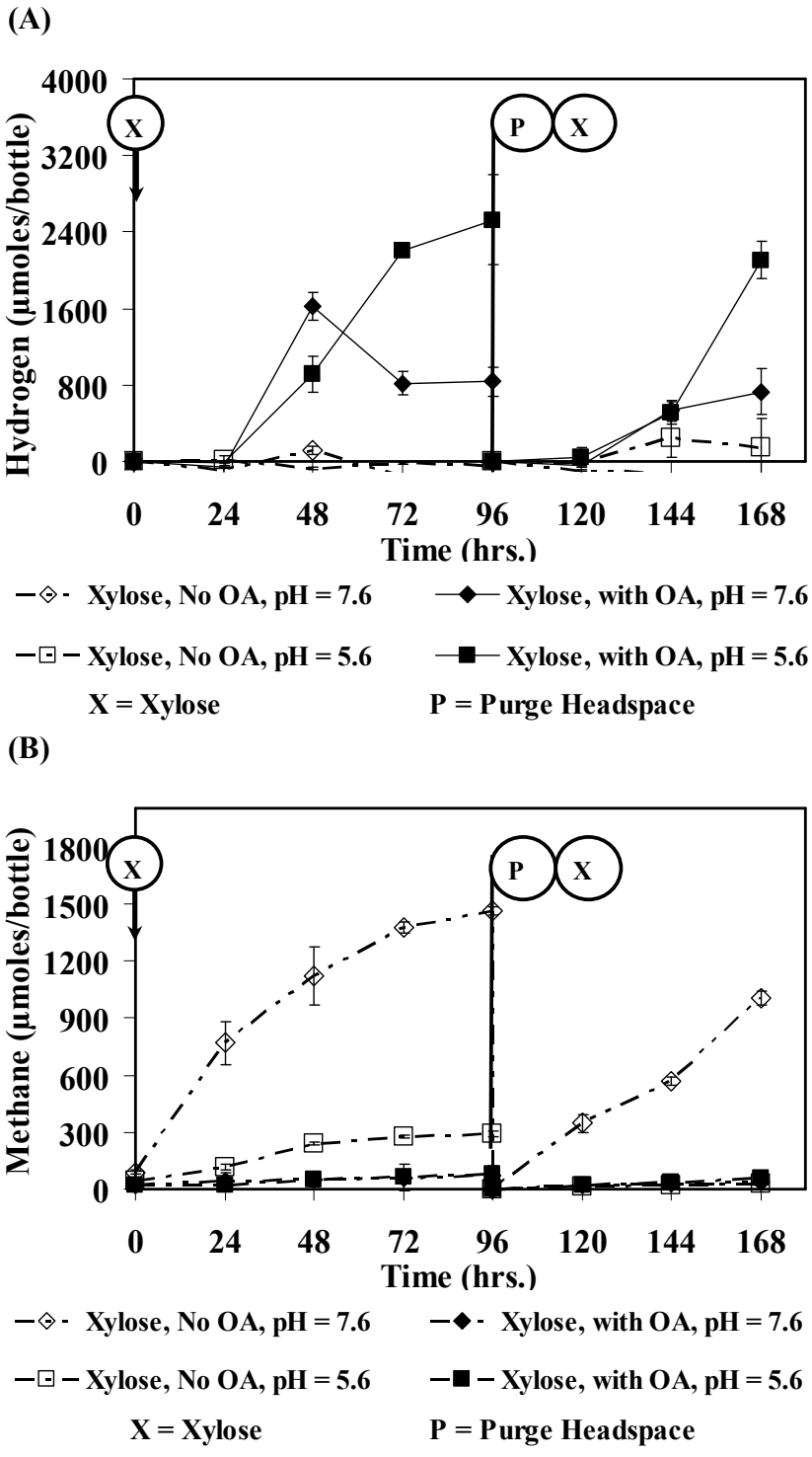


Figure 5.2 Gas production profiles for xylose (5,000 mg/L) degradation under different pH conditions in the presence of OA at 2,000 mg/L. Xylose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated by the standard deviation). (A) Hydrogen production and (B) Methane production.

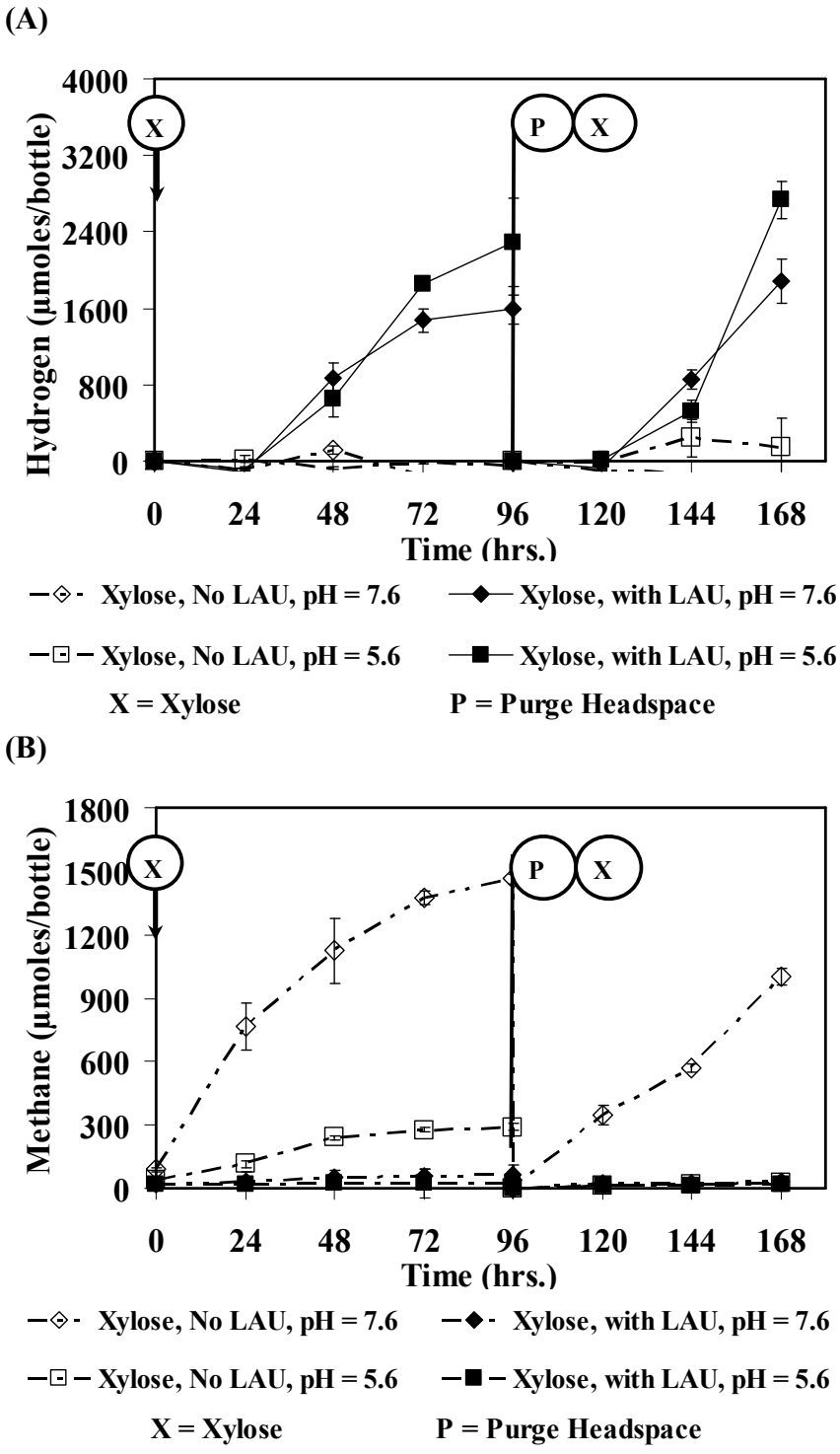


Figure 5.3 Gas production profiles for xylose (5,000 mg/L) degradation under different pH conditions in the presence of LAU at 2,000 mg/L. Xylose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated by the standard deviation). (A) Hydrogen production and (B) Methane production.

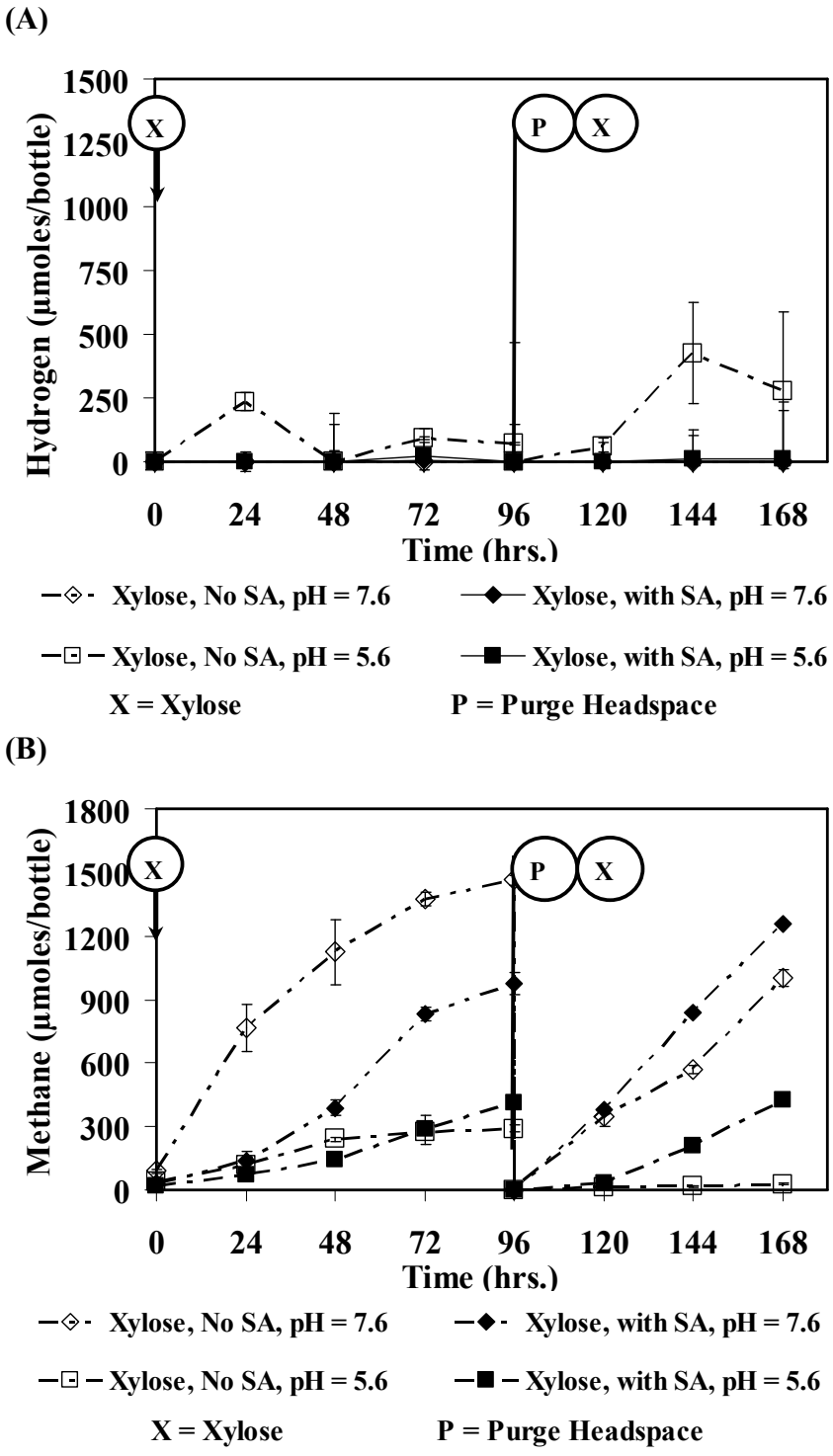


Figure 5.4 Gas production profiles for xylose (5,000 mg/L) degradation under different pH conditions in the presence of SA at 2,000 mg/L. Xylose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated by the standard deviation). (A) Hydrogen production and (B) Methane production.

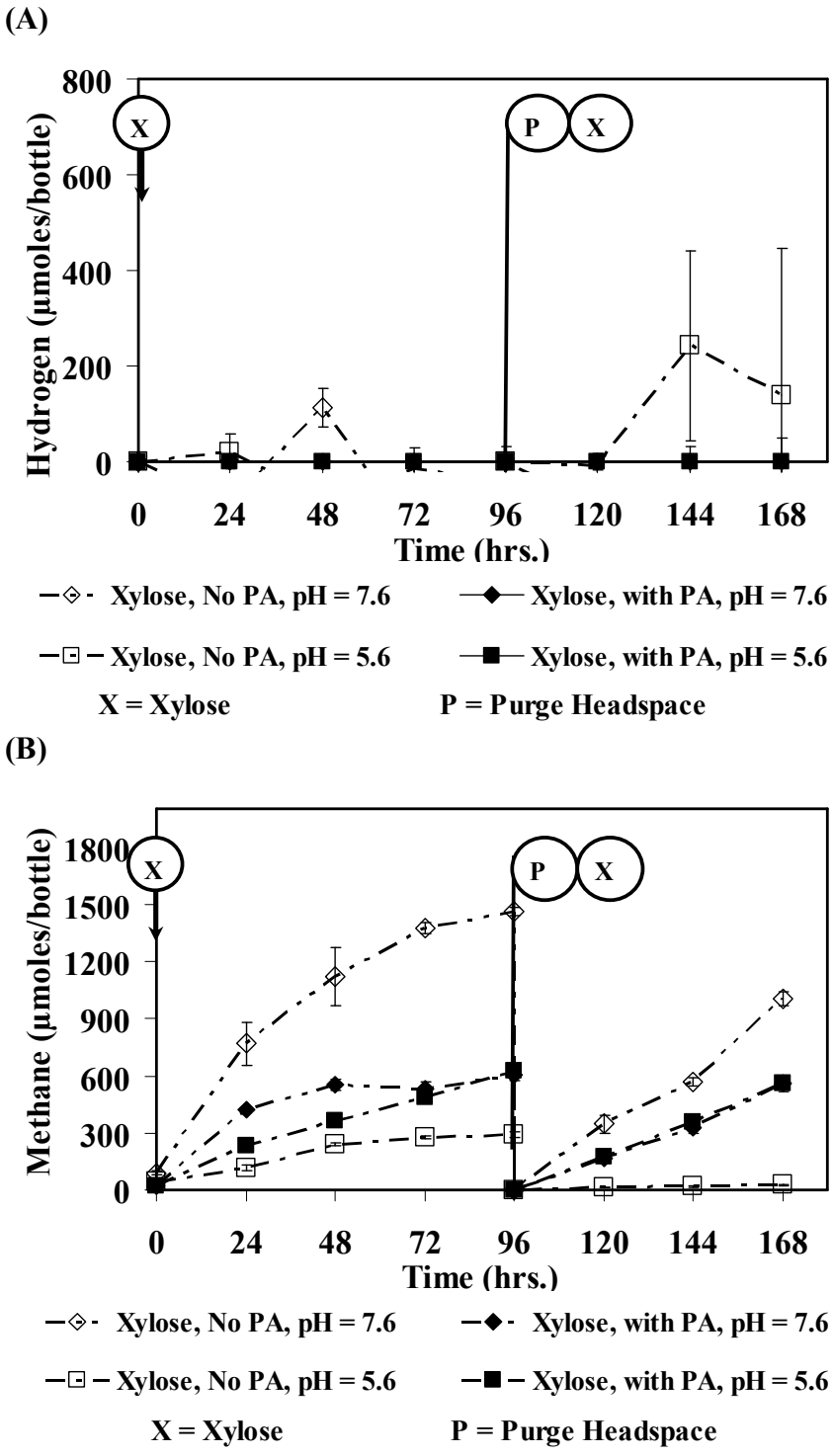


Figure 5.5 Gas production profiles for xylose (5,000 mg/L) degradation under different pH conditions in the presence of PA at 2,000 mg/L. Xylose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated by the standard deviation). (A) Hydrogen production and (B) Methane production.

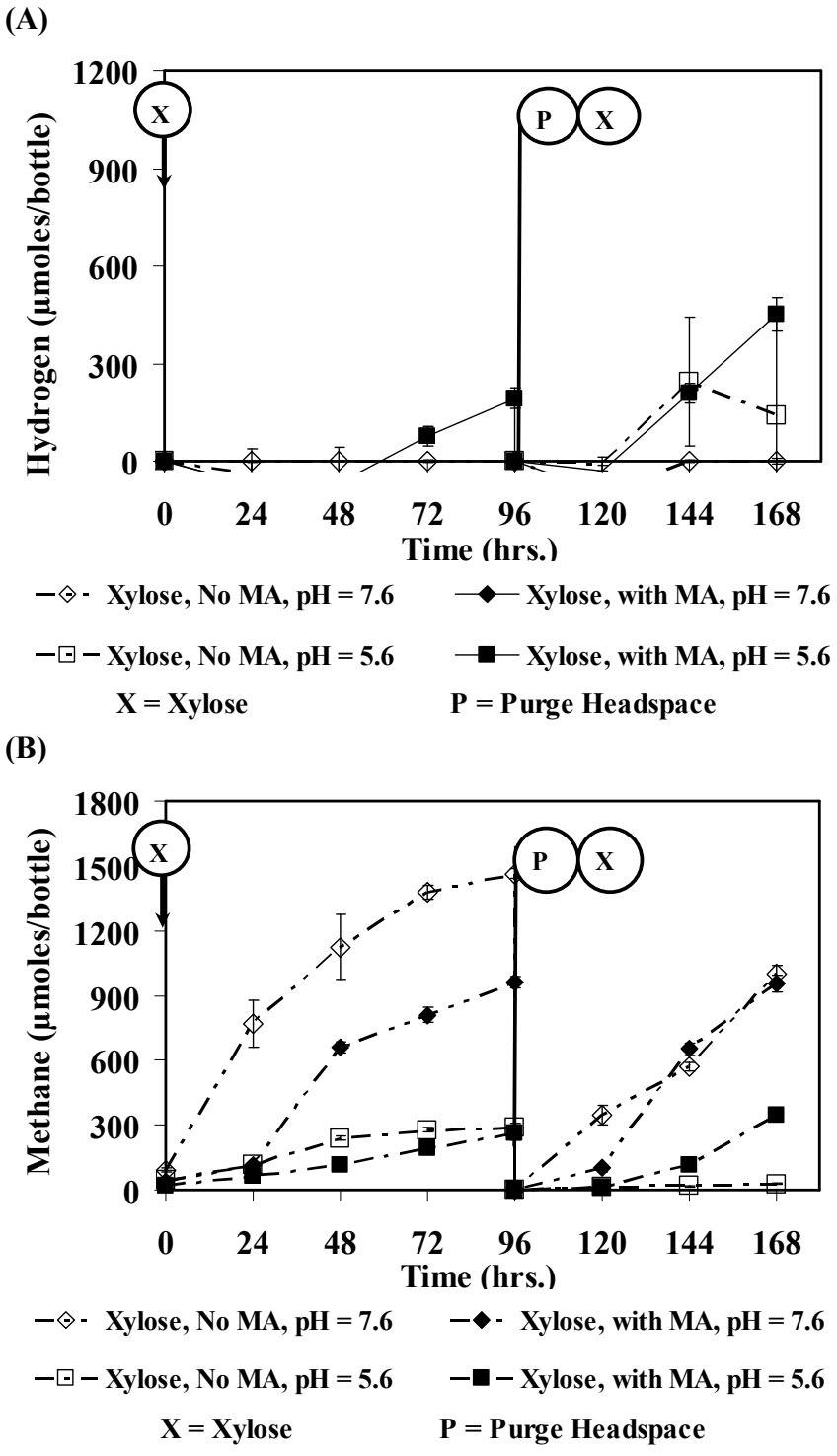


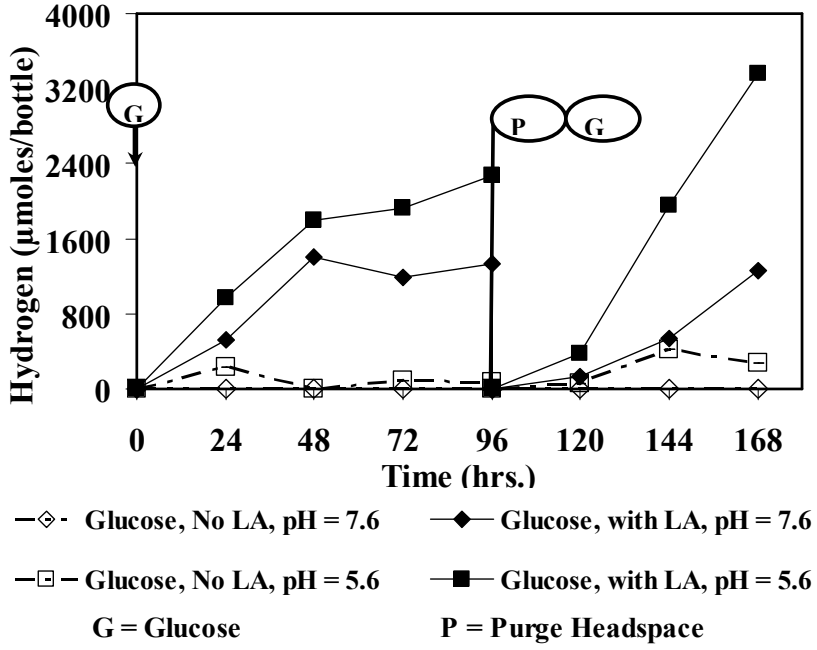
Figure 5.6 Gas production profiles for xylose (5,000 mg/L) degradation under different pH conditions in the presence of MA at 2,000 mg/L. Xylose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated by the standard deviation). (A) Hydrogen Production and (B) Methane Production.

5.3.2 Degradation of Glucose and Glucose/Xylose Mixtures in the Presence of LA

5.3.2.1 Hydrogen and Methane (75%/25%, 50%/50%, 25%/75% Xylose/Glucose, Glucose)

Methane inhibition with hydrogen production at pH 5.6 and 7.6 was observed in cultures fed with glucose (Figure 5.7). With 3,260 μmol of hydrogen produced at pH 5.6 and 3,400 μmol at pH 5.0 (see Chapter 4) yields of 2.42 mol H_2/mol glucose and 2.46 mol H_2/mol glucose, respectively, were observed. The hydrogen yield for cultures maintained at pH 5.6 and fed only xylose was similar to cultures receiving only glucose or the 50**%**/50**%** xylose and glucose mixture at the same pH (Table 5.2). The xylose study was conducted in phase one of this chapter and the results were converted from a mol basis to a mass basis to compare with the results obtained from studies with only glucose to those with the glucose plus xylose mixtures.

(A)



(B)

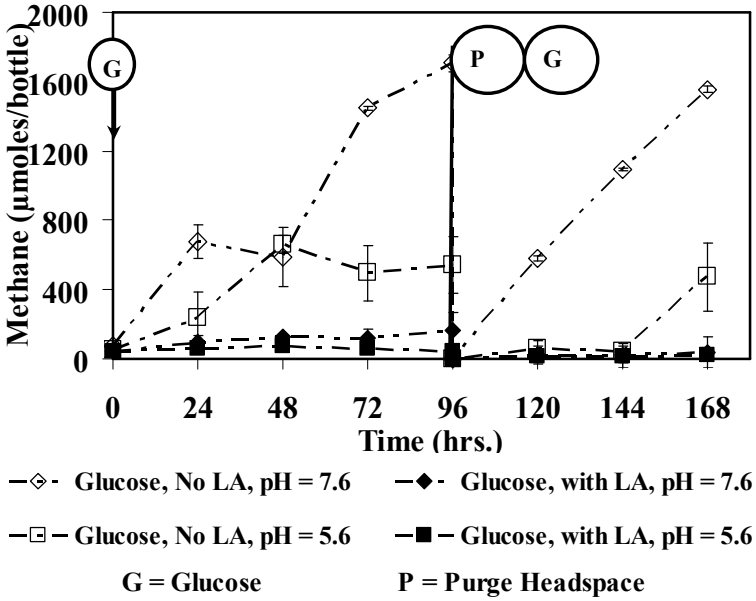


Figure 5.7 Gas production for glucose (5,000 mg/L) degradation under different pH conditions in the presence of LA at 2,000 mg/L. Glucose injected at t = 0 and again after purging at t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen production and (B) Methane production.

Table 5.2 Comparison of hydrogen yields (mmol H_2/g sugar) from 5,000 mg/L sugar injected into cultures at pH 5.6 with LA (2,000 mg/L) (X= xylose and G=glucose) (the data points represent triplicate samples; errors are indicated using the standard deviation)

Sugar	Xylose	75/25 X/G	50/50 X/G	25/75 X/G	Glucose
Yield	14.20±0.05	14.06±0.17	14.08±0.04	13.91±0.13	13.65±0.08

A Tukey's analysis was performed on the difference in the means between the different sugar ratios and the maximum and minimum yield. The analysis performed for the different ratios yielded q_s (1.308) < $q_{critical}$ (2.615) indicating the difference in the means is not significant. The analysis performed for the maximum and minimum values yield q_s (6.879) > $q_{critical}$ (3.2) indicating the difference is significant.

Figures 5.8 through 5.10 summarize hydrogen and methane data for various sugar mixtures under consideration. The methane production plots show methane inhibition with negligible quantities produced. These results are consistent with data for cultures

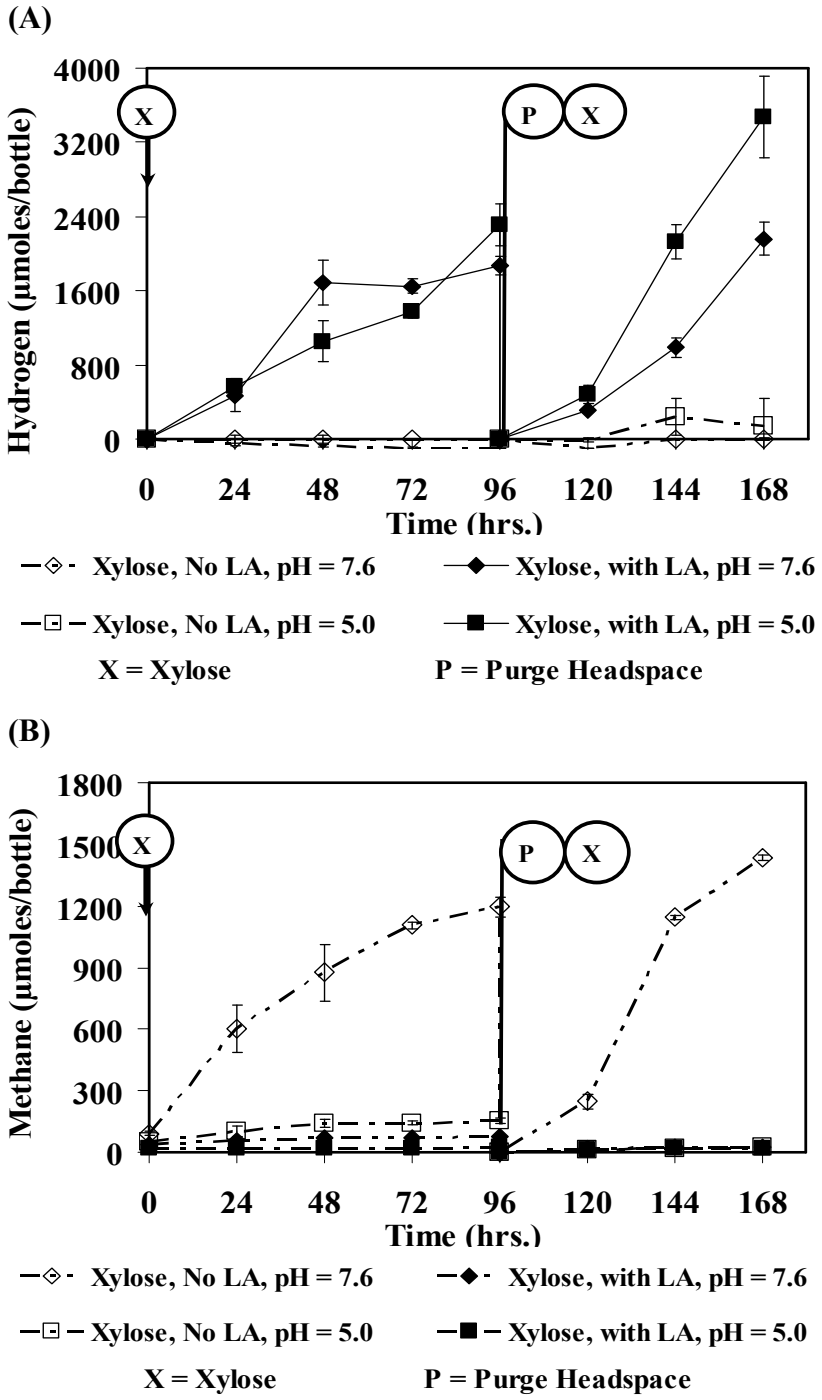
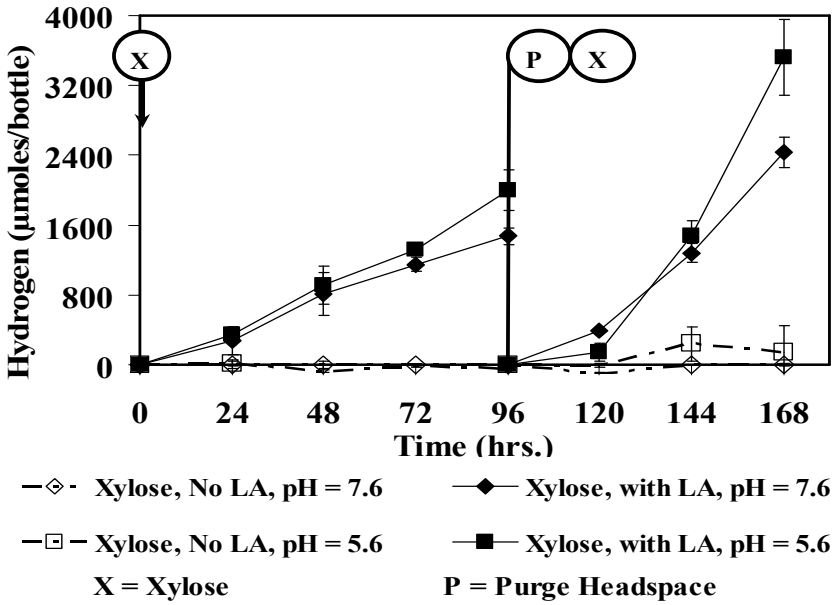


Figure 5.8 Gas production for the degradation of a 25%/75% mixture of xylose plus glucose (5,000 mg/L) under different pH conditions in the presence of LA at 2,000 mg/L. Sugars injected at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen production and (B) Methane production.

A)



(B)

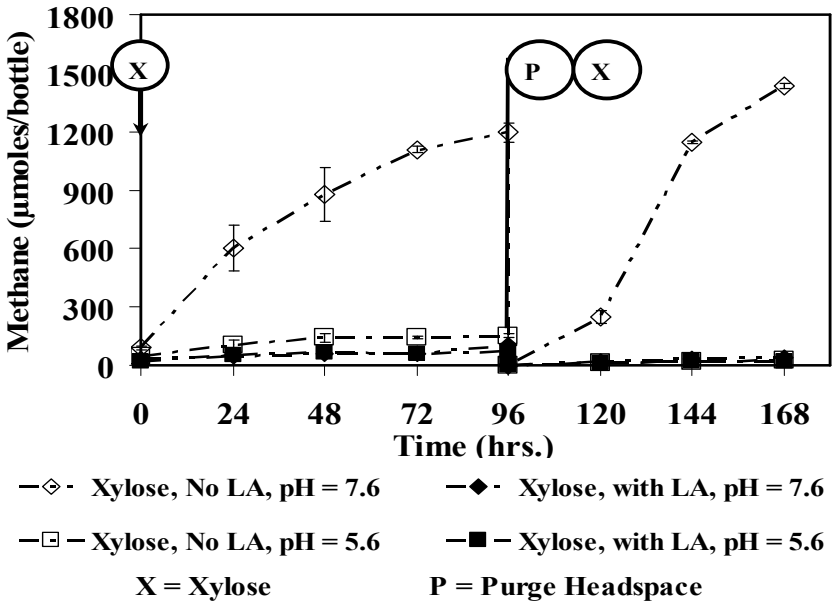


Figure 5.9 Gas production for the degradation of a 50%/50% mixture of xylose plus glucose (5,000 mg/L) under different pH conditions in the presence of LA at 2,000 mg/L. Sugars injected at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen production and (B) Methane production.

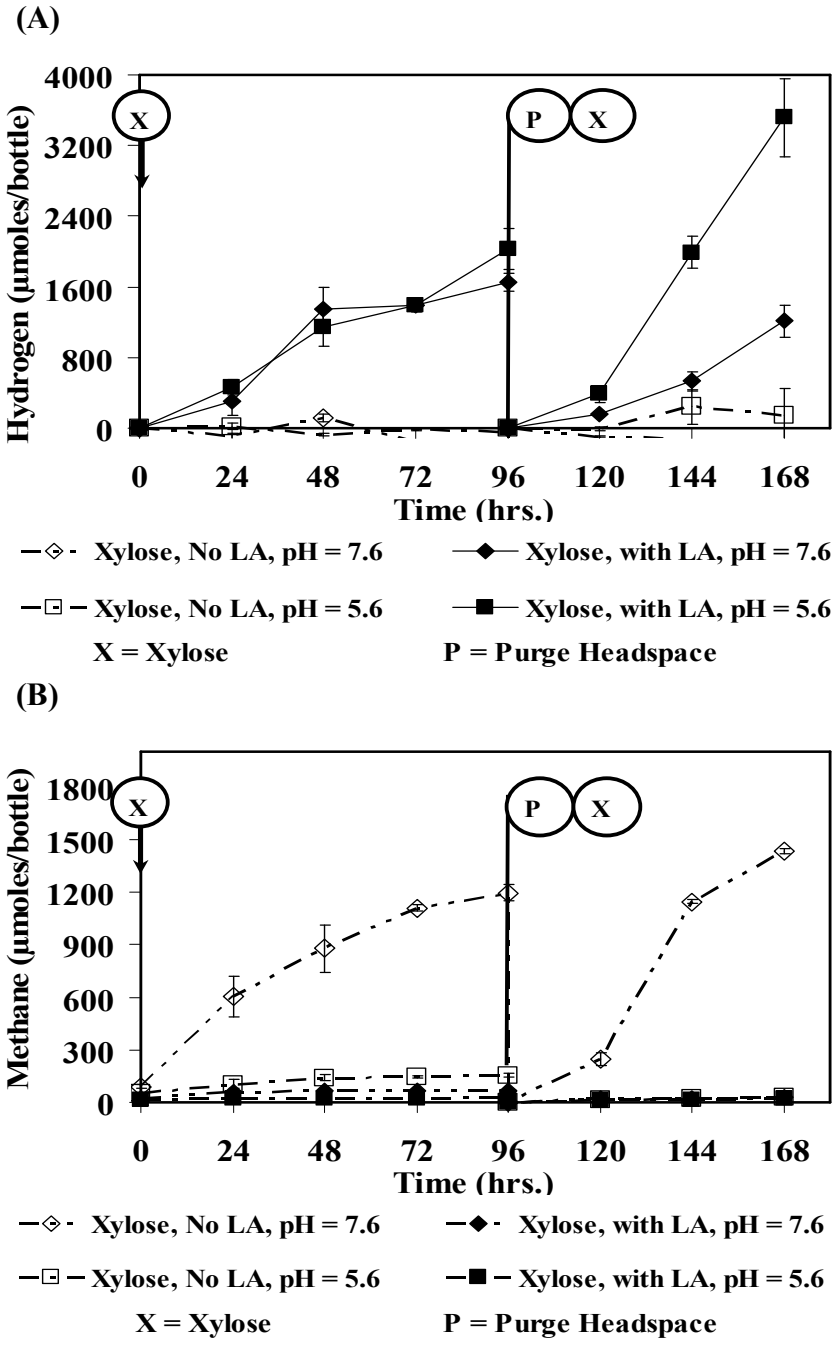


Figure 5.10 Gas production for the degradation of a 75%/25% mixture of xylose plus glucose (5,000 mg/L) under different pH conditions in the presence of LA at 2,000 mg/L. Sugars injected at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Hydrogen production and (B) Methane production.

Table 5.3 Hydrogen yield (mol H_2/mol xylose) from 5,000 mg/L xylose injected into cultures at pH 5.6 and 7.6 with LCFA (0 and 2,000 mg/L) (the data points represent triplicate samples; errors are indicated using the standard deviation)

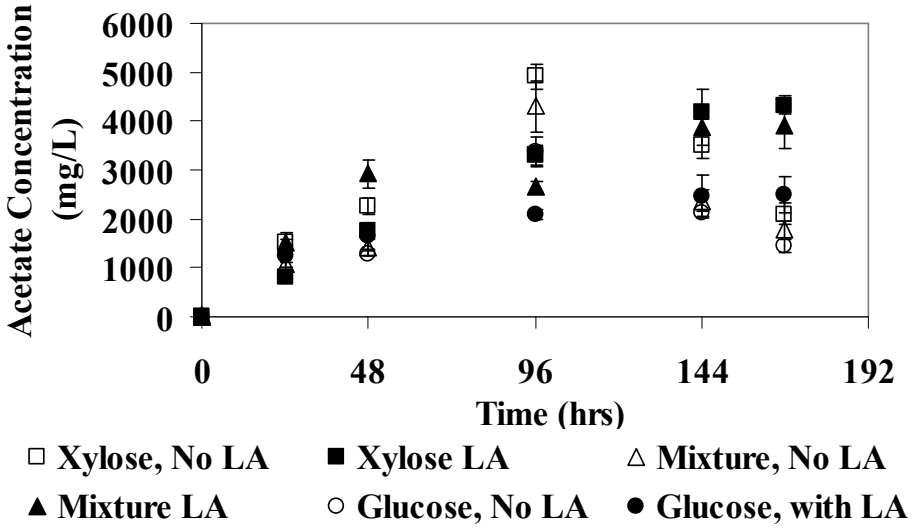
LCFA	Injection	0 mg/L		2000 mg/L	
pH		7.6	5.6	7.6	5.6
LA (C18:2)	1st	0	0	0.53±0.20	1.84±0.15
	2nd	0	0.1±0.12	1.34±0.13	2.13±0.05
OA (C18:1)	1st	0	0	0.54±0.31	1.65±0.11
	2nd	0	0.1±0.12	0.48±0.10	1.37±0.13
SA (C18:0)	1st	0	0	0	0
	2nd	0	0.1±0.12	0	0.01±0.01
PA (C16)	1st	0	0	0	0
	2nd	0	0.1±0.12	0	0
MA (C14)	1st	0	0	0	0.12±0.17
	2nd	0	0.1±0.12	0	0.27±0.11
LAU (C12)	1st	0	0	1.02±0.27	1.50±0.01
	2nd	0	0.1±0.12	1.21±0.05	1.78±0.18

fed glucose or xylose individually. High accumulation of hydrogen in cultures at pH 5.6 is observed. Hydrogen yields of 2.40, 2.41 and 2.32 mol H_2/mol substrate is observed for the 25%/75%, 50%/50% and 75%/25% xylose plus glucose mixtures, respectively. The control cultures show higher production of methane for cultures operating at pH 7.6 compared to those at pH 5.6.

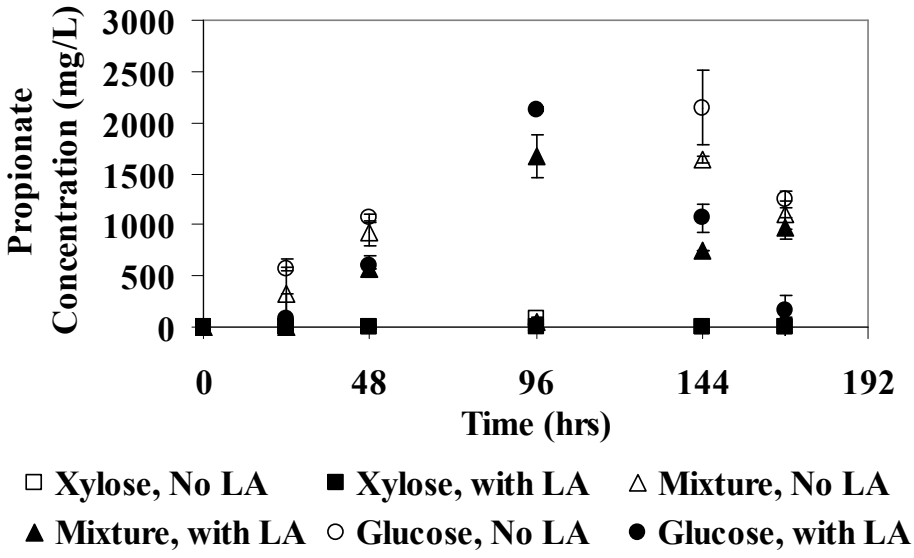
5.3.3 Volatile Fatty Acids (VFA) (100%Xylose, 50%/50% Xylose/Glucose, 100% Glucose)

Acetate, propionate and butyrate were produced in cultures fed with LA and those without any LA (Figure 5.11). In experiments conducted with xylose, acetate production occurs mainly from t = 24 hrs to t = 96 hrs with minimal production from t = 96 hrs to t = 168 hrs. Cultures without LA showed acetate degradation after t = 96 hrs (4,900 mg/L to 2,100 mg/L). Acetate data for cultures receiving the glucose plus xylose mixture with

(A)


Acetate Concentration (mg/L)
6000
5000
4000
3000
2000
1000
0
0
48
96
144
192
Time (hrs)
Xylose, No LA
Xylose LA
Mixture, No LA
Mixture LA
Glucose, No LA
Glucose, with LA

(B)


Propionate Concentration (mg/L)
3000
2500
2000
1500
1000
500
0
0
48
96
144
192
Time (hrs)
Xylose, No LA
Xylose, with LA
Mixture, No LA
Mixture, with LA
Glucose, No LA
Glucose, with LA

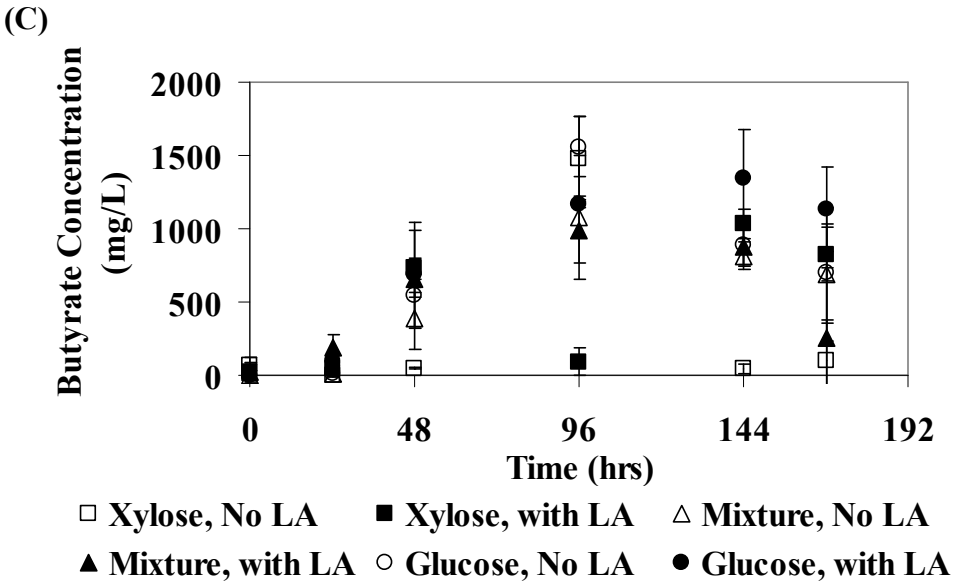


Figure 5.11 Volatile fatty acid production for cultures at pH 5.6 and fed 2,000 mg/L LA plus 5,000 mg/L sugars (glucose, xylose and glucose plus xylose). Sugars injected on t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Acetate Production, (B) Propionate Production, and (C) Butyrate Production.

LA show an overall increase in the amount of acetate up to t = 168 hrs of approximately 3,900 mg/L. The sugar mixture control shows a peak value at t = 96 hrs of approximately 4,300 mg/L then decreases to 1,800 mg/L at t = 168 hrs. Cultures fed glucose showed considerably less acetate production than those fed xylose and the sugar mixture. The acetate profile was similar for cultures fed xylose and the sugar mixture plus LA. The cultures fed LA had an overall acetate production up to t = 168 hrs of 2,500 mg/L and in the controls, acetate degraded after t = 96 hrs from 3,370 mg/L to 1,450 mg/L at t = 168 hrs.

Propionate production (Figure 5.11B) was very different for the xylose, glucose and 50/50 glucose plus xylose mixtures. In cultures fed xylose plus LA, only very small quantities were detected. In the control culture only a small amount (70 mg/L) of propionate was produced at t = 96 hrs and it was consumed by t = 144 hrs. Propionate production fin the control culture was delayed until t = 48 hrs. Propionate production in cultures fed the sugar mixture showed two different profiles. In cultures fed LA, a delay in production was observed up to t = 24 hrs then reaching a peak value at t = 96 hrs

(1,670 mg/L). At t = 144 hrs, the levels decreased and increased to 970 mg/L at t = 168 hrs. The control culture showed immediate propionate production with two peaks. The first peak occurred at t = 48 hrs (1,070 mg/L) and the second peak occurred at t = 144 hrs (2,150 mg/L). Propionate production in cultures fed glucose showed increased production and subsequent degradation as the experiment progressed. This trend was similar to the cultures fed the sugar mixture.

Butyrate production profiles are shown in Figure 5.11C. In the xylose plus LA fed cultures, a delay in production until t = 24 hrs was observed. Two peaks were observed at t = 48 hrs (730 mg/L) and at t = 168 hrs (1,030 mg/L). In the control cultures, a delay in butyrate production up to t = 48 hrs was detected and one major peak was observed at t = 96 hrs (1,480 mg/L). The butyrate production profiles for the sugar mixture show that the culture fed LA reached a peak value at t = 96 hrs (990 mg/L) and subsequently degraded at t = 168 hrs. In the controls, the peak at t = 96 hrs (1,080 mg/L) was degraded up to t = 168 hrs. The glucose fed cultures showed similar profiles with LA inhibition and without LA inhibition. The quantity of butyrate decreased after t = 96 hrs for cultures without LA and after t = 144 hrs for the culture fed LA and reached maximum values of 1,560 mg/L and 1,340 mg/L, respectively.

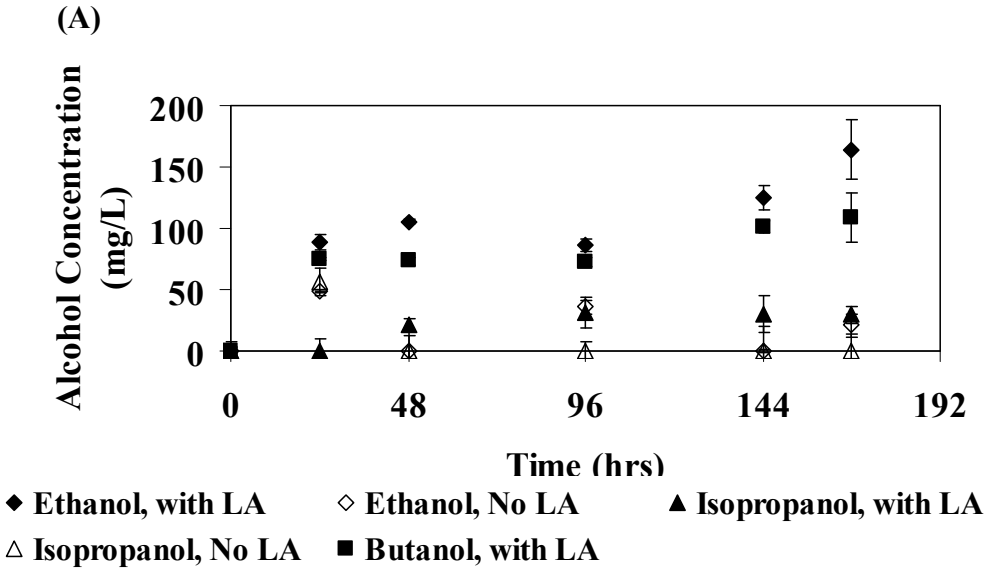
5.3.4 Alcohols (Xylose, 50%/50% Xylose/Glucose, Glucose)

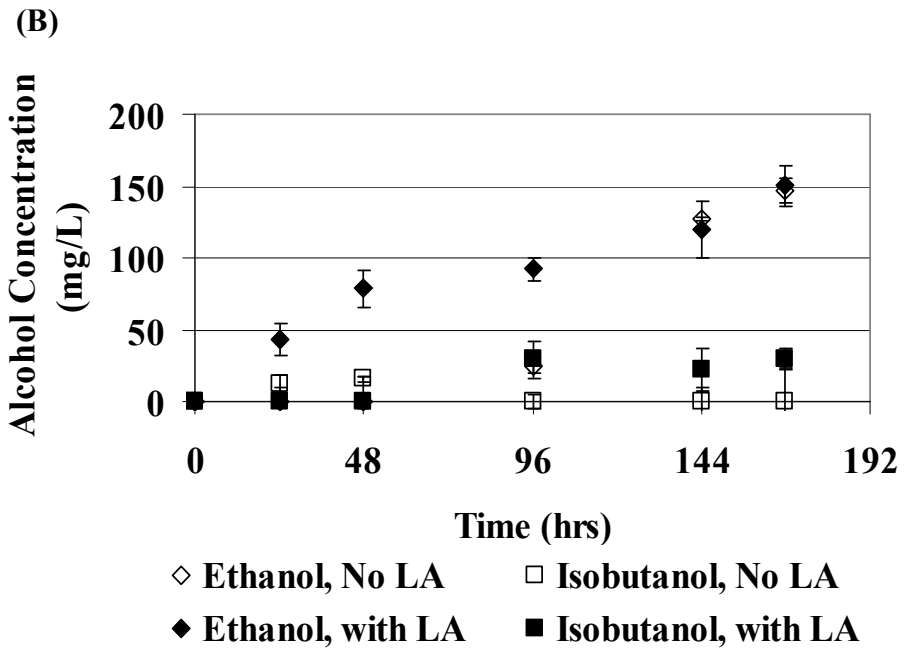
In the xylose controls, the alcohol production was very low. Ethanol and i-propanol were the only alcohols detected in the controls and in the cultures fed LA, the alcohol levels were relatively low (Figure 5.12). The alcohols detected in cultures fed LA were i-propanol and n-butanol, with ethanol being the most abundant alcohol (165 mg/L).

Alcohol production in glucose fed cultures was observed with LA addition and without LA addition. In cultures with LA, the alcohols were ethanol (150 mg/L) and i-butanol (30 mg/L). In cultures without LA, the alcohols detected were the same as those cultures fed LA.

In cultures fed the sugar mixture (glucose plus xylose) and LA, ethanol, n-butanol and i-butanol were detected; however, the levels were very low with ethanol showing the highest value (45 mg/L). In the control cultures, the same alcohols were produced. The

ethanol level reached 40 mg/L with n-butanol and i-butanol showing lower production levels than in cultures fed LA.





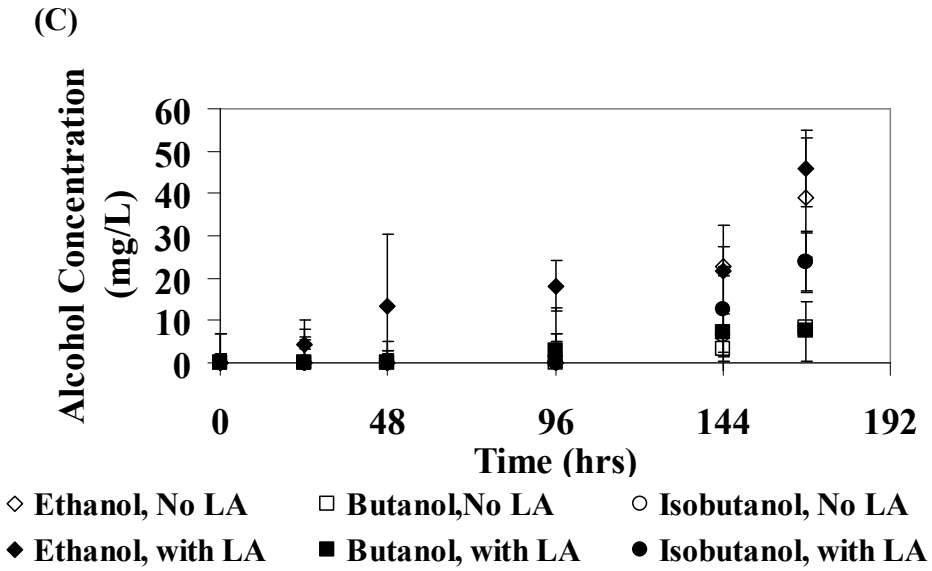


Figure 5.12 Alcohol production for cultures at pH 5.6 and fed 2,000 mg/L LA plus sugars (glucose, xylose and glucose plus xylose). Sugars injected at t = 0 and t = 96 hrs (the data points represent triplicate samples; errors are indicated using the standard deviation). (A) Samples fed 5,000 mg/L xylose. (B) Samples fed 5,000 mg/L glucose. (C) Samples fed 2,500 mg/L xylose and 2,500 mg/L glucose together.

5.3.5 Sugar Degradation

No glucose was detected after 24 hrs period; however, xylose was detected (235 mg/L) in the culture fed LA (Figure 5.13). Adding the LCFA decreased the initial degradation rate (Table 5.4). Glucose was degraded after 24 hrs and its rate of degradation was greater than that of xylose.

Table 5.4 Initial sugar degradation rates (µg/mg VSS/min) for cultures containing 5,000 mg/L 50/50 glucose and xylose mixture with and without LA at pH of 5.6 (each data point are for triplicate samples; errors are indicated using the standard deviation).

Sugar	Control (0 mg/L LA)	2,000 mg/L LA
Glucose	5.35±0.72	4.23±0.67
Xylose	2.42±0.47	1.94±0.19

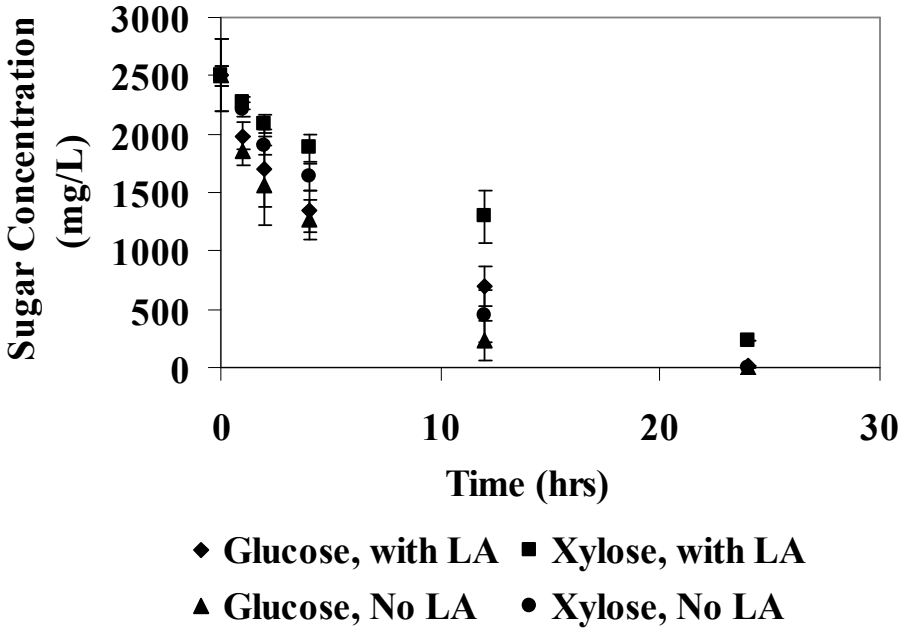


Figure 5.13 Xylose and glucose (5,000 mg/L total) degradation plus 2,000 mg/L LA at a pH of 7.6 (glucose and xylose added at t = 0, the data points represent triplicate samples; errors are indicated using the standard deviation).

A Tukey's analysis was performed to establish if any statistical differences exists in the means for the initial degradation rates. The analysis compared the date set for the controls (no LCFAs) and cultures fed xylose or glucose with LA. The analysis showed insignificant differences in means for cultures fed glucose with LA compared to those fed glucose without LA (q_s (1.67) < $q_{critical}$ (2.15)). In addition, insignificant differences were also observed between the means for cultures fed xylose with LA and those fed xylose without LA (q_s (2.53) < $q_{critical}$ (4.95)). However, the analysis showed a significant statistical difference between the means for cultures fed glucose versus xylose with LA and without LA (q_s (12.05, 6.23) > $q_{critical}$ (7.05, 3.06)).

5.3.6 Electron Balance

The electron balance is based on the transfer of electrons from glucose, xylose and a mixture of both xylose and glucose to the various byproducts (CH_4, H_2, acetate, butyrate, ethanol, i-propanol, n-propanol, i-butanol and n-butanol). LCFAs is a relatively slowly degrading substrate in comparison to xylose and glucose. Alosta (2004) reported a LCFA degradation rate of approximately 0.035 µg/mg VSS/min and the sugar

degradation from this work is between approximately 5.35 – 1.94 µg/mg VSS/min. Hence, the electron contribution from LA degradation is relatively small and is ignored from the electron mass balance. The electron balance (Figure 5.14) shows that approximately 86%±19% of the total electrons from xylose are present in the degradation by-products and approximately 10% is assumed to be used in cell synthesis. The electron mass balance was also developed for the sugar mixture and glucose.

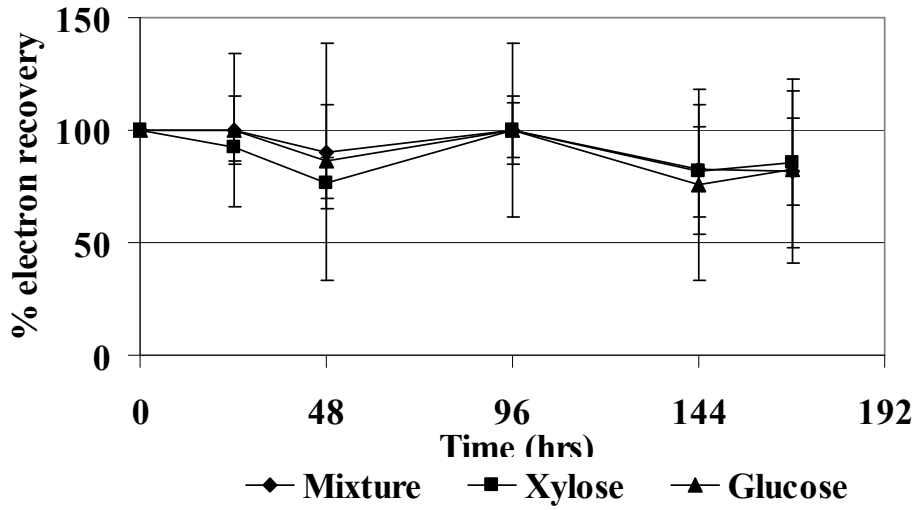


Figure 5.14 Electron mass balance for cultures fed glucose, xylose and a 50%:50% mixture of xylose plus glucose plus 2000 mg/L LA at pH 5.6 (the data points represent triplicate samples; errors are indicated using the standard deviation).

Equations 5.1, 5.2 and 5.3 are based on combining reactions for the electron donors and electron acceptors. The half-reactions for the products and reactants are combined and normalize for xylose, glucose and the 50%/50% glucose plus xylose mixture. Approximately 10% of the electrons from the substrate are assumed to be converted into cell mass. With these values there is a shortfall of electrons in the products totaling 4%, 8% and 7% for cultures fed xylose, the sugar mixture and glucose, respectively.

Xylose

$$C_5H_{10}O_5 + 0.1\ NH_4^+ + 1.382\ HCO_3^- \rightarrow 0.058\ CH_4 + 2.068\ H_2 + 1.134\ CH_3COO^- + 0.148\ CH_3CH_2CH_2COO^- + 0.056\ CH_3CH_2OH + 0.008\ CH_3CH_2CH_2OH + 0.023\ CH_3CH_2CH_2CH_2OH + 0.1\ C_5H_7O_2N + 2.736\ CO_2 + 0.823\ H_2O \quad (5.1)$$

50% Xylose 50% Glucose Mixture

$$0.455C_6H_{12}O_6 + 0.545C_5H_{10}O_5 + 0.109NH_4^+ + 1.554HCO_3^- \rightarrow 0.109CH_4 + 2.151H_2 + 1.159CH_3COO^- + 0.233CH_3CH_2COO^- + 0.052CH_3CH_2CH_2COO^- + 0.036CH_3CH_2OH + 0.007CH_3CH_2CH_2CH_2OH + 0.011CH_3CH_2CHOHCH_3 + 0.109C_5H_7O_2N + 2.983CO_2 + 0.988H_2O \quad (5.2)$$

Glucose

$$C_6H_{12}O_6 + 0.120NH_4^+ + 1.494HCO_3^- \rightarrow 0.038CH_4 + 2.297H_2 + 0.810CH_3COO^- + 0.314CH_3CH_2COO^- + 0.250CH_3CH_2CH_2COO^- + 0.053CH_3CH_2OH + 0.008CH_3CH_2CHOHCH_3 + 0.120C_5H_7O_2N + 3.157CO_2 + 1.120H_2O \quad (5.3)$$

5.4 Discussion of Results

LCFAs are inhibitory to several anaerobic populations. According to several reports, these populations include acidogens, acetogens, acetoclastic methanogens and hydrogenotrophic methanogens (Hallenbeck and Benemann, 2002; Joubert and Britz, 1987; Mara and Horan, 2003; Gujer and Zehnder, 1983). The hydrogen producers and consumers are especially important in this present study because the net outcome is to minimize any inhibition on the hydrogen producing populations and maximize the inhibition imposed upon the hydrogen consumers by adding inhibitors. LCFAs and varying pH values were used to accomplish this task.

Of the LCFAs that showed methanogen inhibition and a sizeable amount of hydrogen gas production, LA at pH 5.6 showed the greatest effect with a value of 2.13 mol H_2/mol xylose and 2.32 mol H_2/mol substrate for the fermentation of xylose and

50/50 xylose to glucose mixture respectively. These optimal conditions are consistent with the results reported in Chapter 4. The data from Chapter 4 showed when LA was fed to cultures maintained at an initial pH of 5.0, the highest hydrogen yield was observed. The hydrogen yield results are significantly higher than values reported by Lin et al. (2008). These authors reported a hydrogen yield of 1.3 mol H_2/mol xylose for a system operating at 40°C and pH 7.1. Lin et al. (2006) also reported a yield of 1.3 mol H_2/mol xylose at 35°C and pH 6.5. A yield of 0.8 mol H_2/mol xylose at 50°C and pH 6.5 under steady state continuous tests was reported by Wu et al. (2008). While Konjan et al. (2009) reported a yield of 1.36 mol H_2/mol xylose at 70°C and neutral pH conditions. All experiments in these reports were performed using heat shocked cultures in CSTR reactors operating at pH values well above the pH 5.6 value used in this study.

The pH value of the culture had a significant effect on the quantity of hydrogen produced. As shown in the glucose, xylose and glucose and xylose mixture gas profiles, cultures operating at pH 7.6 yielded a significantly lower quantity of hydrogen than those operating at pH 5.6. Lin et al. (2006) reported an optimal pH value of 6.5 and hydrogen production of 1.3 mol H_2/mol xylose from xylose fermentation using a heat shocked culture at 35°C. Results from data presented in Chapter 4 and the results of studies in this chapter show the low pH condition as optimal for producing hydrogen. A hydrogen yield of 2.13 mol H_2/mol xylose at pH 5.6 suggests that using LCFAs along with low pH conditions have a positive synergistic effect for increasing the hydrogen production. Synergistic effects on hydrogen production have been reported by Van Ginkel and Logan (2005) where a combination of acetic and butyric acid along with a pH value of 5.5 yield a higher amount of hydrogen from glucose fermentation than either the acids or low pH value alone.

Other LCFAs evaluated for xylose fermentation to hydrogen had varied results. LAU and OA showed complete methane suppression; however, lower yields of hydrogen were observed (1.78 and 1.65 mol H_2/mol xylose respectively) when compared to the effects of LA. Cultures fed MA, PA and SA all showed methane production and in the case of MA fed cultures, a small hydrogen was produced (0.27 mol H_2/mol xylose). Cultures fed PA and SA produced only negligible amount of hydrogen. The results from cultures fed LAU (Figure 5.3) and MA (Figure 5.6) are consistent with data reported by

Soliva et al. (2003). They suggest that LAU is able to impose a 96% suppression of methanogenic while MA alone has negligible effects. Cultures fed OA showed consistent results with Hwa and Lettinga (1997). They reported an OA concentration of 3.7 mM or 1,000 mg/L caused an 80% reduction in specific methanogenic activity. The results of this study showed complete inhibition of methanogenic activity at 2,000 mg/L or 7.1 mM OA.

The LCFAs used had a significant effect on hydrogen production. The trend for increasing amounts of hydrogen produced was as follows: LA (2.13 mol H_2/mol xylose) > LAU (1.78 mol H_2/mol xylose) > OA (1.65 mol H_2/mol xylose) > MA (0.27 mol H_2/mol xylose). No detectable quantity of hydrogen was produced in cultures fed PA or SA. One aspect of the LCFA addition is that the LCFAs were heated using a hot water bath until they became a liquid and then injected into the culture. LA did not have to be heated and OA and LAU had only to be warmed to approximately 40°C. Whereas the MA had to be heated up to approximately 60°C while for PA and SA, the temperature had to increased to 100°C before they transitioned into the liquid state. When the LCFA was injected into the culture which was kept at approximately 23°C, the hot solution containing MA, PA and SA immediately became a solid and did not mix properly with the culture. Therefore, mass transfer with the microorganisms was impaired and could have affected their inhibitory effect on different microorganisms.

Hydrogen production from the fermentation of different sugars (glucose, fructose and sucrose) have been reported by many researchers. Fang et al. (2002) along with Chen et al. (2001) reported a hydrogen yield of 1.88 mol H_2/mol hexose using a sucrose feedstock at pH 5.5 at 37°C. Khanal et al. (2004) reported a yield of 1.62 mol H_2/mol hexose for a culture fed sucrose at a pH of 4.5 and operating at 37°C. Hussey et al. (2005) used a wastewater stream containing sugar beet at a pH of 5.2 and 32°C and reported a yield of 1.66 mol H_2/mol hexose. A consistent yield of hydrogen gas is important when using different sugars or a combination of sugars for the process to be economically feasible.

Low cost feedstocks are important for economical reasons and cellulose based biomass is a low cost feedstock. Cellulose based biomass is composed of 55-65% hexose sugars and 35-45% pentose sugars (Huang and Logan, 2008; Wu et al., 2008) and for

biological reactors to be operated successfully, they will have to process both of these substrates. Glucose and xylose were fed at three different ratios in order to determine if the ratio had an effect on the hydrogen yield. The gas production profiles (Figure 5.8-5.10) show consistent hydrogen yields regardless of the ratio of sugars used. Hydrogen yields of 14.07±0.17, 14.08±0.04, and 13.91±0.13 mmol H_2/g sugar using 25%/75%, 50%/50% and 75%/25% mixture of glucose and xylose, respectively, at pH 5.6 and plus LA were observed consistently. This indicates that a mixed anaerobic culture can use two different carbon sources (hexose and pentose sugars) in different ratios simultaneously. The hydrogen yields convert to an average of 2.38±0.10 mol H_2/mol substrate. Ren et al. (2008) reports a yield of 2.42 mol H_2/mol substrate using a pure culture at pH 6.5 and 60°C. In this work, hydrogen production from fermentation of a combination of different hexose and pentose sugars was feasible.

The alcohol produced in the largest quantity during xylose fermentation in the presence of LA was ethanol followed by n-butanol and i-propanol. The concentration of the alcohols produced in this work was very low compared to data reported by Lin et al. (2006). They reported an ethanol production of 2.6 g/L with a xylose feed concentration of 20 g/L and a hydrogen yield of 1.3 mol H_2/mol xylose. In comparison, the amount of ethanol produced in this study from xylose fermentation was 0.165 g/L with a xylose feed concentration of 5,000 mg/L and a hydrogen yield of 2.13 mol H_2/mol xylose.

When glucose was the electron donor, acetate was the major VFA byproduct produced followed by butyrate and propionate. Ray et al. (2008) reported that acetate (2,800 mg/L) was a significant VFA produced in batch reactors during glucose fermentation using LA at 37°C and pH 5.0. In comparison, Lin and Chang (2004) reported the major VFAs produced were as follows: butyrate (4,200 mg/L), acetate (3,300 mg/L) and propionate (1,000 mg/L). The major difference in VFA production from xylose fermentation as opposed to glucose fermentation could is attributed to no significant amount of propionate production. These results are consistent with xylose fermentation studies reported by other researchers. Konjan et al. (2009) and Lin et al. (2006) reported <1% propionate production for 70°C and neutral pH and at 35°C and pH 6.5, respectively. The butyrate profiles for xylose fermentation show very different profiles between cultures fed LA and cultures not fed LA. In the cultures fed LA there

was very small quantities of butyrate breakdown and in the cultures not fed LA there were large amounts of butyrate were degraded.

VFA data from the 50%/50% glucose plus xylose mixture study indicates that acetate was the main product followed by butyrate and propionate. The propionate profile for the sugar mixture plus LA (Figure 5.11B) show a similar trend as the propionate profiles for the cultures fed glucose plus LA. Notice, the propionate levels in xylose fed cultures with and without LA was negligible. While propionate production varied from xylose to glucose fermentation, butyrate production of xylose and glucose (1,030 and 1,133 mg/L, respectively) (Figure 5.11C) was similar during fermentation of the two sugars.

The electrons liberated during the fermentation process are diverted into the production of by-products, cell biomass and energy. The percent electron recovery for xylose, glucose and the sugar mixture are 86%±19%, 83%±34% and 82%±41%, respectively. Kongjan et al. (2009) reported a 86% COD recovery from xylose fermentation. The unaccounted electrons could be either tied up in products which were not measured and also a fraction used in biomass synthesis. As stated in Chapter 2 an anaerobic bacterial cell can use approximately 10% of the available energy in cell synthesis. The assumed value of 10% biomass is slightly lower than the fraction reported by Kotsopoulos et al. (2006) of 15%.

The degradation profiles of xylose follow a similar trend to that of the glucose profiles as the control cultures have the highest initial degradation rate (Table 5.3) in the case of both sugars. The samples fed LA showed a slower initial breakdown rate and in the culture fed LA, xylose was not fully degraded within 24 hrs. Lalman and Bagley (2002) reported the inhibitory effects of LA, OA and SA on glucose degradation using mixed cultures while in this study, LA inhibited xylose and glucose fermentation.

5.5 Conclusion

Finding the optimal LCFA to maximize the hydrogen yield from xylose fermentation and determining the effect of varying ratios of xylose to glucose on hydrogen production were the objectives for the work described in Chapter 5. The LCFAs examined were LA, OA, SA, PA, MA, and LAU at pH values 7.6 and 5.6. In another part of the study three ratios (25%/75%, 50%/50% and 75%/25%) of xylose to glucose were selected to study the effect on the hydrogen yield. The results from work conducted with different LCFAs demonstrated that adding 2,000 mg/L LA at an initial pH of 5.6 caused the microbial system to produce a maximum yield of 2.13±0.05 mol H_2/mol xylose. Varying the sugar ratio did not show a significant effect on the hydrogen yield. This suggests that chemical feedstocks consisting of a variety of sugars could serve as electron donors for hydrogen production from low value biomass.

Chapter 6
CONCLUSIONS

Many reports have shown that glucose fermentation to hydrogen using mixed cultures is a possible route for producing hydrogen. However, adding flexibility through the use of a variety of feedstocks could improve the process economics. Xylose is a sugar which is present in large quantities in low value biomass and it could be used as an electron donor. In this study, the hydrogen yields from xylose fermentation were comparable to those from glucose. Both sugars were examined independently and combined in the presence of different LCFAs and pH values for the production of hydrogen. LCFAs were used to prevent hydrogen uptake by hydrogen consumers and hence, increase the hydrogen yield.

The purpose of the glucose fermentation described in Chapter 4 was to establish the optimal conditions for hydrogen production in the presence of LCFAs. The LCFAs selected (LA and OA) were based on data from past studies (Ray et al., 2008; Chowdhury et al., 2007). The optimal conditions of pH 5.0 and LA at 2,000 mg/L yielded 2.46 mol H_2/mol glucose. These conditions are used in the selection of operating conditions in the experiments using glucose plus xylose mixtures. Based on these conditions, values for experimental variables were selected to assess the impact of different ratios of glucose and xylose on hydrogen production.

Selecting an appropriate LCFA for xylose fermentation was conducted by assessing the impact of LCFAs bearing 18, 16, 14 and 12 carbons on hydrogen production. Adding LA and adjusting the pH to 5.6 yielded 2.13 mol H_2/mol xylose. These conditions were used to assess hydrogen production from different ratios of glucose to xylose.

The sugar mixture ratios used in this study were 25%/75%, 50%/50% and 75%/25%. Data from this work demonstrated that differences in the sugar ratios did not show any significant effects on the hydrogen yield.

The major conclusions of this study are as follows:

1. The maximum yield of 2.46 mol H_2/mol glucose was observed in cultures fed 2,000 mg/L LA at a pH of 5. The yield of 2.46±0.08 mol H_2/mol glucose was 62% efficient based on maximum yield of 4 mol H_2/mol glucose.
2. A lower hydrogen yield (1.25±0.19 mol H_2/mol glucose) was observed in cultures fed with 2,000 mg/L OA and maintained at pH 5.0
3. A FFD was performed on the results from Chapter 4. The model was used to generate an equation which described the effect of LCFA concentration and pH on hydrogen yield. The equations were as follows: 1. For LA, H_2 yield = 2.7902 + 0.0007*[LA] - 0.3391*(pH) and 2. For OA, H_2 yield = 2.3651 + 0.0003*[OA] - 0.3347*(pH).
4. The maximum yield of hydrogen from xylose fermentation was 2.13±0.05 mol H_2/mol xylose with a conversion efficiency of 64.0% compared to a maximum yield of 3.33 mol H_2/mol xylose. The conditions for the xylose fermentation study were 2,000 mg/L LA and at a pH of 5.6.
5. In cultures fed with xylose plus different LCFAs, the hydrogen production trend was as follows: LA > LAU.> OA > MA. No significant quantities of hydrogen were observed in cultures fed SA and PA.
6. The hydrogen yields for glucose and xylose fermentation on a mass of sugar basis were 13.65±0.08 mmol H_2/g sugar and 14.20±0.05 mmol H_2/g sugar, respectively. These yields were obtained for cultures fed with 2,000 mg/l LA and maintained at a pH of 5.6
7. The hydrogen yield for cultures fed different sugar ratios of 25%/75%, 50%/50% and 75%/25% xylose to glucose were 13.91±0.13, 14.08±0.04 and 14.060.17 mmol H_2/g sugar, respectively. These yields suggest that varying the sugar ratio did not show significant effect on the hydrogen yield.

Chapter 7
Engineering Significance and Future Recommendations

The significance of this study was to demonstrate that sugar mixtures composed of glucose and xylose can be fermented to hydrogen. Hydrogen is known to be a clean and efficient energy source in which has gained popularity as energy prices have increased and pollution associated with the use of fossil fuels has increased. Cellulosic materials are becoming a possible feedstock source for hydrogen production. The breakdown of cellulosic material yields a mixture of hexose and pentose sugars. A process able to ferment both of these sugars to hydrogen would be cost effective. The use of low value biomass as a feedstock source could lead to the development of a process to produce hydrogen from renewable sources.

Using LCFAs and pH to inhibit methanogenic growth will likely eliminate other methods such as heat shock. Heat shocking cultures can become energy intensive if the reactor size is very large. With the cultures only requiring LCFA addition and pH adjustment this technology could be adapted to existing anaerobic reactors.

Based on the results of this study further research could involve moving to pilot scale studies. Recommendations for future research are as follows.

1. Optimize the conditions of a large batch process to determine if the results are consistent with small batch reactors.
2. Develop and optimize a process to maximize the sugar mass loading and constantly remove the hydrogen gas.
3. In the current process development work, VFAs are not degraded. Hence a secondary process could be added to degrade the by-products from the hydrogen production reactions, namely the acids and the alcohols.
4. Optimize the operating conditions of a bacterial culture in order to break down the unwanted products of hydrogen fermentation
5. Establish the effects of different inoculum sources on hydrogen production

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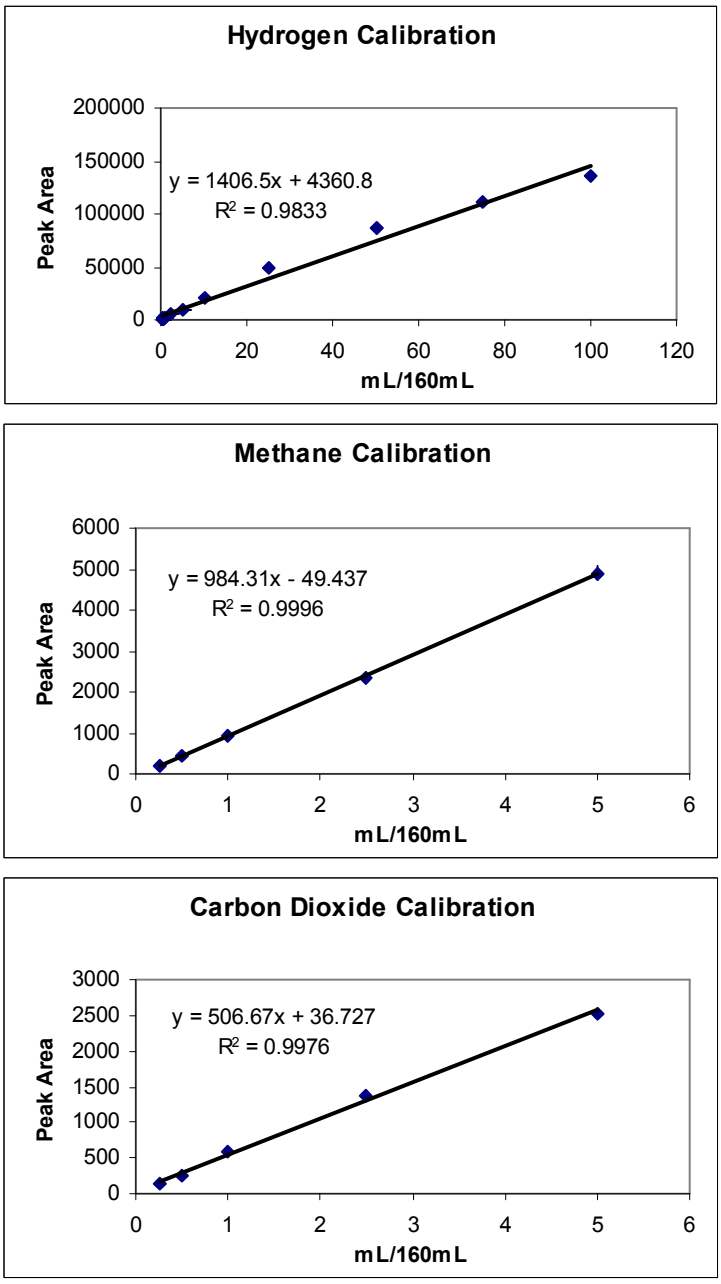
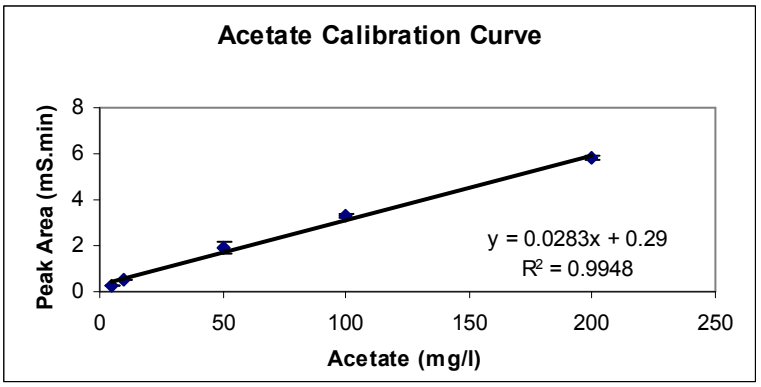
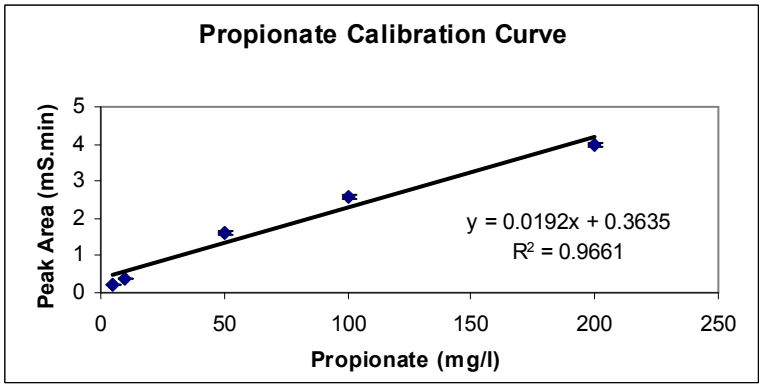
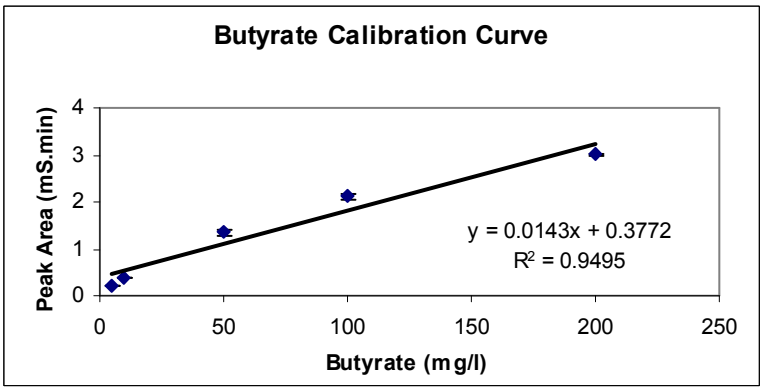
Appendix A: Hydrogen, Methane and Carbon Dioxide Calibration Curves



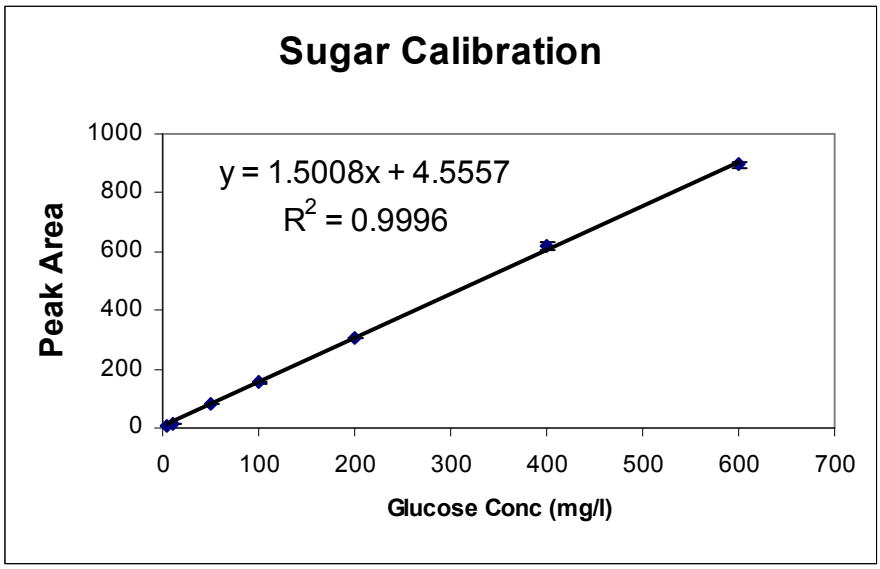
Appendix B: Volatile Fatty Acid Calibration Curves



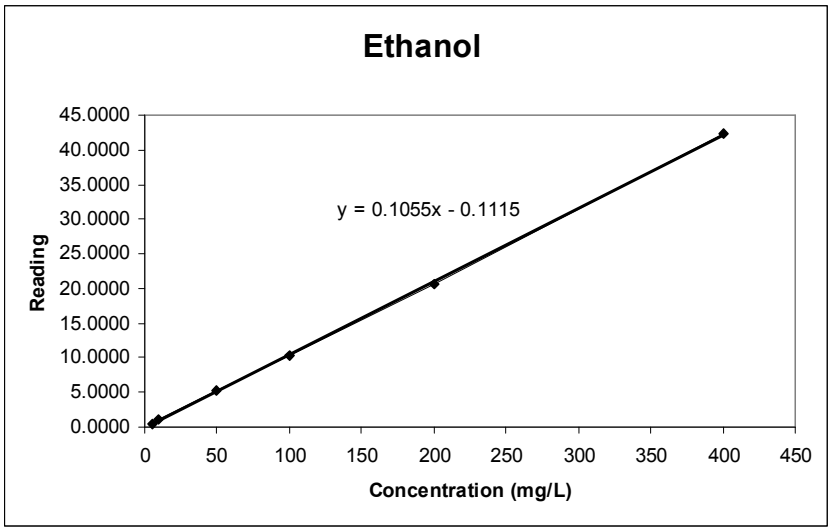


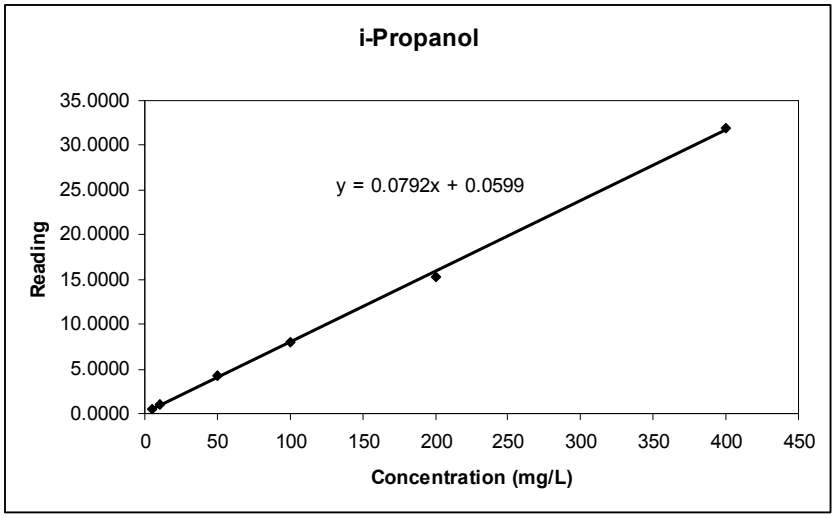


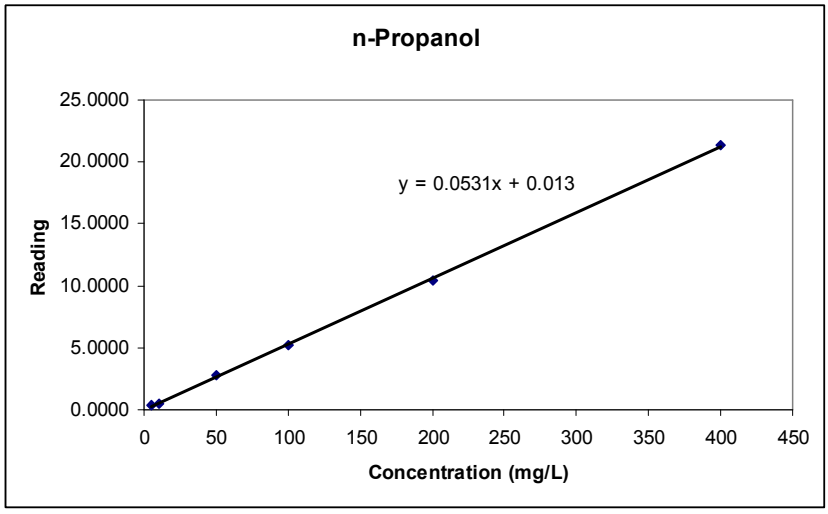
Appendix C: Sugar Calibration Curves

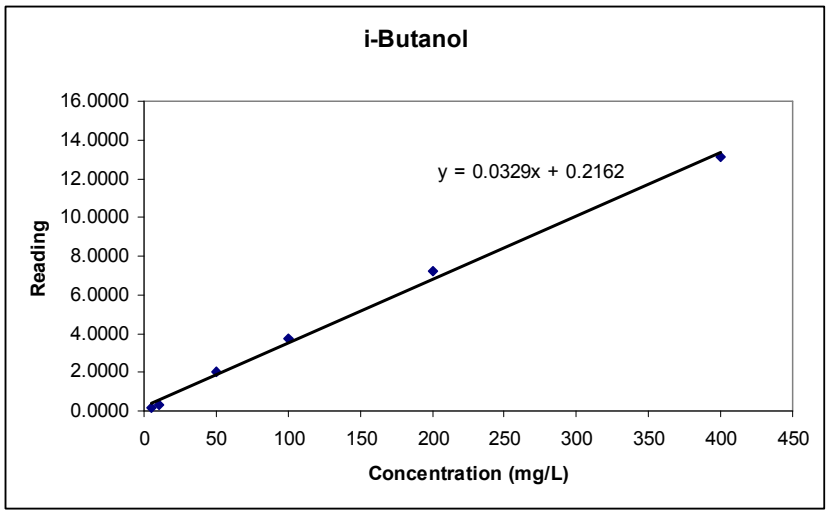


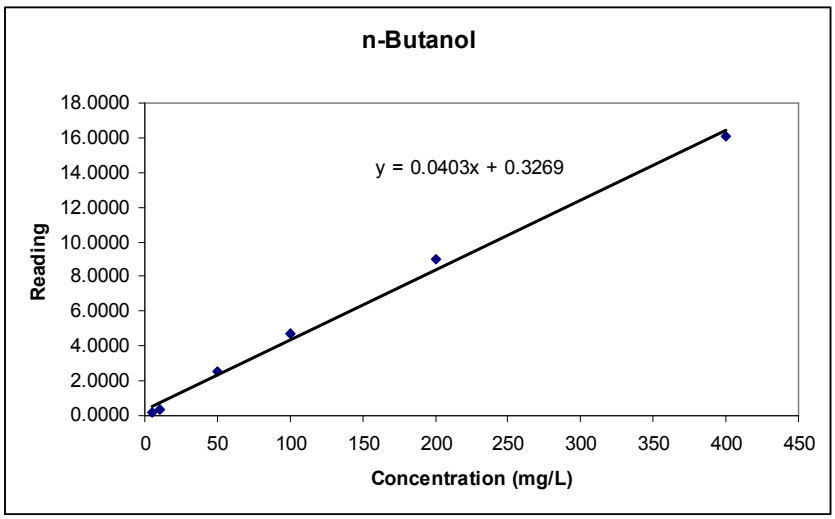
Appendix D: Alcohol Calibration Curves






n-Propanol
y = 0.0531x + 0.013
Reading
Concentration (mg/L)
25.0000
20.0000
15.0000
10.0000
5.0000
0.0000
0
50
100
150
200
250
300
350
400
450


i-Butanol
y = 0.0329x + 0.2162
Reading
Concentration (mg/L)
16.0000
14.0000
12.0000
10.0000
8.0000
6.0000
4.0000
2.0000
0.0000
0
50
100
150
200
250
300
350
400
450


n-Butanol
y = 0.0403x + 0.3269
Reading
Concentration (mg/L)
18.0000
16.0000
14.0000
12.0000
10.0000
8.0000
6.0000
4.0000
2.0000
0.0000
0
50
100
150
200
250
300
350
400
450

Appendix E: Sample Calculations

Hydrogen Yield:

Sample Calculation for hydrogen yield in cultures receiving 5,000 mg/L xylose with 2,000 mg/L LA in figure 5.1

On day 7 amount of hydrogen in the sample bottle was 3,549 umol = 0.003549 mol

Amount of sugar added was 5,000 mg/L * 0.05 L * 1/150 g/mol * 1/1,000 = 0.00167 mol

So, Hydrogen yield is 2.129 mol H_2/mol xylose

On mass basis of sugar hydrogen = 3.549 mmol

Xylose = 5,000 mg/L * 0.05L *1/1,000 = 0.25 g xylose

Hydrogen yield = 14.197 mmol H2/g xylose

Degradation Rate: Sample Calculation

Xylose degradation profile for samples injected with 2500 mg/L xylose figure 5.12

The initial degradation rate is based on the first hour of sugar consumption based on VSS concentration of 2,000 mg/L

$C\ (mg/L) = 2{,}500 - 211t + 17.4t^2$

-dC/dt = 211 – 17.4t2 = 211 mg/L/hr

At t = 0; initial degradation rate = 211 mg/L/hr

= 1.758 μg/mg VSS/min

Based on 2,000 mg/L VSS concentration

Electron Balance: Sample Calculation

The Electron Balance was done on the following assumption:

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

Sample Calculation computed for 5,000 mg/L xylose in the presence of 2,000 mg/L LA

figure 5.1

At t=0

$\Sigma Substrate_0$ = 5,000 mg/L * 2 * 0.05L *1/150mg/mmol * 20e^-/mmol = 66.7 e^-

At t = 7 days

ΣProducts = 1.48 e^- (methane) + 13.24 e^- (Hydrogen) + 28.8 e^- (acetate) + 9.39 e^- (butyrate) + 2.14 e^- (ethanol) + 0.44 e^- (isopropanol) + 1.76 e^- (butanol)
= 57.25

ΣSubstrate = 0

Total Electrons = 57.27 mol

% recovery = 57.27/66.7 = 85.9%

Generation of Chemical Equations

Ex. Breakdown of 2 moles of Xylose to CH_4 in anaerobic fermentation by use of oxidation reduction reactions

$2C_5H_{10}O_5 + 10H_2O \rightarrow 10CO_2 + 40\ H^+ + 40e^-$ Oxidation reaction

$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$ Reduction reaction

* next step is to balance out the electrons

$2C_5H_{10}O_5 + 10H_2O \rightarrow 10CO_2 + 40\ H^+ + 40e^-$

$5CO_2 + 40H^+ + 40e^- \rightarrow 5CH_4 + 10H_2O$

* add two reactions

$2C_5H_{10}O_5 \rightarrow 5CH_4 + 5CO_2$

Appendix F VSS, TSS and pH data for feed reactor

Date	VSS (mg/L)	TSS (mg/L)	VSS/TSS	pH
09/04/2008	8500	10900	0.78	6.4
10/10/2008	7900	10000	0.79	6.8
11/27/2008	8800	11100	0.79	6.8
12/13/2008	8900	11300	0.79	7.1
01/14/2009	9200	11400	0.81	6.8
02/12/2009	8400	10500	0.80	6.9

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