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Abstract

If H_2 yield rates from dark fermentation are to improve, methods must be designed that prevent interspecies H_2 transfer to methanogens. Long chain fatty acids (LCFAs) have been proven to an inexpensive, natural methanogenic inhibitors. BES (2-bromoethanesulfonate), a synthetic chemical inhibitor, is also an effective methanogenic inhibitor. A BES concentration of approximately 50 mM was needed to reduce methane production to the level observed with 2000 mg/l LA. Maximum yields of 3.22 and 3.24 mol H_2/mol glucose were observed in cultures containing 3000 mg/l LA and 100 mM BES at an initial pH 5.5, respectively. Cultures fed with LAU produced more H_2 than cultures containing LA during the first glucose injection period. The breaking of inhibitor additions into two equal increments separated by 24 hours did not improve H_2 yields. However, methane reduction of 46% and 42% was observed for LA and BES, respectively.

Dedication

To my loving and patient family

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Nomenclature

ATP	adenosine tri-phosphate
ADP	adenosine di-phosphate
BES (BESA)	2-bromoethanesulfonate (2-bromoethanesulfonic acid)
Co-A	Coenzyme A
CSTRs	continuously stirred tank reactors
Fd	Ferrodoxin
GC	gas chromatography
HRT	hydraulic retention time
IC	ion exchange chromatography
LA	linoleic acid
LAU	lauric acid
LCFA	long chain fatty acid
mol	moles
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
TCD	thermal conductivity detector
TSS	total suspended solids
VFA	volatile fatty acid
VSS	volatile suspended solids
µS	micro-Siemens (10^{-6})
nC	nano-Coulombs (10^{-9})
µmol	micromol (10^{-6})

Chapter 1: Introduction

1.1 Background

Environmental awareness has increased over the past decade as it becomes clear that global climate change and the world's dependency on fossil fuels are interrelated. Between 80-90% of world's energy consumption is supplied from fossil fuel sources (B.P. Statistical Review of World Energy, 2009). This is an alarming statistic considering energy consumption continues to increase while the earth's available fossil fuel reserves continue to diminish. The development of an alternative energy source is undeniable. However, society's dependency on fossil fuels is deep-rooted. The infrastructure exists and there is strong resistance to change. It is important that this not persuade governments and research groups from pursuing the development of alternative energy sources. Fossil fuel combustion has caused substantial environmental damage to plant and animal life, the lithosphere, hydrosphere and atmosphere. High atmospheric levels of greenhouse gases, such as CO_2, have caused increasing surface temperatures. Other detrimental byproducts from fossil fuel combustion include particulate matter and oxides of nitrogen and sulfur (Das and Veziroglu, 2001). These compounds can lead to serious health effects when exposed to plants and animals. From an environmental perspective, a clean and sustainable alternative to fossil fuels should already be supporting most of the worlds energy demand.

Hydrogen (H_2) is widely considered to possess the most advantages of any alternative energy carrier (Hoffman, 2001). The combustion of H_2 is clean, producing only water as a product. Hydrogen is an energy rich molecule having the highest energy content per unit mass of any fuel (143 GJ/tone) (Boyles 1984). Hydrogen can also be used directly by fuel cells to

produce electricity. The majority of H_2 used in industrial processes is produced from fossil fuels. Four methods which are used to produce H_2 are as follows (Rosen and Scott, 1998):

1. Steam reforming of natural gas. H_2 and carbon monoxide are formed from the mixing of natural gas (methane) and steam at extreme temperature (700-1000°C) in the presence of a metal catalyst;
2. Thermal cracking of natural gas. H_2 and carbon monoxide are formed by heating natural gas in the presence of a catalyst;
3. Partial oxidation of heavier than naphtha hydrocarbons. H_2 is formed by heating hydrocarbons in an oxygen deprived environment;
4. Coal gasification. H_2 and carbon monoxide are formed by the heating and pressurizing of coal and water.

Over 90% of the total H_2 produced is derived from fossil fuels (Das and Veziroglu, 2001; Rosen and Scott, 1998). Methods 1 and 2 alone, which involve the use of natural gas, account for approximately 80% of total H_2 production (Rosen and Scott, 1998). In addition to consuming fossil fuels, these processes are energy intensive, requiring high operating temperatures and pressures. The usefulness of these processes does not extend past industrial application. Production of H_2 from fossil fuels on the scale needed to have an impact on the world's energy supply is completely unsustainable. Other common methods of H_2 production include (Hallenbeck and Benemann, 2002):

- Electrolysis. Water is split into H_2 and oxygen gases using an electrical current to force a non-spontaneous reaction;

- Thermochemical treatment. H_2 gas and other molecules are formed from the breakdown of organic material under extreme environmental and chemical conditions.

There are key limitations to both of these production methods. An energy input of 120 kJ is needed to produce 1 mol of H_2 by electrolysis. Thermochemical processes are energy intensive, and may not always be environmentally friendly. In the case of thermal treatment, the conversion efficiency is a critical parameter to consider. Conversion efficiency relates the amount of useful output energy to the amount of input energy. The methods mentioned above are inefficient producers of H_2 due the amount of energy input required.

1.2 Biological H_2 Production

Biological methods offer an attractive alternative of H_2 production that is environmentally friendly and less energy intensive. Over a century has passed since the first observation of H_2 production from microalgae and bacteria (Hallenbeck and Benemann, 2002). The past quarter century has seen a substantial amount of publications relating to H_2 production from biological methods; however, progress towards practical applications have been slow (Hallenbeck and Benemann, 2002). Biological H_2 production methods can be divided into two distinct categories: light fermentation and dark fermentation. In light fermentation, H_2 is produced by photosynthetic bacteria which use light to convert water into H_2. This light driven process has been the focus of most early research work. Low conversion efficiencies and light dependency are the crucial limiting factors of light fermentation. Dark fermentation has been shown to offer many advantages over light fermentation. Dark fermentation can utilize a wide range of reduced carbon substrates found in municipal waste, agricultural residues and industrial effluents. This ability to turn low value material into energy makes dark fermentation sustainable. The microorganisms that control these reactions have relatively high growth rates.

This ensures that the necessary enzymes required for rapid H_2 production are abundant (Tanisho et al., 1994). Dark fermentation has the advantage of having faster reaction rates compared to light fermentation (Nath and Das, 2004; Hallenbeck and Benemann, 2002). However, there are still many issues that need to be addressed before H_2 produced from dark fermentation can become a significant contributor to the worlds energy supply.

Hydrogen yield rates from dark fermentation are not near the rates needed to be commercialized. A H_2 yield rate represents the number of mol of H_2 produced per mol of feedstock (substrate). The presence of H_2 consuming bacteria plays a determining role in the H_2 yield. Effectively inhibiting these bacteria can significantly improve H_2 yields (Reaume, 2009; Ray et al., 2008; Chowdhurry, 2005; Gurukar, 2005). Hydrogen is produced at several steps in the dark fermentation reaction process and is converted into methane under thermodynamically favourable conditions by hydrogenotrophic methanogens (Kumar and Das, 2000).

Methanogens are inhibited by many chemical inhibitors. Long chain fatty acids (LCFAs) are a safe and inexpensive methanogenic inhibitor (Koster and Cramer, 1987; Lalman and Bagley, 2000). LCFAs are found in wastewater effluent from many food processing plants and can be produced from renewable agricultural sources. 2-bromoethanesulfonic acid (BES) is a synthetic inhibitor which also inhibits methanogens (Zinder et al., 1984; Cheong and Hansen, 2006; Liu et al., 2011). BES is a specific methanogenic inhibitor which competes with coenzyme M during methane formation (Zinder et al., 1984). Coenzyme M is necessary for the methyl-transfer reaction in the metabolism of methanogens. BES competitively inhibits this methyl transfer, the final stage of methanogenesis, using CO_2 and H_2. Several other variables play essential roles in determining H_2 yield, such as, pH temperature, substrate type, H_2 partial pressure, and culture type (i.e. species of bacteria present). Engineering design variables, which

also affect H_2 yield, include factors such as, hydraulic retention time (HRT), continuous or batch reactor operation, and H_2 partial pressure. Research thus far has been aimed at generating H_2 yield data for a variety of environmental/engineering design variables and inhibition methods to determine the optimal conditions for H_2 production.

Hydrogen can make its largest impact when considered as a fuel for automobiles. However, the proton exchange membrane (PEM) fuel cell, which uses H_2 to power automobiles, requires advancement through research and development in order to attain the efficiencies needed for widespread commercial use (Johansson and Ahman, 2002). Other issues include the lack of needed infrastructure, primarily the need for H_2 filling stations, and the need for a safe on-vehicle H_2 storage tank. With increasing research being put into H_2 production technologies along with advancements in fuel cell technology, an economy powered by H_2 is not so far away.

1.3 Objectives

The primary objective of this study was to compare the inhibition potential of BES to LCFAs in a mixed anaerobic culture. All experimental conditions used BES (98%, Sigma-Aldrich, 2010) and LCFAs (Tokyo Chemical Industry Co, Ltd., 2010). Linoleic acid (LA) and lauric acid (LAU) were selected for the study. LA (18:2) and LAU (12:0) contain 18 and 12 carbon chains, respectively. LA has two double bonds while LAU has zero. The variation in bond structure between LA and LAU allows for an assessment on whether saturated (LAU) or unsaturated (LA) LCFAs are preferred inhibitors. In addition, the size difference between LA (18C) and LAU (12C) allows for an evaluation on how the structural size of LCFA affects inhibition. The secondary objective of this study was to determine the impact of adding equal portions of LCFA and BES inhibition at two different time periods. To accomplish this, an injection plan has been

devised where an inhibitor is added in two equal increments separated by 24 hours. The complete list of objectives for the research work is as follows:

1. Determine optimal operating pH of mixed culture;
2. Assess the impact of BES on H_2 production;
3. Evaluate the effects if BES and LA are added in two equal increments separated by 24 hours;
4. Determine what concentration of BES, LA and LAU result in equivalent methane inhibition and H_2 production; and,
5. Construct full stoichiometric reactions, including cell synthesis, for all experiments by method of electron-equivalent balances. Examine which byproducts dominate when H_2 yields reach a maximum value.

Chapter 2: Literature Review

Interest in H_2 as an alternative to fossil fuels has increased exponentially over the past few years. H_2 production through biological processes presents an environmentally friendly means of energy production in an era where environmental concerns and becoming severe and fossil fuel inventories are declining. Dark fermentation is regarded as the biological H_2 production method with the most benefits. The dark fermentation process can utilize a variety of low cost and abundant organic substrates to produce valuable energy that other processes cannot. Low H_2 yield are the main aspect researchers are working to improve in the dark process. In fermentation reactions with mixed anaerobic cultures, the accumulation of H_2 in normally coupled with rapid consumption by methanogens. If yields are to improve, methods must be designed to prevent interspecies H_2 transfer. Researchers are tasked with the job of determining the ideal method of pretreatment, optimal environmental conditions, efficient types of inhibition and effective engineering design to obtain maximum H_2 yield. The generation of H_2 from dark fermentation in a cost-effective manner could play a large role in a future H_2 economy.

2.1 Anaerobic Degradation Process

Dark fermentation (or anaerobic degradation) is a complex multi-stage process where large organic compounds are degraded in the absence of oxygen and light. In an aerobic or oxic environment, oxygen would act as the reactions primary electron acceptor and is reduced to water. In the absence of oxygen, other electron acceptors are required to dispose of the electrons generated from the oxidation reactions. In intermediary steps, protons (H^+) act as electron acceptors and are reduced to molecular hydrogen (H_2). Bacteria operating under normal conditions would ultimately divert all electron equivalences from the oxidation of large organic

compounds to methane (CH_4), carbon dioxide (CO_2) and biomass (cell growth/repair) as final electron sinks. The anaerobic degradation process can be divided into four sequential phases:

1. Hydrolysis;
2. Acidogenesis;
3. Acetogenesis; and,
4. Methanogenesis.

2.1.1 Hydrolysis

In hydrolysis, complex organic polymers are broken down into oligomers and monomers. Of the four phases of anaerobic degradation, hydrolysis is the rate limiting step. The breakdown of lipids and high molecular weight proteins is slow since they do not readily dissolve into the aqueous phase because of their hydrophobic components. Hydrolysis is dependent on factors, such as, pH, temperature, and the availability of hydrolytic enzymes (Jordan and Mullen, 2007).

2.1.2 Acidogenesis

In acidogenesis, the products of hydrolysis (sugars, long chain fatty acids, amino acids) are converted into volatile fatty acids, alcohols, H_2 and carbon dioxide (Veeken et al., 2000). Acidogenesis is facilitated by an extensive group of fermentative bacteria. Acidogens are the most abundant bacteria group in biological reactors due to their high growth rate and resistance to toxins and inhibitors (Joubert and Britz, 1987). *Enterobacter aerogenes* and *Escherichia coli* (E. coli) are examples of acidogens.

2.1.3 Acetogenesis

During acetogenesis, products of acidogenesis (volatile fatty acids excluding acetic acid and alcohols) are converted into acetic acid, hydrogen and carbon dioxide. This process is driven by acetogenic, H_2 producing bacteria, of which *Clostridium thermoacetium* is a well-known species.

The bacteria mediating acetogenesis are strongly influenced by end-product concentration and pH (Joubert and Britz, 1987). If H_2 partial pressure is elevated, greater than 98 Pa in solution, or if the pH is below 4.3, acetogenic reactions will shift to alcohol formation to offset unfavourable thermodynamic effects (Mara and Horan, 2003; Kim et al., 2004).

2.1.4 Methanogenesis

Methanogenesis is the final stage in the anaerobic degradation process. There are two main classes of methanogenic bacteria. Acetoclastic methanogens convert acetate (acetic acid) formed during acidogenesis and acetogenesis into methane and carbon dioxide. Hydrogenotrophic methanogens convert hydrogen and carbon dioxide. According to Speece (1996), approximately 70% of the methane produced from the anaerobic degradation of organic substances is derived from the decarboxylation of acetate by acetoclastic methanogens. Methanogens are slow growers and are sensitive to changes in environmental conditions. The inhibition of H_2-consuming methanogens is a popular technique of increasing the H_2 yields when using mixed anaerobic bacteria.

The entire multi-stage anaerobic degradation pathway is shown in Figure 2.1. Notice how products created by of one the bacteria groups are used by another group of bacteria. Methanogens utilize the byproducts of acidogenic and acetogenic bacteria. This is an example of a syntrophic relationship.

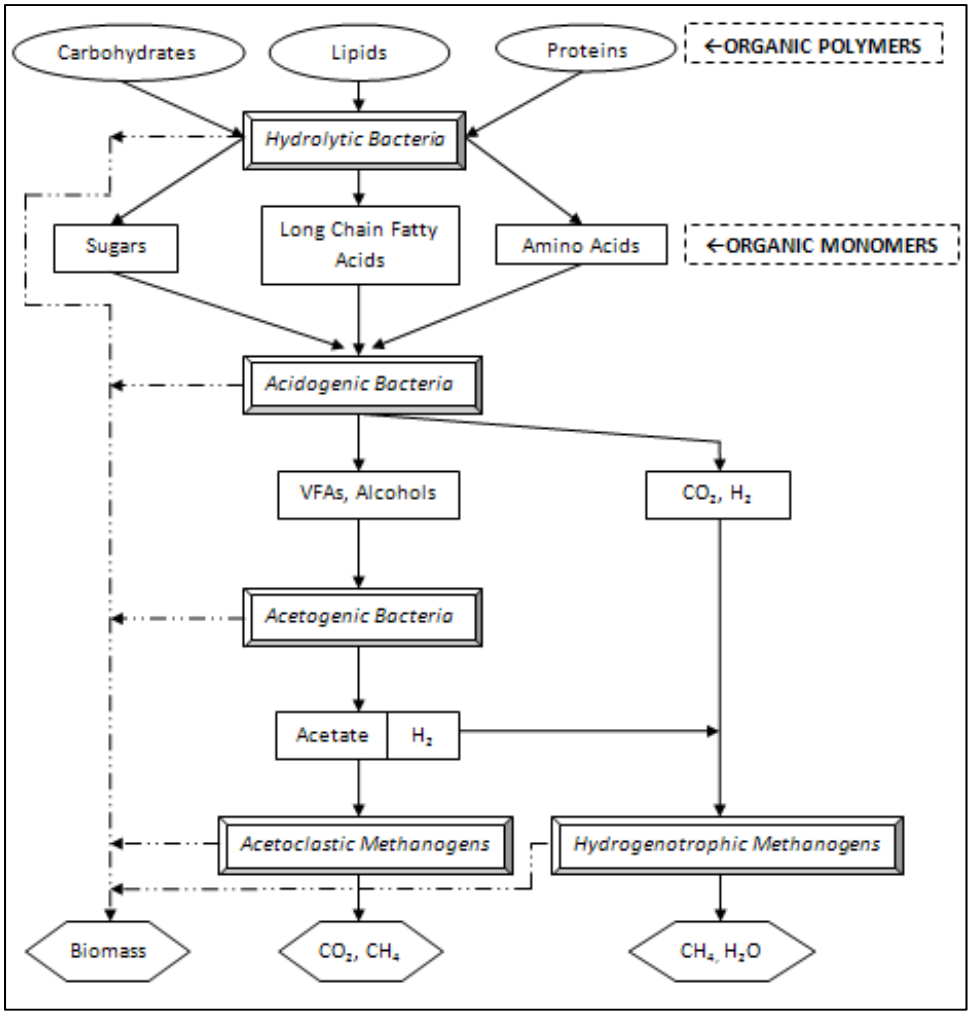


Figure 2.1: Anaerobic degradation pathway of organic substances

2.2 Anaerobic H_2 Production

The majority of fermentative H_2 production is driven by the anaerobic degradation of pyruvate. Pyruvate is a three carbon long molecule which is produced from the breakdown of sugars and some other compounds. Pyruvate degradation can occur in one of two enzyme systems. In the first system, the degradation of pyruvate into acetyl-CoA with H_2 production is catalyzed by pyruvate ferrodoxin oxidoreductase (PFOR) (Equations 2.1 and 2.2) (Hallenbeck and Benemann, 2002). These reactions are common in many *Clostridium* species (Mortenson and Chen, 1974). The acetyl-CoA generated from pyruvate in equation 2.1 can be further converted into acetyl-

phosphate (equation 2.3). Acetyl-phosphate is then oxidized to acetate by ADP which is reduced to ATP (Equation 2.4) (Nath and Das, 2004).

$$Pyruvate + 2Fd(ox) + CoA \xleftrightarrow{PFOR} Acetyl\text{-}CoA + CO_2 + 2Fd(red) \quad \textbf{Eqn 2.1}$$

$$2Fd(red) \leftrightarrow 2Fd(ox) + H_2 \quad \textbf{Eqn 2.2}$$

$$Acetlyl\text{-}CoA \leftrightarrow Acetyl\text{-}phosphate \quad \textbf{Eqn 2.3}$$

$$Acetyl\text{-}phosphate + ADP \leftrightarrow Acetate + ATP \quad \textbf{Eqn 2.4}$$

In the second system, formate is formed through the degradation of pyruvate which is catalyzed by pyruvate formate lyase (PFR) (Equation 2.5). Formate is further degraded to produce H_2 (Equation 2.6) (Gottschalk and Andreeson, 1979; Hallenbeck and Benemann, 2002).

$$Pyruvate + CoA \xleftrightarrow{PFR} Acetyl\text{-}CoA + formate \quad \textbf{Eqn 2.5}$$

$$Formate + H^+ \leftrightarrow H_2 + CO_2 \quad \textbf{Eqn 2.6}$$

In another H_2 production pathway, pyruvate is not involved. This route is commonly referred to as the NADH (nicotinamide adenine dinucleotide, reduced form) pathway. In this pathway, NAHD oxidation and NAD^+ reduction, is catalyzed by NADH ferrodoxin oxidoreductase. Proton (H^+) reduction leads to the formation of molecular hydrogen (Equation 2.7) (Tanisho et al., 1998). In aerobic conditions, oxygen is the final electron acceptor and is reduced to water.

$$NADH + H^+ \leftrightarrow H_2 + NAD^+ \quad \textbf{Eqn 2.7}$$

Figure 2.2 summarizes the main H_2 production pathways via pyruvate fermentation.

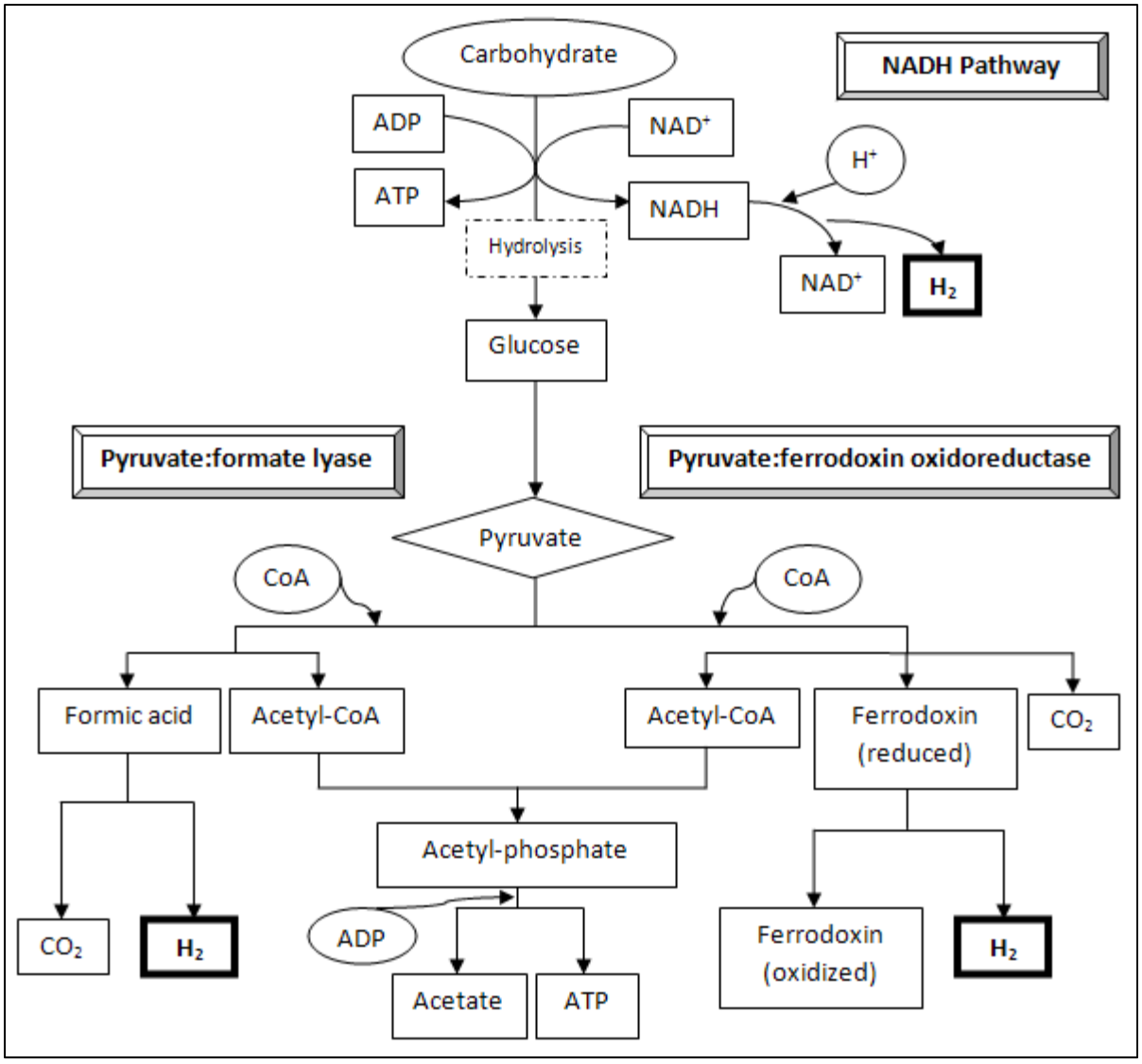


Figure 2.2: H_2 production pathways via pyruvate fermentation (adapted from Nath and Das, 2004)

2.3 Product Formation and Distribution

Analyzing end products and their distribution gives a strong indication of the efficiency of the degradation process. The distribution of VFAs and alcohols formed is regularly used to monitor H_2 production. The VFAs produced are dependent on the type of substrate. The most common VFAs produced in anaerobic degradation include acetic acid, butyric acid, and propionic acid (Rittmann and McCarty, 2001). The distribution of VFAs corresponds to how much H_2 can be

expected. As seen in Table 2.1, when acetic acid is the only reduced carbon byproduct of glucose acidification, the maximum H_2 yield is 4 mol·H_2/mol·glucose (Equation 2.8). However, when butyric acid is the only reduced carbon byproduct, the H_2 yield is 2 mol·H_2/mol·glucose (Equation 2.9). The H_2 yield is zero when lactic acid, propionic acid, or ethanol are the only recuded carbon byproducts of glucose fermentation (Equation 2.10-2.12). During experiments, a single VFA or alcohol are not observed in the fermentation media. There would be a mixture of VFAs and alcohols. Mixtures dominated by acetic and butyric acid are associated with the highest amount of H_2 production. While mixtures containing high amounts of alcohols, and lactic or propionic acid are associated with low H_2 production. End-products, such as, ethanol, butanol, propanol and lactic acid, contain electrons that are not present in more oxidized end-products, such as, acetic acid and butyric acid (Hawkes et al., 2002).

Table 2.1: Reaction pathways from the acidification of glucose (adapted from IWA, 2002)

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

The H_2 partial pressure plays a major role in determining the end products of anaerobic fermentation. Hydrogen synthesis pathways are sensitive to elevated H_2 partial pressure and subjected to a form of end-product inhibition. Based on equation 2.8, it is favourable to optimize the fermentation process for acetic acid formation. Reduced products, such as, acetic acid, can be fermented only at low H_2 partial pressures. Butyric acid and propionic acids can be fermented only when H_2 partial pressure is less than 32.04 Pa and 40.34 Pa, respectively. Ethanol and lactic acid can be fermented at partial pressures 2 to 3 times higher (Fennell et al., 1997).

The byproducts of glucose acidification, Table 2.1, can be further degraded as shown in the reactions in Table 2.2. These reactions are part of the acetogenesis stage of anaerobic degradation. If the H_2 partial pressure is sufficiently low, it is possible for the ethanol and lactic acid to be converted into acetic acid (Equations 2.13, 2.14). It is also possible, for lactic acid to be converted into propionic acid if H_2 partial pressure is elevated (Equation 2.15). This process consumes one mol of H_2 for every mol of lactic acid converted.

Table 2.2: Acetogenic reactions of the by-products of glucose acidification (adapted from Bagley and Brodkorb, 1999)

Substrate	Product	Reaction	Equation
Lactic acid	Acetic acid	$CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + 2H_2 + CO_2$	2.13
Ethanol	Acetic acid	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	2.14
Lactic acid	Propionic acid	$CH_3CHOHCOOH + H_2 \rightarrow CH_3CH_2COOH + H_2O$	2.15
Butyric acid	Acetic acid	$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$	2.16
Propionic acid	Acetic acid	$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$	2.17

In addition to the type of substrate and H_2 partial pressure, product distribution is dependent on many environmental conditions (temperature, pH) and engineering design variables. Alcohol production is more prevalent at pHs below 5. Higher concentrations of ethanol were reported by Zoetemeyer and coworkers (1982) at thermophilic temperatures (50-75°C).

2.4 Thermodynamics

A firm knowledge of microbial thermodynamics can assist in identifying the inherent limitations of the anaerobic degradation process. Thermodynamics is the study of the relationships between heat and different forms of energy. The Gibb's free energy (ΔG) is the amount of energy available to do work. The change in Gibbs free energy of a reaction can be calculated by using Equation 2.18.

$$\Delta G = -n \cdot F \cdot \Delta E \qquad \textbf{Eqn 2.18}$$

ΔG = change in Gibbs free energy (J)

n = number of electrons transferred (mol)

F = number of Coulombs/Faraday (96485 C/mol)

Reactions that consume energy in order to move forward have a positive ΔG value. A reaction that has a negative ΔG value releases energy and proceeds spontaneously. Cell synthesis, the building and repairing of cells, requires an energy input and has a positive ΔG value. Cell synthesis is coupled with the degradation of carbohydrates, an energy releasing process, to meet its energy demand. Most microbial oxidation-reduction reactions are coupled in order to accomplish various tasks.

Energy is needed to build and repair biomass. These reactions are thermodynamically unfavourable with a $\Delta G^{\circ'}$ value of +31.4 kJ/electron equivalence (Yang and Okos, 1987). This energy is derived from the breakdown of organic substances. In aerobic systems, which use oxygen as an electron acceptor, 60% of the energy released from the substrate can be used to build and repair biomass. The remaining 40% is expelled as heat or goes into products such as H_2O and CO_2 (Mara and Horan, 2003). In anaerobic systems which use CO_2 and other organic substances as electron acceptors, 10% of the energy released is used to build and repair biomass. The remaining 90% is expelled as heat or is diverted into products such as organic substances, H_2O and CO_2 (Mara and Horan, 2003). The smaller amount of energy diverted to cell maintenance in anaerobic fermentation is a major advantage when H_2 production is the goal. Cell maintenance is considered an electron sink. When more energy is diverted to product formation, there is more potential for H_2 production.

Reactions are typically classified as oxidation or reduction reactions. These terms relate to the transfer of electrons within a reaction. Recall that an electron represents a negative charge. Therefore, the substances that accept the electrons are reduced, while the substances that donate the electrons are oxidized. Many common microbial oxidation and reduction half-reactions along with their $\Delta G^{\circ'}$ value are listed in Table 2.3. In the anaerobic degradation process, many organic substances are electron donors. They are usually carbohydrates, lipids (fats and oils), and proteins. Oxygen and nitrate are the preferred electron acceptors in many microbial reactions because of their high negative $\Delta G^{\circ'}$ values associated with these electron acceptors (Table 2.3). Sulfate, carbon dioxide and cell synthesis are other forms electron acceptors. These are less favourable acceptors due to their positive $\Delta G^{\circ'}$ values in Table 2.3. Complete reactions are a combination of an electron donor half-reaction and an electron acceptor half-reaction. The $\Delta G^{\circ'}$ values of each half-reaction involved are added together to arrive of the $\Delta G^{\circ'}$ value for the complete reaction. Reactions that are the most thermodynamically favourable (i.e. highly negative ΔG°' value) proceed first.

Table 2.3: Common microbial half-reactions with free energy values

Half Reaction	**$\Delta G^{\circ'}$ (aq) (kJ/electron equivalence)**
Electron Donor (Oxidation)	
Carbohydrates $1/4CH_2O + 1/4H_2O \rightarrow 1/4CO_2 + H^+ + e^-$	-41.8
Fats and Oils $1/46C_8H_{16}O + 15/46H_2O \rightarrow 4/23CO_2 + H^+ + e^-$	-27.6
Protein $1/66C_{16}H_{24}O_5N_4 + 27/66H_2O \rightarrow 8/33CO_2 + 2/23NH_4^+ + 31/33H^+ + e^-$	-32.2
Acetate $1/8CH_3COO^- + 3/8H_2O \rightarrow 1/8CO_2 + 1/8HCO_3^- + H^+ + e^-$	-27.6
Ethanol $1/12CH_3CH_2OH + ¼H_2O \rightarrow 1/6CO_2 + H^+ + e^-$	-31.8
Electron Acceptor (Reduction)	
Oxygen $1/4O_2 + H^+ + e^- \rightarrow 1/2H_2O$	-78.2
Nitrate $1/5NO_3 + H^+ + e^- \rightarrow 1/10N_2 + 3/5H_2O$	-71.6
Carbon Dioxide $1/8CO_2 + H^+ + e^- \rightarrow 1/8CH_4 + 1/4H_2O$	+24.3
Sulphate $1/8SO_4^{2-} + 19/16H^+ + e^- \rightarrow 1/16\ H_2S + 1/16HS^- + 1/2H_2O$	+21.3
Cell Mass $1/20NH_4^+ + 1/20HCO_3^- + 1/5CO_2 + H^+ + e^- \rightarrow 1/20C_5H_7O_2N + 9/20H_2O$	+31.4

Reaction rates are another aspect of microbial thermodynamics which can provide insights into the efficiency of the degradation of electron donors. Reaction rate depends on many variables, such as, environmental conditions (pH and temperature), and the concentration of the reactants and products. However, to drive many of these enzymatic reactions, the reaction must have relatively low activation energy. Activation energy represents an energy barrier that must be overcome such that the reaction proceeds in the forward direction. If the activation energy is large, the rate of reaction is slow. Forging the reaction into the forward direction requires the use of a catalyst. Catalysts lower the activation energy of a reaction; however, they do not change the ΔG value. Enzymes act as catalysts and are a critical component in all living systems (Mara and

Horan, 2003). Without enzymes many reactions will not proceed. All biological production of H_2 is dependent on the presence and activity of H_2 producing enzymes (hydrogenase enzymes). Active hydrogenase enzymes are needed to maximize H_2 production rates (Hallenbeck and Benemann, 2002). Figure 2.3 depicts the free energy profile of a reaction with an enzyme catalyzed reaction versus the reaction coordinate.

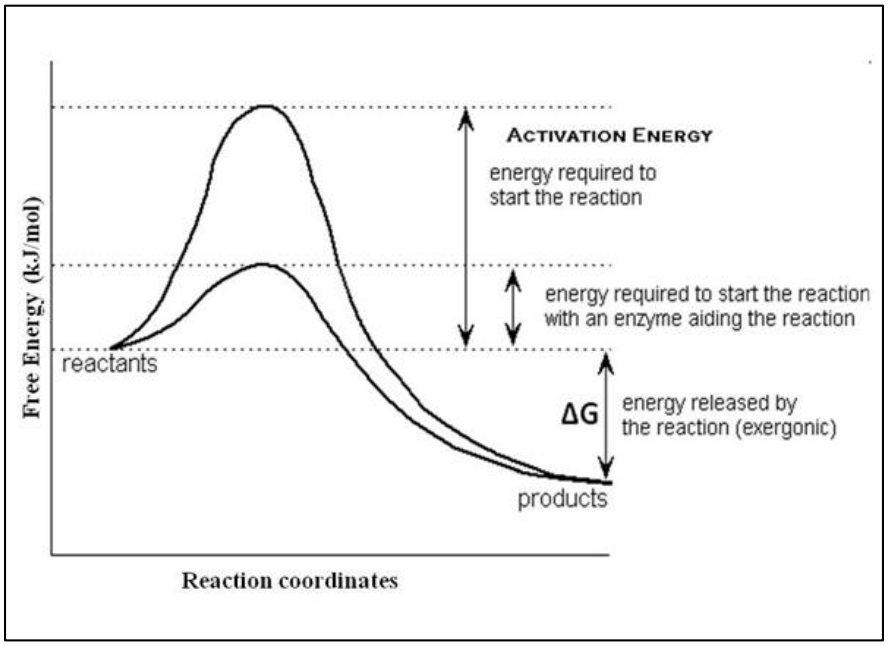


Figure 2.3: Activation energy profile of a reaction aided by an enzyme

2.5 Factors Affecting Bio-H_2 Production

2.5.1 Effect of Nutrients

Bacteria require macro- and micro- nutrients to function. A critical aspect of maintaining a bacterial culture is to provide the necessary nutrients. Nitrogen and phosphorus are primary macronutrients. Nitrogen is vital for protein and DNA synthesis. Phosphorus is essential in energy storage and in DNA synthesis. It is also important for its buffering capacity (Lin and Lay, 2005). However, large quantities of phosphorus can cause overgrowth. Trace amounts of heavy metals are also important. Micronutrients, such as, magnesium, sodium, zinc and iron are part of

the cofactors needed for enzyme function (Lin and Lay, 2005). All these metals can have toxic effects if added at elevated levels (Li and Fang, 2007). Other metals, such as, cadmium, chromium, copper, lead and nickel can inhibit enzymatic function or disrupt transportation pumps in the cell membrane. According to Fang et al. (2004), these protein pumps are essential in controlling substrate transportation.

2.5.2 Effect of Temperature

The effect of temperature on H_2 production has been studied in depth over the past several years. A majority of studies have shown that H_2 production increases with increasing temperature. Lin and Chang (2004a,b) examined the affect for a temperature range of 15-34°C. They reported that the range from 30-34°C produced the maximum H_2 yield (192 ml H_2/g hexose). Li and Fang (2007) have reported H_2 yield data at different temperatures from a variety of published sources. The following three temperature ranges were examined: ambient (15-30°C), mesophilic (32-39°C), and thermophilic (50-64°C). For carbohydrate substrates, thermophilic and mesophilic temperature ranges produced very comparable yields, while the yields were much lower at ambient temperatures. The highest yield in the mesophilic and thermophilic range were 333 ml H_2/g hexose (Van Ginkel et al., 2001) and 327 ml H_2/g hexose, respectively. With wastewater as the substrate, the highest yield was observed at 60°C in the thermophilic range (Ueno et al., 1996). For solid waste as the substrate, the highest yield was observed at 55°C for a mixed food and paper waste source (Valdez-Vazquez et al., 2005). Zhang et al. (2002) observed that more H_2 can be produced from starch at a thermophilic temperature (55°C) than at a mesophilic temperature (37°C). It is clear that the type of substrate used dictates what temperature provides the greatest H_2 yield. Researchers studying carbohydrates as the substrate commonly use temperatures in the range of 36-40°C to maximize the H_2 yield.

At temperatures above the ambient range, an energy input is needed to increase the reaction temperature. Weighing the benefits of increased H_2 yields to the cost of energy is an important factor to consider when decisions are made to operate at elevated temperatures. At elevated temperatures, the diffusion of compounds by passive or active transport into and out of the cell becomes thermodynamically feasible. The cell membrane is more permeable due to increased fluidity and flexibility in the phospholipid layer (Cirne et al., 2007). Henry's gas constant is higher at elevated temperatures. Consequently, H_2 gas solubility decreases with increasing temperature. This is an advantage for H_2 production because low amounts of dissolved H_2 correspond to low H_2 partial pressures and this is favourable for H_2 production. In addition, the reaction rate is directly proportional to the temperature. Rittmann and McCarty (2001) observed that for every 10°C increase in temperature, the reaction rate increases 2-fold. A faster reaction rate allows for higher loading rates (i.e. more substrate). Rittmann and McCarty (2001) noted that thermophilic systems are more sensitive to temperature fluctuations. Temperature also affects the metabolic pathway of reactions which result in different by-product formation.

2.5.3 Effect of pH

Several aspects of anaerobic fermentation are affected by pH. The aspects relate directly to the function of bacteria. It affects their utilization of energy sources, efficiency of substrate degradation, synthesis of proteins and release of metabolic products from the cell (Bailey and Ollis, 1996). Most importantly, the pH directly affects hydrogenase enzyme activity (Dabrock et al, 1992). The pH is a crucial factor in the suppression H_2-consuming methanogens. Methanogenesis decreases or stops at pH lower than 6.3 and higher than 7.8 (Chen et al., 2002). Fang and Liu (2002) examined the effects of pH on H_2 yield over the range of 4.0-7.0 in increments of 0.5. Their results showed that a maximum yield occurred at a pH of 5.5 with a yield of 286 ml H_2/g hexose. The yields were 190 ml H_2/g hexose and 41 ml H_2/g hexose for pH

4.0 and 7.0, respectively. For H_2 production from carbohydrates, the optimal pH ranged from 5.2-7.0, with an average of 6.0 (Li and Fang, 2007). The optimal pH ranges from 5.2-5.6 for the H_2 conversion of wastewater effluents (Noike, 2002) while Lee et al. (2002) reported an optimal pH of 9 for H_2 production using batch fermentation of sucrose. According to Lay (2000), pH can also affect metabolism pathways during H_2 production. Acetate and butyrate are the two main byproducts, with butyrate production favourable at lower pH values. At a pH above 7, propionate formation increases significantly. The propionate formation reaction (Equation 2.10) consumes H_2 and consequently decreases the yield (Li and Fang, 2007). Fang and Liu (2002) examined product profiles from pH 4.0 to 7.0 and found that at pH 6.0 or below, butyrate was observed as the predominant product (46%) while at pH 6.5 and above, acetate was predominant (up to 34%).

2.5.4 Effect of H_2 Partial Pressure

Hydrogen can inhibit its own production and at elevated levels, it can cause the breakdown of VFAs to become thermodynamically unfavourable. Acetogenic reactions (Table 2.2) producing H_2 have positive ΔG values. For these reactions to proceed, the H_2 partial pressure must remain low. Ahring and Westermann (1988) showed that a H_2 partial pressure of 2.0 kPa prevents butyrate consumption (Equation 2.16). Maintaining a low H_2 concentration in solution (i.e. a low partial pressure) allows the equilibrium reaction to proceed in the forward direction. H_2 producing and H_2 consuming reactions common to anaerobic degradation are shown in Table 2.4. Based on the ΔG values, H_2 consumption is more feasible than H_2 than production. Producing one mol of methane from 4 mol of H_2 is thermodynamically favourable with a $\Delta G^{o'}$ value of -131.0 kJ/mol. In comparison, acetate degradation to 4 mol of H_2 has a $\Delta G^{o'}$ value of +94.9 kJ/mol and hence, this reaction is thermodynamically unfavourable. This is the most desired pathway since it produces the most H_2. Thermodynamics govern which reaction

pathways are to be taken; therefore, developing techniques to overcome the limitations of thermodynamics are importance.

Table 2.4: H_2 producing and consuming reactions (Schink, 1997)

Reaction	$\Delta G^{\circ'}$ (kJ/mol)
H_2-producing reactions	
$CH_3CH_2CH_2COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + H^+ + 2H_2$	+43.6
$CH_3CH_2COO^- + 2H_2O \leftrightarrow CH_3COO^- + CO_2 + 3H_2$	+73.6
$CH_3COO^- + H^+ + 2H_2O \leftrightarrow 2CO_2 + 4H_2$	+94.9
$CH_3CH(CH_3)CH_2COO^- + CO_2 + 2H_2O \leftrightarrow 3CH_3COO^- + 2H^+ + H_2$	+25.5
$CH_3CH_2OH + H_2O \leftrightarrow CH_3COO- + H^+ + 2H_2$	+1.9
H_2-consuming reactions	
$4H_2 + 2CO_2 \leftrightarrow CH_3COO^- + H^+ + 2H_2O$	-94.9
$4H_2 + CO_2 \leftrightarrow CH_4 + 2H_2O$	-131.0
$H_2 + HCO_3^- \leftrightarrow HCOO^- + H_2O$	-1.3
$H_2 + S \leftrightarrow H_2S$	-33.9
$4H_2 + SO_42^- + H^+ \leftrightarrow HS^- + 4H_2O$	-151.0
$H_2C(NH_3^+)COO- + H_2 \leftrightarrow CH_3COO^- + NH_4^+$	-78.0
Fumarate + H_2 ↔ succinate	-86.0

Figure 2.4 shows the affect H_2 partial pressure has on the thermodynamics of a reaction. ΔG values change by varying H_2 partial pressure. The H_2 production via butyric acid degradation is more favourable than methane production via H_2 consumption at partial pressure below 9.8 Pa. At any partial pressure above this value, methane production is thermodynamically favoured.

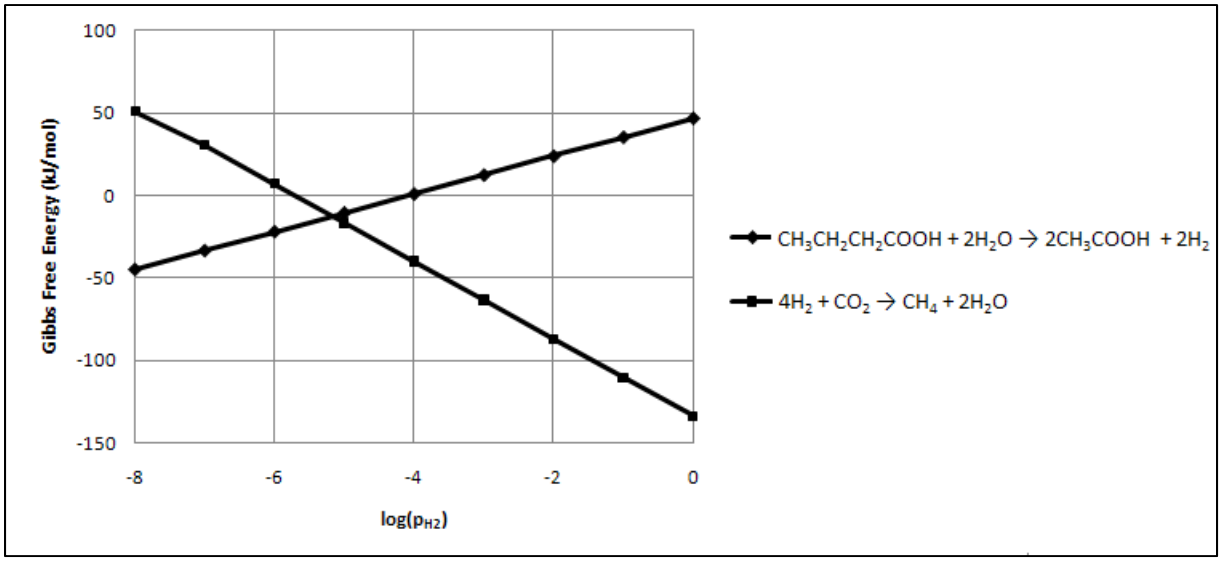


Figure 2.4: $\Delta G^{\circ'}$ values for H_2 producing and H_2 consuming reactions as a function of H_2 partial pressures

Researchers have attempted many techniques to reduce the aqueous H_2 partial pressure in many H_2 producing microbial cultures. Applying a vacuum was found to have little effect on H_2 yield (Kataoka, 1997). In another approach, sparging with an inert gas, such as nitrogen or argon, assisted in forcing H_2 out of solution. Hussy et al., (2003) observed that sparging with nitrogen increased the H_2 yield from 172 to 254 ml H_2/g hexose. Another method used a specially designed membrane filter that is only permeable to H_2. Due to its small molecular size, dissolved H_2 can be separated from the mixed liquor (Liang et al., 2002). Continuous pressure release was also shown to have substantial effects on H_2 yield. In comparison to experiments that released pressure intermittently, continuous release showed a 43% improvement in the H_2 yield (Logan et al., 2002). The most simple and commonly used method is vigorous mixing. Lay (2000) observed substantial improvement when employing vigorous mixing to the reactor. An increase of stirring from 100 to 700 revolutions per minute more than doubled the daily rate of H_2 production from starch degradation.

2.5.5 Hydraulic Retention Time

Hydraulic retention time (HRT) is an important engineering design variable in the anaerobic conversion process. HRT is defined as the average time it takes a volume element to enter and leave a reactor. It is important to first distinguish between batch reactors and continuously stirred tank reactors (CSTRs). HRT does not apply to batch reactors since there is no continuous effluent stream. When a feed is introduced into the batch reactor, the system remains closed as the reaction proceeds to completion. For a CSTR, when a liquid is introduced, an impeller is used to remove a continuous stream of reactor effluent. Many studies are conducted using batch reactors for their simplicity and ease of control. However, batch studies are impractical for large scale production of H_2. From an engineering standpoint, a continuous production process is the most logical option for large scale production (Li and Fang, 2007).

A short hydraulic retention time means the influent spends very little time in the reactor. A HRT that is shorter than optimal leads to unfermented substrate in the effluent and as a result, the full H_2 yield is not acheived. Short HRTs are used to washout methanogens and reduce the amount of methane formation. Reactors operating under long HRT conditions are inefficient because they promote methane production. According to Li and Fang (2007), the optimal HRT values for glucose and sucrose were in the range of 3-8 hours. However, Fang and Liu (2004) reported high values at 13.7 hours while Chang et al. (2002) have demonstrated H_2 production at values as low as 1 hour. In studies with starch, optimal HRT values of 15-17 hours were used because of the rate limiting hydrolysis step (Hussy et al., 2003; Lay, 2000).

2.5.6 Effect of Microbial Source

The different bacteria species in a biological reactor play a critical role in determining the variety of byproducts formed during anaerobic degradation of organic substances. During the oxidation of organic substances, electrons are release and captured by electron acceptors. In anaerobic

systems, the electron acceptors include protons, carbon dioxide, nitrate, sulfate and other organic intermediates. The activity of the hydrogenase enzyme, in certain species of bacteria, is responsible for producing H_2 through a specific mechanism to dispose of excess electron equivalents.

2.5.6.1 Pure Cultures

Bacteria species such as *Enterobacter* and *Clostridium* are often the first choice of researchers using pure cultures (Li and Fang, 2007). A number of studies have shown these species to be the ideal H_2 producing bacteria. *Clostridium* species are gram-positive, rod-shaped, endospore formers and strict anaerobes. *Enterobacter* species, on the other hand, are gram-positive, rod-shaped, facultative anaerobes. With the absence of H_2 consuming bacteria, H_2 yields are higher when using pure culture systems. However, pure cultures require constant maintenance and are very sensitive to changes in environmental conditions. Contamination from organisms in non-sterile feedstocks can be a nuisance and may result in low yields.

2.5.6.2 Mixed Cultures

Mixed culture contains a variety of bacteria species which can be found in many natural communities. These communities can be found in rice fields, landfills, wastewater facilities, sludge composts and soil. The disadvantage of mixed cultures when compared to pure cultures is the presence of H_2 consuming bacteria. Mixed cultures operate in a syntrophic relationship and there is also no need for concern of contamination from a non-sterile feedstocks.

2.5.7 Effect of Substrate Source and Concentration

Carbohydrates (sugars), cellulose, and starch are an abundant source of electron donors for producing H_2 using mixed anaerobic cultures. These substances are renewable, abundant and readily available at relatively low cost. The food to microorganism (F/M) ratio is an important value to consider during the operation of continuous flow reactors. According to Lay (2001), the

ideal F/M ratio ensures proper operational efficiency. Van Ginkel et al. (2001) theorizes that when high substrate concentrations causes the F/M ratio to become outside of the normal operating range, an inhibitory effect is experienced due to increased acid production in the form of VFAs. The increased acid formation lowers the pH and increases the H_2 partial pressure and subsequently decreases the H_2 yield. Lay (2001) observed that when the cellulose concentration exceeded 25 g/l, the H_2 producing ability of a bacterial culture at 37°C and pH 5 significantly decreases. In this study, the maximum H_2 yield was observed with an F/M ratio of 8 g cellulose/g VSS.

The type of substrate can control the H_2 yield. Work published by Li and Fang (2007) compared H_2 yields from different substrates such as glucose, sucrose, molasses, solid waste and cellulose. A large component of the work by Li and Fang (2007) has shown large H_2 yields using substrates such as glucose. Glucose is the most studied substrate because it is widely available and it is easily degradable under aerobic and anaerobic conditions.

Hydrogen yields can often be predicted by analyzing the substrates chemical formula. For example, consider the H_2 production pathways for glucose and xylose. The most common H_2 production pathways are through acetate and butyrate degradation. Equations 2.19 and 2.20 represent the acetate and butyrate pathways for glucose while equations 2.21 and 2.22 represent the acetate and butyrate pathways for xylose. The stoichiometric H_2 yield for acetate degradation is 4 mol and 3.33 mol for glucose and xylose, respectively. Similarly, the stoichiometric H_2 yield for butyrate degradation is 2 mol/mol hexose and 1.67 mol/mol pentose for glucose and xylose, respectively. Since glucose has two extra H_2 atoms, one extra carbon atom, and one extra oxygen atom, when compared to xylose, its degradation is expected to produce more H_2.

$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$ **Eqn. 2.19**

$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COO^- + 2HCO_3^- + 3H^+ + 2H_2$ **Eqn. 2.20**

$C_5H_{10}O_5 + 3.33H_2O \rightarrow 1.67CH_3COO^- + 1.67HCO_3^- + 3.33H^+ + 3.33H_2$ **Eqn. 2.21**

$C_5H_{10}O_5 + 1.67H_2O \rightarrow 0.83CH_3(CH_2)_2COO^- + 1.67HCO_3^- + 2.5H^+ + 1.67H_2$ **Eqn. 2.22**

The majority of research to date has been directed at using expensive pure substrates (i.e. sugars and starch). Large scale production of H_2 would consume enormous amount of this feedstock. To achieve pure sustainability and meet societies demand for renewable energy, greater focus should be shifted to sustainable feedstocks. These feedstocks can include corn stock, wheat, sugar beet or fodder grass (Hawkes et al., 2002). Using low cost feedstocks that are abundant in nature or are considered waste can offer a very sustainable large scale H_2 production process.

2.6 Microbial Inhibition

In a mixed culture, the H_2 produced during acidogenesis and acetogenesis is consumed by H_2 consuming bacteria during methanogenesis, the final stage of fermentation. The primary H_2 consumers in a mixed anaerobic microbial system are hydrogenotrophic methanogens, sulfate-reducing bacteria and homoacetogens. Sulfate-reducing bacteria do not play a major role since their effect is limited beyond a substrate threshold limit (Liamleam and Annachhatre, 2007). Methanogenic H_2 consumption is thermodynamically favoured over the homoacetogenesis process. Therefore, the bacteria responsible for the largest percent of H_2 consumption are hydrogenotrophic methanogens. Methanogens change the distribution of products by producing methane and reducing the amounts of acetate, H_2 and carbon dioxide. To recover the H_2 produced in acidogenesis and acetogenesis, efficient methods are required to suppress or eliminate the H_2 consuming bacteria. Researchers use a variety of techniques to accomplish this

task. The majority of techniques involve subjecting the mixed bacterials culture to a treatment process prior to feeding. As a result, they are referred to as 'pretreatment' methods.

The simplest types of pretreatment take advantage of the spore forming ability of most H_2 producing bacteria. *Clostridium* species have been shown to have this spore forming ability (Dabrock et al., 1992). When spore forming bacteria are subjected to harsh environmental conditions, the cells replicate their genetic material and surround it with a tough outer coating. When the outer part of the cell is destroyed, the released spore is well protected from the harsh conditions. These most widely used forms of pretreatment include: heat treatment, acid/base treatment, electric current, aeration, and chemical addition.

2.6.1 Heat Treatment

Heat treatment is the oldest and most common method for elimination of H_2 consuming bacteria. The temperatures and durations used vary from 71 to 121°C and 15 minutes to 2 hours, respectively. Li and Fang (2007) report that heating at 100°C for 15 minutes is most common. There has been no study to date to identify the optimal temperature and duration. Heat treatment has been shown to not always be 100% effective at eliminating all H_2 consumers. Oh et al. (2003) observed that homoacetogenic bacteria can survive heat treatment. Homoacetogens consume H_2 and produce acetate. Homoacetogens are normally outcompeted by methanogens due to the thermodynamic of the reaction pathway (equations 2.23 and 2.24, Table 2.5). However, in the absence of methanogens, the homoacetogens dominate and could produce large amounts of acetate.

Table 2.5: H_2 consuming reactions for methanogens and homoacetogens with $\Delta G^{\circ'}$ values

Type of Bacteria	Reaction	$\Delta G^{\circ'}$ (kJ/mol)	Equation
Homoacetogens	$4H_2 + 2CO_2 \leftrightarrow CH_3COO^- + H^+ + 2H_2O$	-94.9	2.23
Methanogens	$4H_2 + CO_2 \leftrightarrow CH_4 + 2H_2O$	-131.1	2.24

2.6.2 Acid/Base Treatment

Acid/base treatment is another common pretreatment method to eliminate H_2 consumers. Methane production rates can decrease significantly at pH values below 6.3 and above 7.8 (van Haandel and Lettinga, 1994; Chen et al., 2002). A substantial pH decrease or increase in bacterial communities, which are continuously maintained at a pH of 7, can effectively inhibit methanogenic activity. This pH adjustment may not affect spore forming bacteria such as H_2 producers. Chen et al. (2002) reported the impact of acid/base treatment on the H_2 yield after treating the bacteria culture at a pH of 3 for 24 hours. They reported the H_2 yield increased by a factor of 333; however, in comparison at a pH of 10 for 24 hours, they also observed an increase by a factor of 200.

2.6.3 Electric Current

Roychowdhurry (2000) observed that an application of low voltage (3.0-4.5 V) electric current to an anaerobic culture was able to as separate H_2 producing bacteria. These researchers tested cellulosic landfill sludge and sewage sludge and reported H_2 production without methane.

2.6.4 Aeration

Methanogens are pure anaerobes and are very oxygen sensitive. Purging with oxygen into a reactor impairs the function of methanogens. Ueno et al. (1995) observed a strong H_2 yield of 330 to 340 ml H_2/g hexose for a compost culture when aeration was used to inactivate methanogens.

2.6.5 Chemical Inhibition

2.6.5.1 Acetylene and Chloroform

Acetylene at a partial pressure of above 500 Pa has been shown to reduce the activity of pure methanogenic cultures (Sprott et al., 1982). Sparling et al. (1997) provided evidence that acetylene can affect H_2 production for an anaerobic digested sludge fed with paper waste.

Chloroform has also been shown to inhibit H_2 consuming bacteria. Chloroform inhibition was examined by Cheng et al. (2003) and Liang et al. (2002) with peptone and glucose, respectively.

2.6.5.2 2-Bromoethanesulfonate (BES)

BES is a specific methanogen inhibitor. It is an analog of coenzyme M, a cofactor found in all methanogens (DiMarco et al., 1990; Liu et al., 2011). Coenzyme M mediates the final steps in methane formation (Figure 2.5; Steps 6 and 7). BES competitively inhibits the methyl transfer reaction during the last reductive step of methane production. Gunsalus et al. (1978) observed that BES was inhibitory to the reduction of methyl coenzyme-M to methane in cell extracts of *Methanobacterium thermoautotrophicium*. A comparison of the chemical structure of BES to Coenzyme M is shown in Figure 2.6.

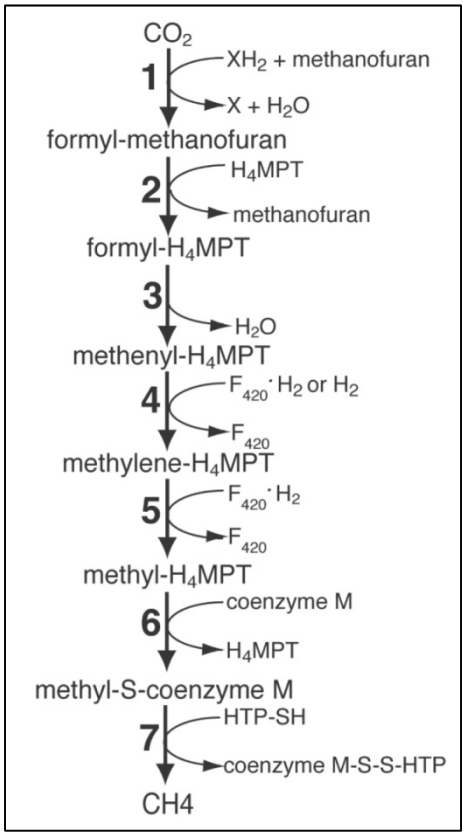
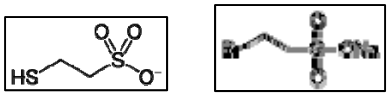


Figure 2.5: Methanogenesis reaction pathway



Coenzyme M BES

Figure 2.6: Coenzyme M and BES chemical structure

Using BES at a concentration of 25 mM (Sparling et al., 1997) and 100 mM (Wang et al., 2003) has been proven effective for H_2 production. According to Danko et al. (2008), BES inhibition is more effective than heat treatment. BES inhibition has not been successful in 100% of the studies in which it has been used. Santoro and Konisky (1987) found BES resistant strains of methanogens and Smith (1983) observed the reversal of BES inhibition. Moreover, treating bacteria cultures with these concentrations of BES is extremely costly. According to Li and Fang (2007), large-scale H_2 production using BES is uneconomical.

2.6.6 Long-Chain Fatty Acids (LCFAs)

LCFAs can be classified as either saturated or unsaturated fatty acids. Saturated fatty acids have no carbon-carbon double bonds and are typically solid at room temperatures. On the other hand, unsaturated fatty acids have one or more carbon-carbon double bonds on their carbon chain. The double bonds create backbone branching causing the molecules not to stack with each other Therefore, unsaturated fatty acids are liquid at room temperature. LCFAs contain both a hydrophobic end and a hydrophilic end. The LCFAs are attached to a glycerol backbone by ester bonds to form glycerides. Glycerides can exist in mono-, di- and tri- forms, which contain one, two and tree, fatty acids, respectively. Figures 2.7-2.9 show the three possible forms of glycerides.

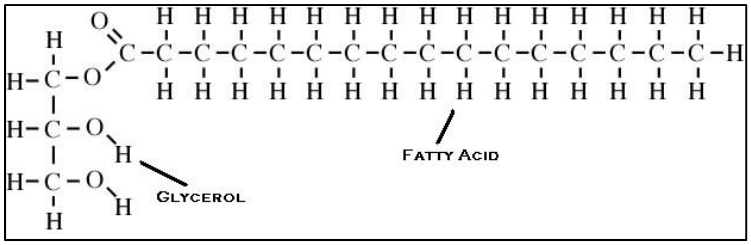


Figure 2.7: Typical mono-glyceride chemical structure

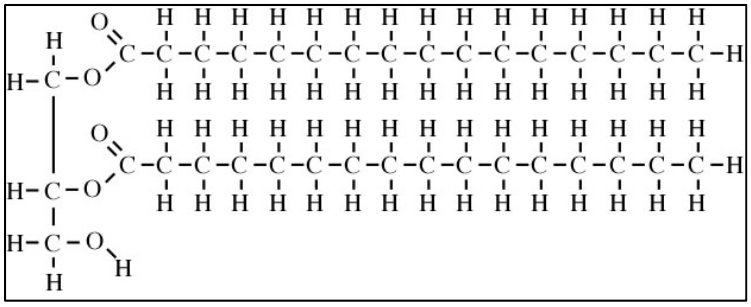


Figure 2.8: Typical di-glyceride chemical structure

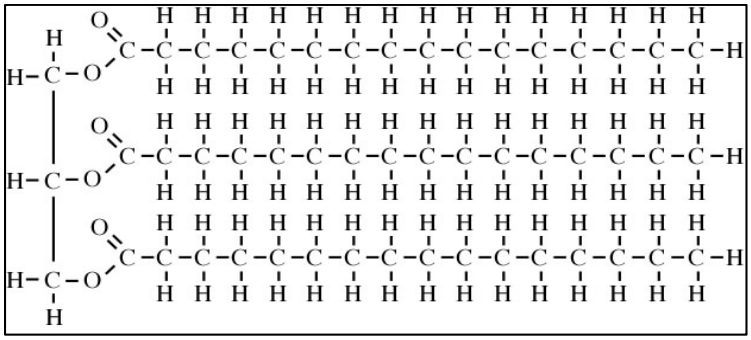


Figure 2.9: Typical tri-glyceride chemical structure

Nieman (1954) observed that unsaturated fatty acids have antibacterial effects on gram-positive bacteria but not on gram-negative bacteria. Neiman also reported that the inhibitory effect increases as the number of double bonds increases. Both LA and oleic acid (OA) are unsaturated and have 18 carbons. Work by Fuller and Moore (1967) has shown that LA (2 C=C bonds) has a greater inhibitory effect than OA (1 C=C bond) on gram-positive bacteria. Figure 2.10 and 2.11 show the difference in chemical structure between LA and LAU, the two LCFAs used in this

research work. Methanogens have a gram-positive cell wall and are susceptible to LCFA inhibition.

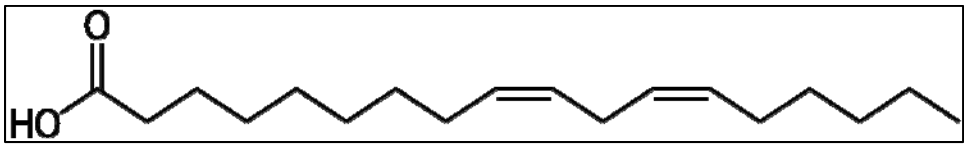


Figure 2.10: LA molecular structure

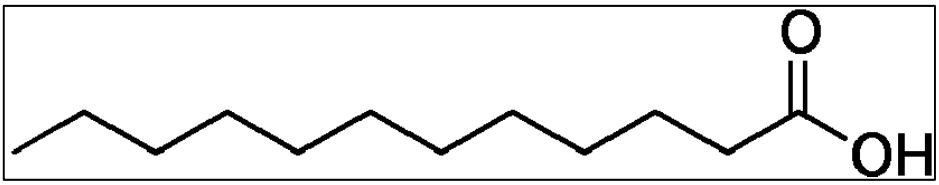


Figure 2.11: LAU molecular structure

LCFAs act by a number of different mechanisms to inhibit different microorganisms. They can cause death by surrounding the cell in a lipid layer. In CSTRs, this lipid layer causes the bacteria to float with subsequent wash out from the bioreactor (Alves et al., 2001). LCFA absorption on the surface of the anaerobic bacteria cultures has been reported to decrease the substrate degradation rate by reducing the permeability of the cell wall and ultimately limiting the transport of soluble substrate (Sayed et al., 1988; Rinzema et al., 1993; Demeyer and Hendrrickx, 1967). LCFAs also act as a membrane disruptor across the cell membrane of gram-positive bacteria, causing leakage of proteins and ions (Greenway and Dyke, 1979). After entering the cytoplasm, the LCFAs are able to dissociate and cause acidification (Baird-Parker, 1980). The end result is a change in pH across the cell membrane. With disrupted transfer of important molecules in and out of the cell membrane, the cell cannot function properly and eventually they die (Cirne et al., 2007). At pH values below 7 within the cell, most enzymes are inactivated, and subsequently, cell functions are impaired (Lehninger et al., 1999).

Experiments performed by Koster and Cramer (1987) were the first to recognize the ability of LCFAs to inhibit methanogenesis. More recent experiments have been conducted by a number of researchers (Lalman and Bagley, 2000; 2003; Chowdhurry, 2005; Gurukar, 2005; Ray et al., 2008; and Reaume, 2009). Lalman and Bagley (2000) examined the impact of LA on acetoclastic and hydrogenotrophic methanogens at 21°C. They observed that a threshold of 30 mg/l LA inhibited acetoclastic methanogens. Concentrations slightly higher than 30 mg/l were required to inhibit hydrogenotrophic methanogens. Lalman and Komjarova (2004) reported increases to both substrate degradation rate and the inhibitory effect caused by the LCFAs when the temperature was increased from 21°C to 37°C. LCFAs present many advantages over expensive chemical substances such as BES, acetylene and chloroform. LCFA inhibitors are less expensive, less toxic and they do not cause severe damage is discharged to the environment (Ray et al., 2008). In comparison to BES, acetylene and other chemical inhibitors, LCFAs can be derived agricultural crops.

Chapter 3: Materials and Methods

3.1 Experimental Plan

The experiments were divided into four phases to achieve the research objectives. The first phase was designed to determine the optimum pH for glucose degradation by the mixed anaerobic culture used. The optimum pH was selected when the quantity of H_2 produced reached a maximum. This optimum pH was used as the initial pH for all subsequent experiments. Phase II examined H_2 production from glucose degradation with varying BES concentrations. The concentrations of BES studied were 0, 10, 25, 50, and 75 mM. Included in Phase II was a control set containing 0 mM BES and 0 g/l glucose. The controls were used to determine culture degradation over the course of the 8-day experiment. Phase III was designed to determine the effects of adding BES and LCFA in incremental quantities. As an alternative to adding a specific amount of BES in one injection, the amount was divided into two equal injections separated by 24 hours. The same procedure was used for LA. This incremental inhibitor injection study for BES and LA was conducted in combination with single inhibitor injections for direct comparison. Phase IV of the experimental plan was designed to compare the inhibition potential LAU and LA to BES. The objective was to determine what concentration of LA and LAU inhibition equates to BES inhibition in terms of H_2 and methane formation. Special importance was placed on varying LCFA and BES concentrations above and below what had been reported in other studies. Reaume (2009) found that 2000 mg/l LA was the most effective in inhibiting methanogens in the mixed cultures. In Phase IV, LAU and LA concentrations of 1000, 2000 mg/l and 3000 mg/l were selected. A large amount of research to date has reported using BES concentrations between 25 and 50 mM. As a result, BES concentration of 25, 50 and 100 mM were selected for this study. Tables 3.1-3.4 outline the four experimental phases in this study.

Table 3.1: Phase I - pH Optimization Study

Cultures	pH	1st Glucose injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	5.5	5 g/l	5 g/l
4, 5, 6	6.5	5 g/l	5 g/l
7, 8, 9	7.7	5 g/l	5 g/l

Table 3.2: Phase II – H_2 production from glucose degradation in the presence of BES

Cultures	BES Conc., mM (g/l)	1st Glucose Injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	0	0	0
4, 5 ,6	0	5 g/l	5 g/l
7, 8 , 9	10 (2.11)	5 g/l	5 g/l
10, 11, 12	25 (5.275)	5 g/l	5 g/l
13, 14, 15	50 (10.55)	5 g/l	5 g/l
16, 17, 18	75 (15.825)	5 g/l	5 g/l

Table 3.3: Phase III – H_2 production from glucose degradation in the presence of BES and LA added in increments

Cultures	Inhibitor	1st Addition (t=0 hr)	2nd Addition (24 hr)	1st Glucose Injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	BES	0	50 mM	5 g/l	5 g/l
4, 5, 6	BES	25 mM	25 mM	5 g/l	5 g/l
7, 8, 9	LA	0	2000 mg/l	5 g/l	5 g/l
10, 11, 12	LA	1000 mg/l	1000 mg/l	5 g/l	5 g/l

Table 3.4: Phase IV – H_2 production from glucose degradation with varying concentrations of LAU, LA and BES

Cultures	LAU conc., mg/l	LA conc., mg/l	BES conc., mM	1st Glucose Injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	1000	0	0	5 g/l	5 g/l
4, 5, 6	2000	0	0	5 g/l	5 g/l
7, 8, 9	3000	0	0	5 g/l	5 g/l
10, 11, 12	0	1000	0	5 g/l	5 g/l
13, 14, 15	0	2000	0	5 g/l	5 g/l
16, 17, 18	0	3000	0	5 g/l	5 g/l
19, 20, 21	0	0	25	5 g/l	5 g/l
22, 23, 24	0	0	50	5 g/l	5 g/l
25, 26, 27	0	0	100	5 g/l	5 g/l
28, 29, 30	0	0	0	5 g/l	5 g/l
31, 32, 33	0	0	0	0 g/l	0 g/l

3.2 Inoculum Source and Maintenance

A 4-L batch reactor with a liquid volume of 3.5 L was used in this study. The reactor was covered with aluminum foil to prevent biological growth upon exposure to light. The reactor was maintained at a VSS concentration of 5000-7000 mg/l. Table 3.5 lists the inoculum sources which were used to make up final microbial populations in the mother reactors.

Table 3.5: Inoculum sources of reactor by %VSS

Inoculum source	%VSS of Reactor
Municipal Primary Sludge (Chatham)	25
Municipal Digested Sludge (Chatham)	10
Industrial Bioethanol Sludge (Guelph)	15
Industrial Wastewater Treatment Sludge (Cornwall)	50

Reactor maintenance and feeding was performed once a week. Mixing was stopped for a few hours to allow the solids in the reactor to settle to the bottom. This caused phase separation between the liquid and solids. The top water layer (approximately 2L) was decanted and fresh basal medium was added to adjust the reactor volume to 3.5 L. Basal medium is a nutrient solution which promote culture growth. Resazurin in the basal medium served as indicator of aerobic conditions. The colorless solution converts into a pale pink if oxygen contaminates the reactor. Once this maintenance was completed, the pH of the reactor was recorded to ensure the culture was not overly acidic. A pH between 6 and 7.6 was considered acceptable. Lastly, 5000 mg/l of glucose was added (i.e. 17.5 grams for 3.5 L volume) and the reactor was purged with nitrogen gas (N_2) gas for approximately 2-3 minutes, and then sealed. The temperature of the reactor was recorded once per month to ensure the culture was consistently maintained at 37°C throughout the course of the study.

3.3 Basal Media

The basal media used in bottle preparation and reactor maintenance was constituted by adding the list of ingredients in Table 3.6 to tap water (Weigant and Lettinga, 1985).

Table 3.6: Ingredients of basal medium

Substance	Concentration, mg/l
$NaHCO_3$	6000
NH_4HCO_3	70
K_2HPO_4	14
$(NH_4)_2SO4$	10
Yeast extract	10
Resazurin	1.0
$CuCl_2 \cdot 2H_20$	0.03
Na_2SeO_3	0.1
$CoCl_2 \cdot 6H_20$	0.15
$MnCl_2 \cdot 4H_20$	0.5
$NiCl_2 \cdot 6H_20$	0.05
H_3BO_3	0.05
KCl	25
$ZnCl_2$	0.05
$MgCl_2 \cdot 4H_20$	9
EDTA	1.0
$(NH_4)_6MoO_7 \cdot 4H_20$	0.09
$FeCl_2 \cdot 4H_20$	2.0
Na_2S	3.0

3.4 Experimental Details

All experiments were prepared in 160 ml serum bottles covered in aluminum foil to prevent biological growth of exposure to light. Within a Coy® anaerobic chamber, each bottle was injected with specific amounts of culture and basal medium to achieve a VSS concentration of 2000 mg/l. The anaerobic chamber used in this study is shown in Figure 3.1. The composition of the gas mixture in the anaerobic chamber was approximately 80% N_2 and 20% CO_2. The pH of each culture was adjusted to the optimal pH determined in Phase I using 1 M HCl and 1 M NaOH. The bottles were capped using Teflon®-lined silicon rubber septas and aluminum crimp caps. To avoid negative pressure from forming during headspace sampling, 20 ml of anaerobic

chamber gas mixture (80%N_2/20%CO_2) was injected using a gastight syringe. The capped and pressurized bottles were removed from the anaerobic chamber and placed into a temperature controlled room. The room temperature was maintained at 37°C and the bottles were kept constant mixed at 200 rpm using an orbital shaker (Lab Line Instruments). Prior to receiving inhibitor (LA, LAU, BES) each bottle was left mixing for approximately 24 hours. After receiving inhibitor, the serum bottles contents were allowed to mix for another 24 hours to remove any H_2 which was added from the anaerobic chamber's head space before glucose was injected. The time when glucose was injected is considered time 0 hour. The total liquid volume of all the bottles at time 0 hour was 50 ml. After sampling at time 96 hrs, each bottle was uncapped, and the pH was adjusted to the initial pH. The bottles were purged for 3 minutes with N_2 gas, recapped, pressurized with 20 ml of carbon dioxide (CO_2) and re-injected with glucose at a concentration of 5000 mg/l. All experiments were conducted in triplicates.

Figure 3.1: Coy® anaerobic chamber

3.5 Analytical Methods

3.5.1 VFAs

VFAs were measured using a DX-500 Ion Chromatograph (IC) (Dionex, Oakville, ON) equipped with an automated sampler (AS40), a gradient pump (GP50), a conductivity detector (CD20), a

liquid chromatography oven (LC10), and an ASRS-ULTRA (4-mm) anion self-regenerating suppressor. The IC was configured with an IonPac®24-cm x 4-mm diameter AS11-HC analytical column and IonPac® AG11-HG guard column with a sample loop of 25 µL. The VFA analysis method was adapted from Lalman and Bagley (2000) and used the following three eluents:

Eluent A: Milli-Q® grade water (18 ΩM);

Eluent B: 5 mM sodium hydroxide (NaOH); and,

Eluent C: 50 mM NaOH.

Eluents B and C were prepared with Milli-Q® (18 ΩM) grade water. The total eluent flow rate was 1 ml/min for the 20 minutes analysis time. Figure 3.2 shows the individual flow rates of each eluent, as a percent of total flow, over the analysis time of a sample.

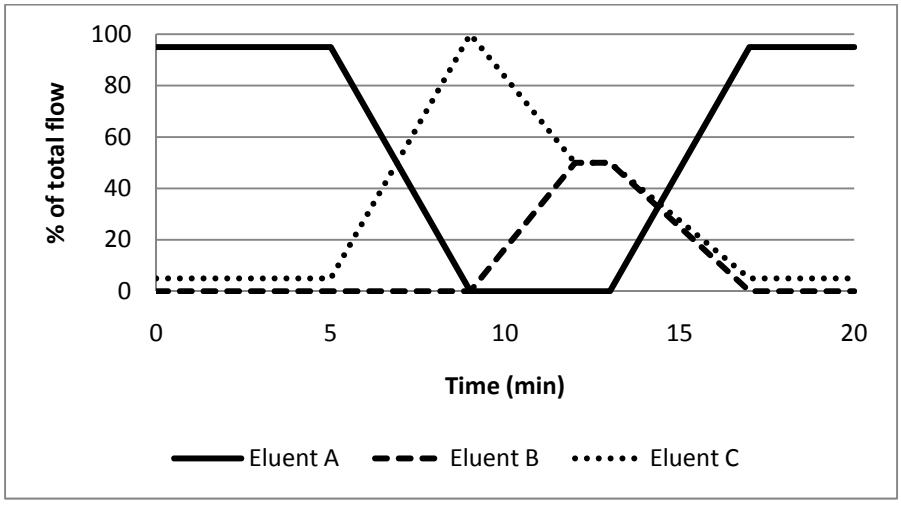


Figure 3.2: Eluent flow rate by analysis time

A VFA calibration curve was generated by analyzing six standards prepared in triplicate; 5, 10, 25, 50, 75, 100 mg/l. The six standards were prepared using acetic acid (99.7%), butyric acid (99%), propionic acid (99%), lactic acid (90%) and formic acid (95%). All standards were

prepared with basal medium diluted 20 times with Milli-Q® grade water (18 ΩM) in order to simulate experimental conditions. Samples containing MQ water were inserted every 10-15 injections to ensure to proper calibration of the IC. All VFAs had a lower detection limit of 5 mg/l. With a dilution factor of 20X, the analyte concentrations from samples taken from the fermentation media fell within the calibration curve range from 100 to 2000 mg/L. The VFAs calibration curves are shown in Appendix I.

3.5.2 Alcohols

Alcohols were measured using a DX-600 Ion Chromatograph (IC) (Dionex) equipped with an automated sampler (AS40), a gradient pump (GP50), a liquid chromatography oven (LC10), and a conductivity detector (ED50). The IC was configured with a 4-mm x 250-mm CarboPac™ MA1 analytical column (Dionex) and a 4-mm x 50-mm CarboPac™ MA1 guard column (Dionex) with a sample loop of 10 μL. The 480 mM NaOH eluent was prepared using Milli-Q® grade water. The eluent flow rate was set at 0.4 ml/min for 30 minute analysis time. An alcohol calibration curve was generated by analyzing the following 5 standards in triplicate: 25, 50, 75, 100, 200 mg/l. The 5 standards were prepared from ethanol (95%), propanol (99%), iso-propanol (99.9%), butanol (99.4%) and iso-butanol (99.9%). All standards were prepared by diluting basal medium 5 times with Milli-Q® water in order to simulate experimental conditions. Control samples containing Milli-Q® grade water were inserted every 10-15 injections to ensure to proper calibration of the IC. All alcohols had a lower detection limit of 25 mg/l. With a dilution factor of 5X, the analyte concentrations from samples taken from the fermentation media fell within the calibration curve range from 125 to 1000 mg/L. The alcohol calibration curves are shown on Appendix II

3.5.3 Glucose

Glucose degradation was measured using a DX-600 Ion Chromatograph (IC) (Dionex) equipped with an automated sampler (AS40), a gradient pump (GP50), a liquid chromatography oven (LC10), and a conductivity detector (ED50). The IC was configured with a 3-mm x 150-mm CarboPac™ PA20 analytical column (Dionex) and a 3-mm x 30-mm CarboPac™ PA20 guard column (Dionex) with a sample loop of 10 µL. The eluent used was a 30 mM NaOH solution, which was prepared using Milli-Q® grade water. The total eluent flow rate was 0.2 ml/min for the 20 minute analysis time. A glucose calibration curve was generated by analyzing the following eleven triplicate standards: 1, 5, 10, 25, 50, 100, 200,300, 400, 500 mg/l. The eleven standards were prepared using pure glucose (dextrose monohydrate) (Sigma-Aldrich, Toronto, ON). All standards were prepared in basal medium which was diluted 10 times with Milli-Q® grade water in order to simulate experimental conditions. Control samples containing Milli-Q® grade water were inserted every 10-15 injections to ensure proper calibration of the IC. Glucose had a lower detection limit of 1 mg/l. With a dilution factor of 10X, the analyte concentrations from samples taken from the fermentation media fell within the calibration curve range from 10 to 5000 mg/L. The glucose calibration curves are shown on Appendix III.

3.5.4 Headspace Gas Measurement

Gases (H_2, CH_4, CO_2) were measured using a Varian 3800 gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a 2-mm x 1-mm diameter packed Shincarbon ST (Restek) column. The operational temperature set points of the TCD, injector and oven were 200°C, 100°C, and 200°C, respectively. Nitrogen gas (99.99%, Praxair) was used as the carrier at a flow rate of 20 ml/min with a total analysis time of 2 minutes. The GC was calibrated with standards prepared using H_2 (99.99%, Praxair), methane (99.99%, Praxair) and carbon dioxide (99.99%, Praxair). The standards were prepared in 160 ml serum bottles which had been purged

with N_2 (99.99%, Praxair) for 3 minutes then sealed and capped with Teflon®-lined silicon rubber septas and aluminum crimp caps. The standard concentrations for H_2, CO_2 and CH_4 were as follows: 0.25, 0.5, 1, 2.5, 5, and 10 ml gas/160 ml. Additional standards of 25, 50, 75 and 100 ml/160 ml were analyzed with only H_2. All gases sampled from the serum bottles reactors were detected at levels greater than the lower detection limit of 0.25 ml/160 ml. The gas calibration curves are shown on Appendix IV.

3.5.5 VSS/TSS and pH Measurement

The volatile suspended solids (VSS) and total suspended solids (TSS) concentration in the reactor was measured following the Standard Method of Analysis (APHA, AWWA, WEF, 1992). VSS and TSS measurements were conducted at the beginning of all experiments to ensure the VSS concentration was 2000 mg/l in the serum bottle reactors. When experiments were not run, VSS and TSS measurements were taken periodically to ensure the health of the reactor. The measurements were conducted in triplicates using a liquid sample between 4-6 ml and filtered using 0.45 μm pore size glass fiber filters (VVR, ON). A VWR SR40C, Symphony pH meter (Orion) was used in bottle preparations and reactor maintenance. The meter was calibrated before each use with pH 4 and pH 7 standard buffer solutions.

3.6 Sampling Plan and Sampling Process

3.6.1 Sampling Plan

Table 3.8 displays the sampling plan used in all five phases of experiments. In phase IV, VFA and alcohol samples were not taken at 72, 120 and 168 hours.

Table 3.7: Sampling Plan

Group	Substance	Sampling Intervals (hr)																
Sugars	Glucose	Bottle Preparation (t = -48 hr)	Inhibitor injection (BES, LA, OA or Omega-3) (t = -24 hr)	5 g/l Glucose Injection (t = 0)	0	2	4	6	8	12	16	24				Bottles opened - pH adjusted to initial value - N_2 purged for 3 min - pressurized w/ 20 ml CO_2 - 5 g/l glucose injected (t = 96 hr)				
Alcohols	Iso-propanol				0							24	48	72	96		120	144	168	192
	Ethanol																			
	Propanol																			
	Iso-butanol																			
	Butanol																			
VFAs	Lactic acid				0							24	48	72	96		120	144	168	192
	Acetic acid																			
	Formic acid																			
	Propionic acid																			
	Butyric acid																			
Gases	H_2									12		24	48	72	96		120	144	168	192
	Carbon Dioxide																			
	Methane																			

3.6.2 Sampling Process

The following steps were followed to prepare VFA, alcohol and glucose samples for IC analysis. The samples were diluted to allow for IC peak resolution. Alcohol samples were diluted 5X; VFA samples were diluted 20X and glucose samples were diluted 10X. Even though no separation was needed for glucose samples, the 10X dilution kept the IC, specifically, the electrochemical detector (ED50), in a range where it was most accurate. The output data generated by the IC was converted into a concentration using the corresponding calibration curve and then multiplied by the appropriate dilution factor to arrive at the true concentration in the sample.

3.6.2.1 Alcohols

1. At the sampling times shown in Table 3.8, a 1 ml sample was extracted from a bottle using a 1-ml syringe;
2. The 1 ml sample was injected into 4 ml of Milli-Q® grade water (Millipore) previously added in a 10-ml glass vial;
3. Steps 1 and 2 were repeated for all bottles in the experiment;
4. All 10-ml glass vials containing the 5-ml liquid volume were centrifuged at 4000 rpm for 10 minutes;
5. The supernatant was extracted from the glass vial using a 5-ml syringe. The 5-ml syringe was used to filter the sample through a double filtering process and into a 5-ml plastic IC vial. The first filtering process used a 25-mm diameter 0.45 μm polypropylene membrane. This was connected to the second filtering method, a 1-ml polypropylene cartridge with a 20 μm PE frit and filled with Chelex® 100 to 200 mesh silicon resin;
6. Step 5 was repeated for all centrifuged 10-ml glass vials. The end result was a set of 5-ml IC vials containing 5X diluted samples ready for alcohol analysis.

3.6.2.2 VFAs

7. A 1 ml sample was removed from an IC vial (Step 6) using a 1-ml syringe and was injected into 3 ml of Milli-Q® grade water previously added in another 5-ml IC vial;
8. Step 7 was repeated for all 5-ml IC vials. The end result was a set of 5-ml IC vials containing 20X diluted samples ready for VFA analysis;
9. Both sets (Alcohol-Step 6 and VFA-Step 8) of the 5-ml IC vials were capped.

3.6.2.3 Glucose

1. At the sampling times shown in Table 3.8, a 0.5 ml sample was extracted from a bottle using a 0.5-ml syringe;
2. The 0.5 ml sample was injected into 4.5 ml of Milli-Q® grade water previously added in a 10-ml glass vial
3. Steps 1 and 2 were repeated for all bottles in the experiment;
4. All 10-ml glass vials containing 5-ml liquid volume were centrifuged at 4000 rpm for 10 minutes;
5. The supernatant was extracted from the vials using a 5-ml syringe. The 5-ml syringe was used to push the sample through a double filtering process and into a 5-ml plastic IC vial;
6. Step 5 was repeated for all 10-ml glass vials. The end result in a set of 5-ml IC vials containing 10X diluted samples ready for glucose analysis;
7. The 5-ml IC vials were capped.

3.6.2.4 Gas Samples

Headspace gas samples of 35-50 µL were removed using a 100 µL Hamilton gastight syringe (VVR). Part of the gas sample was expelled from the syringe until 25 µL remained and the sample was then manually injected into the GC. A pressure meter was used to measure the pressure in each bottle at the time of injection. The gas calibration curve was used to convert the

GC output into analyte mass. This pressure measurement was used to convert the mass of gas into mol using the ideal gas law.

3.7 LCFA Delivery Method

The LCFAs used in this study possess 12 and 18 carbons and are relatively insoluble in water. In order to properly disperse the LFCA in the fermentation media, they were dissolved in water prior to use in an experiment. A delivery method developed by Angelidaki and Ahring (1992) was used to make the LCFA stock solutions used in this study. Stock solutions of 50,000 mg/l were prepared. The LCFAs were melted *au bain-marie* and dissolved in 50°C vigorously stirred sodium hydroxide (NaOH) solution. The amount of NaOH used, expressed as grams NaOH per grams of LCFA, is shown in Table 3.8.

Table 3.8: Amount of NaOH used in LCFA stock preparation

LCFA	NaOH (g NaOH/g LCFA)
LAU	0.200
LA	0.142

3.8 Batch Reactor Operation and Culture Acclimation

The reactor set-up used for all experimental work is shown in Figure 3.3. A gas counter was used to measure the amount of gas production after each glucose feeding. The round plastic compartment contains a tipping balance submerged in water. The gas produced from the reactor travels through a connecting tube and enters the gas counter. The gas is released directly below the tipping bucket causing it to rise to one side. Once the tip reached to a certain level, it contacts a magnetic sensor connected to a counter which closes a circuit. Figure 3.4 shows the internal components of the gas counter. The counter is reset to zero prior to every weekly feeding and monitored on a daily basis.

The gas counter was calibrated after installation by injecting a known amount of gas and observing how many counts are recorded. An average was taken after five injections. Every count corresponded to 4.6 ml of gas production. Figure 3.5 displays gas counts taken over a period of five weeks. Approximately 17.5 grams of glucose was added to the reactor at time=0 (this equates to a concentration of 5 g/l for a 3.5 L liquid volume). The counts were recorded at approximately the same time each day (±1 hour). The counts were multiplied using the volume per count value obtained from the calibration.

Figure 3.3: Reactor Set-up

Figure 3.4: Internal View of Gas Counter

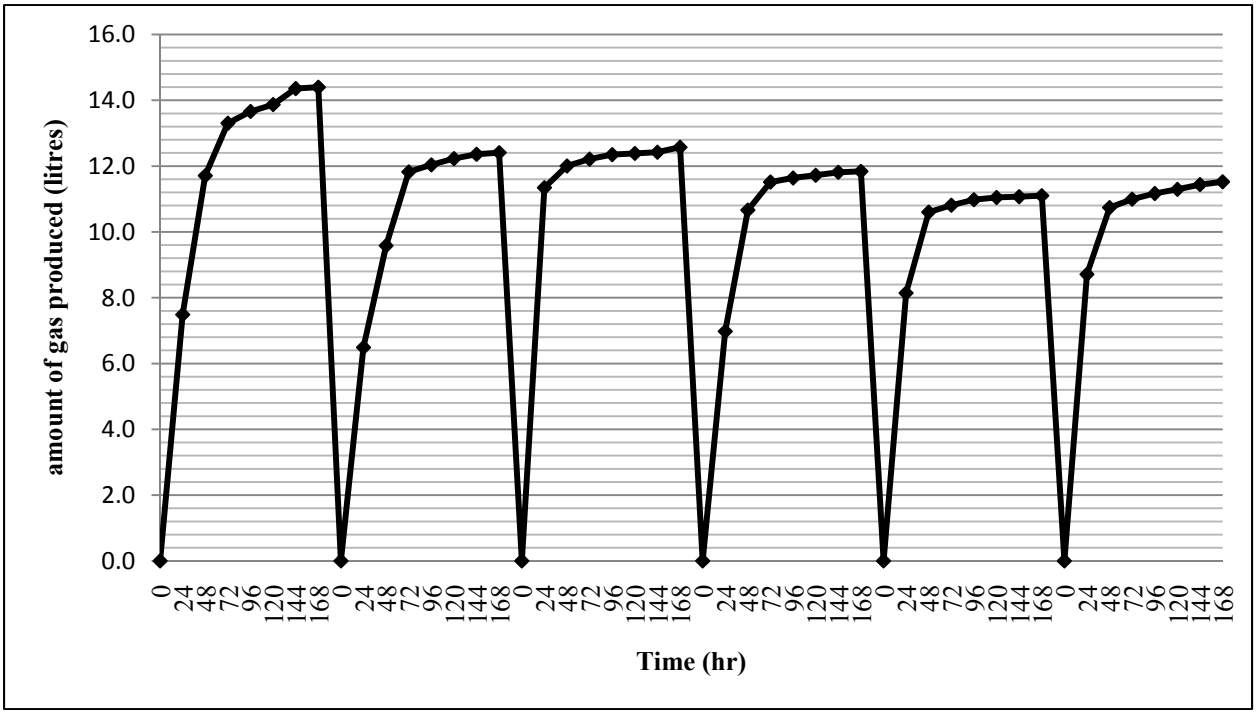


Figure 3.5: Weekly Reactor Gas Production

3.9 Generating Stoichiometric Reactions by Method of Electron Balancing

One of the objectives of this work was to develop balanced stoichiometric reactions for phase II, III and IV. Two reactions were generated for each culture set; one set of liquid and gas samples were removed and analyzed during the first glucose injection period and the other during the second glucose injection. In phase II and III at 72 and 168 hours, liquid and gas sample concentrations were removed and analyzed and the analyte concentrations were used to generate the stoichiometric reactions. In phase IV, reactions were generated using gas and liquid concentrations at 96 and 192 hours (VFA and alcohol results were not collected at 72 and 168 hours). Balanced stoichiometric reactions were generated by adding electron donor and electron acceptor half-reaction. All balanced reactions are shown in the last section of Chapters 5, 6 and 7 for phases II, III and IV, respectively.

The following steps outline the process used to generate balanced reactions. Gas results, in μmol/bottle, are converted into mmol. Liquid byproducts (VFAs and alcohols), in mg/l, are converted in mmol by multiplying by reactor volume (0.05 litres) and divided by their molecular weight (mg/mmol). The specific m_{eq}/mmol for each product was determined from their half reaction. Electron equivalences (m_{eq}) were obtained for all gas and liquid products by multiplying the mmol of each product by their specific m_{eq}/mmol. Electron equivalences (m_{eq}) are normalized based on the input of electron equivalences. Glucose was the electron donor used in all experiments. Glucose contains 24 m_{eq}/mmol. An addition of 5000 mg/l glucose equals 1.388 mmol electron equivalents. Therefore, 33.304 m_{eq} [24 × 1.388] are available for product formation. The m_{eq} for each product is divided by 33.304. This quotient is then multiplied by the quotient of 33.304 and the sum of all m_{eq} for all products (gas, VFAs and alcohols). The

summation of all normalized electron equivalences values (x_{eq}) equals 1. A chart at the end of Appendix V shows this process for one culture set.

To develop balanced stoichiometric reactions based on these electron equivalences (x_{eq}), half reactions (on a 1 electron basis) for all products (gases, VFA and alcohols) are required. The coefficients in the half reactions were multiplied by the electron equivalence (x_{eq}) of the corresponding product. All adjusted half reactions were added together to get a reaction R_a (This is the total acceptor reactor). R_a is multiplied by 0.9. Assuming 10% of the electron equivalences are diverted to cell synthesis allows the remaining 90% to be used in producing the different metabolites. This allows the half-reaction for cell synthesis (R_c) to be multiplied by 0.1. The glucose half reaction represents the donor reaction (R_d). Therefore, $R_{total} = (0.9R_d+0.1R_c) + R_d$. The carbon dioxide ($CO_2$) on the product side of R_{total} was removed from the reaction. This was done by adding the amount of CO_2 produced to H_2O (reactant side), HCO_3^- (product side) and H+ (product side). The final reaction, R_{total}, represents the final balanced reaction. An element and charge count was done on the final reaction to ensure it was balanced properly.

Chapter 4: Culture Characterization and pH Optimization

The optimal pH for H_2 production depends on the composition of the mixed culture. All species of bacteria have a preferred operating pH which is ideal for optimizing the production of specific byproducts. Based on the literature review, a pH of 5.5 is expected for optimum H_2 production. At this pH, the activities of most H_2 consuming bacteria are reduced. To confirm this expectation, the mixed culture to be used in all experiments was tested at three initial pH values; 5.5, 6.5, and 7.7. A pH of 7.7 represents the unaltered pH of the mixed culture. A full 8-day experiment was conducted, with two glucose injections (t=0 and t=4 days). All cultures were prepared in triplicates. The principal objective of most bio-H_2 research was to obtain the highest H_2 yield possible. Therefore, the pH producing the highest H_2 yield was selected to perform all subsequent experiments. Table 4.1 summarizes the experimental design conditions for this study. Gas, VFA, alcohol and glucose profiles are shown in Figures 4.1-4.14.

Table 4.1: Phase I - pH optimization study

Cultures	pH	1st Glucose injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	5.5	5 g/l	5 g/l
4, 5, 6	6.5	5 g/l	5 g/l
7, 8, 9	7.7	5 g/l	5 g/l

4.1 H_2 and Methane Production

Very low quantities of H_2 accumulated at initial pH values of 6.5 and 7.7. Only in cultures at an initial pH of 5.5 were substantial quantities of H_2 observed. Maximum yields of 0.86 and 1.40 mol H_2/mol glucose were obtained at an initial pH of 5.5 during the first and second glucose injection periods, respectively (Figure 4.1; Table 4.2). Note the H_2 yields were greater during the second glucose injection period. Hydrogen yields peaked 3 days after glucose injection (72 hours and 168 hours). As expected, methane production was dominant in cultures with an initial pH of

7.7; reaching a maximum yield of 2.15 mol CH_4/mol glucose at 192 hours (Figure 4.2; Table 4.3). At an initial pH of 6.5, a lower amount of methane was produced. The quantities of methane reached a maximum yield of 0.54 mol CH_4/mol glucose after 72 hours. The amount of methane production remained fairly equal in both first and second glucose injections at initial pH values of 6.5 and 7.7. Methane production at initial pH 5.5 was considerably lower than pH 6.5 and 7.7. At pH 5.5, the methane yield peaked at 0.18 mol CH_4/mol glucose at 96 hours. Methane did not accumulate during the second glucose injection in cultures with an initial pH of 5.5. As anticipated, the most H_2 was produced in cultures with an initial pH of 5.5. Therefore, an initial of 5.5 was used for all subsequent experiments. Table 4.1 summarizes all the yield data for H_2 and methane over the duration of this experiment. A bar chart showing the maximum methane concentration per bottle for each culture set over the duration of the experiment is shown in Figure 4.4.

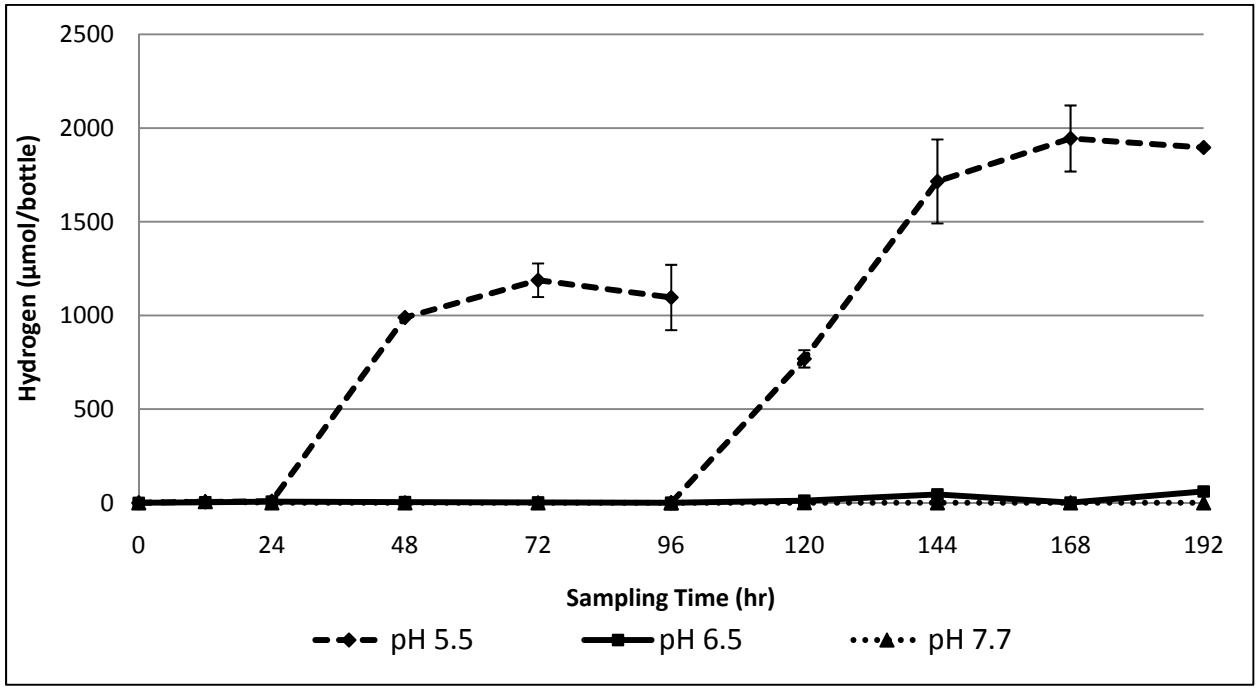


Figure 4.1: H_2 production in cultures at different initial pH values

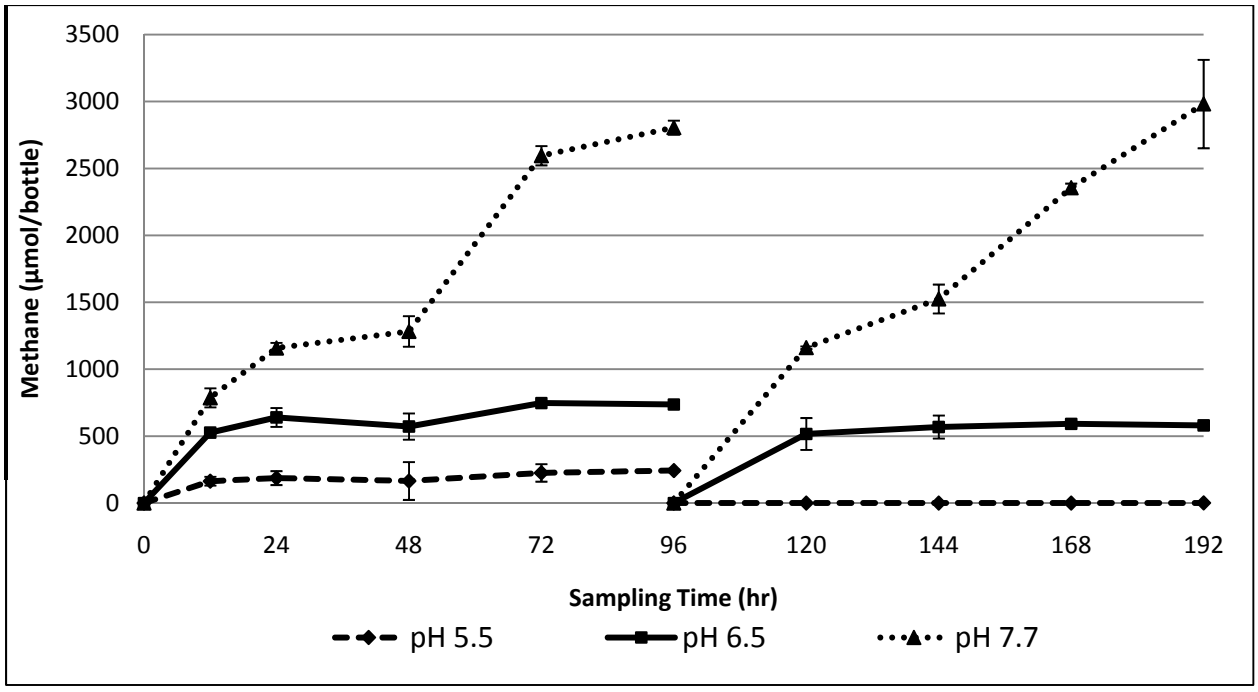


Figure 4.2: Methane production in cultures at different initial pH values

Table 4.2: H_2 and methane yields in cultures at different initial pH values

	mol H_2/mol glucose			mol METHANE/mol glucose		
Time(hr)	**pH 5.3**	**pH 6.5**	**pH 7.7**	**pH 5.3**	**pH 6.5**	**pH 7.7**
12	0.004	0.002	0.005	0.118±0.02	0.379±0.01	0.567±0.05
24	0.006	0.005	0.000	0.135±0.04	0.461±0.05	0.835±0.03
48	0.713	0.003	0.000	0.119±0.10	0.412±0.07	0.924±0.08
72	0.856±0.06	0.002	0.000	0.162±0.05	0.538±0.01	1.870±0.05
96	0.790±0.13	0.000	0.000	0.175	0.531±0.01	2.021±0.04
120	0.554±0.03	0.008	0.001	0.000	0.372±0.09	0.837±0.01
144	1.236±0.16	0.032	0.000	0.000	0.409±0.06	1.099±0.08
168	1.401±0.13	0.001	0.000	0.000	0.426±0.01	1.698±0.02
192	1.367±0.01	0.044±0.01	0.000	0.001	0.418±0.02	2.149±0.24

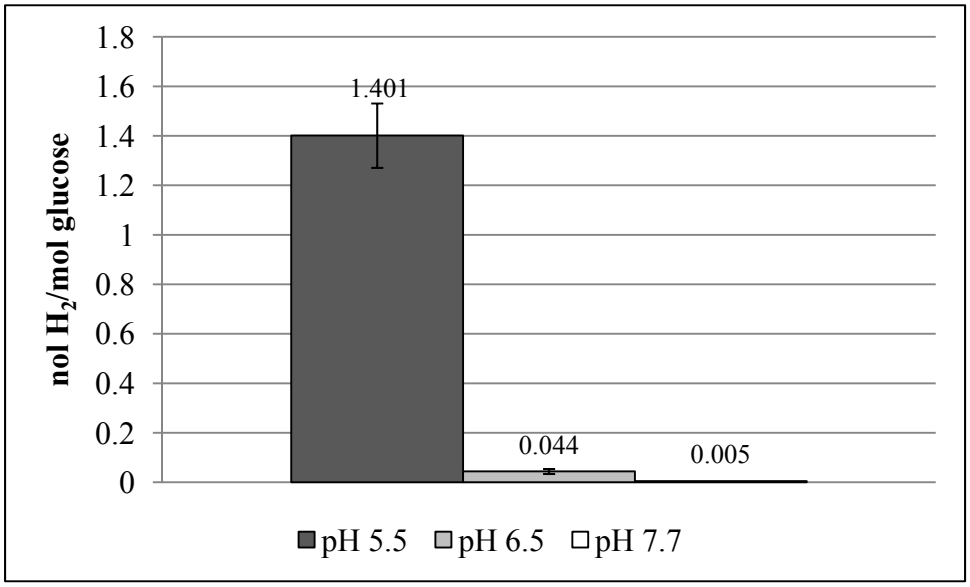


Figure 4.3: Maximum H_2 yield observed at different pH conditions

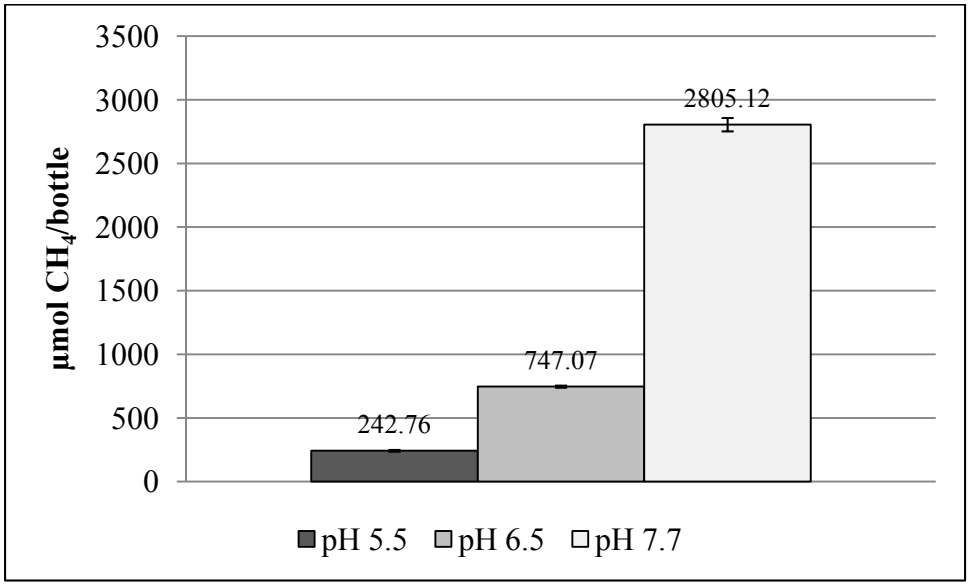


Figure 4.4: Maximum methane concentration per culture observed at different pH conditions

4.2 VFA Production

The initial pH values the culture affected the VFA distribution. At an initial pH of 5.5, lactic acid formation was dominant (Figure 4.5). Lactic acid accumulation increased and reached a maximum concentration of 1660 mg/l at 192 hours. Lactic acid formation was nearly none

existent at initial pH values of 6.5 and 7.7. At initial pH values of 5.5 and 6.7, acetic acid accumulation increased slowly over the duration of the 8-day experiment (Figure 4.6). Maximum acetic acid concentrations observed were 1210 mg/l and 670 mg/l at initial pH of 6.5 and 5.5, respectively. At initial pH of 7.7, the acetic acid accumulation reached a maximum 24 hours after glucose injection and declined to nearly 0 mg/l four days later. This trend occurred in both first and second glucose injections. Propionic acid accumulation was relatively equal in all cultures (Figure 4.7). The maximum propionic acid concentrations ranged from approximately 800-1200 mg/l. Formic acid production was very low in all cultures (Figure 4.8). At initial pH of 6.5, formic acid reached a maximum concentration of 100 mg/l at 144 hours. The formic acid concentrations remained below 40 mg/l in cultures at initial pH of 5.5 and 7.7 over the entire experiment. Butyric acid accumulation was significantly higher in cultures at an initial pH of 5.5 and 6.5 compared to 7.7 (Figure 4.9). The maximum butyric acid concentrations were 2950 and 2910 mg/l at initial pH values of 5.5 and pH 6.5, respectively. At an initial pH of 7.7, the butyric acid accumulation peaked at around 1000 mg/l at time 144 hours.

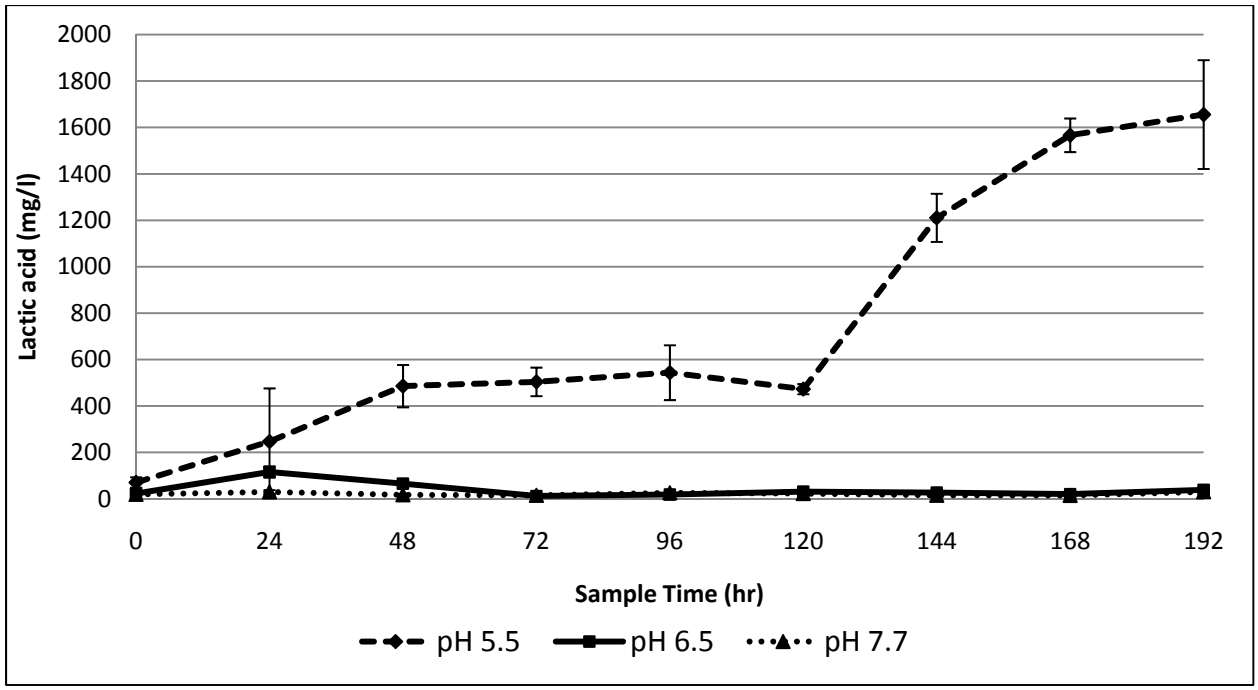


Figure 4.5: Lactic acid production in cultures at different initial pH values

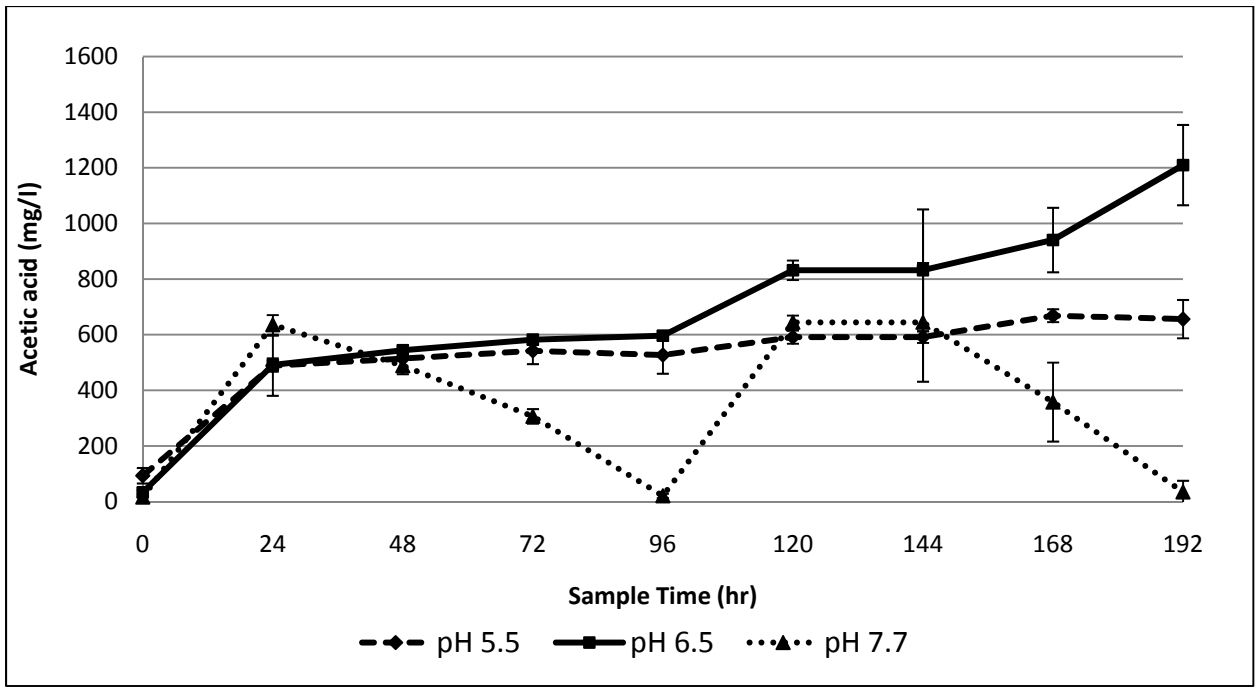


Figure 4.6: Acetic acid production in cultures at different initial pH values

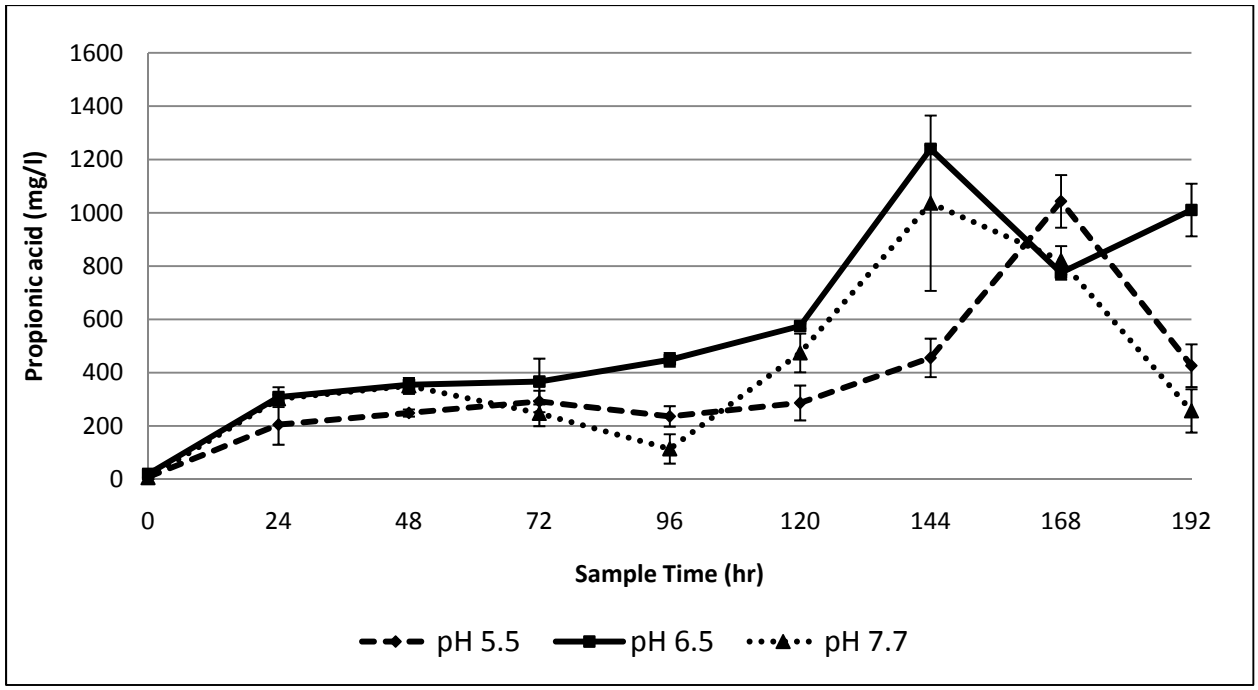


Figure 4.7: Propionic acid production in cultures at different initial pH values

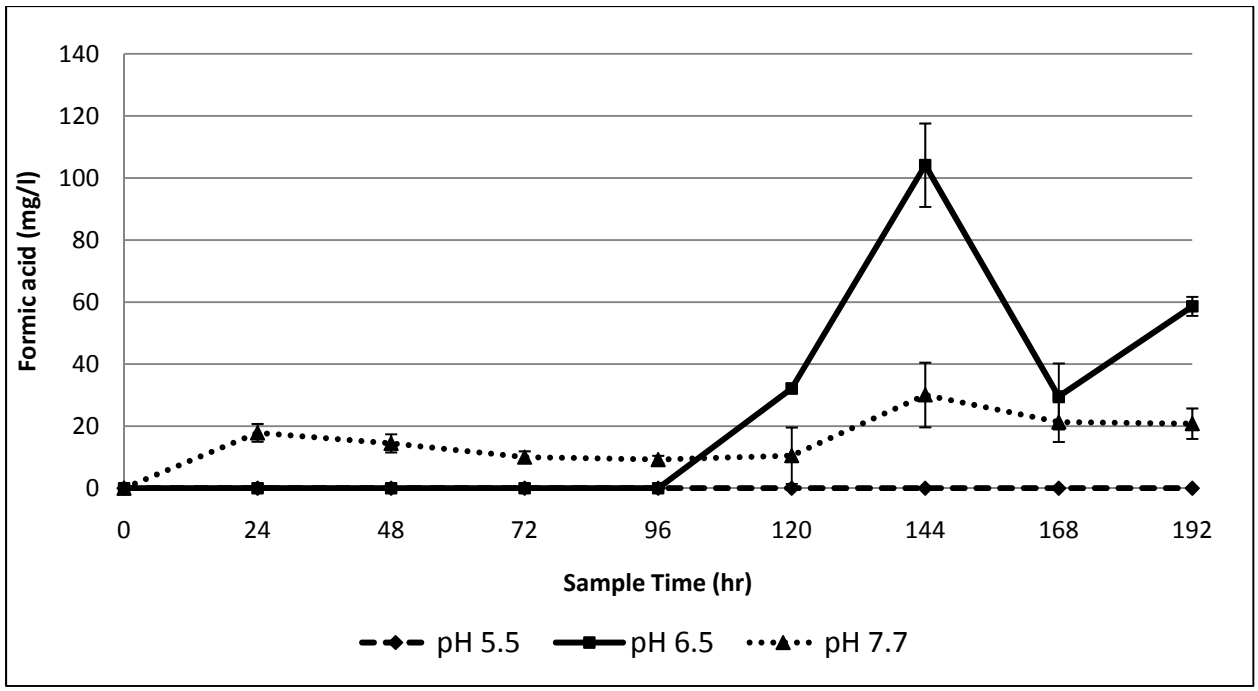


Figure 4.8: Formic acid production in cultures at different initial pH values

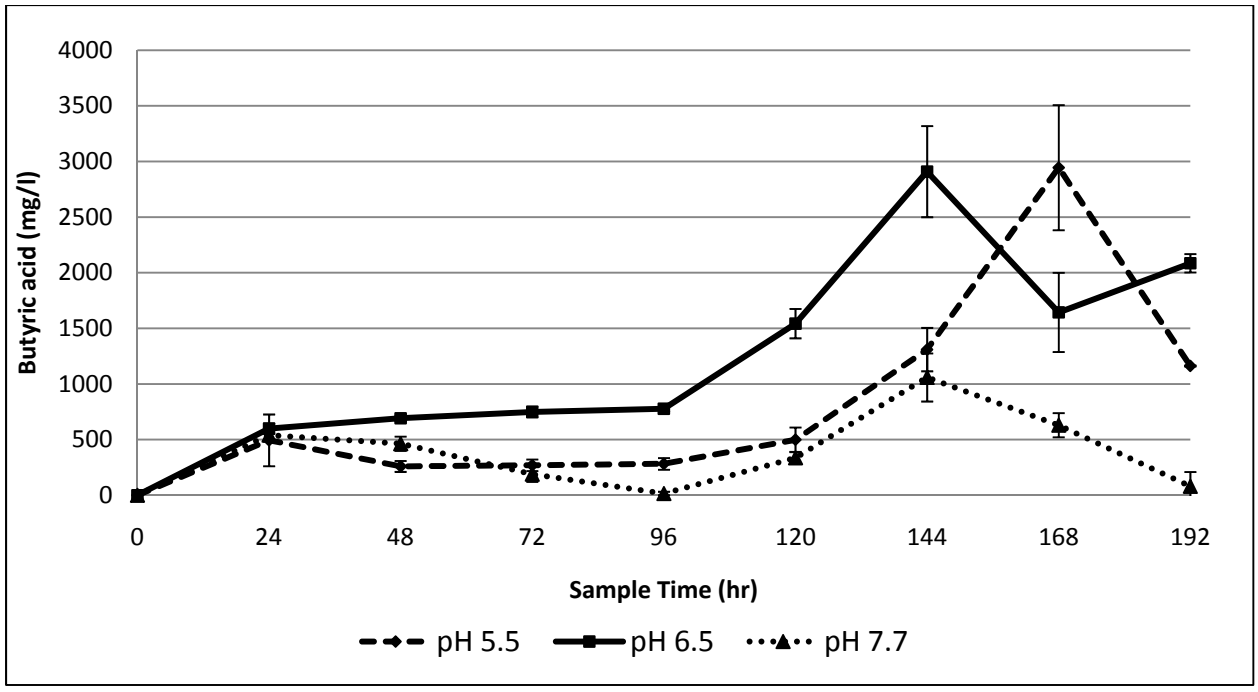


Figure 4.9: Butyric acid production in cultures at different initial pH values

4.3 Alcohol Production

Much like VFAs, alcohol distribution was strongly influenced by the initial pH value. A significant quantity of iso-propanol was produced at initial pH of 5.5 (Figure 4.10). The iso-propanol concentration reached a maximum of 2020 mg/l at 24 hours at an initial pH of 6.5. At an initial pH of 5.5, the iso-propanol concentration increased slowly till it reached a maximum concentration of 1280 mg/l after 192 hours. The iso-propanol concentration did not exceed 500 mg/l when the initial pH was set at 7.7. No ethanol concentrations surpassed 450 mg/l (Figure 4.11). Small amounts of ethanol did accumulate in cultures at an initial pH of 5.5 and at an initial pH of 6.5, ethanol reached a maximum concentration of 340 mg/l after 24 hours and then sharply declined for the remainder of the experiment. Propanol production was only observed at an initial pH of 5.5 (Figure 4.12). It reached a maximum concentration of 2830 mg/l after 168 hours. Very low iso-butanol production was observed at all initial pH values (Figure 4.13). No iso-butanol was observed at pH 7.7; however, at initial pH values of 5.5 and 6.5, small amounts

of iso-butanol accumulated in the final 3 days of the experiment. No butanol production was observed under any of the conditions examined.

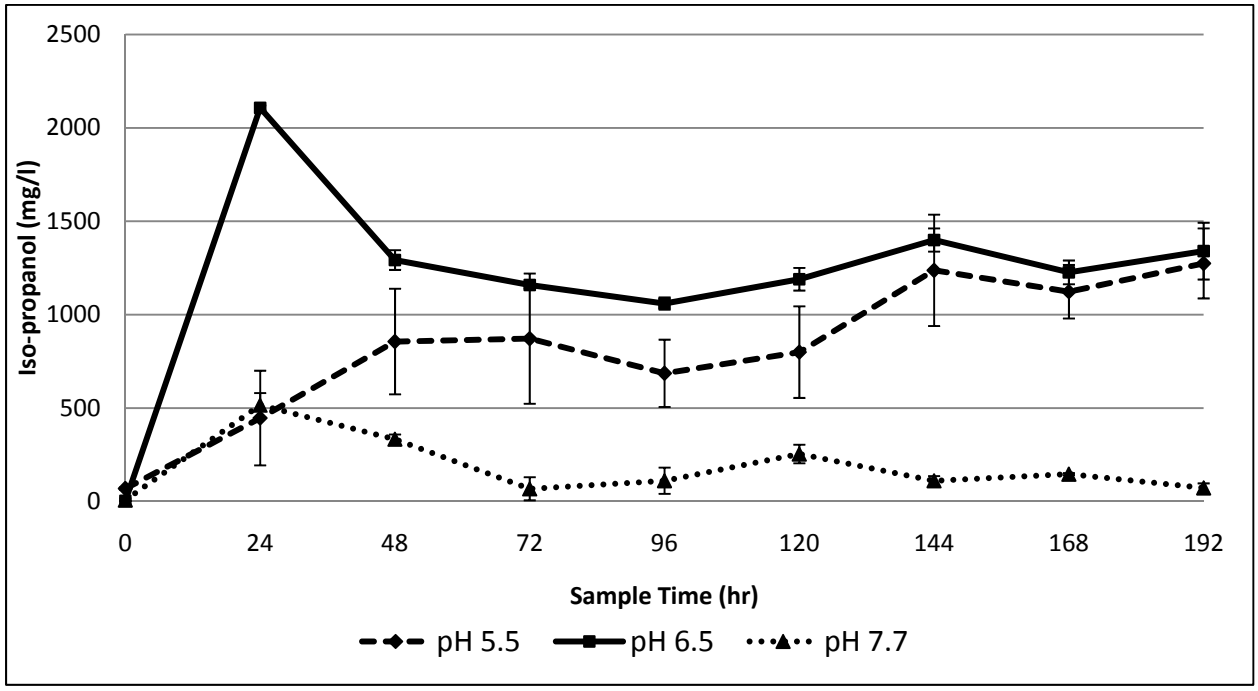


Figure 4.10: Iso-propanol production in cultures at different initial pH values

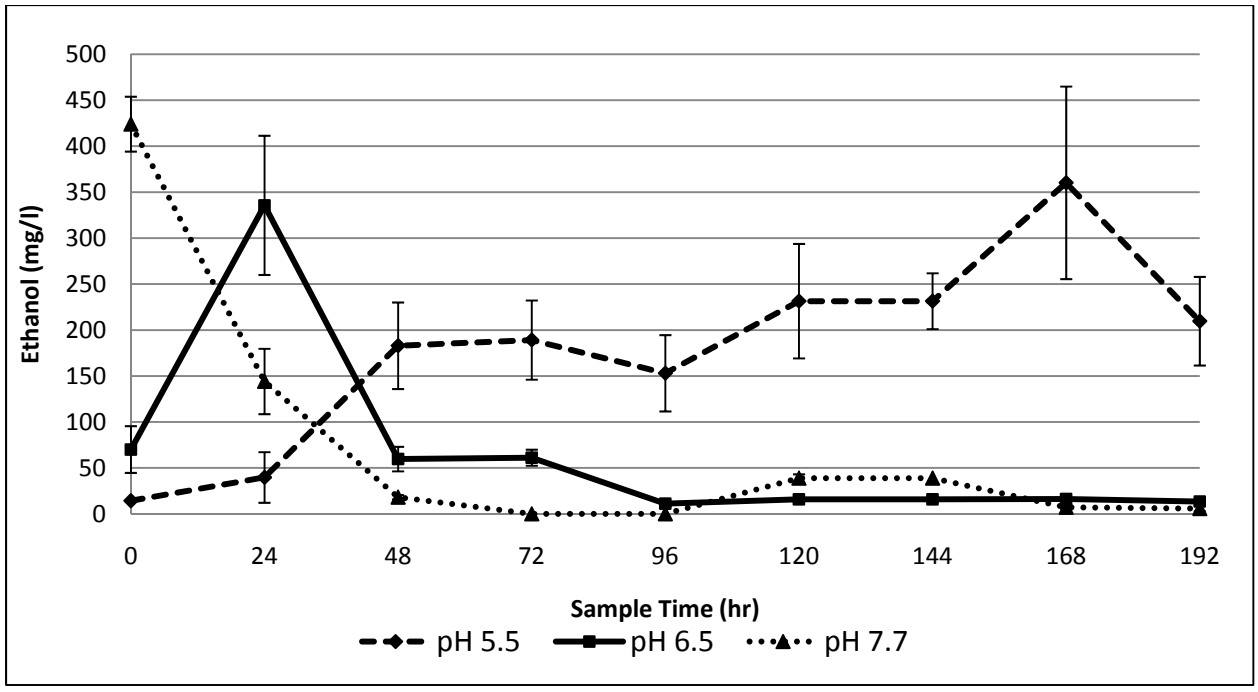


Figure 4.11: Ethanol production in cultures at different initial pH values

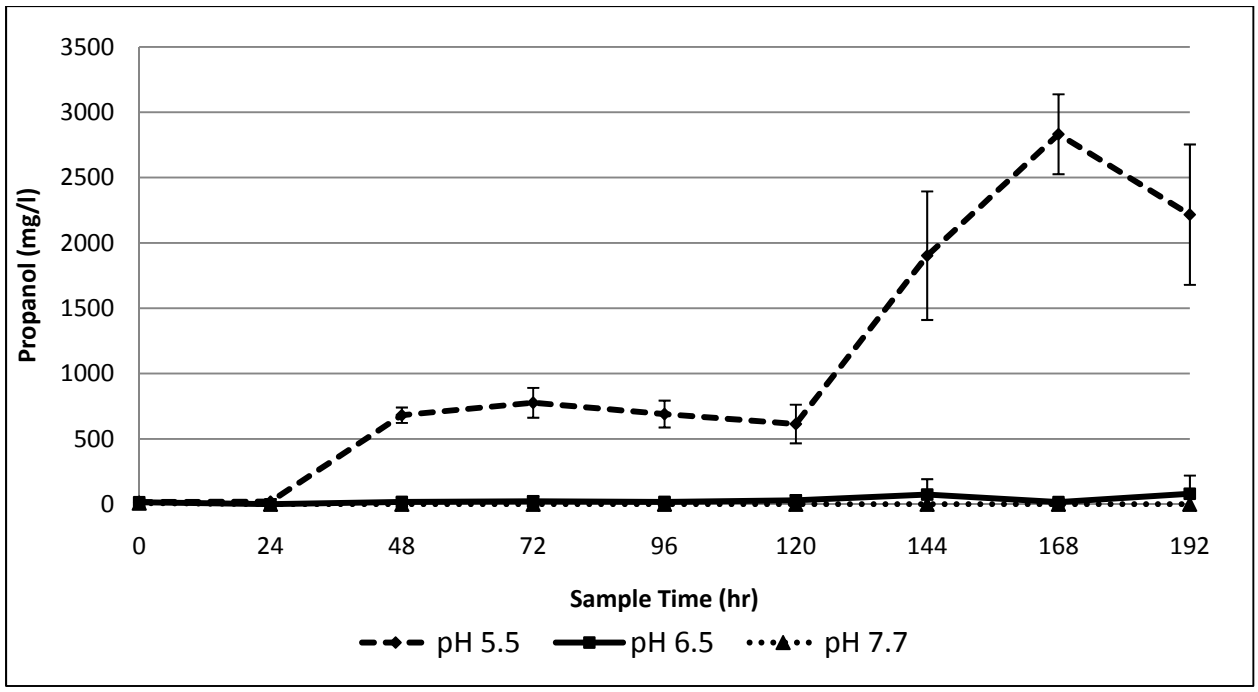


Figure 4.12: Propanol production in cultures at different initial pH values

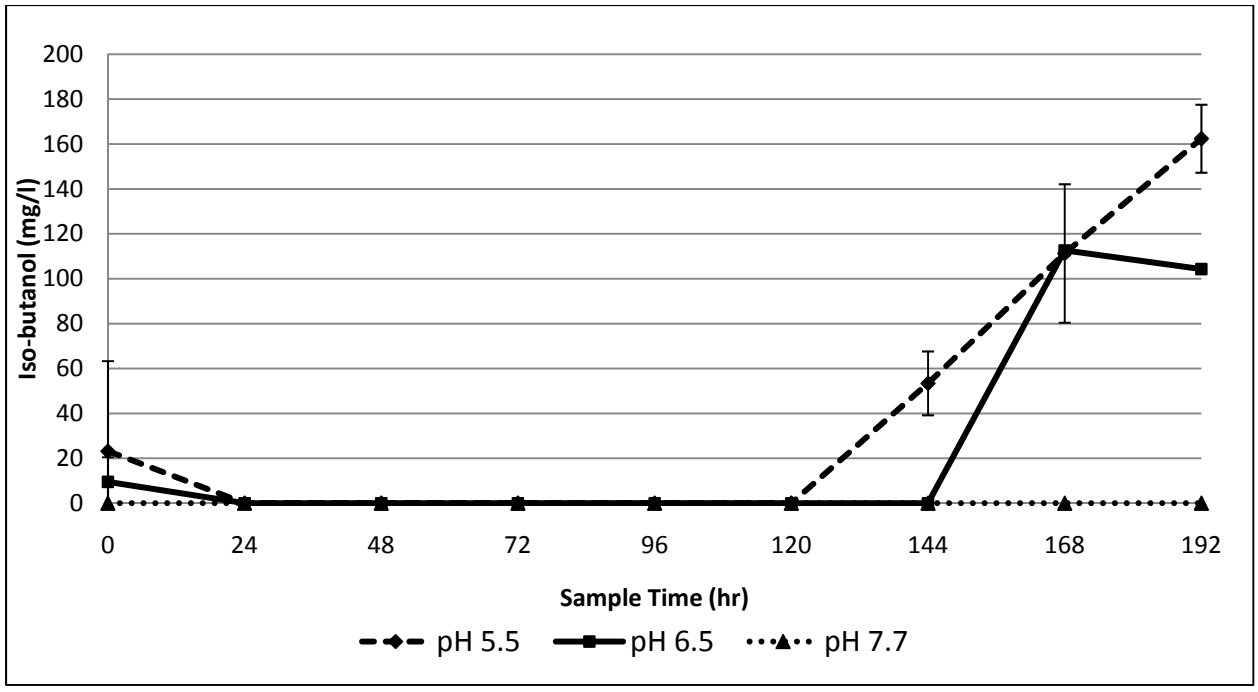


Figure 4.13: Iso-butanol production in cultures at different initial pH values

4.4 Glucose Degradation

Glucose degradation was the greatest at initial pH 7.7. The levels were reduced from 5000 mg/l to 0 mg/l in approximately 12 hours (Figure 4.14). An initial pH of 5.5, all the glucose was consumed within approximately 48 hours.

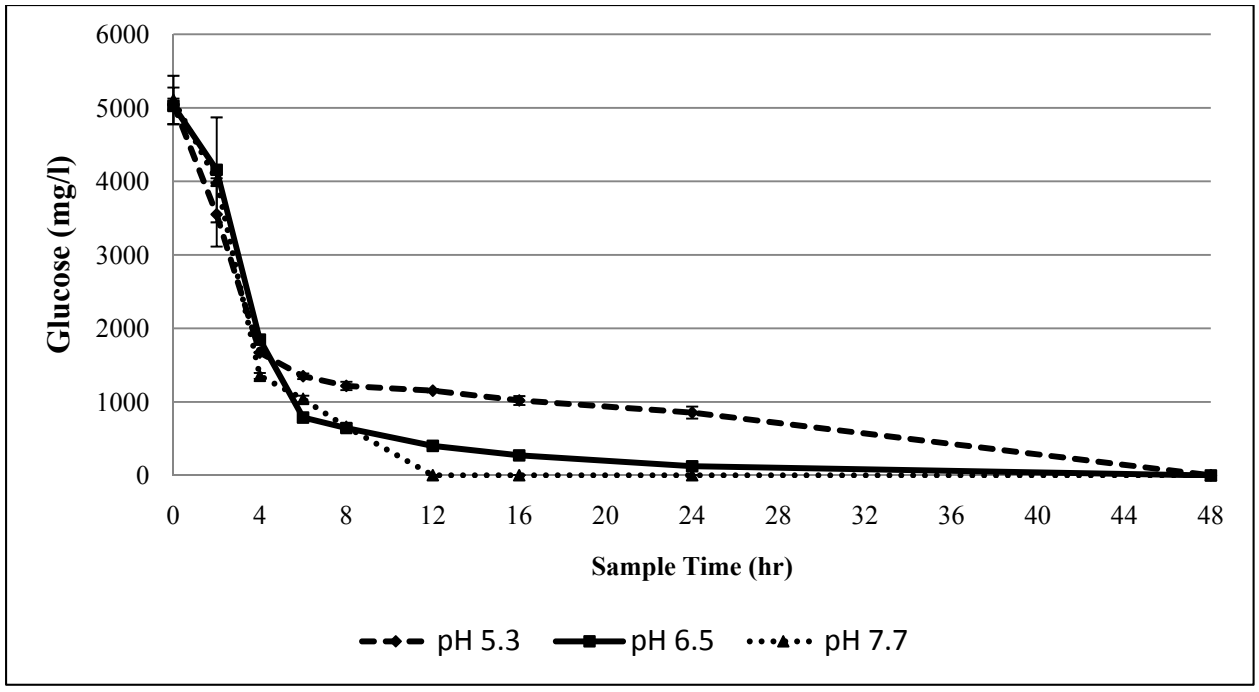


Figure 4.14: Glucose degradation in cultures at different initial pH

Chapter 5: H_2 Production from Glucose Degradation in the Presence of BES

Zinder (1984) reported that a concentration of 50 mM BES is capable of complete methanogenesis inhibition. However, many factors determine what concentration of BES is most effective. The composition of bacteria (i.e. inoculum sources) is the most obvious of these factors. To study the effect of BES inhibition, several concentrations were selected for experimentation. Special consideration was given to use BES concentrations above and below the levels reported in past studies. Control cultures containing no BES were included in addition to cultures containing no BES or glucose. Table 3.2 summarizes the experimental design followed. Gas, VFA, alcohol and glucose profiles from this phase of experimentation are shown in Figures 5.1-5.15.

Table 5.1: Phase II – H_2 production from glucose degradation in the presence of BES

Cultures	BES Conc., mM (g/l)	1st Glucose Injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	0	0	0
4, 5 ,6	0	5 g/l	5 g/l
7, 8 , 9	10 (2.11)	5 g/l	5 g/l
10, 11, 12	25 (5.275)	5 g/l	5 g/l
13, 14, 15	50 (10.55)	5 g/l	5 g/l
16, 17, 18	75 (15.825)	5 g/l	5 g/l

5.1 H_2 and Methane Production

During the first glucose injection period, the maximum yield for cultures containing 25 mM and 10 mM BES concentrations were 2.68 and 2.63 mol H_2/mol glucose, respectively (Figure 5.1; Table 5.2). Cultures containing 50 mM BES and 0 mM BES (no inhibitor) were the next strongest H_2 producers. The lowest amount of H_2 was produced in cultures containing 75 mM BES (highest inhibitor concentration). The maximum yield for cultures containing no inhibitor (0 mM BES) was 1.91 mol H_2/mol glucose. Therefore, an increase of 0.77 mol H_2/mol glucose was

observed between the cultures containing no inhibitor and the cultures containing 25 mM BES (Table 5.2).

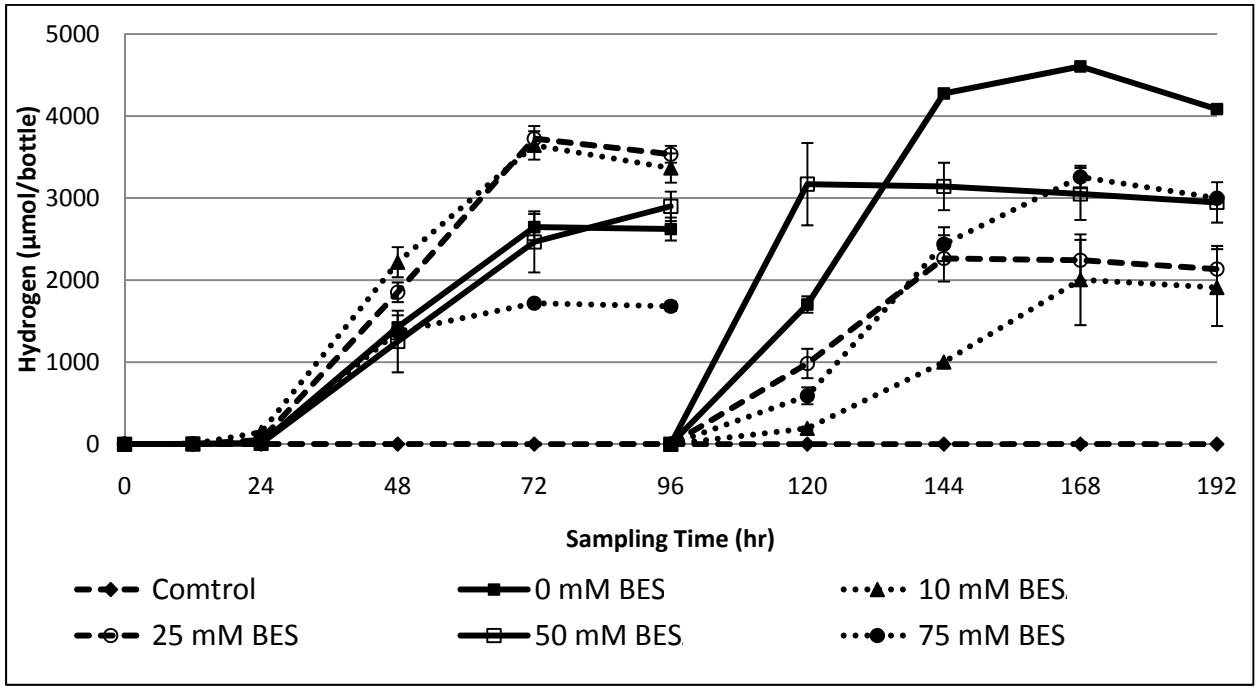


Figure 5.1: H_2 production in cultures receiving varying BES concentrations

During the second glucose injection, changes in the H_2 yields were significant. Cultures containing 0 mM BES were the strongest H_2 producers, reaching a maximum yield of 3.32 mol H_2/mol glucose at 168 hours. The next best H_2 production was observed in cultures containing 50 mM and 75 mM BES. Cultures containing 10 mM and 25 mM BES, which produced the most H_2 during the first glucose injection period, produced the lowest amount of H_2. Cultures containing no inhibitor generated a higher H_2 yield during the second glucose injection period than the maximum yield of 2.68 mol H_2/mol glucose observed during the first glucose injection (Figure 5.1). Cultures containing 50 mM BES reached a yield of 2.29 mol H_2/mol glucose 24 hours after the second glucose injection (120 hours). Such rapid H_2 production was not observed in other cultures.

During the first glucose injection it is clear that BES inhibited methane formation (Figure 5.2). Cultures containing no BES produced a maximum methane concentration of 330 µmol/bottle at 48 hours. The largest amount of methane in cultures containing BES was only 162 µmol/bottle in cultures containing 10 mM BES. There were small differences in methane production between cultures fed with various BES concentrations.

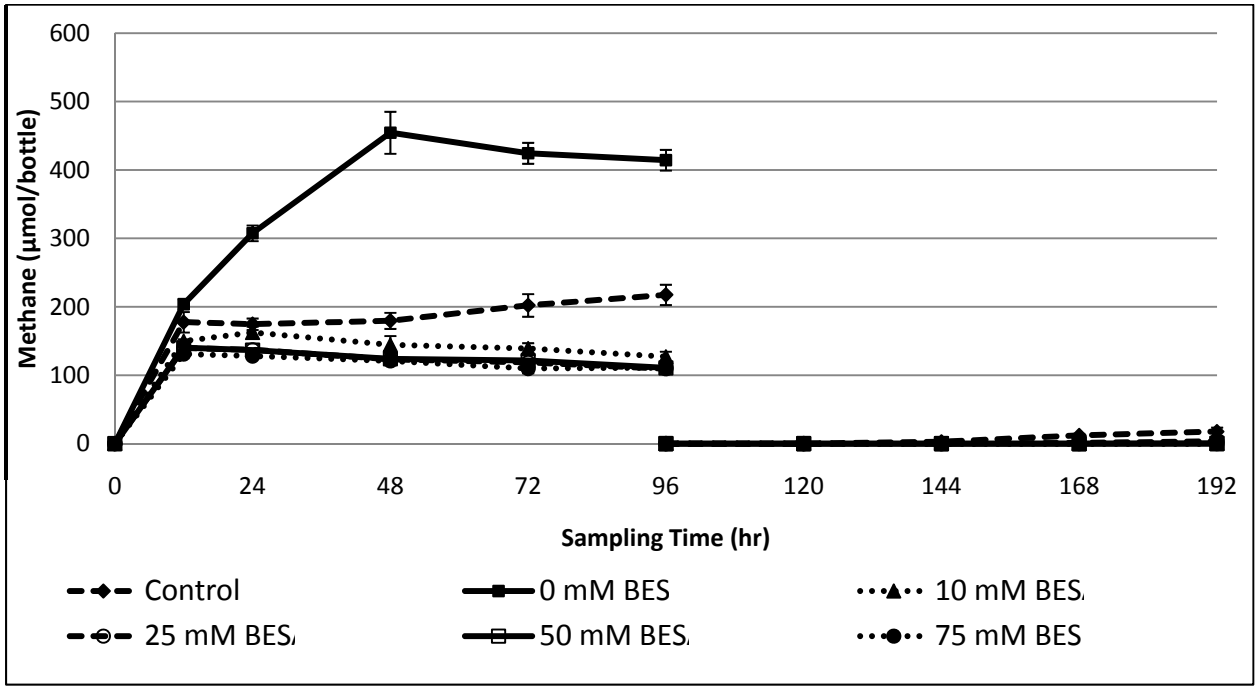


Figure 5.2: Methane production in cultures receiving varying BES concentrations

As expected, cultures containing 75 mM BESA showed the lowest methane production and the largest was produced in cultures containing 10 mM BES. However, the difference was extremely small. At 48 hours, the methane concentration in cultures containing 10 mM and 75 mM BES was 160 and 130 µmol/bottle, respectively. Notice methane production was not detected during the second glucose injection period. Methanogenesis was completely inhibited. Note that in the cultures receiving 0 BES, no methane was produced during the second injection. The rapid difference in methane production between the first and second glucose injection can be attributed

to low pH. The presence of BES had no effect during the second glucose injection. It is clear that the BES behave as an inhibitor of H_2 production in the second glucose injection; however, this mechanism is unclear. Tables 5.2 and 5.3 provide H_2 and methane yields for this phase of study. A bar chart displaying the maximum H_2 yield for each culture set over the duration of the experiment is shown in Figure 5.3. A bar chart displaying the maximum methane concentration per culture for each culture set over the duration of the experiment is shown in Figure 5.4.

Table 5.2: H_2 yields in cultures receiving varying concentrations of BES

	mol H_2/mol glucose				
Time (hr)	**0 BES**	**10 BES**	**25 BES**	**50 BES**	**75 BES**
0	0.00	0.00	0.00	0.00	0.00
12	0.00	0.00	0.00	0.00	0.00
24	0.01	0.10±0.01	0.04±0.01	0.01	0.01
48	1.03±0.11	1.60±0.13	1.33±0.09	0.90±0.27	0.99±0.01
72	1.91±0.11	2.63±0.12	2.68±0.11	1.78±0.27	1.24
96	1.89±0.10	2.43±0.13	2.55±0.07	2.09±0.13	1.21±0.04
120	1.23±0.07	0.14±0.02	0.71±0.13	2.28±0.36	0.42±0.07
144	3.08	0.72	1.63±0.20	2.26±0.21	1.76±0.15
168	3.32	1.44±0.40	1.62±0.18	2.20±0.23	2.35±0.10
192	2.94	1.38±0.34	1.54±0.20	2.12±0.18	2.16±0.01

Table 5.3: Methane yields in cultures receiving varying BES concentrations

	mol METHANE /mol glucose				
Time (hr)	**0 BES**	**10 BES**	**25 BES**	**50 BES**	**75 BES**
0	0.00	0.00	0.00	0.00	0.00
12	0.15±0.01	0.11	0.10	0.10	0.09
24	0.22±0.01	0.12±0.01	0.10±0.01	0.10	0.09
48	0.33±0.02	0.10±0.01	0.09	0.09	0.09
72	0.31±0.01	0.10±0.01	0.09	0.09	0.08
96	0.30±0.01	0.09±0.01	0.08±0.01	0.08	0.08
120	0.00	0.00	0.00	0.00	0.00
144	0.00	0.00	0.00	0.00	0.00
168	0.00	0.00	0.00	0.00	0.00
192	0.00	0.00	0.00	0.00	0.00

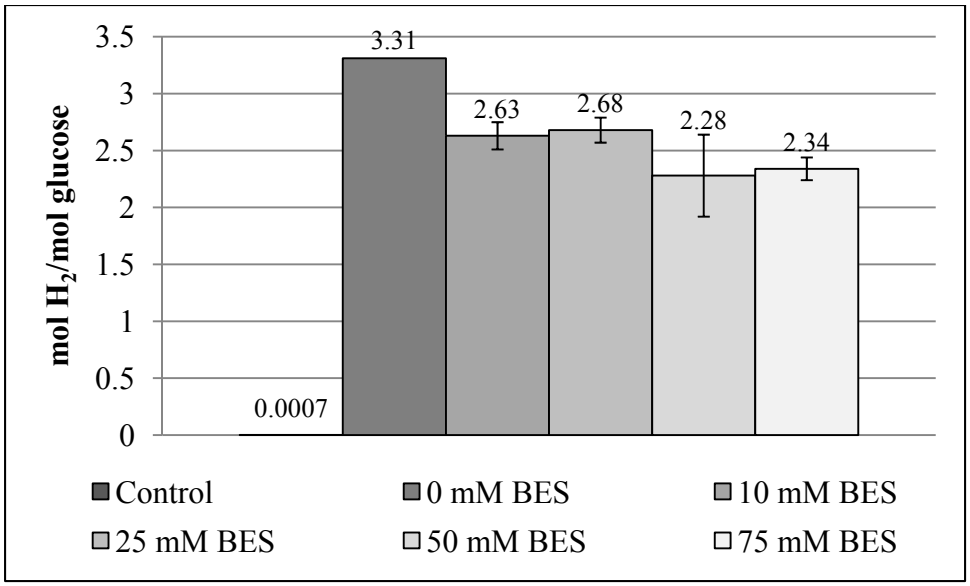


Figure 5.3: Maximum H_2 yield for cultures receiving different BES concentrations

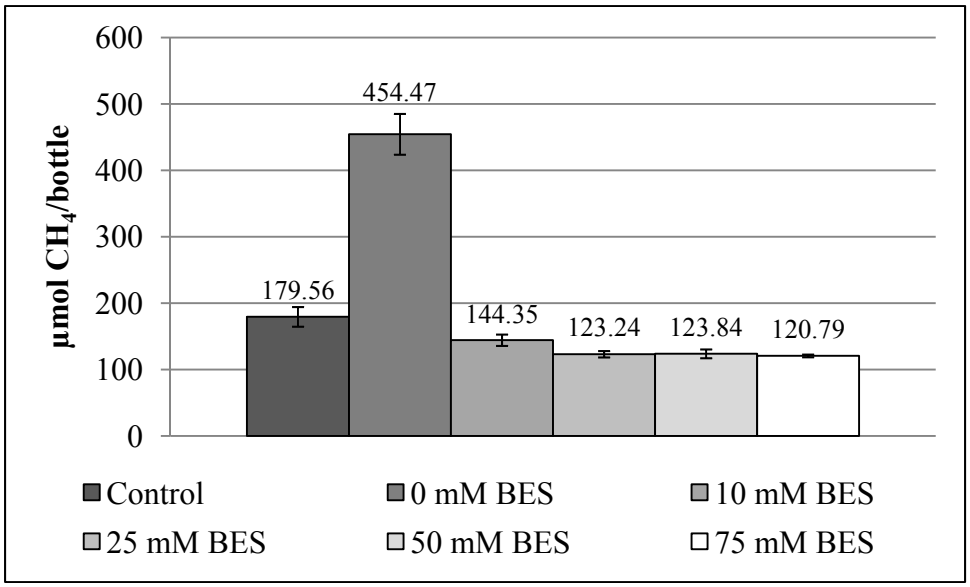


Figure 5.4: Maximum methane concentration per culture for cultures receiving different BES concentrations

5.2 VFA Production

Acetic acid, propionic acid and butyric acid were the dominant VFAs produced under the conditions examined. Lactic acid and formic acid were not observed in significant quantities (Figure 5.5). Acetic acid concentration reached a maximum of 3190 mg/l after 168 hours in

cultures containing 25 mM BES (Figure 5.6). Acetic acid accumulated slowly over the duration of the experiment in all cultures except those containing 50 mM BES. In cultures containing 50 mM BES, the acetic acid levels decreased after 144 hours. Propionic acid concentration reached a maximum of 1970 mg/l at 168 hours in cultures containing 25 mM BES and butyric acid concentration reached a maximum of 1230 mg/l at 144 hours in cultures fed 25 mM BES (Figures 5.8 and 5.9). No variation was observed in VFA production between the different BES concentrations.

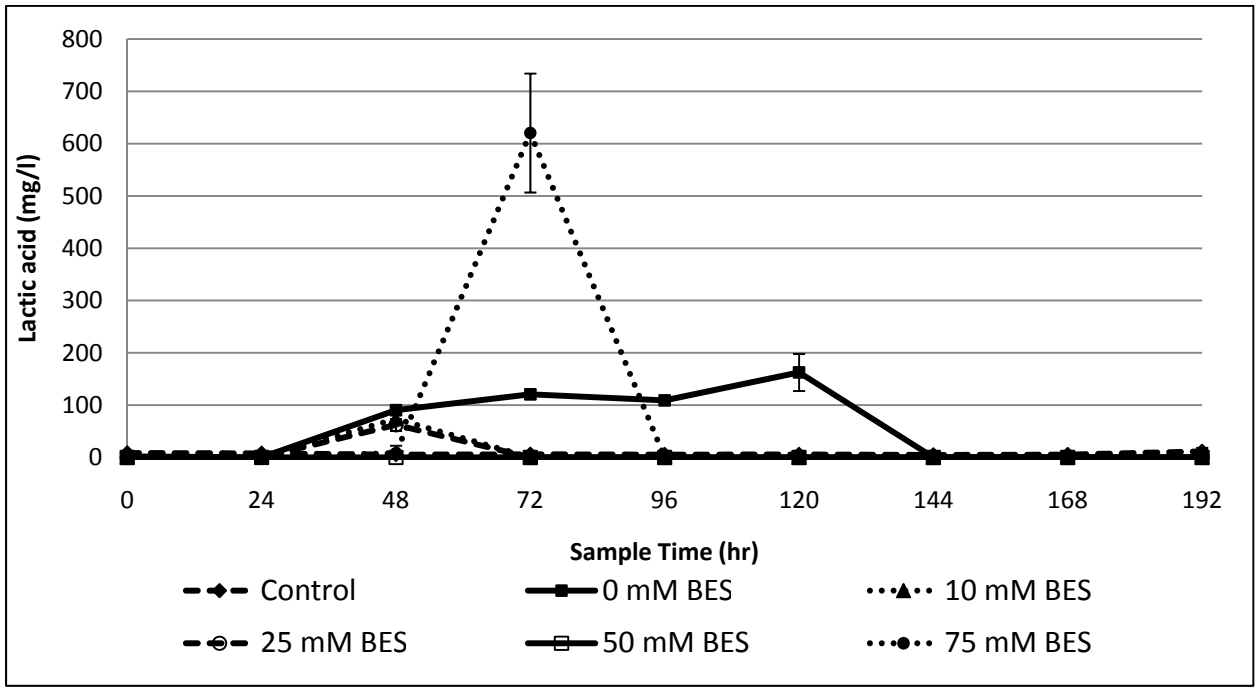


Figure 5.5: Lactic acid production in cultures receiving varying BES concentrations

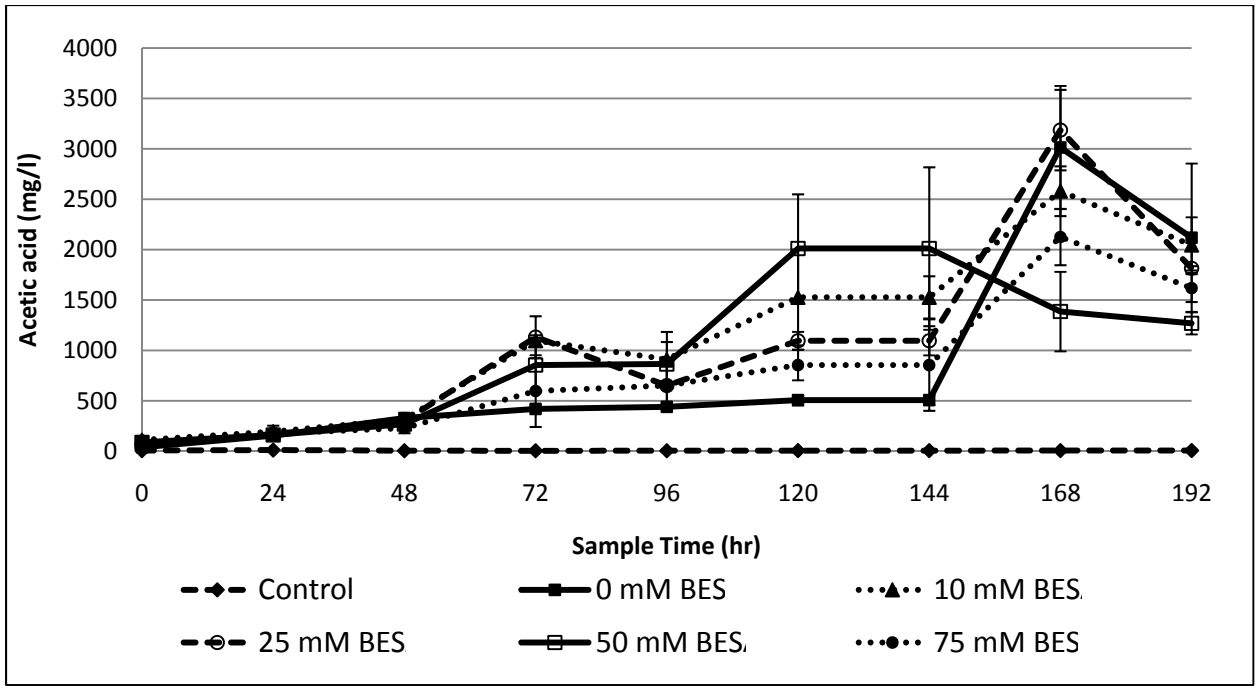


Figure 5.6: Acetic acid production in cultures receiving varying BES concentrations

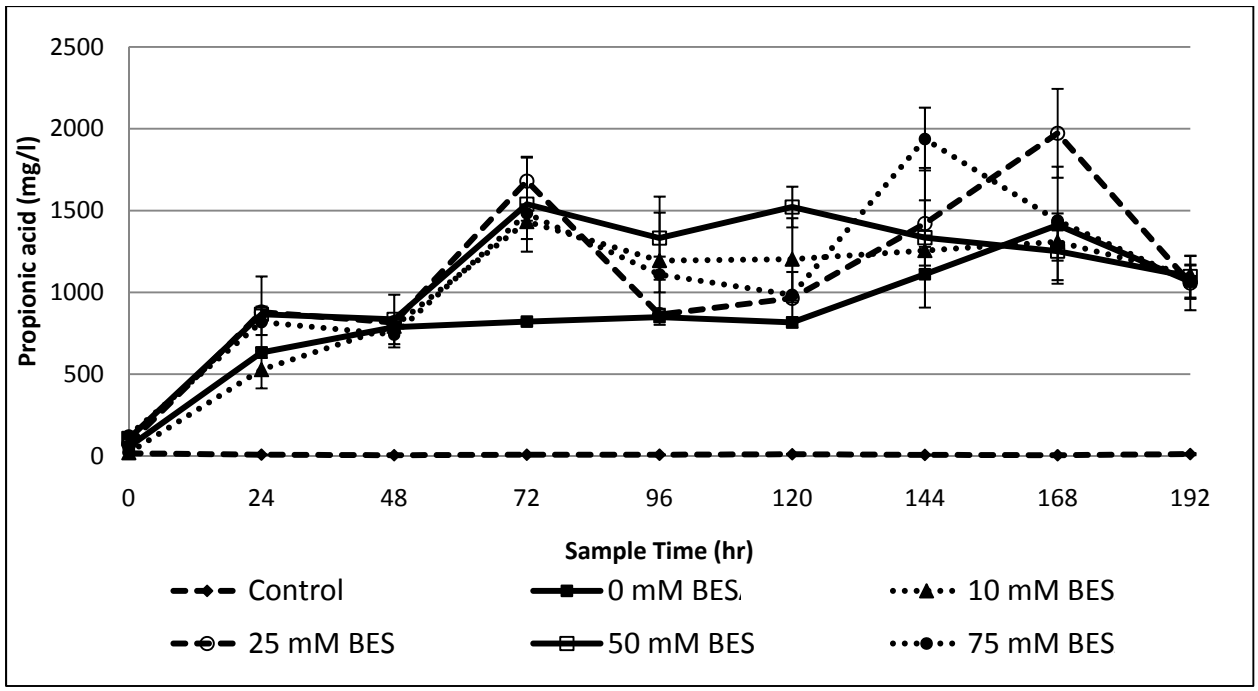


Figure 5.7: Propionic acid production in cultures receiving varying BES concentrations

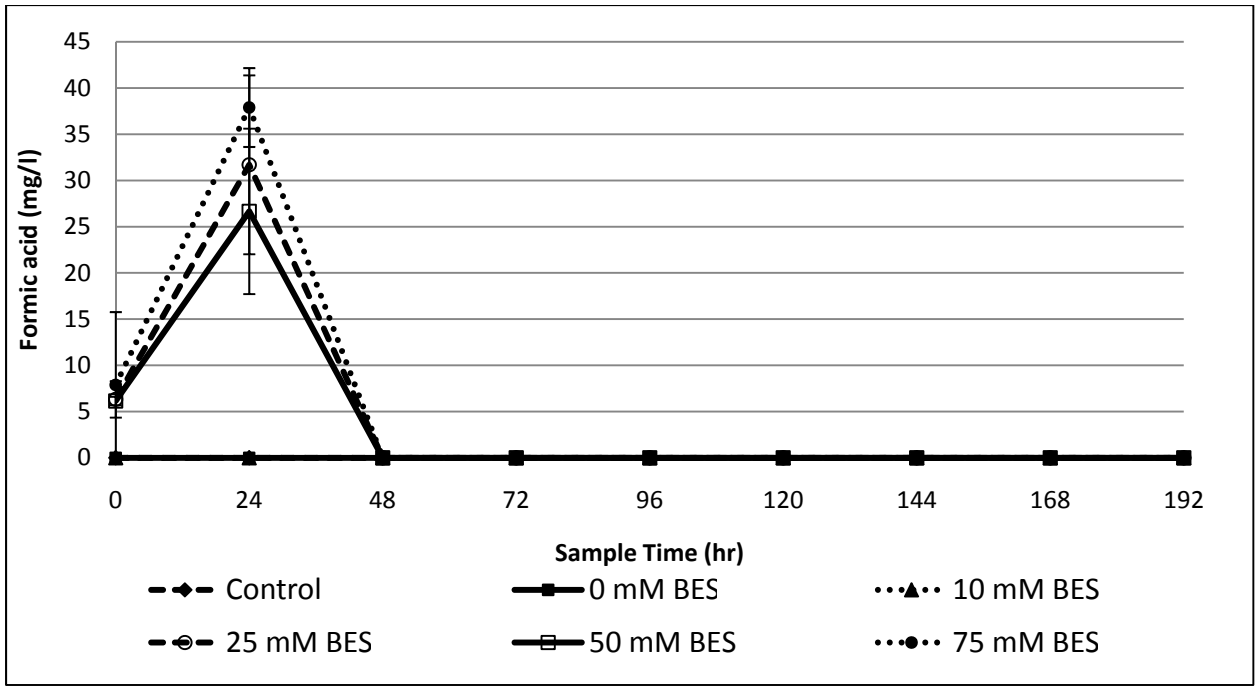


Figure 5.8: Formic acid production in cultures receiving varying BES concentrations

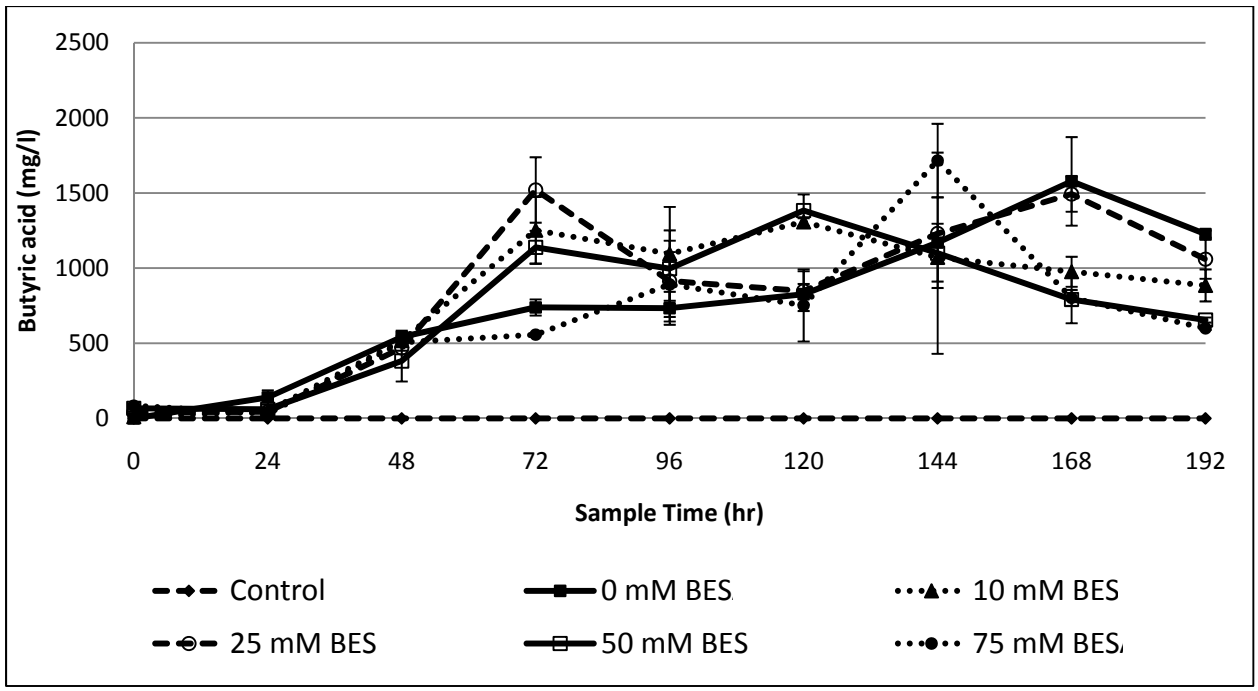


Figure 5.9: Butyric acid production in cultures receiving varying BES concentrations

5.3 Alcohol Production

The major alcohols produced in this phase were iso-propanol, ethanol and propanol. Iso-propanol, ethanol and propanol production peaked between 72 and 144 hours (Figure 5.10). The highest ethanol concentration observed was 930 mg/l at 144 hours in cultures containing no inhibitor (Figure 5.11). Iso-propanol and propanol reached maximum concentrations of 2620 and 4400 mg/l, respectively, at 120 hours in cultures containing 25 mM BES (Figure 5.10 and Figure 5.12). Note low amounts of iso-butanol (less than 280 mg/l) and butanol (less than 80 mg/l) were observed over the duration of the study (Figure 5.13 and Figure 5.14).

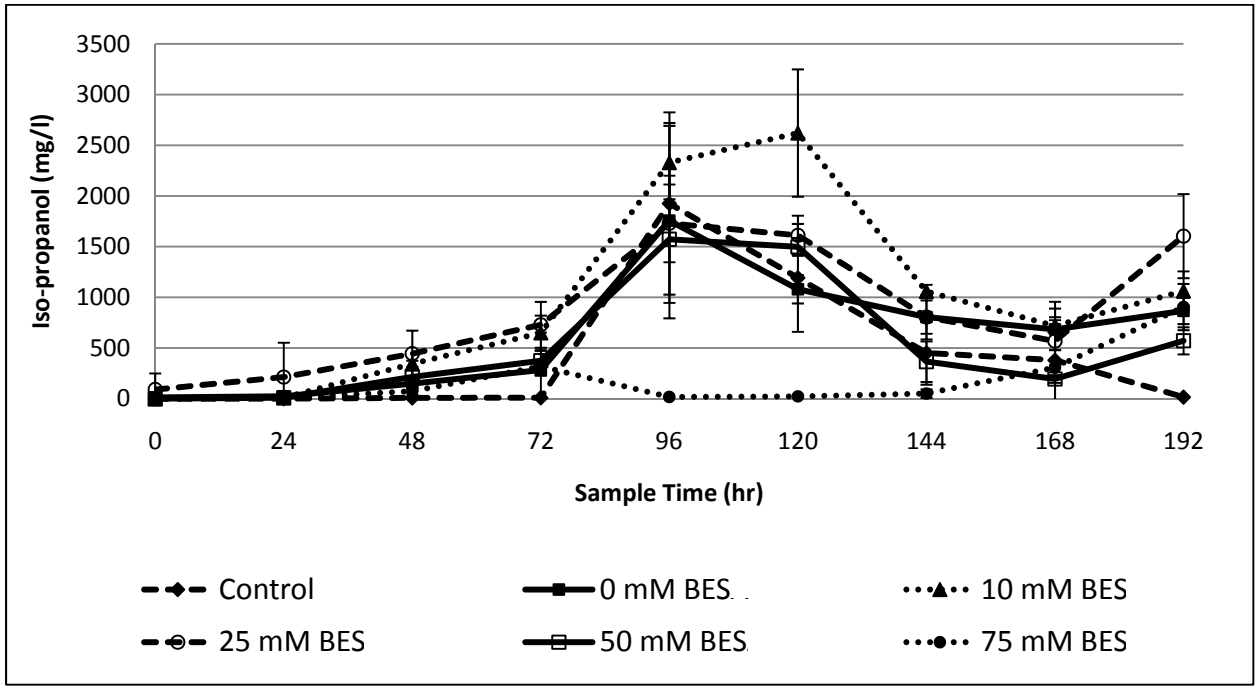


Figure 5.10: Iso-propanol production in cultures receiving varying BES concentrations

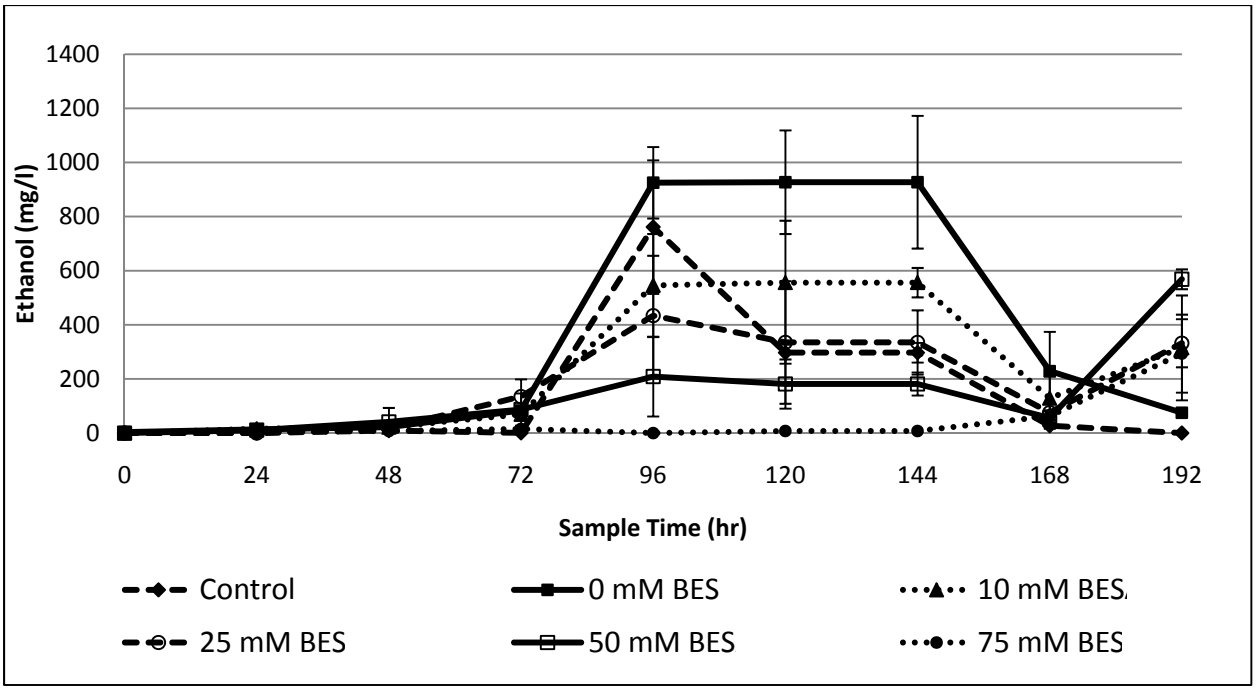


Figure 5.11: Ethanol production in cultures receiving varying BES concentrations

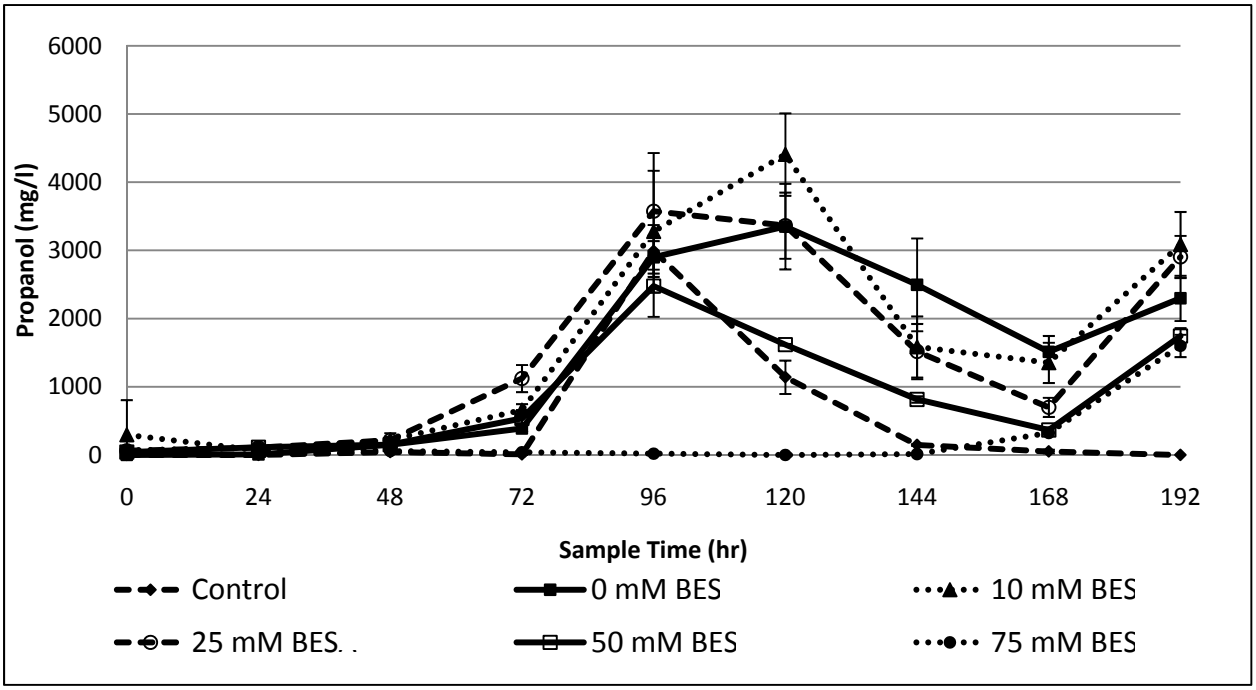


Figure 5.12: Propanol production in cultures receiving varying BES concentrations

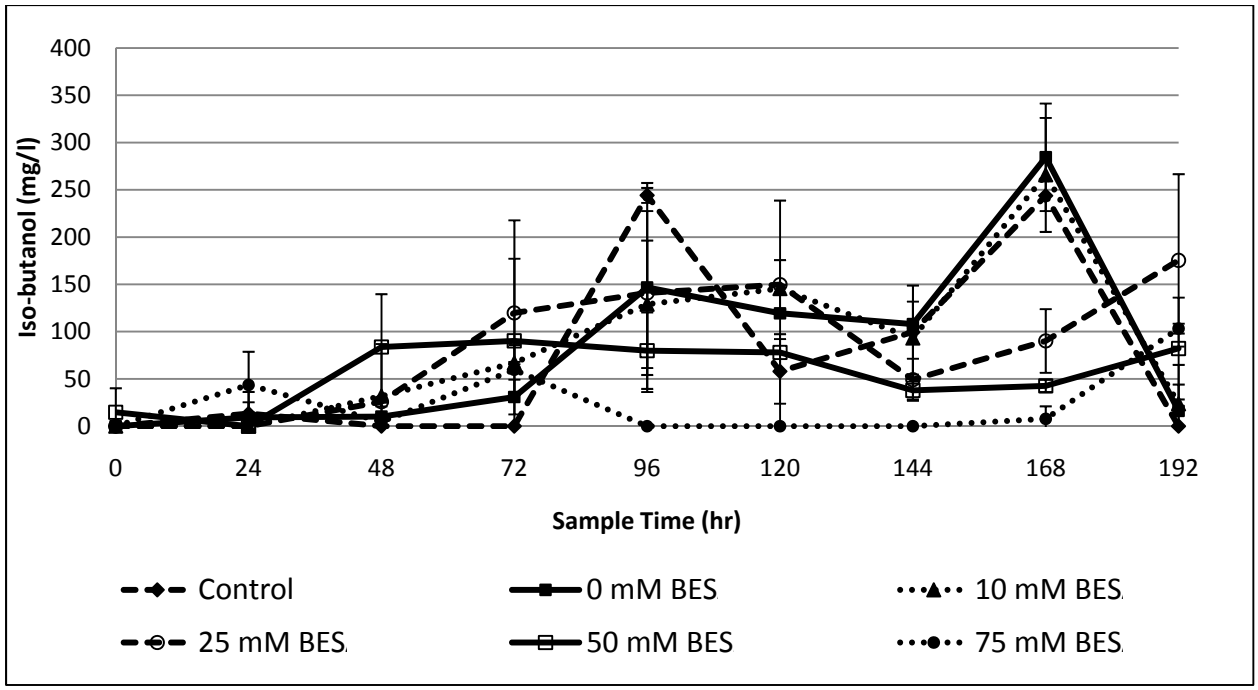


Figure 5.13: Iso-butanol production in cultures receiving varying BES concentrations

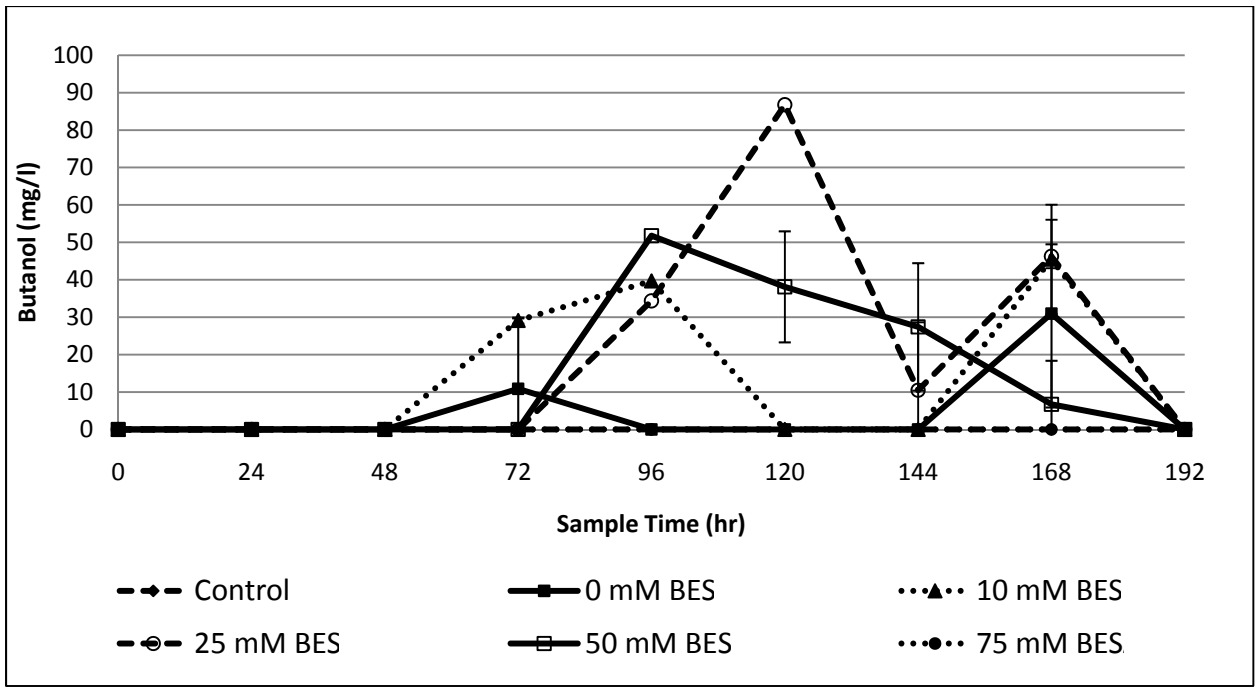


Figure 5.14: Butanol production in cultures receiving varying BES concentrations

5.4 Glucose Degradation

Glucose degradation was relatively consistent in all cultures containing BES (Figure 5.15). However, the controls containing no BES degraded glucose faster over the first 8 hours after glucose addition. From 8 hours onwards, the glucose degradation profile for all the cultures was the same.

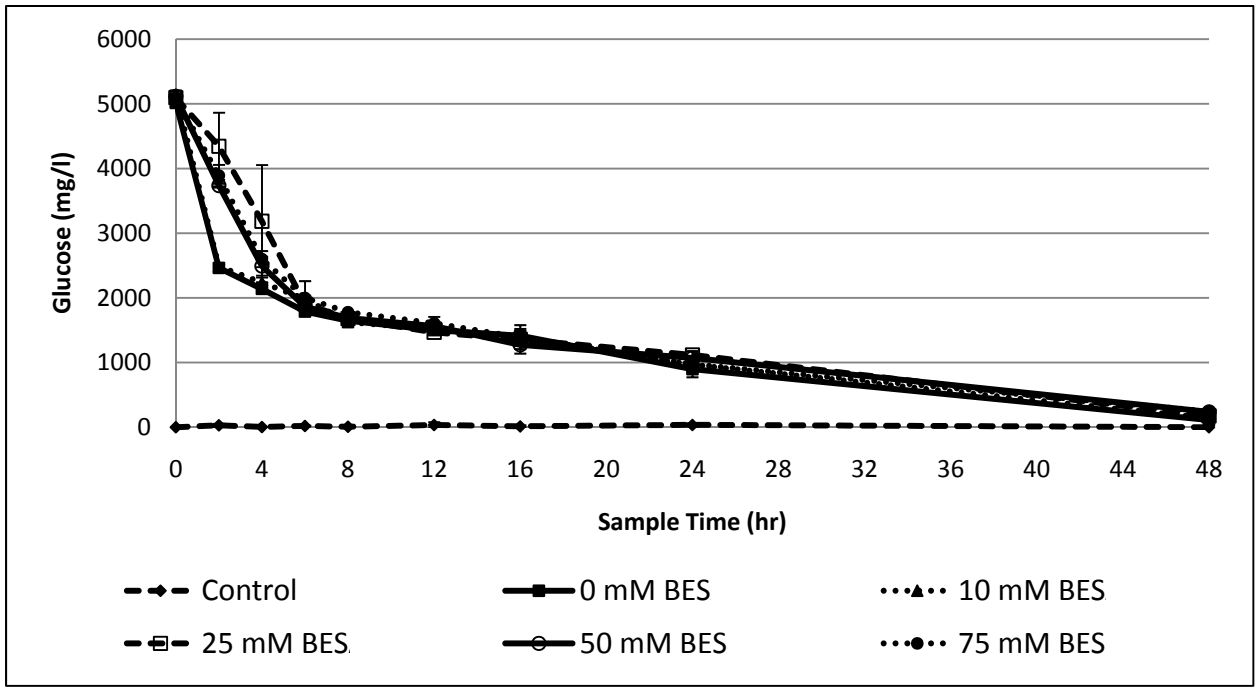


Figure 5.15: Glucose Degradation in cultures receiving varying BES concentrations

5.5 pH Profile

Figure 5.16 shows the pH profile of the cultures over the course of the experiment. All the cultures began with an initial pH of 5.5 with no buffering capacity. After 96 hours, the cultures were opened and the pH recorded. The pH was then re-adjusted to 5.5 prior to the second glucose injection. The pH was again recorded at the end of the experiment (192 hours). The pH profiles of all cultures were extremely close during the first injection. The pH decreased from 5.5 to around 3.6-3.7 in all cultures in a period of approximately 4 days. The pH did not decrease as low in the second injection and more variation was observed. The pH decreased to 3.5 in cultures

containing no inhibitor. This was the only case where the pH drop was lower in the second injection. The pH in cultures containing 50 mM BES decreased to 4.2, compared to 3.7 during the first glucose injection period. In all other cultures, the pH decreased from a pH of 5.5 to approximately 3.65-3.85 during the second glucose injection.

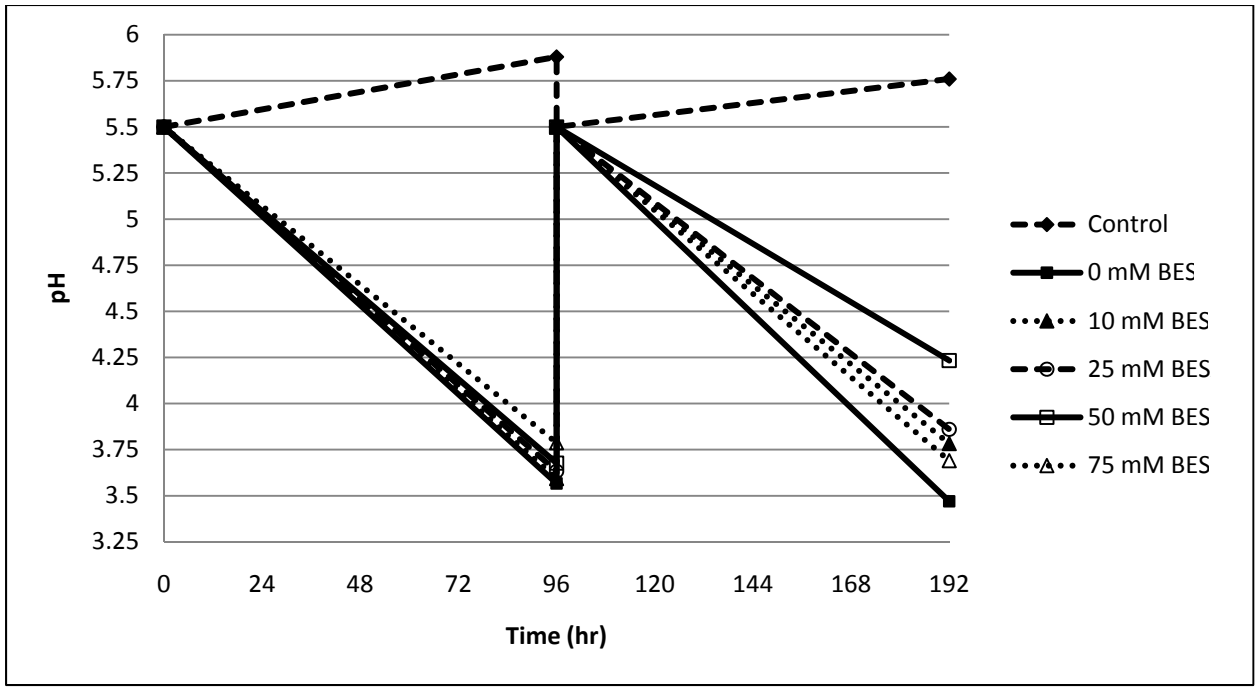


Figure 5.16: pH profile over duration of 8-day experiment

5.6 Reactions Based on Electron Balance

Chemical equations are shown below for varying BES levels and sampling periods. From each equation a biomass molar yield ($Y_{glucose/biopmass}$) and H_2 molar yield ($Y_{H2/glucose}$) can be determined. For example, for 0 mM BES at 72 hours, $Y_{glucose/biopmass}$ and $Y_{H2/glucose}$ are 1.47 and 0.12, respectively.

<u>0 mM BES at 72 hours</u>

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.0693H_2O \rightarrow 0.06149H_2 + 0.00986CH_4 + 0.005C_5H_7O_2N + 0.00157CH_3CH_2OCOO^- + 0.00826CH_3COO^- + 0.0131CH_3CH_2COO^- + 0.00985CH_3CH_2CH_2COO^- + 0.00547CH_3COH_2CH_3 + 0.00749\ CH_3CH_2CH_2OH + 0.07643HCO_3^- + 0.1142H^+$

0 mM BES at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.0812H_2O \rightarrow 0.0943H_2 + 0.005C_5H_7O_2N + 0.04462CH_3COO^- + 0.00788CH_3CH_2COO^- + 0.00993CH_3CH_2CH_2COO^- + 0.00190CH_3CH_2CHOHCH_3 + 0.06482HCO_3^- + 0.1322H^+$

10 mM BES at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.0586H_2O \rightarrow 0.05934H_2 + 0.00214CH_4 + 0.005C_5H_7O_2N + 0.01288CH_3COO^- + 0.01512CH_3CH_2COO^- + 0.01109CH_3CH_2CH_2COO^- + 0.00834CH_3COH_2CH_3 + 0.00465CH_3CH_2\ CH_2OH + 0.06839HCO_3^- + 0.1125H^+$

10 mM BES at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.08626H_2O \rightarrow 0.09680H_2 + 0.005C_5H_7O_2N + 0.06824CH_3COO^- + 0.00382CH_3CH_2COO^- + 0.00446CH_3CH_2CHOHCH_3 + 0.05922HCO_3^- + 0.1363H^+$

25 mM BES at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.04718H_2O \rightarrow 0.04183H_2 + 0.00133CH_4 + 0.005C_5H_7O_2N + 0.01076CH_3COO^- + 0.01291CH_3CH_2COO^- + 0.009802CH_3CH_2CH_2COO^- + 0.006814CH_3COH_2CH_3 + 0.00082CH_3CH_2OH + 0.01048CH_3CH_2CH_2OH + 0.00091CH_3CH_2CHOHCH_3 + 0.06707HCO_3^- + 0.10554H^+$

25 mM BES at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.03686H_2O \rightarrow 0.05184H_2 + 0.005C_5H_7O_2N + 0.04962CH_3COO^- + 0.01753CH_3CH_2COO^- + 0.00768CH_3CH_2CH_2COO^- + 0.04239HCO_3^- + 0.1222H^+$

50 mM BES at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.04277H_2O \rightarrow 0.04258H_2 + 0.00210CH_4 + 0.005C_5H_7O_2N + 0.01249CH_3COO^- + 0.01692CH_3CH_2COO^- + 0.0113CH_3CH_2CH_2COO^- + 0.00539CH_3COH_2CH_3 + 0.0077CH_3CH_2CH_2OH + 0.06277HCO_3^- + 0.1085H^+$

50 mM BES at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.2701H_2O \rightarrow 0.2851H_2 + 0.005C_5H_7O_2N + 0.04122CH_3COO^- + 0.14255HCO_3^- + 0.1888H^+$

75 mM BES at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.03879H_2O \rightarrow 0.04416H_2 + 0.00282CH_4 + 0.005C_5H_7O_2N + 0.00895CH_3CH_2OCOO^- + 0.01071CH_3COO^- + 0.02387CH_3CH_2COO^- + 0.00697CH_3CH_2CH_2COO^- + 0.00680CH_3COH_2CH_3 + 0.05401HCO_3^- + 0.10951H^+$

75 mM BES at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.1020H_2O \rightarrow 0.1008H_2 + 0.005C_5H_7O_2N + 0.03862CH_3COO^- + 0.00699CH_3CH_2COO^- + 0.00790CH_3COH_2CH_3 + 0.00831CH_3CH_2CH_2OH + 0.07819HCO_3^- + 0.1288H^+$

5.7 Discussion of Results

Lower amounts of methane were produced with increasing BES concentrations. The highest BES concentration studied was 75 mM and under this condition, the lowest amount of methane was produced during the first glucose injection period (0 to 96 hours). The methane concentration reached 120 µmol/bottle after 48 hours. Cultures receiving 0 BES produced a maximum methane concentration of 455 µmol/bottle at 48 hours. This represents a 73% decrease in methane production at its peak time of production. However, when comparing the methane production between the several concentrations of BES studied, small differences were observed. At 48 hours, the methane concentration was 145 and 120 µmol/bottle for cultures containing 10 mM and 75 mM BES, respectively. This only represents a difference of 16%.

Inhibiting methane production is important in improving H_2 yields; however, the electrons that are diverted away from methane production must be directed to H_2 production. Unfortunately, this does not appear to be the case with BES inhibition at an initial pH of 5.5. Although 75 mM of BES inhibited 73% of methane production, when compared to 0 BES at 48 hours, the cultures with 0 BES had a higher H_2 yield. In fact, the cultures containing 0 BES had a higher H_2 yield than the cultures containing 75 mM over the duration of the experiment. The highest H_2 producing cultures during the first glucose injection contained 10 and 25 mM BES. These

cultures showed greater methane production during the first glucose injection than cultures containing 50 mM and 75 mM BES. At these BES concentrations, lower methane production was detected. Methane production during the second glucose injection was none existent. Even cultures with 0 BES produced no methane. The cause of this is likely attributed to the low pH. The pH in cultures at 96 hours decreased to values approaching 3.6-3.8 due to acid production. Methane producing bacteria are very sensitive to low pH and it is probable that the majority of methane producing bacteria were killed or inactivated prior to the second glucose injection. Because cultures containing 0 BES produced the highest amounts of H_2, it is likely that BES limited H_2 production. If this were the case, one would expect cultures containing the highest BES concentration to show the lowest H_2 production over the second glucose injection period. However, this is not the case and cultures containing 50 and 75 mM BES were the highest H_2 producers.

Alcohol and VFA production differed slightly with BES inhibition. VFAs were produced immediately and concentrations increased over the duration of the experiment. Acetic, propionic and butyric acid were the dominate VFAs produced. Alternatively, alcohols production started very slowly and after hours 72 and 96 the levels increased drastically. Iso-propanol, ethanol and propanol production was dominant; however, the concentrations began to decrease for these alcohols between 144 and 168 hours.

Chapter 6: H_2 Production from Glucose Degradation in the Presence of Incremental Addition of BES and LA

This experiment was designed to evaluate the changes in methane inhibition due to incremental additions of BES and LCFAs. A significant amount of results have been published showing the effects when a specific concentration of inhibitor is added to a mixed culture prior to glucose injection. The objective here is to take that 'specific concentration' of inhibitor and break it up into two equal injections separated by a specific amount of time. In this study, the injections were separated by 24 hours. Table 6.1 summarizes the experimental design followed for this phase of study. For direct comparison, the traditional method of single inhibitor injection was examined along with the incremental injection. For the purposes of this study, BES and LA were used as inhibitors. A concentration of 50 mM BES was selected based on the results in Chapter 5. Inhibitor concentrations of 50 mM BES and 2000 mg/l LA were selected due to their use in recent publication. All cultures were prepared with an initial pH of 5.5. Gas, VFA, alcohol and glucose results from this phase of experiment are shown in Figures 6.1-6.15.

Table 6.1: Phase III – H_2 production from glucose degradation in the presence of BES and LA added in increments

Cultures	Inhibitor	1st Addition (t=0 hr)	2nd Addition (24 hr)	1st Glucose Injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	BES	0	50 mM	5 g/l	5 g/l
4, 5, 6	BES	25 mM	25 mM	5 g/l	5 g/l
7, 8, 9	LA	0	2000 mg/l	5 g/l	5 g/l
10, 11, 12	LA	1000 mg/l	1000 mg/l	5 g/l	5 g/l

6.1 H_2 and Methane Production

During the first glucose injection period, the cultures which received one direct injection of inhibitor produced greater quantities of H_2 than cultures that received two equal injections. The difference was small for BES; however, the difference was considerably large for LA. At 96

hours, the cultures receiving two portions of 1000 mg/l LA injections had a yield of 1.1 mol H_2/mol glucose, whereas the cultures receiving one 2000 mg/l LA injection reached a yield of 1.67 mol H_2/mol glucose (Figure 6.1). That is a difference of 0.56 mol H_2/mol glucose. When comparing inhibitor type, LA produced H_2 more rapidly than BES. BES did not begin to produce substantial amounts of H_2 until after 48 hours. After 96 hours, cultures receiving BES produced more H_2 than those receiving LA.

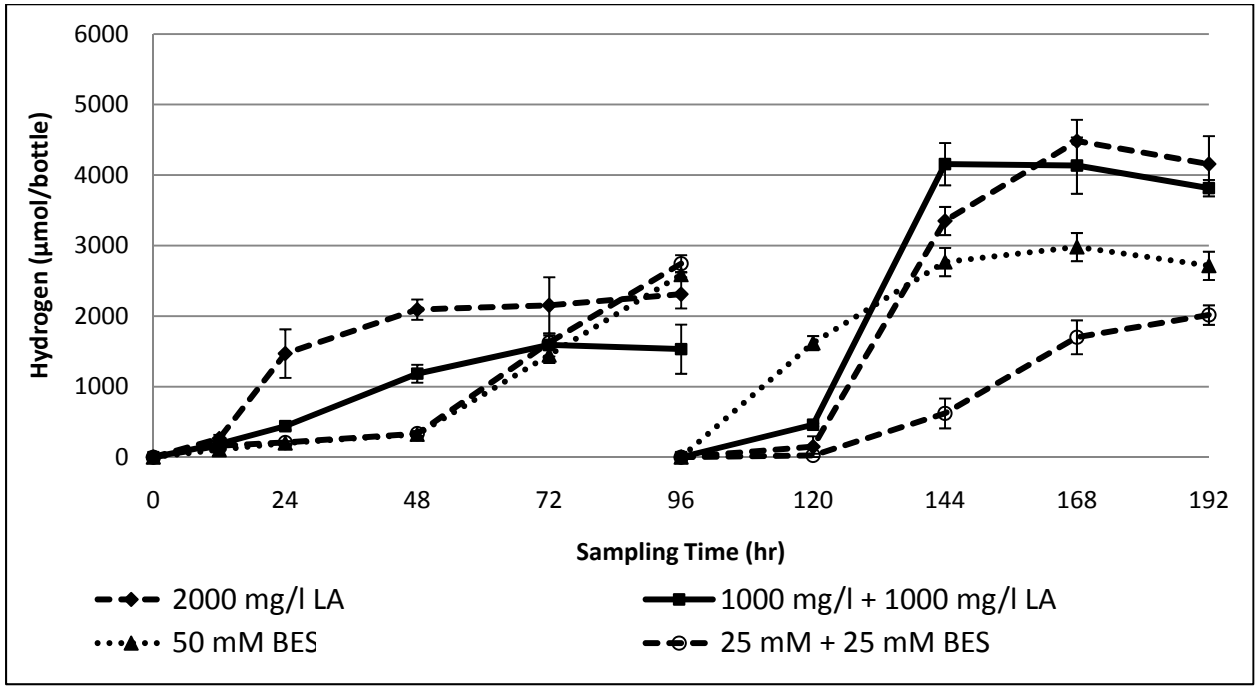


Figure 6.1: H_2 production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

During the second glucose injection period, the cultures receiving one direct injection of inhibitor produced greater quantities of H_2 than cultures that receiving two portions of the inhibitor. However, in this case, the difference was small for LA and large for BES. At 168 hours, the cultures receiving two 25 mM BES injections had a yield of 1.23 mol H_2/mol glucose, whereas the cultures receiving one 50 mM BES injection reached a yield of 2.15 mol H_2/mol glucose (Figure 6.1). In this case, the difference was 0.99 mol H_2/mol glucose. LA produced

more H_2 than BES during the second glucose injection period. The maximum yield attained was 3.23 mol H_2/mol glucose with one direct 2000 mg/l LA injection at 168 hours while the highest yield with BES inhibition was 2.148 mol H_2/mol glucose with one 50 mM injection at 168 hours.

The results clearly showed that addition of inhibitors in two equal increments separated by 24 hours significantly reduced the production of methane. The methane formation was remarkably similar for both 2000 mg/l LA and 50 mM BES cultures where the inhibitor was added in one portion. The cultures containing 1000 mg/l + 1000 mg/l LA and 25 mM + 25 mM BES produced roughly the same amount of methane over the duration of the experiment (Figure 6.2).

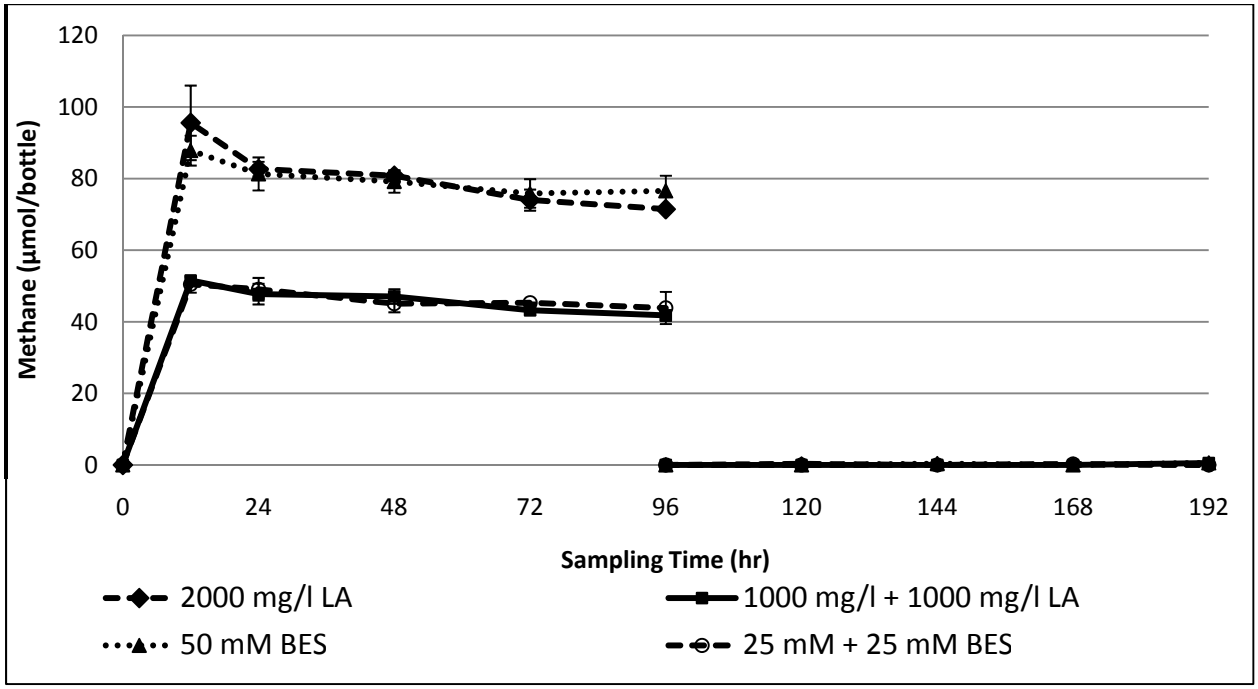


Figure 6.2: Methane production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

Maximum methane production for cultures with 2000 mg/l LA and 1000 + 1000 mg/l was 95 and 50 µmol/bottle at 12 hours, respectively. The maximum methane production for cultures containing 50 mM BES and 25 mM + 25 mM BESA was 90 and 50 µmol/bottle at 12 hours,

respectively. These concentrations account for a methane reduction of 45% and 40% for LA and BES, respectively. Therefore, adding the inhibitors in two separate increments lead to a reduction of approximately half the methane production.

Similar to the previous phase, methane production was essentially non-existent during the second glucose injection. Hydrogen and methane yield for this phase of study are shown in Tables 6.2 and 6.3. Bar charts displaying the maximum H_2 yield and methane production for each culture set over the duration of the experiment are shown in Figures 6.3 and 6.4, respectively.

Table 6.2: H_2 yields in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

	mol H_2/mol glucose			
Time (hr)	**2000 LA**	**1000 + 1000 LA**	**50 BES**	**25 + 25 BES**
0	0.000	0.000	0.000	0.000
12	0.191±0.04	0.135	0.076±0.02	0.117±0.01
24	1.060±0.25	0.317±0.04	0.144±0.02	0.150±0.01
48	1.509±0.10	0.854±0.09	0.233±0.03	0.241±0.02
72	1.552±0.29	1.148±0.12	1.043±0.08	1.170±0.07
96	1.666±0.14	1.105±0.25	1.866±0.03	1.978±0.09
120	0.107±0.11	0.332±0.01	1.167±0.07	0.019±0.02
144	2.414±0.14	2.995±0.22	1.995±0.14	0.448±0.15
168	3.230±0.22	2.980±0.29	2.148±0.14	1.226±0.17
192	2.996±0.29	2.750±0.08	1.956±0.14	1.453±0.10

Table 6.3: Methane yields for cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

	mol METHANE/mol glucose			
Time (hr)	**2000 LA**	**1000 + 1000 LA**	**50 BES**	**25 + 25 BES**
0	0.000	0.000	0.000	0.000
12	0.069±0.01	0.037	0.063	0.036
24	0.060	0.034	0.059	0.035
48	0.058	0.034	0.057	0.032
72	0.053	0.031	0.055	0.033
96	0.052	0.030	0.055	0.032
120	0.000	0.000	0.000	0.000
144	0.000	0.000	0.000	0.000
168	0.000	0.000	0.000	0.000
192	0.000	0.000	0.000	0.000

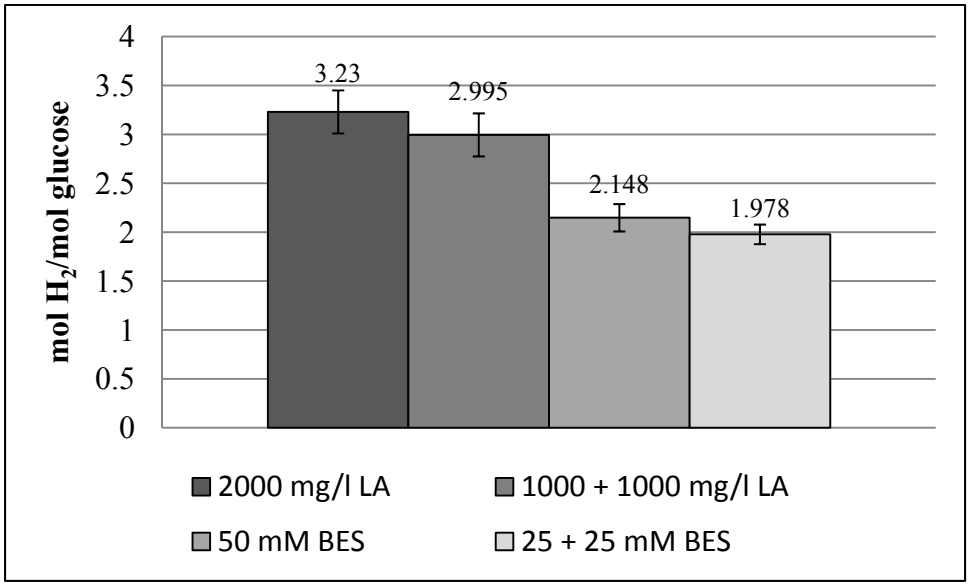


Figure 6.3: Maximum H_2 yield per culture

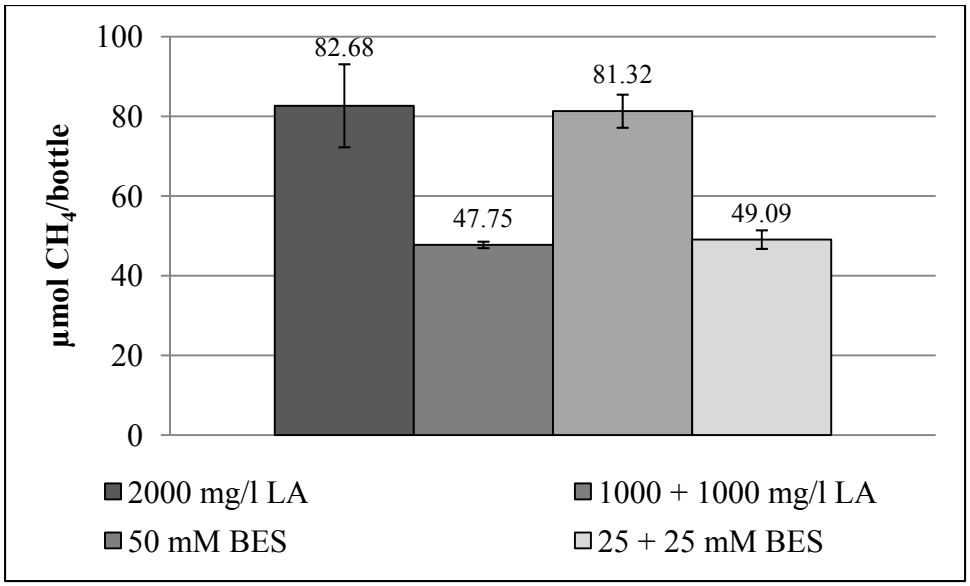


Figure 6.4: Maximum methane concentration per culture

6.2 VFA Production

The VFA observed under the different conditions include lactic, acetic, formic, propionic and butyric acids (Figures 6.5 to 6.9). Lactic acid levels were fairly low with peak levels (90 mg/l) detected at 24 hours (Figure 6.5). The levels after 24 hours were less than approximately 20 mg/l over the remainder of the study. The most prominent acetic acid production was observed between 144 and 168 hours in all the cultures. Peak acetate levels (1200 mg/l) were observed after 96 hours in cultures fed 50 mM BES and at 168 hours increasing levels at 3700 mg/l were attained under the same inhibitory condition (Figure 6.6).

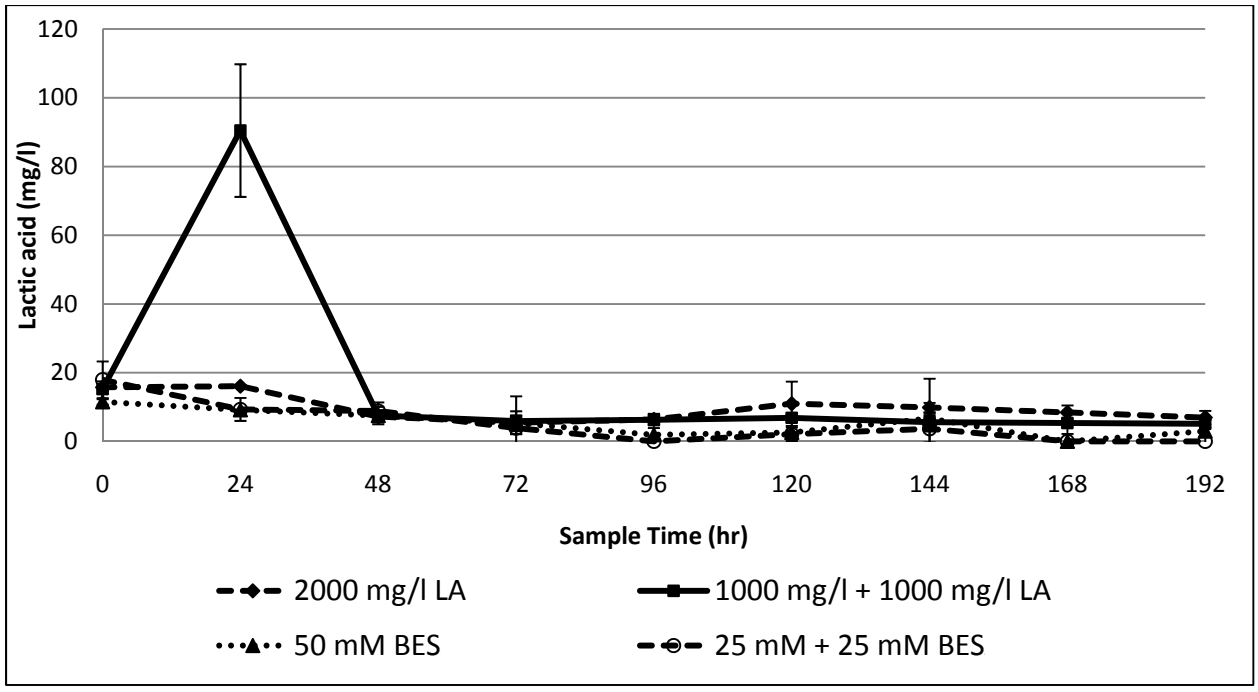


Figure 6.5: Lactic acid production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

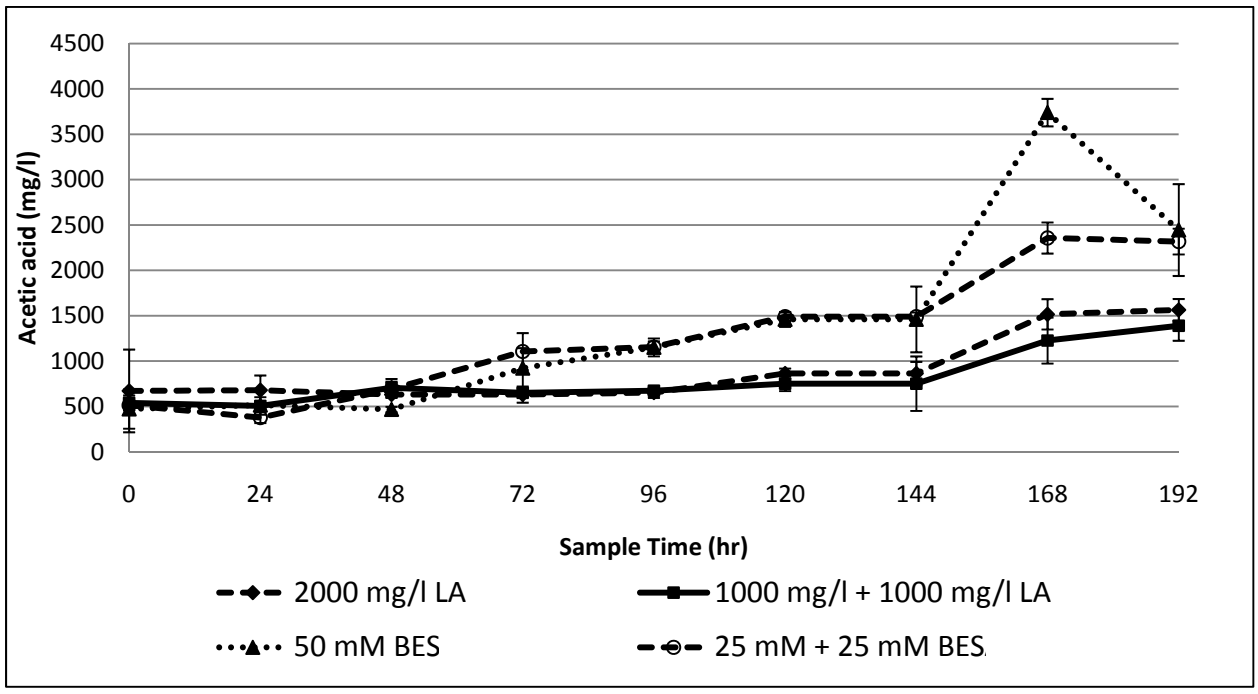


Figure 6.6: Acetic acid production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

In all the cultures, the formic acid concentration increased steadily over the duration of the experiment. The formic acid concentration reached a maximum of 490 mg/l at 168 hours in the cultures containing 2000 mg/l LA (Figure 6.7). At time 0, the propionic acid concentration varied between 2000 and 2500 mg/l in all cultures (Figure 6.8). The reason for this is likely due to VFAs from the mother reactor which was not completely degraded. The propionic acid concentration decreased to approximately 1500 mg/l in all the cultures within the first 24 hours. Between 120 and 144 hours, the propionic acid concentration increased to 2800 and 2300 mg/l in the cultures containing 50 mM BES and 25 mM BES + 25 mM BES, respectively. Over the duration of the study, the butyric concentrations varied between 40 to 150 mg/l (Figure 6.9).

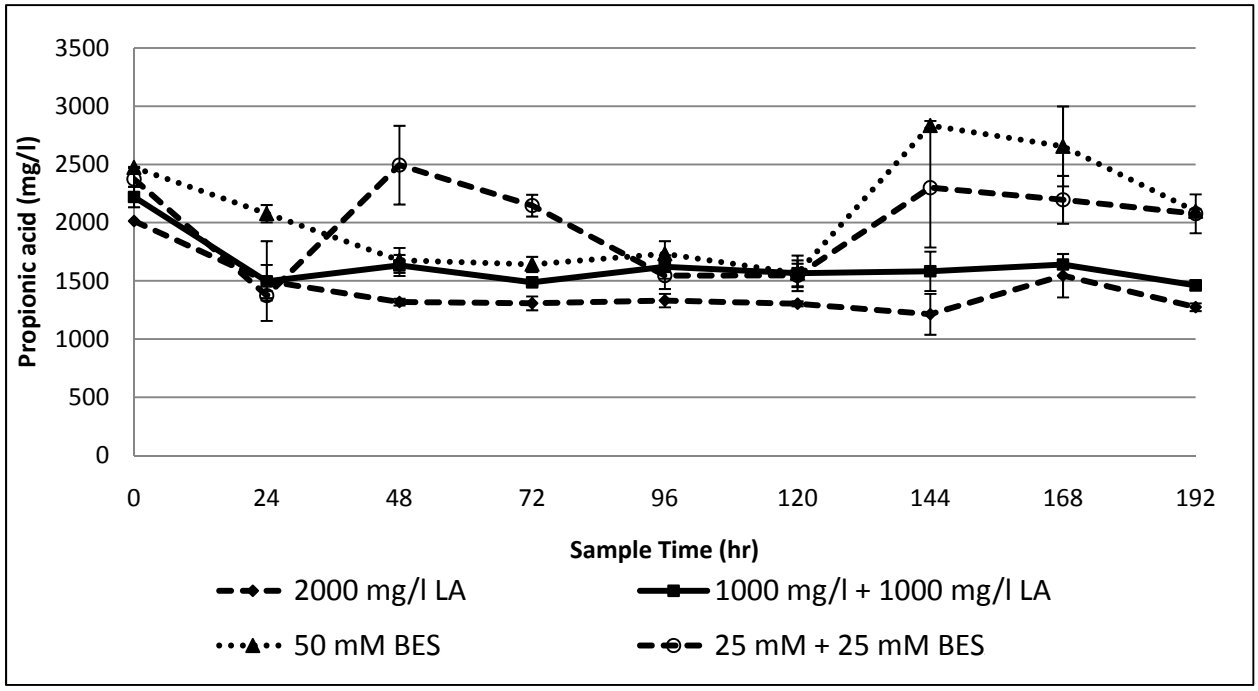


Figure 6.7: Propionic acid production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

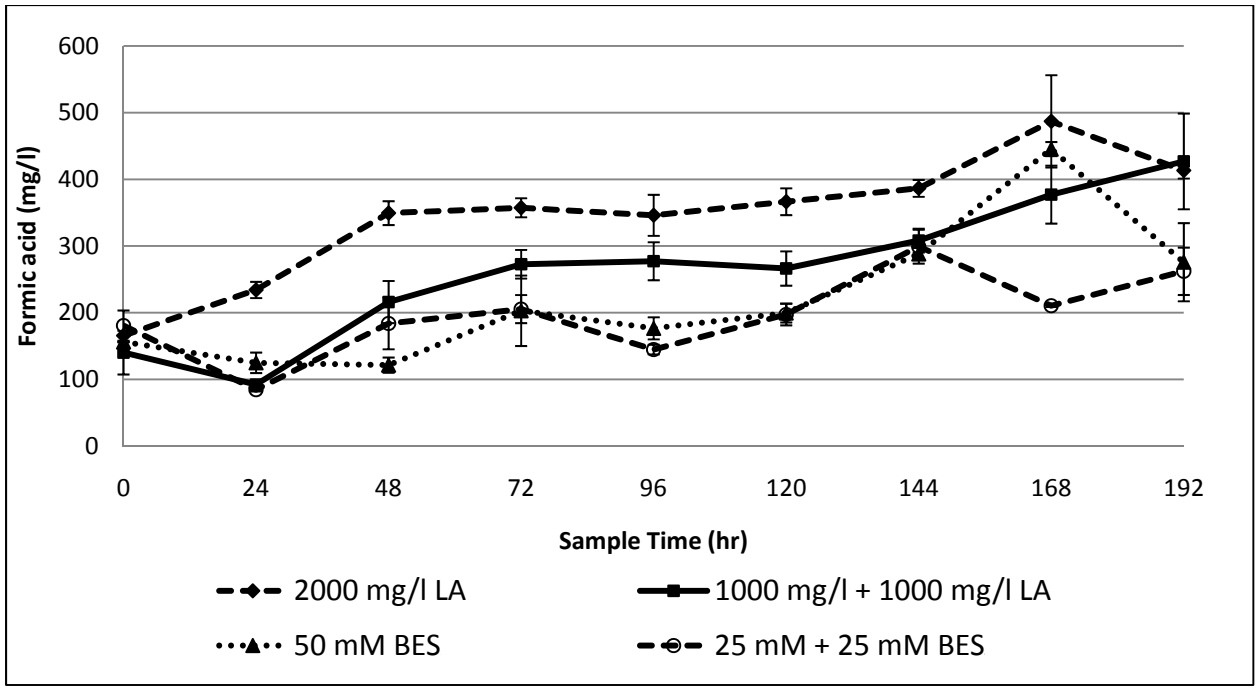


Figure 6.8: Formic acid production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

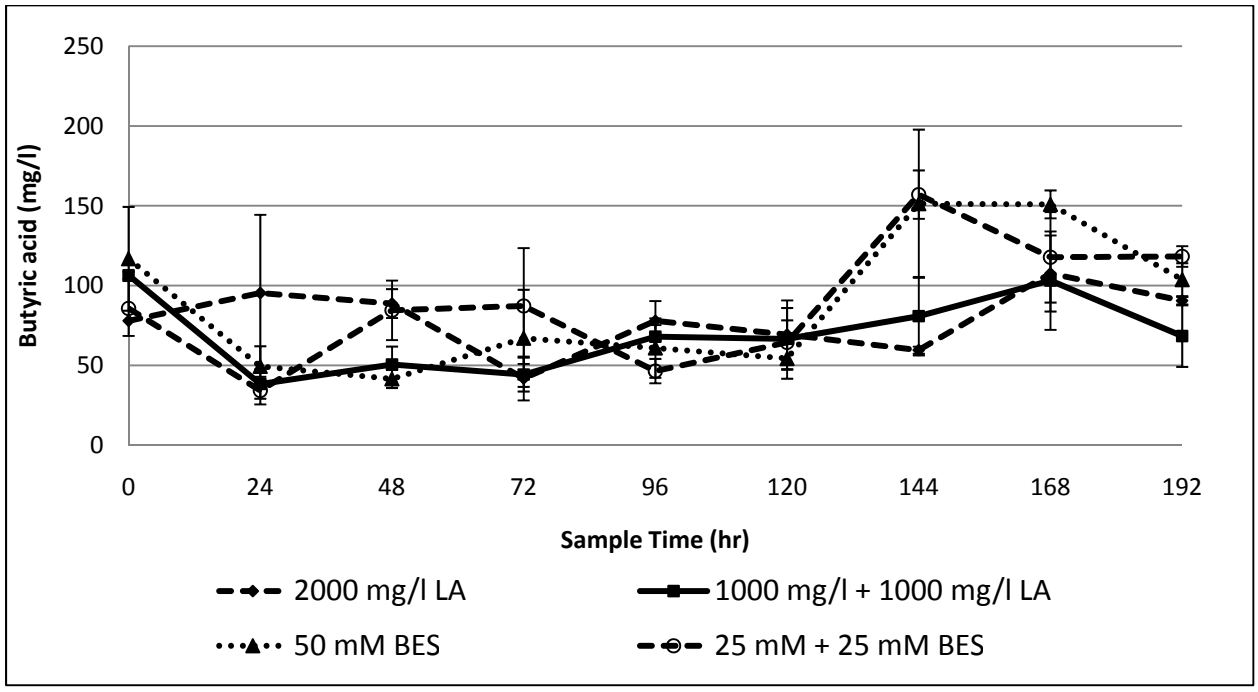


Figure 6.9: Butyric acid production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

6.3 Alcohol Production

The primary alcohols produced in this phase were iso-propanol, ethanol and propanol. Iso-propanol accumulation steadily increased over the duration of the experiment. The maximum iso-propanol concentration observed was 1570 mg/l at 168 hours in cultures containing 1000 mg/l LA + 1000 mg/l LA (Figure 6.10).

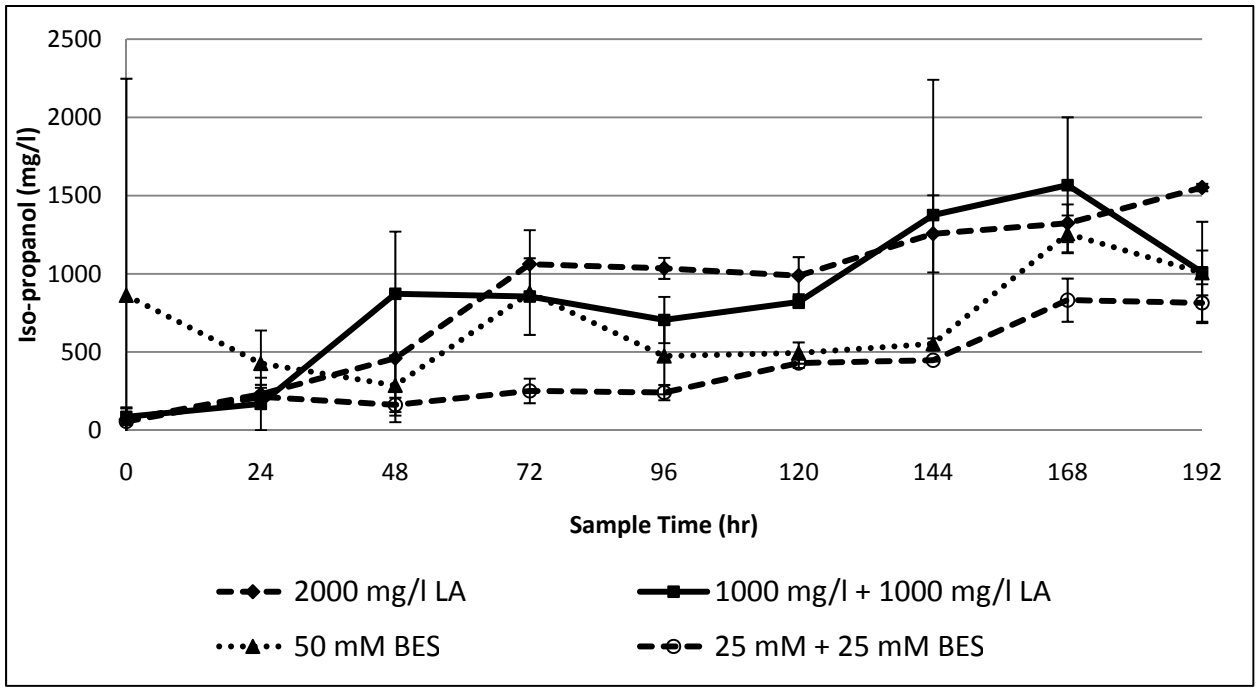


Figure 6.10: Iso-propanol production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

In cultures containing 2000 mg/l LA, the iso-propanol levels reached approximately 1500 mg/l after 192 hours. In the cultures fed BES, the iso- propanol concentrations were slightly lower throughout the experiment. Ethanol and propanol displayed similar production patterns in this phase (Figure 6.11 and Figure 6.12).

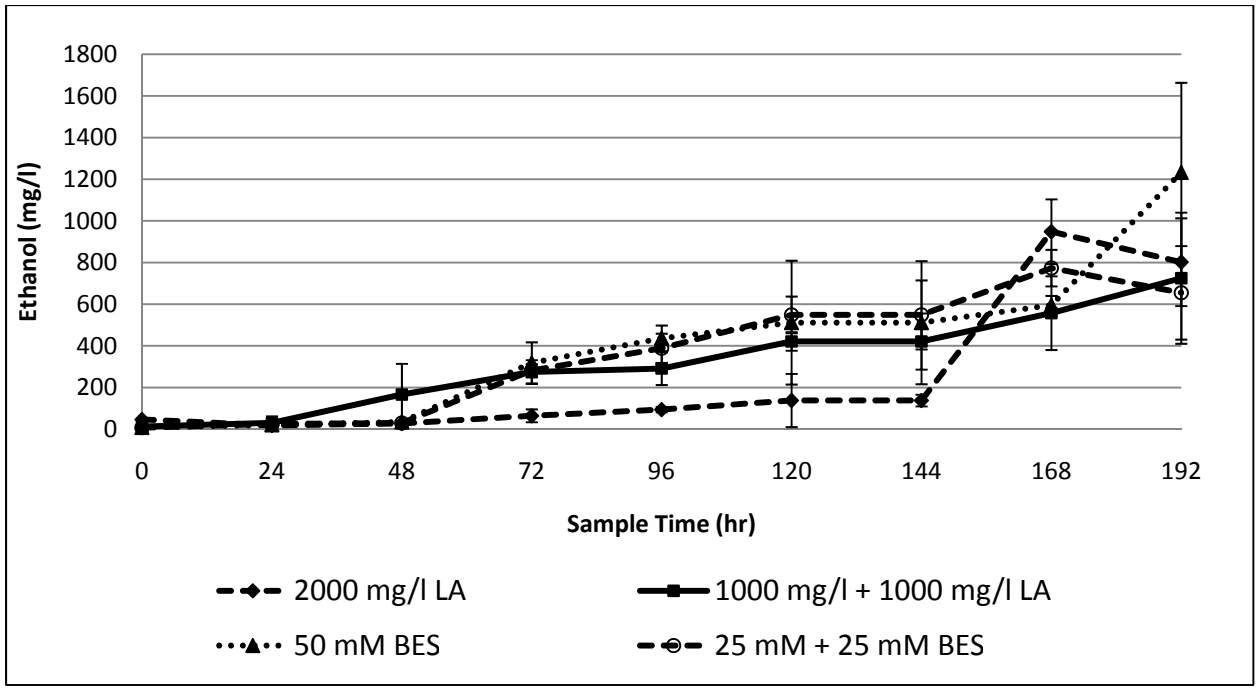


Figure 6.11: Ethanol production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

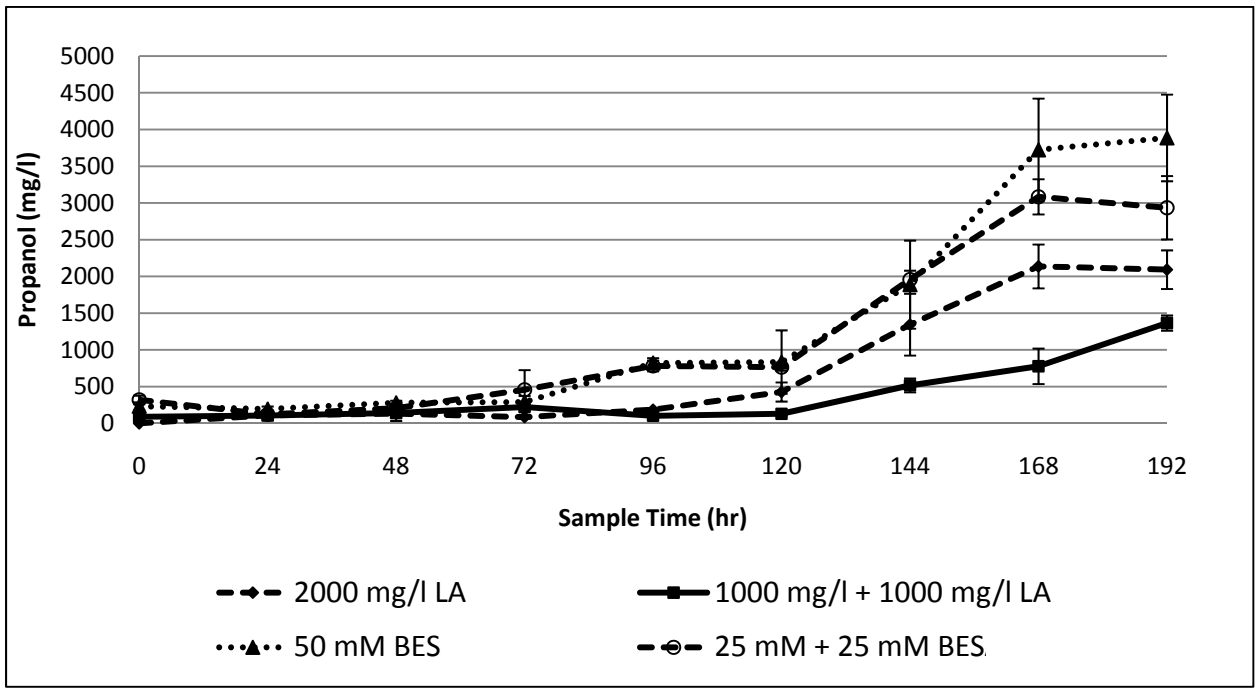


Figure 6.12: Propanol production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

The production of both alcohols increased sharply after 96 hours. The ethanol concentration reached a maximum of 1230 mg/l at 192 hours in cultures containing 50 mM BES (Figure 6.11). The majority of the propanol produced occurred between 120 and 168 hours (Figure 6.12). A maximum propanol concentration of 3900 mg/l was observed at 192 hours in cultures containing 50 mM BES. Small quantities of iso-butanol, reaching 350 mg/l, were produced towards the end of the experiment; however, butanol was not produced at significant levels in this phase (Figure 6.13 and Figure 6.14).

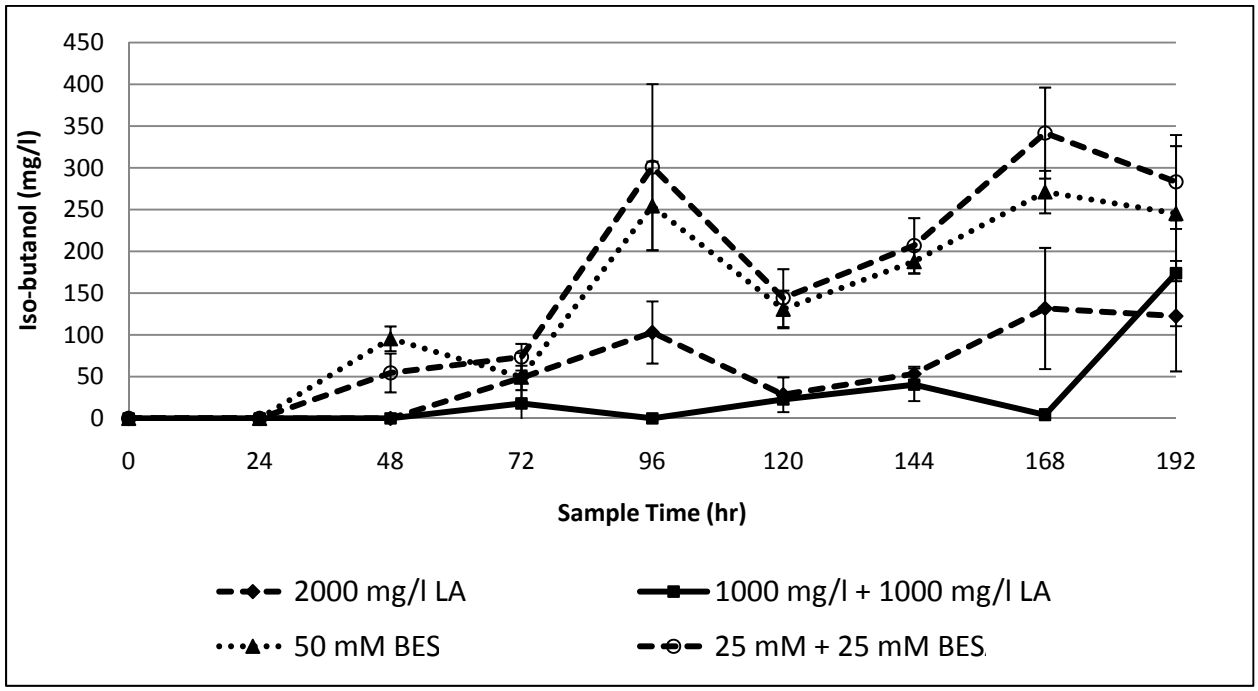


Figure 6.13: Iso-butanol production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

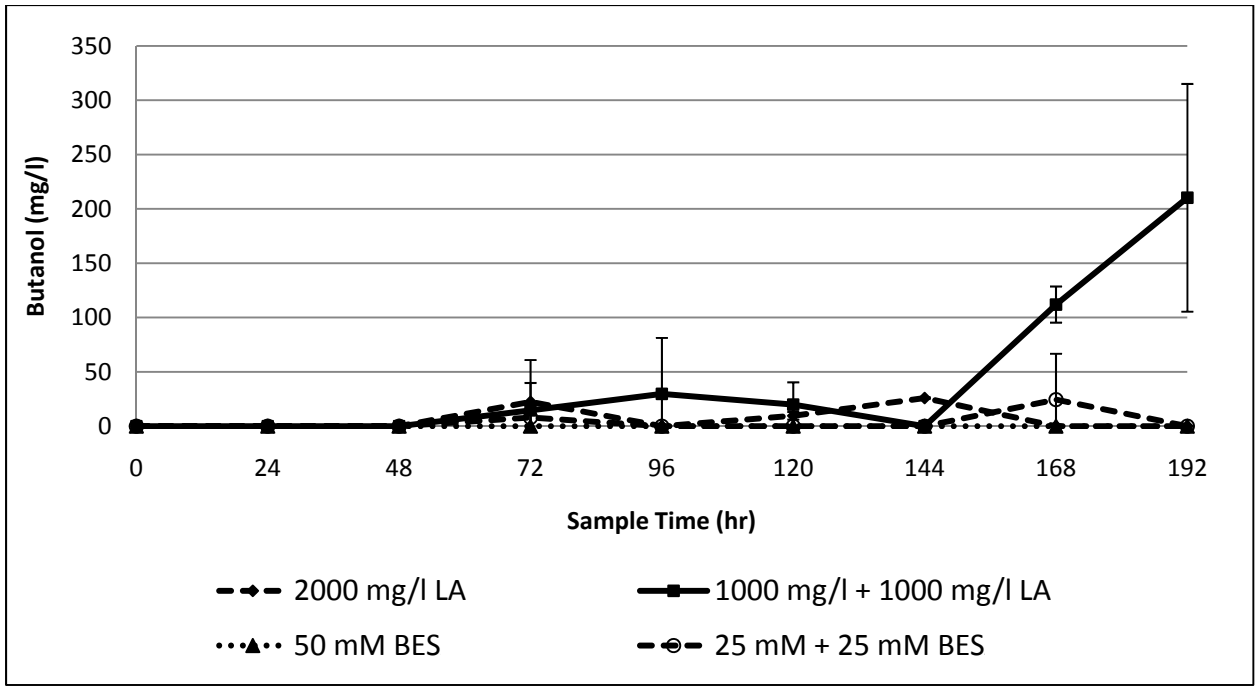


Figure 6.14: Butanol production in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

6.4 Glucose Degradation

The degradation of glucose remained fairly equal for all cultures in this phase. Between 8 and 24 hours, the cultures containing 50 mM BES were observed with slightly less glucose than the other cultures (Figure 6.15).

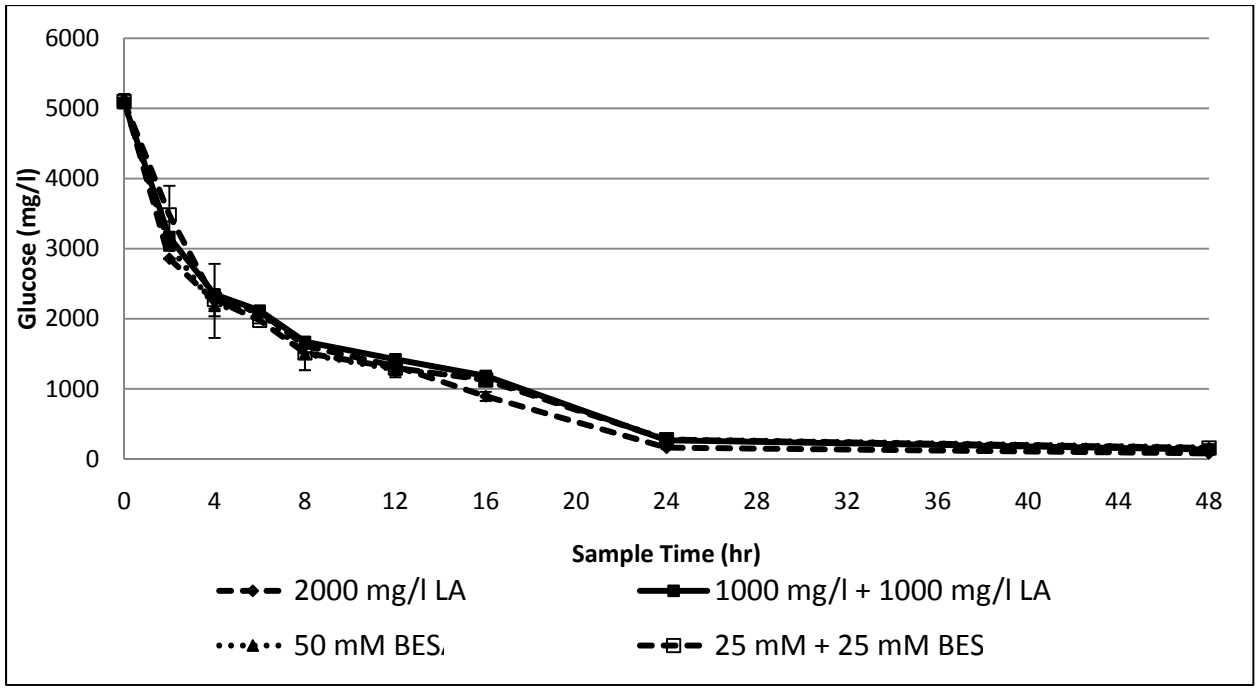


Figure 6.15: Glucose degradation in cultures receiving 2000 mg/l LA and 50 mM BES (Comparing single addition to two equal additions separated by 24 hours)

6.5 pH Profile

Figure 6.16 shows the pH profile of the cultures over the course of the experiment. In all cultures, the initial pH was set at 5.5 with no pH buffer capacity. At 96 hours, the cultures were opened and the pH recorded. The pH was re-adjusted to 5.5 prior to the second glucose injection. The pH was again recorded at the end of the experiment (192 hours). The pH decrease was significantly larger in cultures containing BES. The pH decreased from 5.5 to 3.8 in 96 hours in cultures containing 50 mM BES and 25 mM BES + 25 mM BES. In cultures containing with LA, the pH drop was not nearly as large. The pH decreased from 5.5 to approximately 4.4 in 96 hours in cultures containing 2000 mg/l LA and 1000 mg/l LA + 1000 mg/l LA. The pH difference between cultures with BES and LA inhibition was not as large during the second glucose injection period. The pH in the cultures with BES did not decrease as much as in the first injection. The pH in cultures containing 50 mM BES versus those containing 25 mM BES + 25

mM BES decreased from 5.5 to 4.1 from 96 to 192 hours. The pH drop was larger in cultures containing LA during the second glucose injection. The pH in cultures containing 2000 mg/l LA versus those containing 1000 mg/l LA + 1000 mg/l LA decreased from 5.5 to 4.3 from 96 to 192 hours.

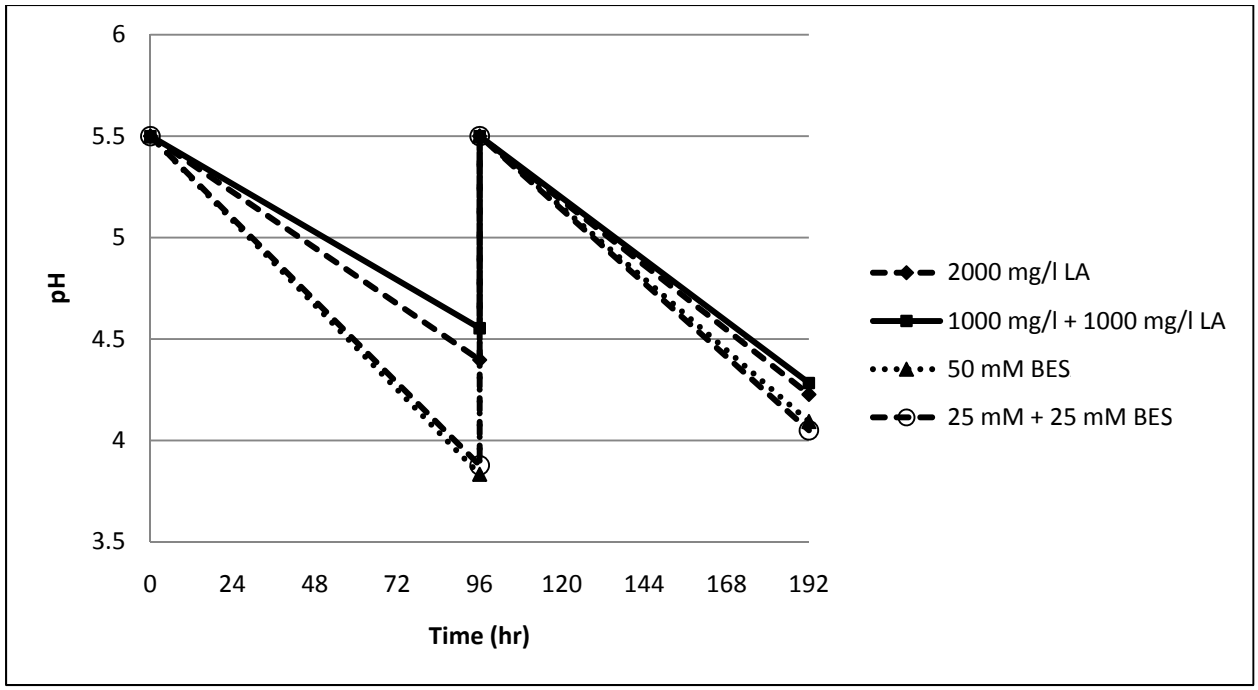


Figure 6.16: pH profile over the duration of 8-day experiment

6.6 Reactions Based on Electron Balance

Chemical equations are shown below for the different inhibition conditions and sampling periods. From each equation a biomass molar yield ($Y_{glucose/biopmass}$) and H_2 molar yield ($Y_{H2/glucose}$) can be determined. For example, for 2000 mg/l LA at 72 hours, $Y_{glucose/biopmass}$ and $Y_{H2/glucose}$ are 2.30 and 0.12, respectively.

2000 mg/l LA at 72 hours

$$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.121H_2O \rightarrow 0.0959H_2 + 0.0033CH_4 + 0.005C_5H_7O_2N + 0.0095HCOO^- + 0.0368CH_3COH_2CH_3 + 0.1017HCO_3^- + 0.1162H^+$$

2000 mg/l LA at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.09843H_2O \rightarrow 0.07448H_2 + 0.005C_5H_7O_2N + 0.008639CH_3COO^- + 0.00223HCOO^- + 0.001026CH_3CH_2CH_2COO^- + 0.003981CH_3COH_2CH_3 + 0.007698CH_3CH_2OH + 0.026953CH_3CH_2CH_2OH + 0.00032246CH_3CH_2CHOHCH_3 + 0.091896HCO_3^- + 0.1010H^+$

1000 mg/l LA + 1000 mg/l LA at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.0999H_2O \rightarrow 0.07238H_2 + 0.00196CH_4 + 0.005C_5H_7O_2N + 0.0043CH_3COO^- + 0.00669HCOO^- + 0.02909CH_3COH_2CH_3 + 0.00646CH_3CH_2OH + 0.00504CH_3CH_2CH_2OH + 0.09245HCO_3^- + 0.108791H^+$

1000 mg/l LA + 1000 mg/l LA at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.1149H_2O \rightarrow 0.0957H_2 + 0.005C_5H_7O_2N + 0.01084CH_3COO^- + 0.00032CH_3CH_2COO^- + 0.00256HCOO^- + 0.00047CH_3CH_2CH_2COO^- + 0.01659CH_3COH_2CH_3 + 0.00334CH_3CH_2OH + 0.01297CH_3CH_2CH_2OH + 0.00128CH_3CH_2CH_2CH_2OH + 0.09744HCO_3^- + 0.1166H^+$

50 mM BES LA at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.1466H_2O \rightarrow 0.1336H_2 + 0.0070CH_4 + 0.005C_5H_7O_2N^- + 0.0352CH_3COO^- + 0.00479HCOO^- + CH_3CH_2CH_2COO- + 0.0013CH_3COH_2CH_3 + 0.0153CH_3CH_2OH + 0.00439CH_3CH_2CH_2OH + 0.0952HCO_3^- + 0.1402H^+$

50 mM BES LA at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.0461H_2O \rightarrow 0.02967H_2 + 0.005C_5H_7O_2N + 0.0218CH_3COO^- + 0.00629CH_3CH_2COO^- + 0.00297HCOO^- + 0.00051CH_3CH_2CH_2COO^- + 0.00647CH_3COH_2CH_3 + 0.00086CH_3CH_2OH + 0.0240CH_3CH_2CH_2OH + 0.00011CH_3CH_2CHOHCH_3 + 0.0638HCO_3^- + 0.1004H^+$

25 mM BES + 25 mM BES LA at 72 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.0420H_2O \rightarrow 0.04420H_2 + 0.00123CH_4 + 0.005C_5H_7O_2N + 0.01366CH_3COO^- + 0.03617CH_3CH_2COO^- + 0.0075HCOO^- + 0.00442CH_3COH_2CH_3 + 0.00405CH_3CH_2OH + 0.00313CH_3CH_2CH_2OH + 0.05643HCO_3^- + 0.1120H^+$

25 mM BES+ 25 mM BES at 168 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.04356H_2O \rightarrow 0.0202H_2 + 0.005C_5H_7O_2N + 0.01209CH_3COO^- + 0.00529CH_3CH_2COO^- + 0.00087HCOO^- + 0.00049CH_3CH_2CH_2COO^- + 0.005841CH_3COH_2CH_3 + 0.00248CH_3CH_2OH + 0.0297CH_3CH_2CH_2OH + 0.000327CH_3CH_2CHOHCH_3 + 0.06925HCO_3^- + 0.09298H^+$

6.7 Discussion of Results

When the inhibitors were divided into two equal portions and added in a 24 hour interval, methane production was significantly reduced. Both sets of LA and BES cultures that received two inhibitor additions showed remarkably similar methane production over the duration of the experiment. In fact, there was never more than a 2 μmol/bottle difference at each sampling time interval. The culture sets of LA and BES which received one injection also showed remarkably similar methane production. All the cultures reached peak methane concentrations 12 hours after glucose was injected. The methane concentration was 95 and 50 μmol/bottle after 12 hours for cultures containing 2000 mg/l LA and 1000 mg/l LA + 1000 mg/l LA, respectively. Moreover, the methane concentration was 90 and 50 μmol/bottle after 12 hours for cultures containing 50 mM BES and 25 mM BES + 25 mM BES, respectively. These represent a 45% and 43% decreases in methane production, for LA and BES, respectively; exclusively by splitting the inhibitor injections into two equal amounts separated by 24 hours. No methane was produced during the second glucose injection.

Unfortunately, this substantial reduction in methane production did not lead to higher H_2 yields. Even with more methane production, cultures containing 2000 mg/l LA produced more H_2 than cultures containing 1000 mg/l LA + 1000 mg/l LA. After 96 hours, cultures containing 2000 mg/l LA reached a H_2 concentration of 2310 μmol/bottle while the cultures containing 1000 mg/l LA + 1000 mg/l LA had a concentration of 1500 μmol/bottle. Hydrogen production between

2000 mg/l LA and 1000 mg/l + 1000 mg/l LA cultures was much closer during the second glucose injection period. In fact, from 96 to 144 hours, cultures containing 1000 mg/l LA + 1000 mg/l LA had more H_2 accumulation than cultures containing 2000 mg/l LA. Hydrogen production in cultures fed LA produced significantly more H_2 during the second glucose injection compared to the first injection. Peak H_2 concentrations at 168 hours reached 2 times the H_2 concentrations at 96 hours in LA inhibited cultures.

Hydrogen production was similar for both BES culture sets during the first glucose injection. Production was initially slow; however, between 48 and 72 hours, H_2 production occurs rapidly. The variation in inhibitor injection pattern had no effect on H_2 production for cultures with BES. Hydrogen production in cultures containing 50 mM BES or 25 mM BES + 25 mM BES was higher than cultures containing LA after 96 hours. A maximum H_2 yield of 1.87 and 1.98 mol H_2/mol glucose was observed at 96 hours for cultures containing 50 mM and 25 mM + 25 mM BES, respectively. During the second glucose injection, H_2 production in cultures containing BES varied greatly. The cultures with 25 mM + 25 mM BES produced substantially less H_2 than cultures containing 50 mM BES. At 144 hours, the difference in yield was 1.55 mol H_2/mol glucose between these cultures. The difference was reduced with increasing time. Unlike the cultures containing LA, cultures containing BES did not produce higher amounts of H_2 during the second glucose injection. This again shows that BES in detrimental during the second injection when there is no longer any methanogens to inhibit.

In cultures containing BES, higher VFA production was observed when compared to cultures containing LA. The lower H_2 yields during the second glucose injection can be likely attributed to higher concentrations of acetic, propionic and butyric acid. Cultures receiving BES also showed high propanol production from 144 to 192 hours. Other alcohols observed a relatively

similar production pattern between all the culture sets. Cultures containing LA produced more formic acid than cultures containing BES; however, the difference was small in comparison to the difference observed for acetic, propionic and butyric acid.

Based on the results of this experiment, it is clear that adding inhibitors in small increments is useful in reducing the methane formation. However, this approach had minimal effect on increasing the H_2 yield. In large scale H_2 producing reactors that employ microbial inhibition, it is suggested that they add inhibitors in increments separated by specific time intervals. Any electron divergence away from methane formation is beneficial. Methane reduction is a large benefit since lesser quantities of gas separation would be needed to obtain pure H_2. Also secondary degradation processes can further degrade other liquid by-products into H_2.

Chapter 7: H_2 Production from Glucose Degradation in the Presence of a LAU, LA and BES

This experiment is designed to compare the effects of BES and LCFA inhibition on H_2 and methane formation. BES is a synthetic compound manufactured in commercial laboratories; unlike organic LCFAs, which can be obtained naturally. BES is a 'specific' methanogen inhibitor and this suggests that its presence should not affect the functioning of other species of bacteria. LCFAs are 'non-specific' methanogenic inhibitors. Their presence affects many bacteria at threshold levels. Two species of LCFAs were evaluated in conjunction with BES; lauric and linoleic acid. Lauric acid (LAU) is a saturated fatty acid, with a 12 carbon long backbone while linoleic acid (LA) is a much larger molecule. LA is an unsaturated fatty acid with an 18 carbon long backbone containing two carbon-carbon double bonds. The double bonds cause LA to become a liquid at room temperature. Comparing two LCFAs with BES provides for a broader range of chemical structure comparison. Reaume (2009) reported that 2000 mg/l LA was the optimal LCFA concentration for H_2 production. However, the results are largely dependent on the species type and quantity of the microorganisms present in the mixed anaerobic culture. Concentrations of 1000, 2000 and 3000 mg/l were tested for both LAU and LA. While in the case of BES, concentrations were 25, 50 and 100 mM. These BES concentrations represent the high and low end of levels which have reported in published literature. Table 7.1 summarizes the experimental design followed for this phase of study. All cultures were prepared with an initial pH of 5.5. Gas, VFA, alcohol and glucose results from this phase of experiment are shown in Figures 7.1-7.22.

Table 7.1: Phase IV – H_2 Production from glucose degradation with varying concentrations of LAU, LA and BES

Cultures	LAU conc., mg/l	LA conc., mg/l	BES conc., mM	1st Glucose Injection (t=0 hr)	2nd Glucose Injection (t=96 hr)
1, 2, 3	1000	0	0	5 g/l	5 g/l
4, 5, 6	2000	0	0	5 g/l	5 g/l
7, 8, 9	3000	0	0	5 g/l	5 g/l
10, 11, 12	0	1000	0	5 g/l	5 g/l
13, 14, 15	0	2000	0	5 g/l	5 g/l
16, 17, 18	0	3000	0	5 g/l	5 g/l
19, 20, 21	0	0	25	5 g/l	5 g/l
22, 23, 24	0	0	50	5 g/l	5 g/l
25, 26, 27	0	0	100	5 g/l	5 g/l
28, 29, 30	0	0	0	5 g/l	5 g/l
31, 32, 33	0	0	0	0 g/l	0 g/l

7.1 H_2 and Methane Production

During the first glucose injection period, cultures containing LAU produced more H_2 that those containing LA. Cultures containing 3000 mg/l LAU were the strongest H_2 producers of all the LCFA cultures (Figure 7.1). The H_2 levels reached a maximum yield of 2.3 mol H_2/mol glucose at 48 hours. Cultures containing 3000 mg/l LAU maintained the highest H_2 concentration throughout the first glucose injection period. Cultures containing 2000 mg/l LAU were the second best producers of H_2. They reached a maximum yield of 2.1 mol H_2/mol glucose at 48 hours. The third best H_2 producing cultures contained 3000 mg/l LA. These cultures reached a maximum yield of 2.0 mol H_2/mol glucose at 96 hours. The strongest H_2 producing cultures contained 3000 mg/l LAU, 2000 mg/l LAU, and 3000 mg/l LA. Under all these conditions, rapid H_2 production was observed 24 hours after was injected. The remaining cultures with LCFA inhibition did not produce anywhere near the amount of H_2 as these cultures in the first 24 hours. Cultures containing 1000 and 2000 mg/l LA produced the lowest amounts of H_2.

Unlike the H_2 results in Chapter 6, cultures containing LCFAs produced lower amounts of H_2 during the second glucose injection period. The only exception was the cultures containing 3000 mg/l LA (Figure 7.1). All other culture sets produced lower amounts of H_2 during the second glucose feeding period. Cultures containing 3000 mg/l LAU, which were the strongest H_2 producers during the first glucose feeding period, were the weakest producer during the second injection. A maximum yield of 0.5 mol H_2/mol glucose was attained at 192 hours. Cultures containing 3000 mg/l LA were outstanding H_2 producers with levels reaching a yield of 3.22 mol H_2/ mol glucose at 168 and 192 hours. The second highest H_2 yield observed with LCFA inhibition during the second glucose injection was less than half the yield of 3.22 mol H_2/mol. Cultures containing 1000 mg/l LA and 1000 mg/l LAU obtained the second and third highest yields of 1.57 and 1.38 mol H_2/mol glucose at 192 hours, respectively.

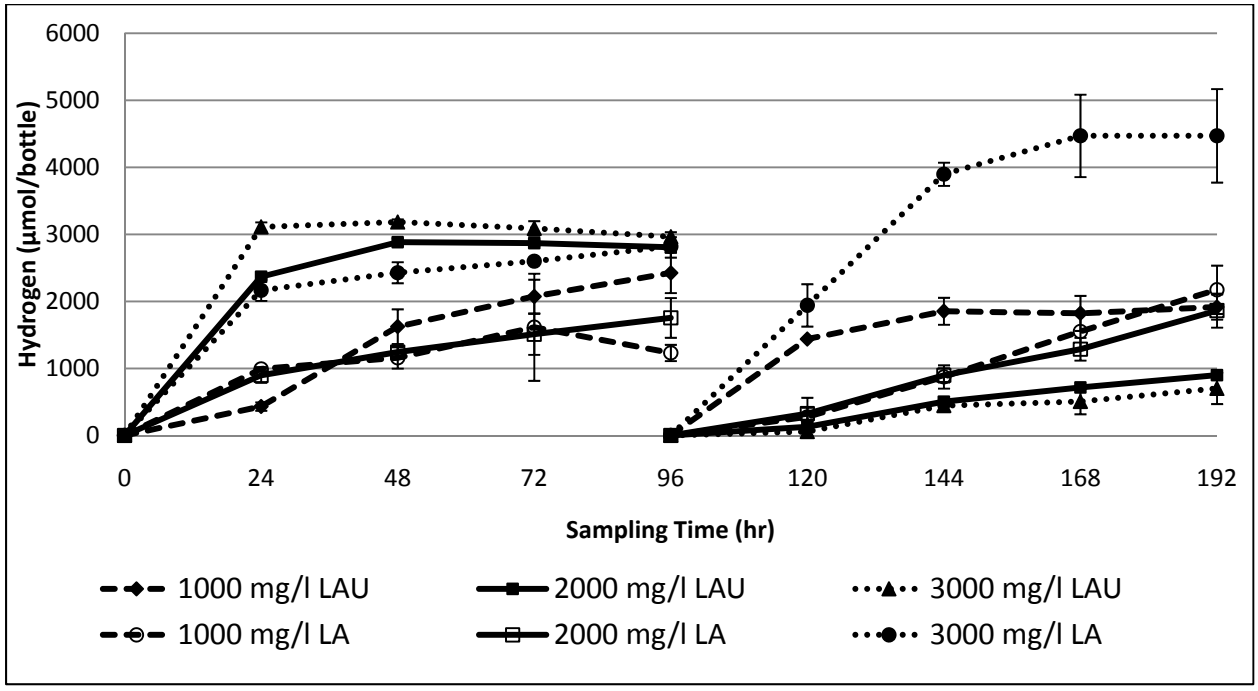


Figure 7.1: H_2 production in cultures receiving varying concentrations of LAU and LA

During the first glucose injection period, cultures containing BES inhibition produced H_2 at very modest levels. Hydrogen accumulation was essentially none existent in all cultures containing BES until the period between 48 and 72 hours (Figure 7.2). It is clear that the higher the BES concentration, the longer the lag in initial H_2 production. After 72 hours, the H_2 yield for cultures containing 25, 50 and 100 mM BES was 0.73, 0.34, and 0.01 mol H_2/mol glucose, respectively. Cultures containing no inhibitor exhibited a similar H_2 production pattern as cultures containing BES. In these cultures, a 48 hour lag was observed until significant quantities of H_2 were produced. At 72 hours, cultures with no inhibitor showed a yield of 0.91 mol H_2/mol glucose. In comparison, this yield was greater than yields for the cultures containing BES. The maximum yield at 96 hours in cultures containing 25 mM BES was 1.33 mol H_2/mol glucose. The yield observed in cultures containing 25 mM BES was similar to those receiving no BES. In cultures containing no inhibitor, the yield reached 1.26 mol H_2/mol glucose at 96 hours.

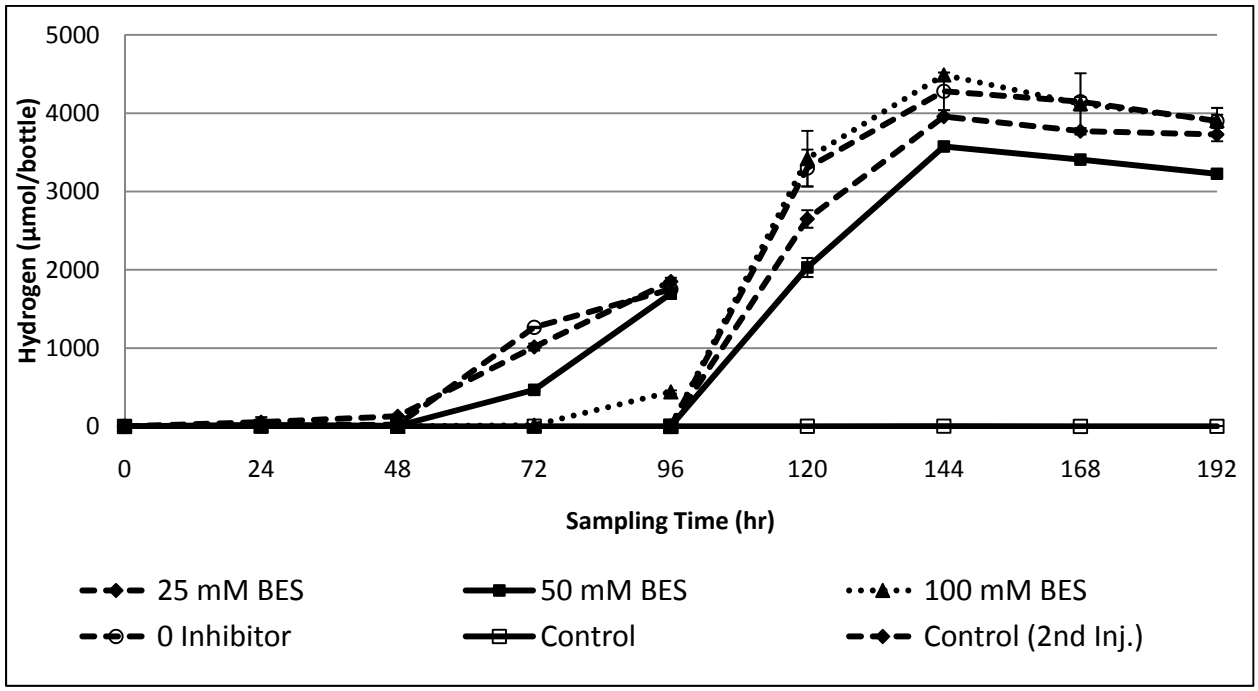


Figure 7.2: H_2 production in cultures receiving varying concentrations of BES

The increase in H_2 production from the first to the second glucose injection period was remarkable in cultures containing BES. No lag in H_2 production was observed as substantial amounts of H_2 were produced 48 hours after injecting glucose again (96-144 hours). Hydrogen accumulation peaked at 144 hours for all cultures containing BES and in cultures receiving no inhibitor. The H_2 production pattern was very similar between all cultures containing BES and those not receiving BES (Figure 7.2). This is expected as BES is a specific methanogenic inhibitor and theoretically does not affect the H_2 producing populations at the levels under consideration. Cultures containing 50 mM BES were weaker H_2 producers than those fed with 25 and 100 mM BES. A maximum H_2 yield of 3.24 mol H_2/mol glucose was attained in cultures containing 100 mM BES at 144 hours. This represents the highest yield recorded in Phase IV. Cultures containing no inhibitor produced the second highest H_2 yield at 3.08 mol H_2/mol glucose at 144 hours while in cultures containing 25 mM BES, a maximum H_2 yield of 2.85 mol H_2/mol glucose was observed at 144 hours.

LAU and LA proved to be extremely effective methanogenic inhibitors because no methane production was observed in cultures containing with these LCFAs (Figure 7.3 and Tables 7.2 and 7.3). In cultures containing BES, methane production was also very low. Methanogenesis was completely inhibited in cultures containing 100 mM BES while in cultures containing 50 mM and 25 mM BES, methanogenesis was not completely inhibited (Figure 7.4 and Table 7.3). Maximum methane concentrations of 23 and 10 μmol/bottle were observed in cultures containing 25 and 50 mM BES, respectively. The highest methane concentration observed in cultures containing no inhibitor was 90 μmol/bottle at 72 hours. The methane concentration in these cultures remained fairly consistent (between 80 and 90 μmol/bottle) after the first glucose injection. No methane formation was observed in all the cultures, excluding the controls after the

second glucose injection (Figure 7.4). Methane and H_2 yields are shown in Tables 7.2 and 7.3, respectively. The maximum H_2 yield for each condition under examination over the duration of the experiment is shown in Figure 7.5. In Figure 7.6, the maximum methane concentration per culture is shown for each culture set over the duration of the experiment.

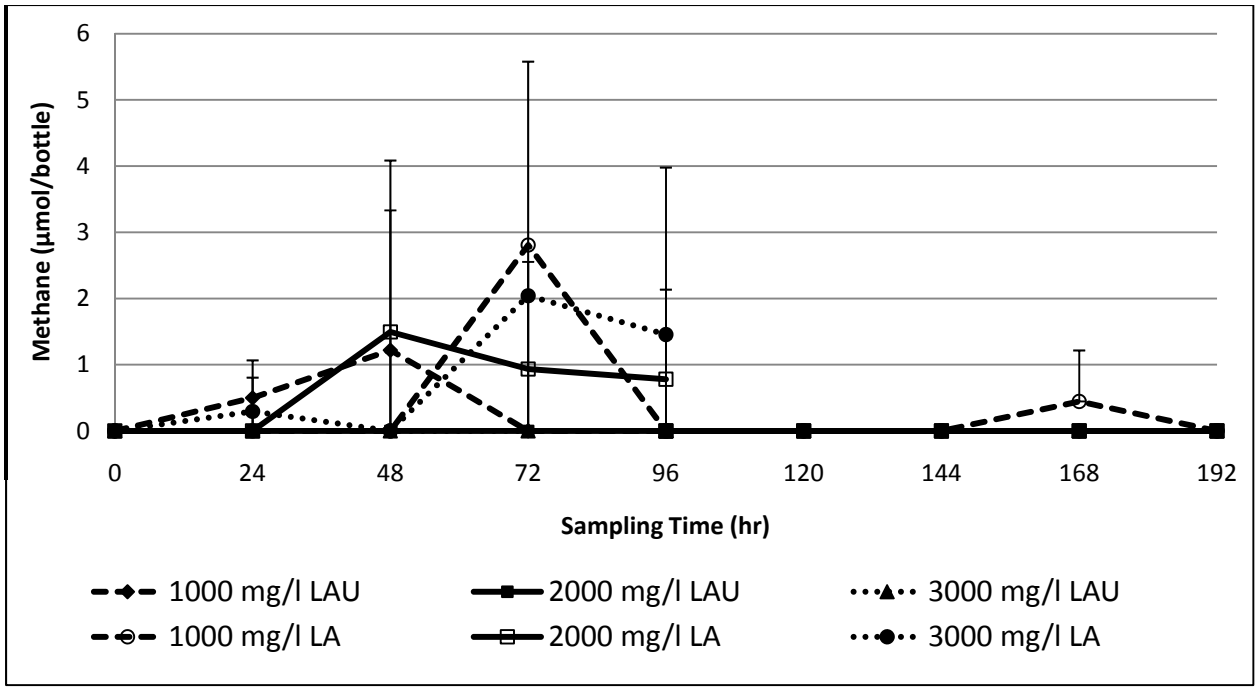


Figure 7.3: Methane production in cultures receiving varying concentrations of LAU and LA

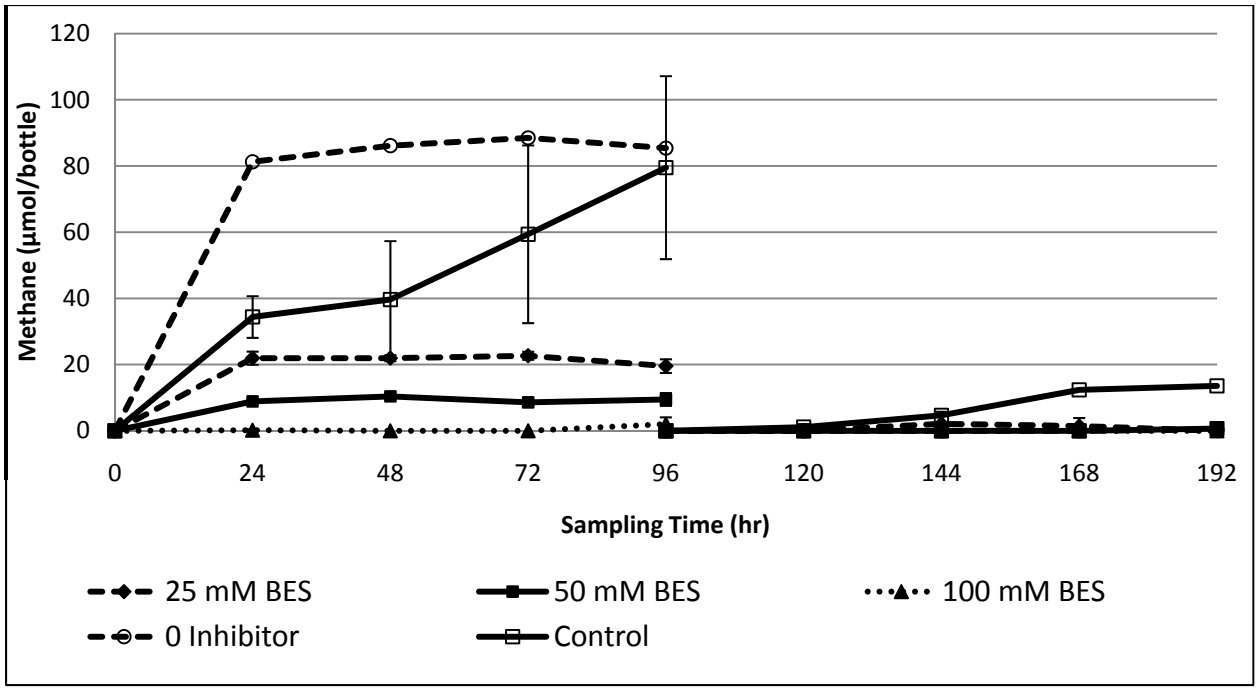


Figure 7.4: Methane production in cultures receiving varying concentrations of BES

Table 7.2: H_2 yield results for glucose degradation with varying concentrations of LAU, LA and BES

Time (hr)	Mol H_2/mol glucose							
	24	**48**	**72**	**96**	**120**	**144**	**168**	**192**
1000 LAU	0.31±0.05	1.17±0.19	1.49±0.18	1.75±0.22	1.04	1.34±0.15	1.31±0.19	1.38±0.14
2000 LAU	1.71±0.02	2.08±0.04	2.07±0.03	2.02±0.11	0.09±0.14	0.36	0.52	0.65
3000 LAU	2.24±0.05	2.29±0.03	2.23±0.08	2.14±0.05	0.04±0.06	0.32	0.36±0.14	0.51±0.17
1000 LA	0.72±0.02	0.83±0.11	1.16±0.58	0.89±0.09	0.20±0.21	0.63±0.13	1.12±0.19	1.57±0.26
2000 LA	0.65±0.08	0.90±0.06	1.09±0.22	1.26±0.21	0.23±0.06	0.65±0.08	0.93±0.12	1.34±0.18
3000 LA	1.56±0.11	1.75±0.11	1.87	2.03±0.03	1.40±0.23	2.81±0.12	3.22±0.44	3.22±0.50
25 BES	0.04±0.05	0.09±0.01	0.73±0.03	1.33±0.04	1.91±0.08	2.85±0.02	2.72±0.03	2.69±0.06
50 BES	0.01	0.00	0.34±0.01	1.22±0.04	1.46±0.09	2.58	2.46	2.33
100 BES	0.01	0.00	0.01±0.01	0.32±0.02	2.47±0.26	3.24	2.97±0.06	2.81±0.12
0 INH	0.01	0.01	0.91±0.01	1.26±0.02	2.38±0.17	3.08±0.17	2.99±0.26	2.81±0.06
Con.	0.001	0.001	0.001	0.001	0.001	0.002	0.000	0.001

Table 7.3: Methane yield results for glucose degradation with varying concentrations of LAU, LA and BES

Time (hr)	mol METHANE/mol glucose							
	24	**48**	**72**	**96**	**120**	**144**	**168**	**192**
1000 LAU	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
2000 LAU	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3000 LAU	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1000 LA	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000
2000 LA	0.000	0.001	0.001	0.001	0.000	0.000	0.000	0.000
3000 LA	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000
25 BES	0.016	0.016	0.016	0.014	0.000	0.002	0.001	0.000
50 BES	0.006	0.007	0.006	0.007	0.000	0.000	0.000	0.000
100 BES	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
0 INH	0.059	0.062	0.064	0.062	0.000	0.000	0.000	0.000
Control	0.025	0.029±0.01	0.043±0.02	0.057±0.02	0.001	0.003	0.009	0.010

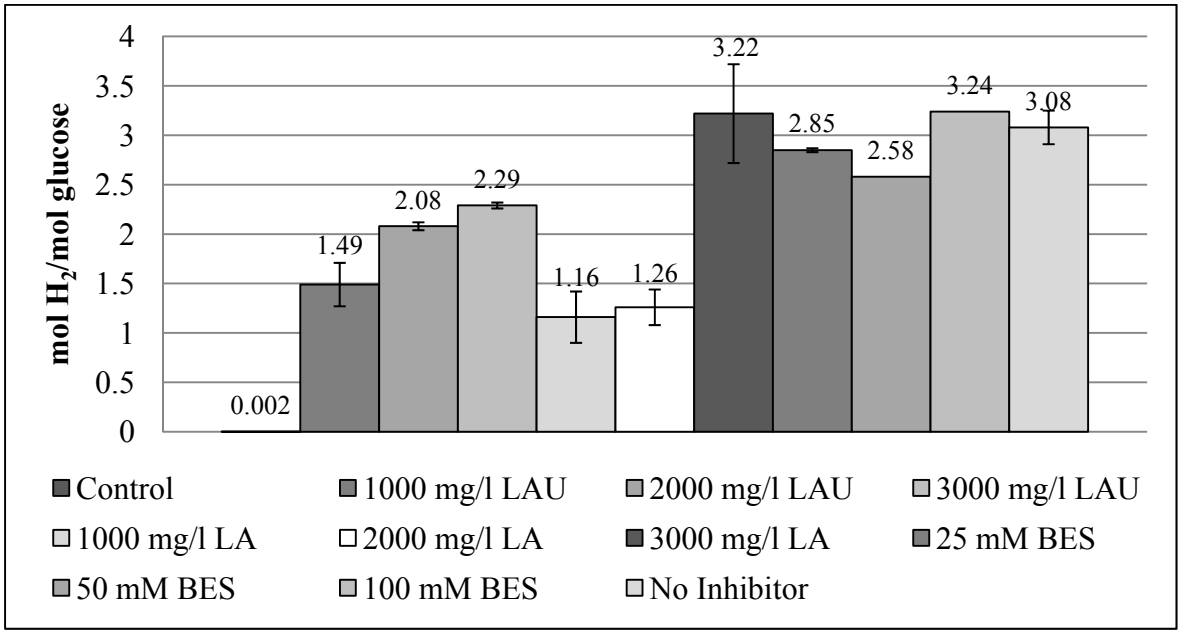


Figure 7.5: Maximum H_2 yield observed in each culture set

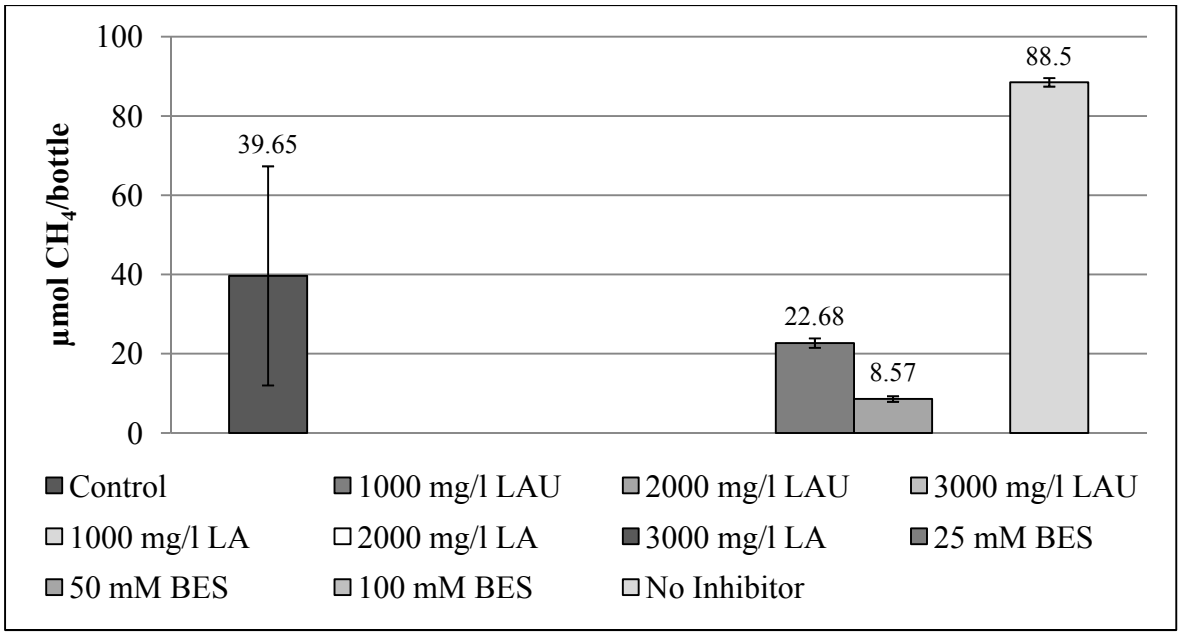


Figure 7.6: Maximum methane concentration per culture observed in each culture set

7.2 VFA Production

Acetic, propionic, formic and butyric acid were produced under all conditions in this phase of the study. All lactic acid concentrations measured were below the lower detection limit for VFAs. A residual level of acetic acid between 250-400 mg/l was present in all the cultures at time 0(Figure 7.7). In a majority of cultures containing LAU or LA, acetic acid was consumed between time 0 and 48 hours after the first injection. In cultures containing 3000 mg/l LAU, 1000 mg/l LA, 2000 mg/l LA, and 2000 mg/l LAU, elevated acetic acid accumulation was observed between 48 and 96 hours. This was the only significant time period where acetic acid was produced in culture containing the LCFA inhibitors. Cultures containing 3000 mg/l LAU reached a maximum acetic acid concentration of 925 mg/l at 96 hours after beginning with a concentration of 250 at time 0. In cultures containing 1000 mg/l LA, the second highest concentration of acetic acid was observed among the cultures containing LCFA; an acetic acid concentration of 650 mg/l was observed at 96 hours after beginning with an initial concentration of 310 mg/l at time 0. In all cultures containing LCFAs, the acetic acid concentration at 192 hours was less than that recorded

at time 0. Cultures containing BES and no inhibitor exhibited two stages of acetic acid accumulation (Figure 7.8). In the first stage, acetic acid accumulated 48 hours after feeding glucose and was then consumed for the next 48 hours. More acetic acid accumulation was observed after the second glucose injection period in cultures containing BES. Cultures containing 50 mM and 100 mM BES reached maximum acetic acid concentration of 910 and 890 at 192 hours, respectively. In these cultures, the acetic acid concentration was approximately 0 mg/l, 96 hours after the second glucose injection. The acetic acid concentration did not decline in these cultures 48 hours after glucose injection (between 144-192 hours) and over the duration of the study, the levels continued to increase. After the second glucose injection, the most rapid acetic acid accumulation occurred between 96 and 144 hours.

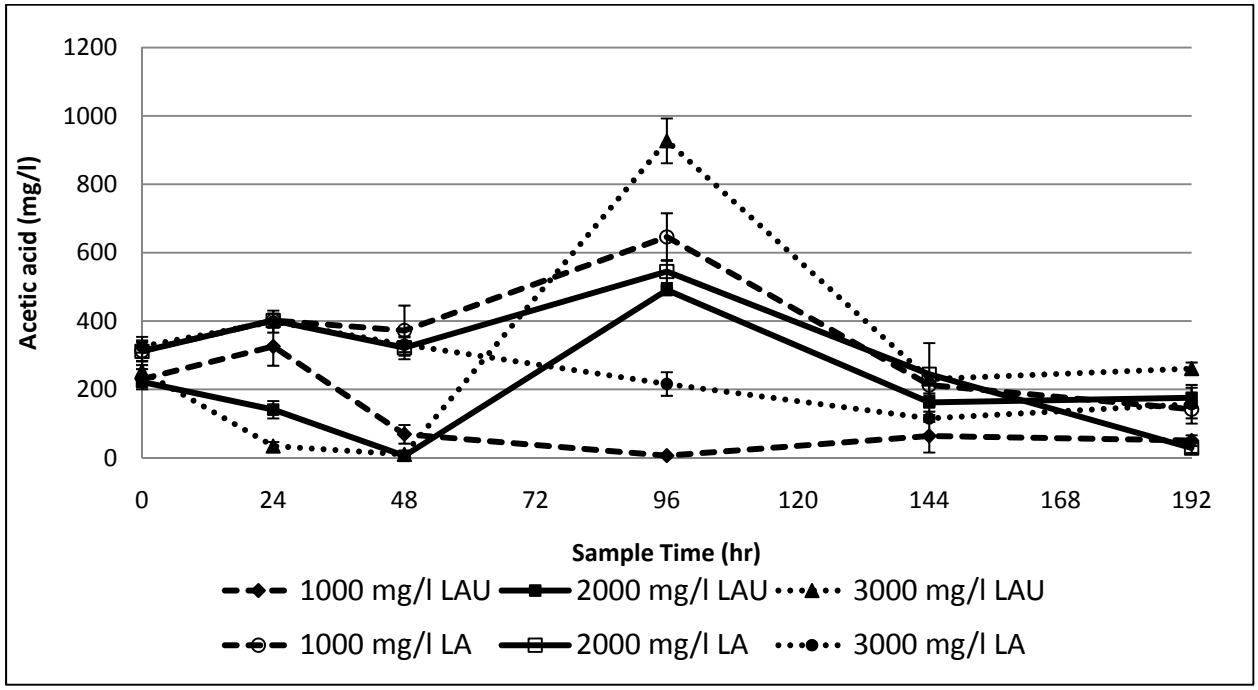


Figure 7.7: Acetic acid production in cultures receiving varying concentrations of LAU and LA

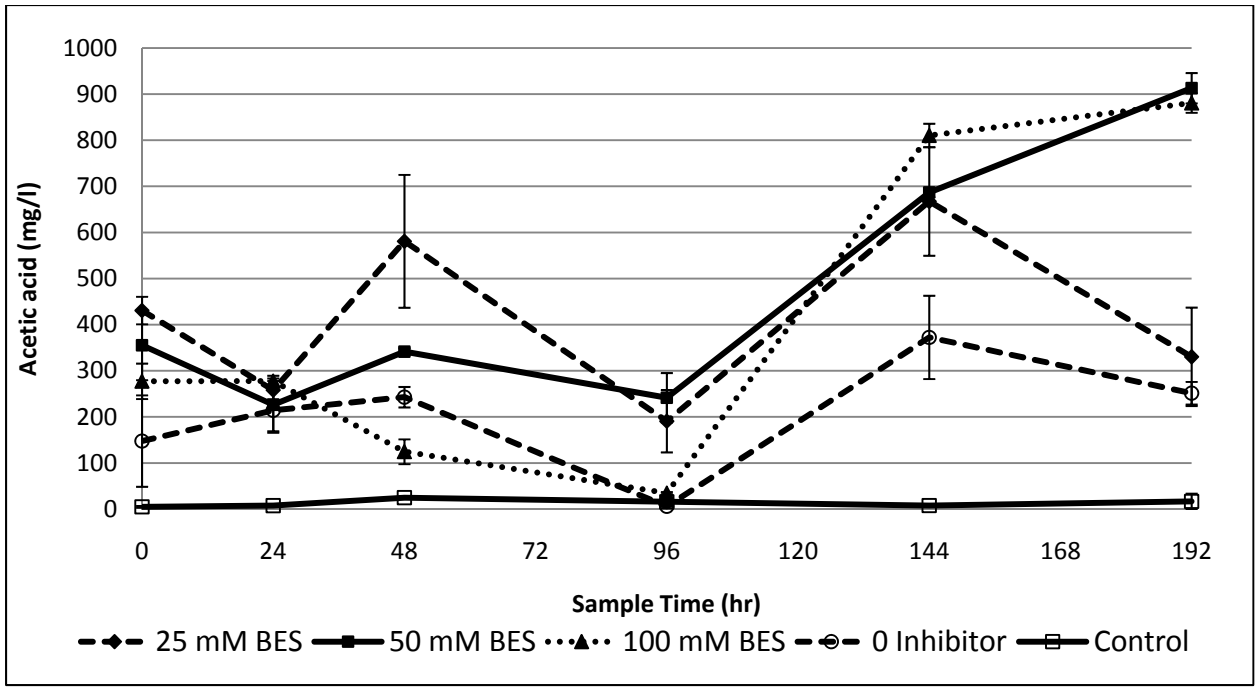


Figure 7.8: Acetic acid production in cultures receiving varying concentrations of BES

At time 0, residual propionic acid concentrations between 1150 and 1300 mg/l were detected in all cultures containing LCFAs (Figure 7.9). In cultures containing BES, the residual propionic concentrations detected at 0 hours was between 1650 and 1750 mg/l. Propionic acid accumulation patterns differed significantly between cultures containing BES or LCFAs. In cultures fed LCFAs, propionic acid production was observed within 24 hours after the first glucose injection. This accounted for approximately all the propionic acid production in cultures containing either LAU or LA. In cultures containing 3000 mg/l LA, the levels of propionic acid accumulated between 96 and 144 hours, reaching 2820 mg/l at 144 hours. In these cultures, the propionic acid concentration at 0 hours was 1240 mg/l. The net increase in propionic acid in these cultures was approximately 1600 mg/l over 144 hours. Very little propionic acid was detected during the first glucose injection, In cultures containing BES and those with no inhibitor, the largest amount of propionic acid accumulated between 96 and 144, 48 hours after

the second glucose injection. From 144 to 192 hours, no additional propionic acid accumulated. In cultures containing no inhibitor, the highest propionic acid concentration attained was recorded at 3400 mg/l at 144 hours (Figure 7.10). In comparison, in cultures containing 25 mM BES, the propionic acid levels reached 3300 mg/l after 144 hours. At time 0, in cultures containing no inhibitor and those fed 25 mM BES, the residual propionate acid levels were 1150 and 1700 mg/l, respectively. Therefore, net increase in propionic acid was approximately of 2250 and 1600 mg/l for cultures containing 0 BES and 25 mm BES, respectively.

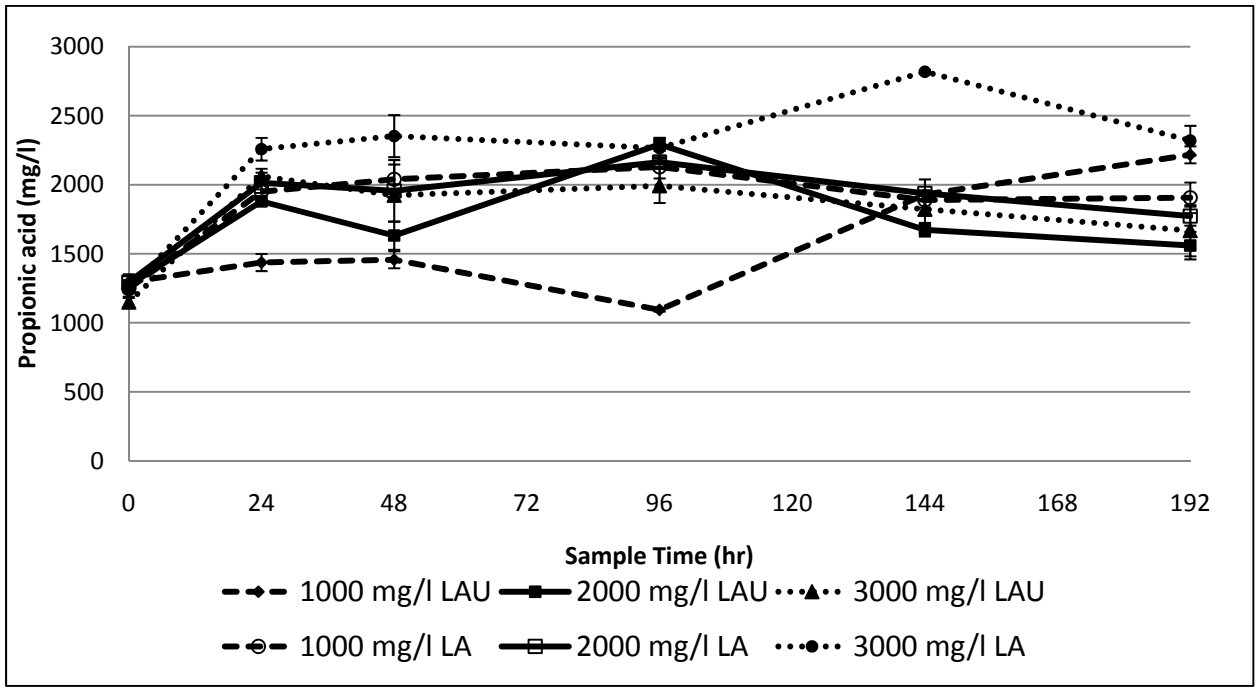


Figure 7.9: Propionic acid production in cultures receiving varying concentrations of LAU and LA

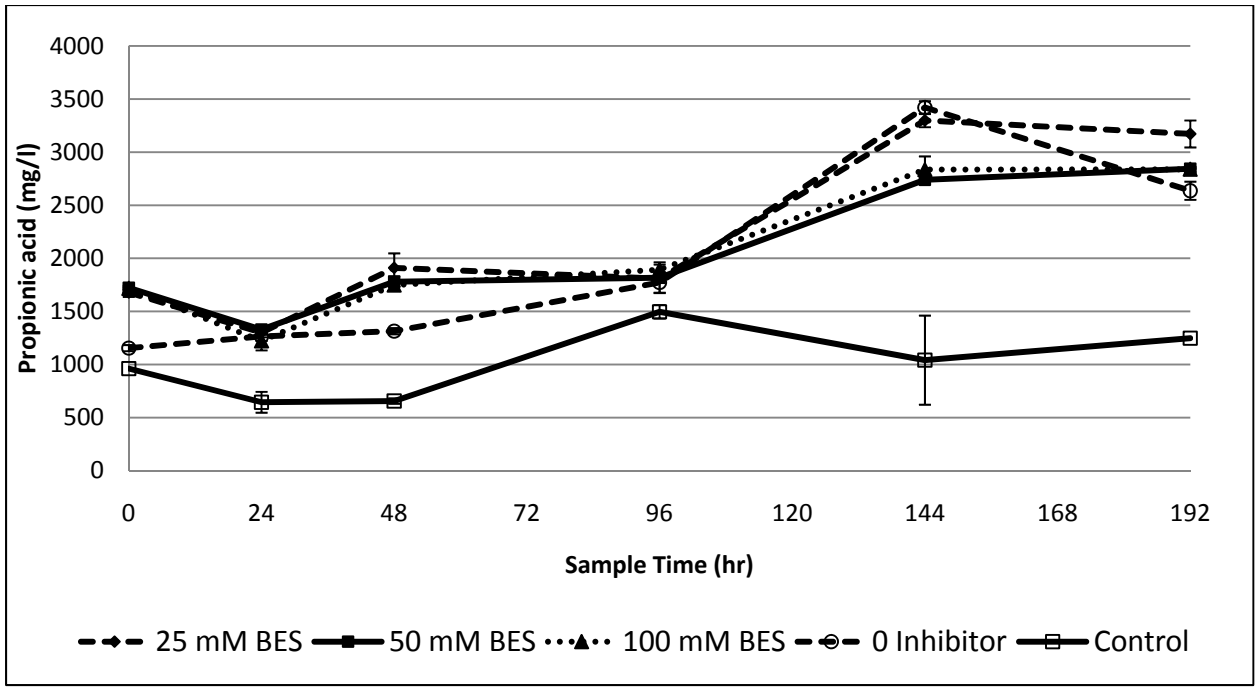


Figure 7.10: Propionic acid production in cultures receiving varying concentrations of BES

Formic acid production patterns in cultures containing LCFA were similar to the acetic acid production profiles. In all cultures, at time 0, the formic acid concentration was approximately 50 mg/l (Figure 7.11). No formic acid accumulation was detected in cultures containing 2000 mg/l LAU or 3000 mg/l LAU 48 hours after glucose was injected. However, a rapid spike in formic acid production was detected between 48 to 96 hours. In cultures containing 3000 mg/l LAU and 2000 mg/l LAU, formic acid concentrations of 340 and 260 were attained at 96 hours, respectively. Cultures containing 1000 mg/l LAU showed very little formic acid accumulation. In these cultures, a minimum concentration of 110 mg/l was attained. In all cultures containing LCFA, but excluding those fed 1000 mg/l LAU, a reduction in formic acid concentration was observed from 144 to 192 hours. Cultures containing BES and no inhibitor exhibited two stages of formic acid accumulation (Figure 7.12). Formic acid accumulation was detected within 48 hours after feeding glucose; however, it was consumed over the next 48 hours. Roughly the same

amount of acetic acid accumulation was observed during the first and second glucose injection periods. However, formic acid consumption was considerably slower during the second glucose injection period. In cultures containing 50 mM BES, a high formic acid concentration of 270 mg/l was detected at 48 hours. Cultures containing 100 mM BES reached a maximum formic acid concentration of 240 mg/l after 48 hours.

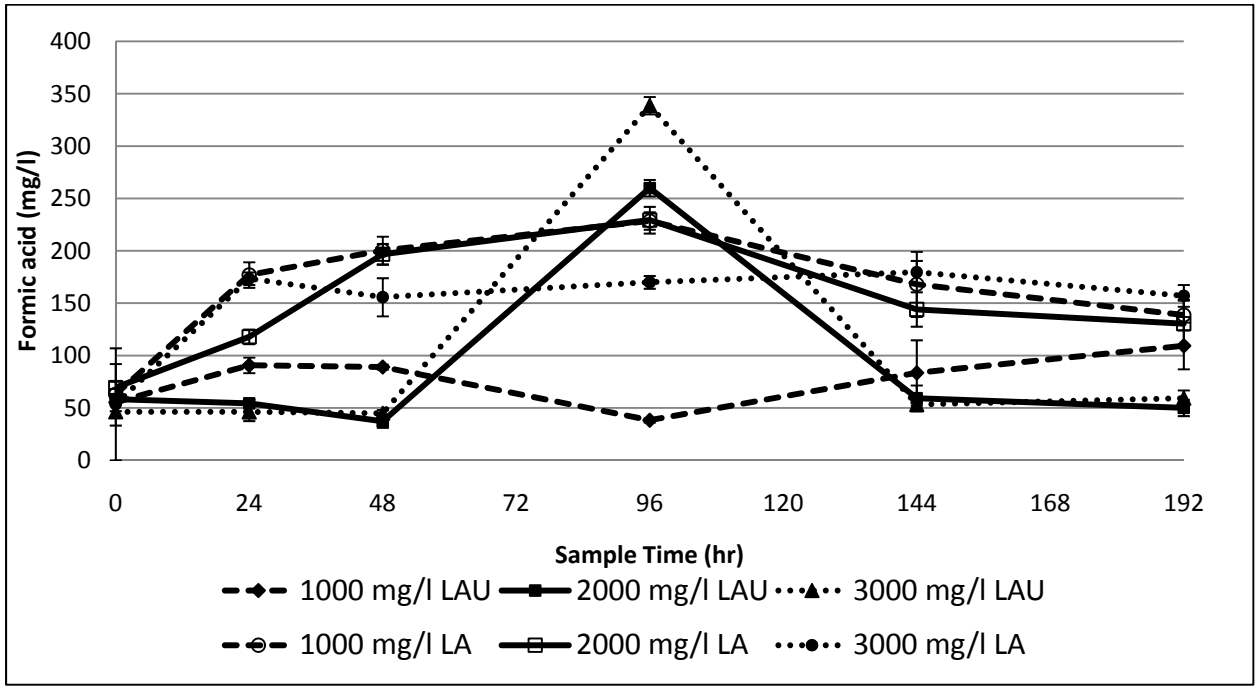


Figure 7.11: Formic acid production in cultures receiving varying concentrations of LAU and LA

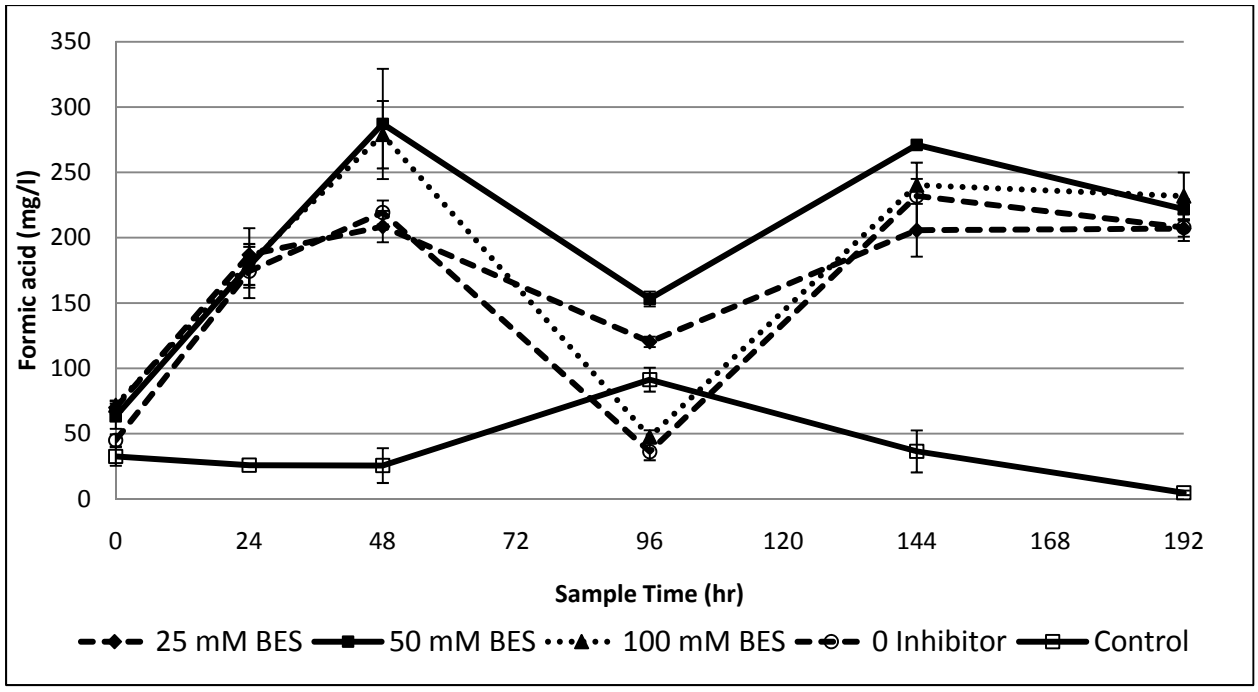


Figure 7.12: Formic acid production in cultures receiving varying concentrations of BES

In cultures containing BES and those containing no inhibitor, a significant amount of butyric acid accumulation was detected between 96 and 144 hours (Figure 7.14). Butyric acid concentrations of 300, 290, and 280 mg/l were detected in cultures containing 25, 50 and 100 mM BES at 144 hours, respectively. In cultures with no inhibitor, a maximum butyric acid concentration of 200 mg/l was attained at 144 hours. Butyric acid formation in cultures containing 2000 mg/l LA was rapid within 24 hours following the first glucose injection (Figure 7.13). The levels reached a maximum concentration of 500 mg/l after 24 hours and it then decreased to 140 mg/l after 48 hours.

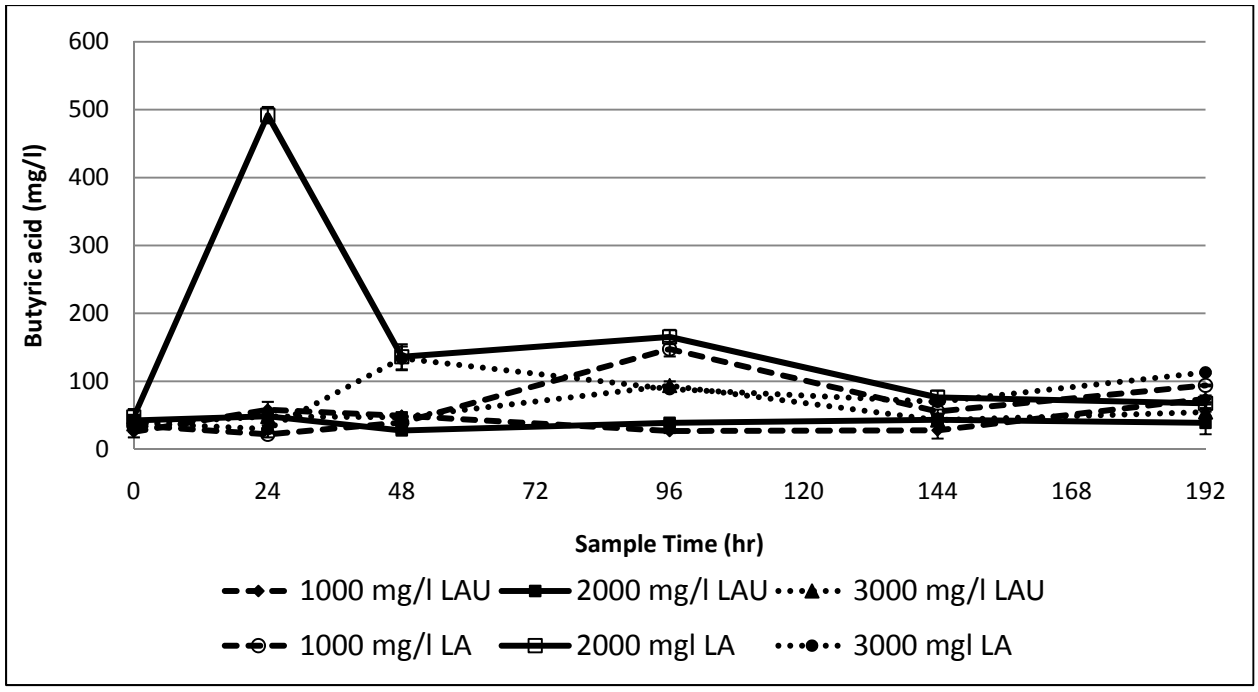


Figure 7.13: Butyric acid production in cultures receiving varying concentrations of LAU and LA

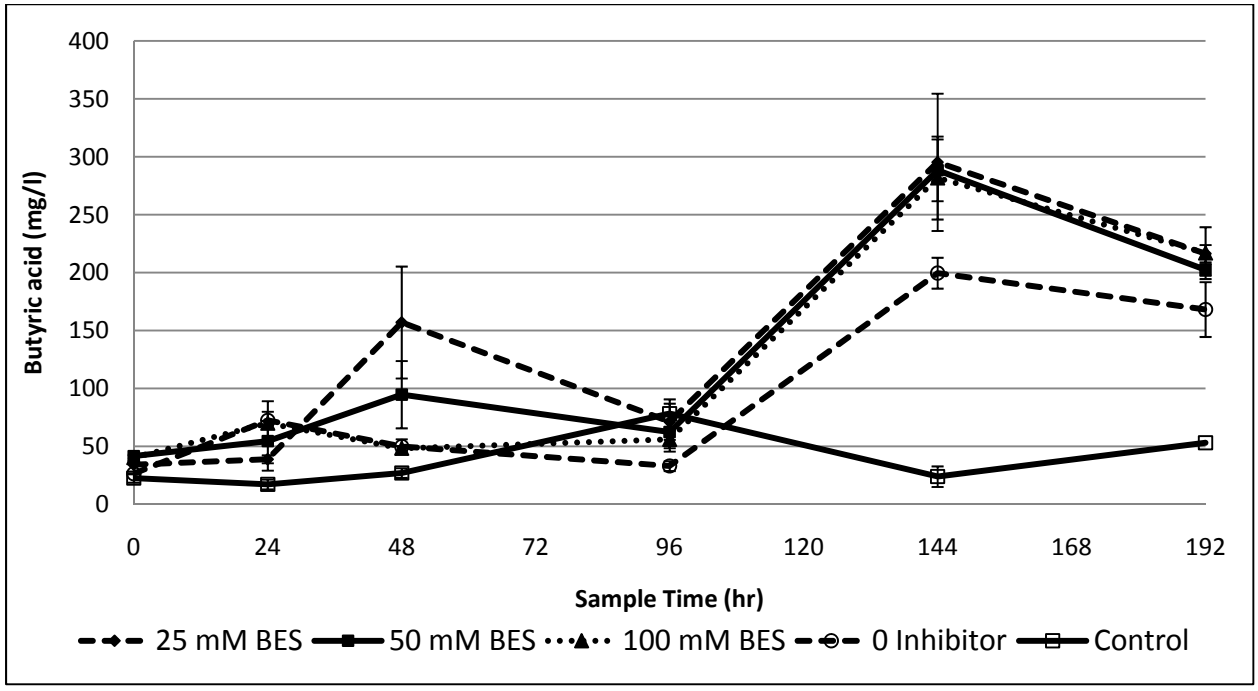


Figure 7.14: Butyric acid production in cultures receiving varying concentrations of BES

7.3 Alcohol Production

Iso-propanol and ethanol were the dominate alcohols produced in this phase of experimentation. In the cultures containing LCFAs, iso-propanol was produced at a relatively consistent rate throughout the 8-day experiment (Figure 7.15). Maximum iso-propanol production at 192 hours was observed in cultures fed LCFAs. Increased iso-propanol production of approximately 3450 mg/l was observed between time 144 and 192 hours in cultures containing 1000 mg/l LAU. In comparison, in cultures containing 3000 mg/l LAU, elevated levels of 1850 mg/l were observed. Iso-propanol levels were greater in cultures containing LAU than those containing LA. The iso-propanol production pattern was slightly different for cultures containing BES. Cultures containing BES observed a steady increase in iso-propanol production from time 0 to 96 hours (Figure 7.16). Between 96 to 144 hours, iso-propanol production was more rapid than in the time period between 0 and 96 hours. From 144 to 192 hours, deceasing iso-propanol levels were observed. Cultures containing no inhibitor exhibited a less pronounced iso-propanol production pattern. The maximum iso-propanol concentrations for cultures containing 25, 50 and 100 mM BES were 935, 1060 and 1180 mg/l at 144 hours, respectively. In cultures containing no inhibitor, a maximum iso-propanol concentration of 615 mg/l was detected at 144 hours.

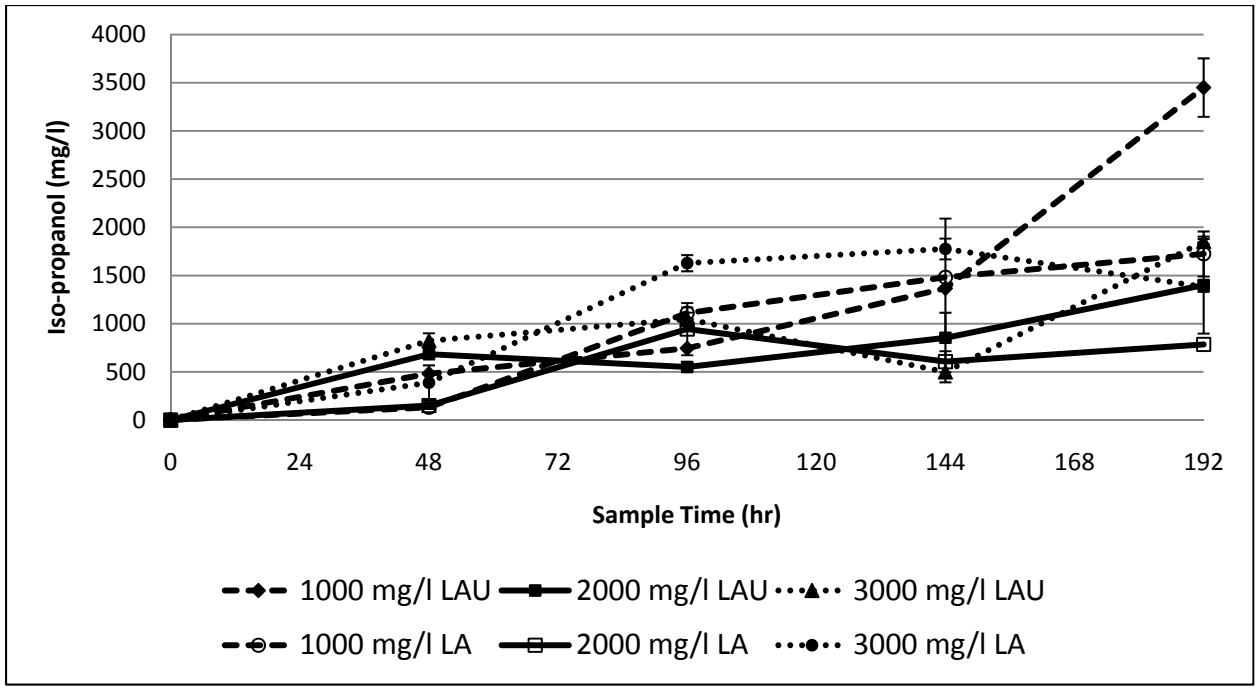


Figure 7.15: Iso-propanol production in cultures receiving varying concentrations of LAU and LA

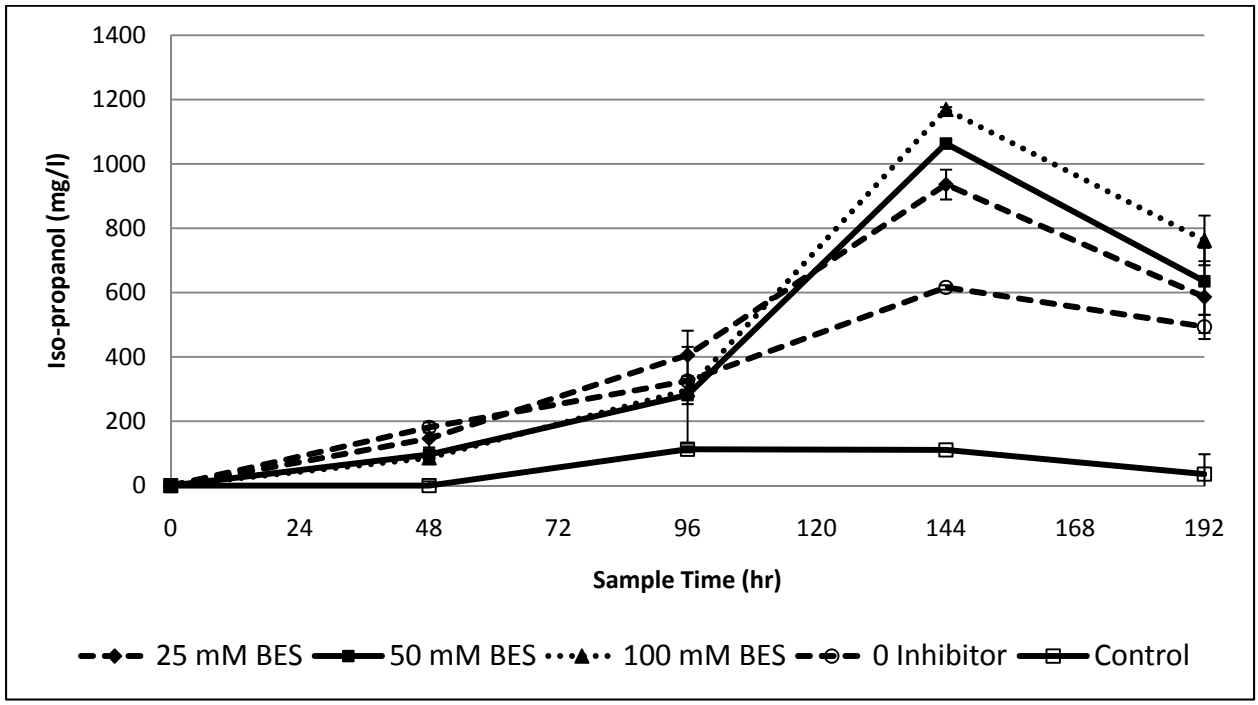


Figure 7.16: Iso-propanol production in cultures receiving varying concentrations of BES

Ethanol production was similar to the iso-propanol production pattern in cultures containing LCFAs. Ethanol concentrations levels increased steadily over the duration of the experiment; however, the ethanol production in most cultures subsided by 144 hours and the concentration remained fairly constant. However, in cultures containing 2000 mg/l LA, ethanol production reached a maximum concentration of 1600 mg/l at 192 hours (Figure 7.17). The ethanol production pattern in cultures containing 3000 mg/l LAU was variable and decreased from 48 to 96 hours and then increased rapidly from 96 to 144 hours. At 144 hours, a maximum concentration was attained and then a decreased was observed until 192 hours. In cultures containing LAU, more ethanol was produced in comparison to cultures containing LA. In cultures containing BES, ethanol production was detected only after 48 hours in a large portion of the cultures (Figure 7.18). In cultures with no inhibitor and 100 mM BES, extremely rapid ethanol production was observed from 48 to 144 hours. Maximum ethanol concentrations of 1530 and 1400 mg/l were observed at 144 hours in cultures containing no inhibitor and 100 mM BES, respectively. Ethanol concentration in these cultures decreased rapidly from 144 to 192 hours. The ethanol production profiles for cultures containing 25 and 50 mM BES were different from 48 to 192 hours. Ethanol production was observed at a fairly constant rate reaching a maximum at 192 hours. At 192 hours, ethanol production in cultures containing 25 and 50 mM BES reached maximum levels of 1100 and 1070 mg/l, respectively.

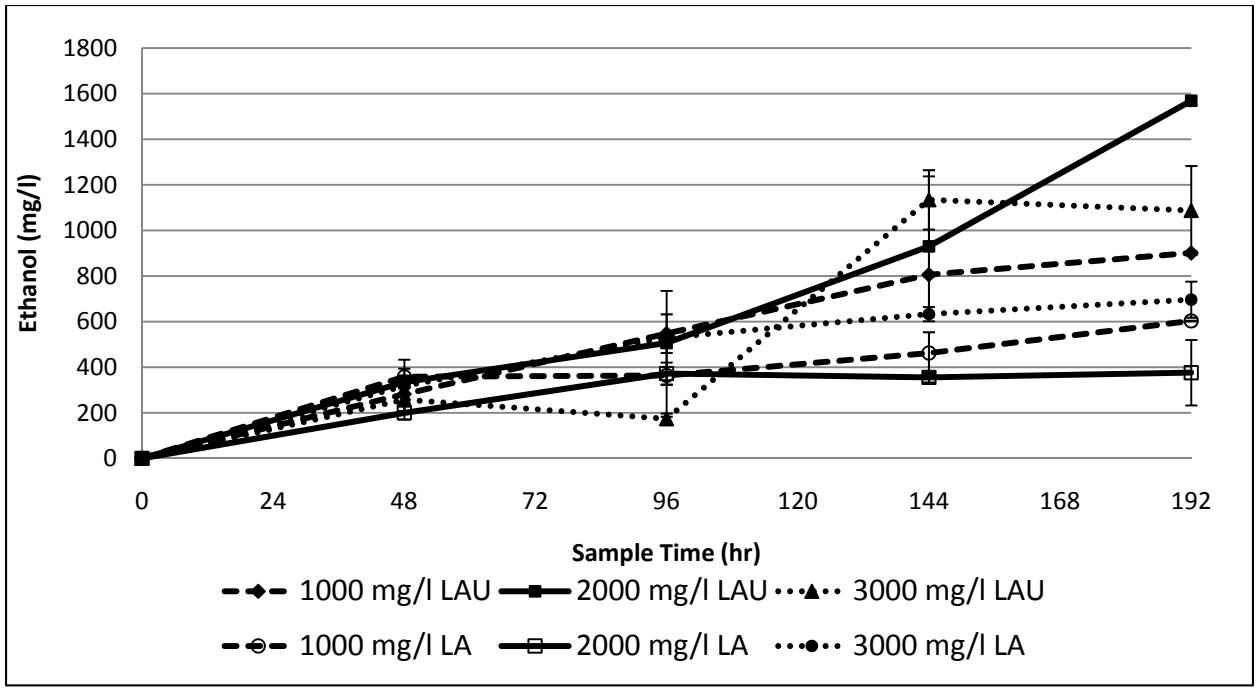


Figure 7.17: Ethanol production in cultures receiving varying concentrations of LAU and LA

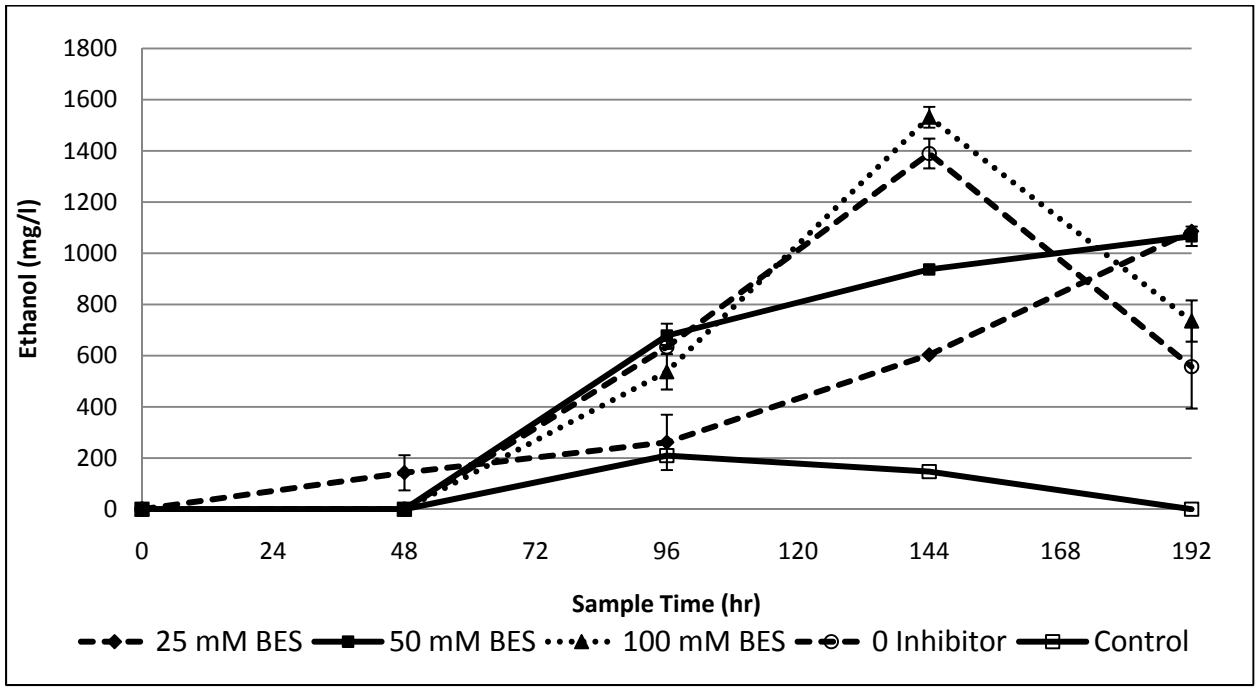


Figure 7.18: Ethanol production in cultures receiving varying concentrations of BES

Modest propanol production was observed towards the end of the experiment. The propanol levels detected were slightly above the lower detection limit for alcohols. Most of the propanol production occurred between 48 and 96 hours. The highest concentration of propanol measured was 310 g/l at 144 hours in cultures containing 100 mM BES (Figure 7.20). Very low levels of iso-butanol and butanol were detected throughout the experiment and all the levels were below the lower detection limits.

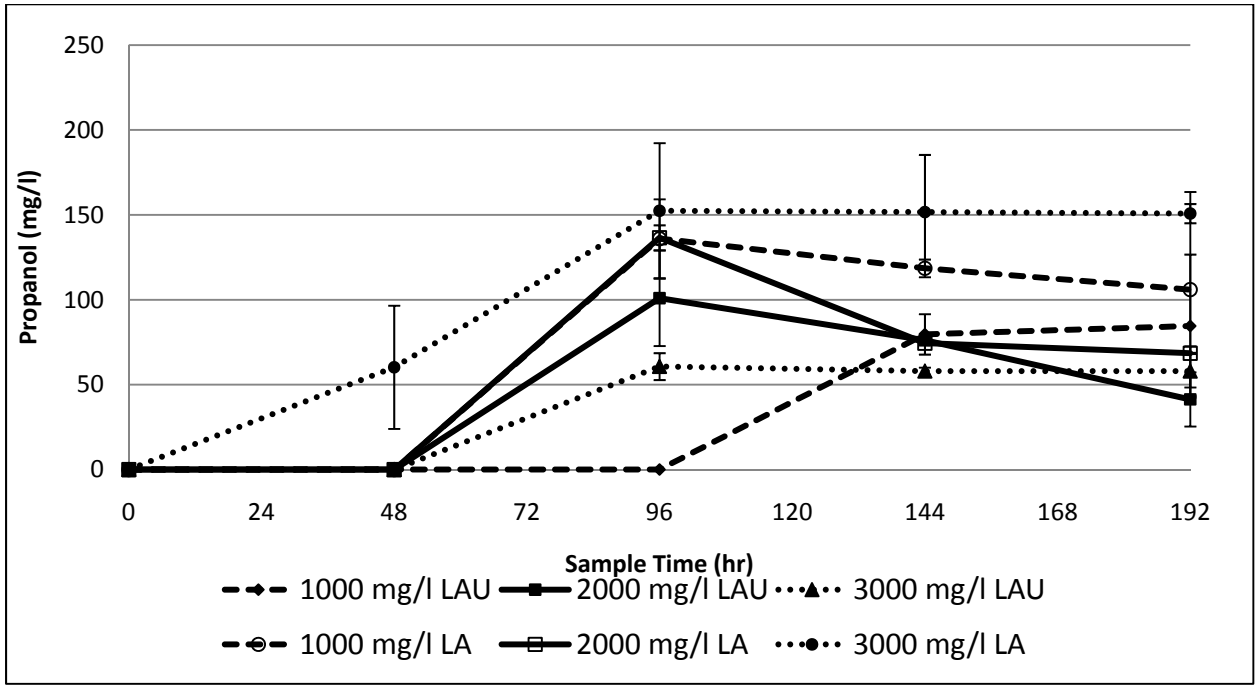


Figure 7.19: Propanol production in cultures receiving varying concentrations of LAU and LA

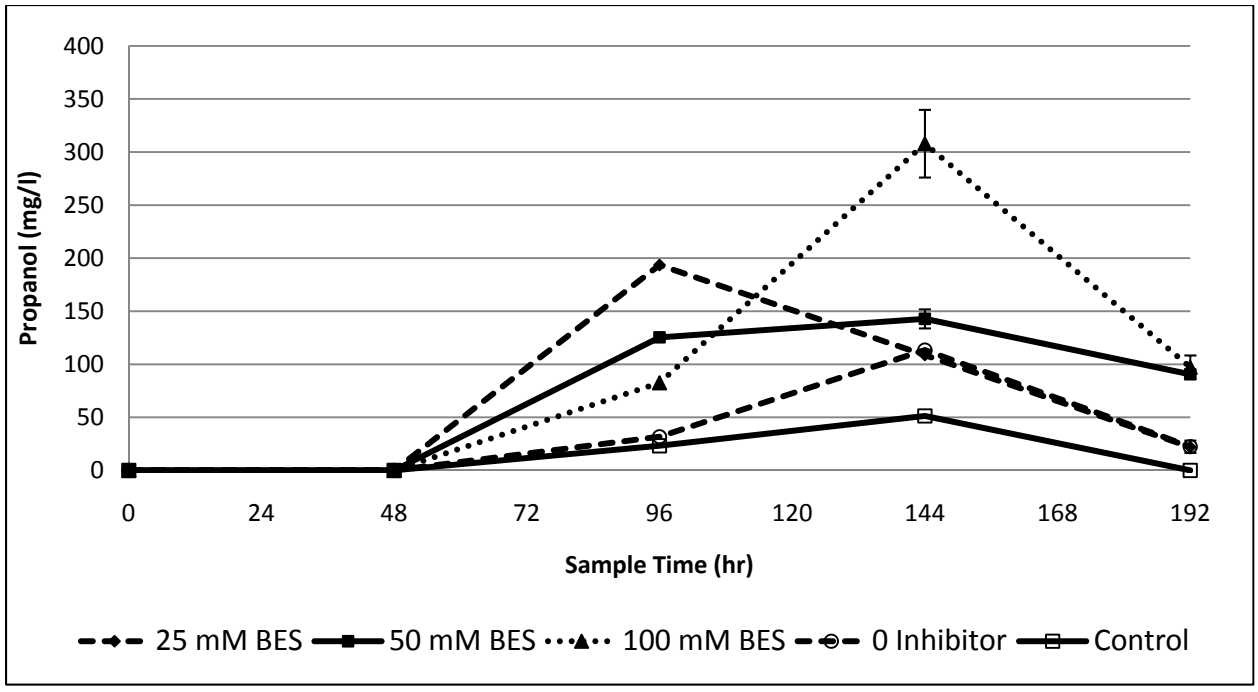


Figure 7.20: Propanol production in cultures receiving varying concentrations of BES

7.4 Glucose Degradation

The glucose degradation data show that high concentrations of LCFA caused a reduction in the glucose degradation rate. Residual glucose levels remained after 96 hours in cultures containing 3000 mg/l LAU and 3000 mg/l LA (Figure 7.21). This is in contrast to controls in which glucose removal is accomplished within 12 hours. After 96 hours, the residual glucose level in cultures containing 3000 mg/l LA was approximately 1000 mg/l. The presence of LA was more detrimental to glucose degradation than LAU. LA is larger molecule than LAU and it has two carbon-carbon double bonds. The inhibitory factor responsible for the reduction of glucose degradation is unclear; however, based on past reports, the increased inhibition can be attributed to the increasing number of unsaturated carbon double bonds. Glucose removal was rapid in cultures containing 1000 mg/l LAU and 2000 mg/l LAU and the glucose levels were undetectable after 48 hours.

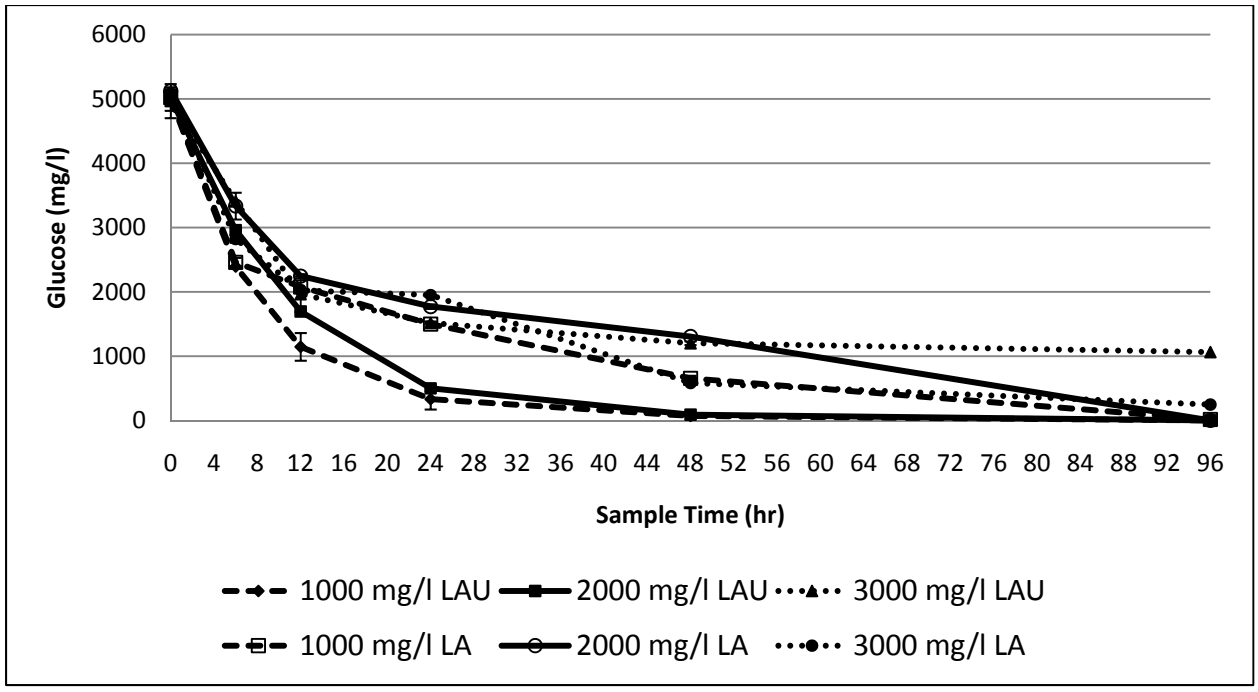


Figure 7.21: Glucose degradation in cultures receiving varying concentrations of LAU and LA

The presence of BES was extremely influential in reducing the glucose degradation rate. In cultures containing BES, the glucose degradation rate after the first glucose injection period was approximately the same. Cultures containing 100 mM BES had the highest residual concentration because of a slower glucose consumption rate. Cultures containing BES had between 1550 to 1590 mg/l glucose remaining at 96 hours (Figure 7.22). The low quantities of gas produced indicate that all the glucose was not completely removed. However, during the second glucose injection period, larger quantities of gas were produced in cultures containing BES. This indicates that all the glucose was likely degraded. As expected, rapid glucose degradation was observed in cultures containing no inhibitor. Only 500 mg/l of glucose remained after 24 hours and no glucose was detected after 48 hours.

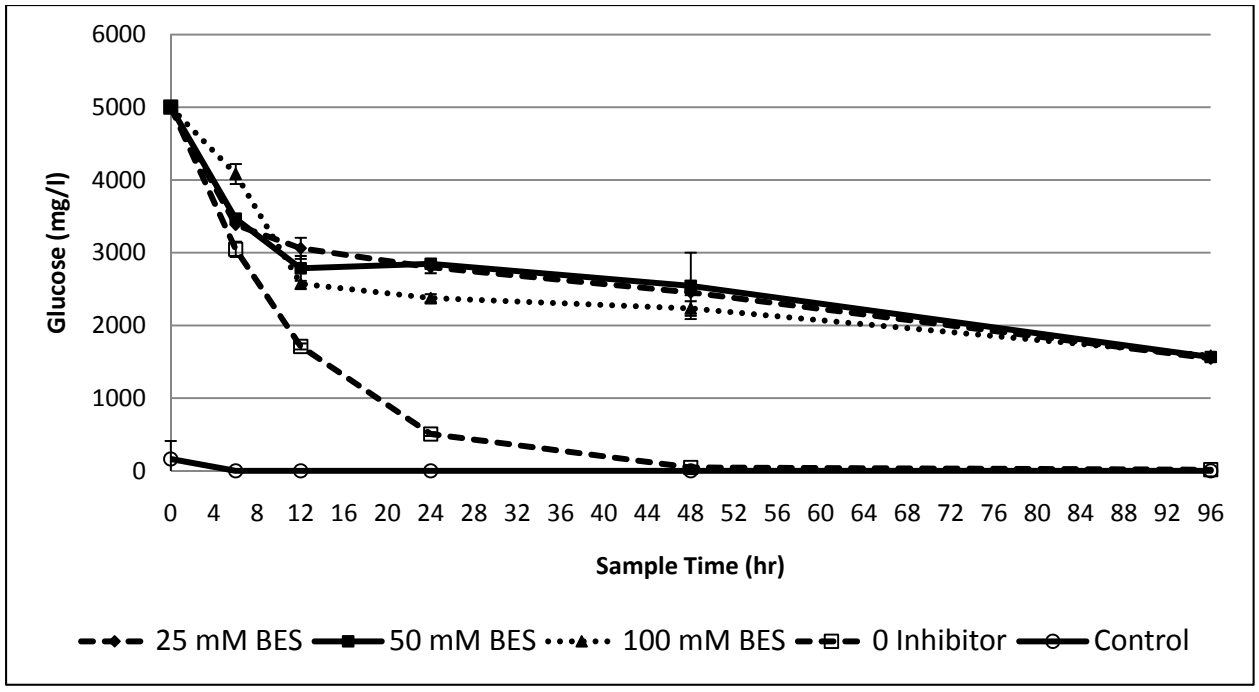


Figure 7.22: Glucose degradation in cultures receiving varying concentrations of BES

7.5 pH Profile

The pH profiles in cultures over the duration of the experiment are shown in Figures 7.23 and 7.24. In all the cultures, the initial pH was set at 5.5 with no pH buffer capacity. After 96 hours, the cultures were opened and the pH recorded. The pH was re-adjusted to 5.5 prior to the second glucose injection. The pH was again recorded at the end of the experiment (192 hours). All cultures containing BES had the greatest pH decrease after both glucose injection periods. The pH drop was greatest in cultures containing 100 mM BES. As expected, the cultures containing 25 mM and 50 mM BES also showed a large pH decrease over the duration of the study. For cultures containing BES, the pH decrease was slightly greater during the first glucose injection. All cultures containing BES, along with the cultures containing no inhibitor, the pH decreased from 5.5 at time 0 to between 4.2 and 4.0 at 96 hours. In comparison, only in cultures containing 1000 mg/l LA did the pH decreased to below 5 at time 96 hours. In all cultures, except those containing 3000 mg/l LAU, the pH decreased to between 5.2 and 5.0. In cultures containing

3000 mg/l LAU, the smallest pH decrease was observed. The decrease was observed from to pH 5.5 to 5.42 and 5.48 from 0 to 96 hours and 96 to 192 hours, respectively. Excluding the cultures containing 2000 mg/l LAU and 3000 mg/l LAU, the cultures containing LCFAs observed a greater pH decrease during the second glucose injection period. Cultures containing 1000 mg/l LA showed the greatest decrease from pH 5.5 at 96 hours to 4.7 at 192 hours. It is clear that the cultures containing LAU have much smaller pH decrease than those containing LA. The LAU stock solution contains more NaOH than LA and this could be a reason for the smaller pH decrease in cultures containing LAU. Moreover, the cultures containing BES have greater pH decrease than both LA and LAU.

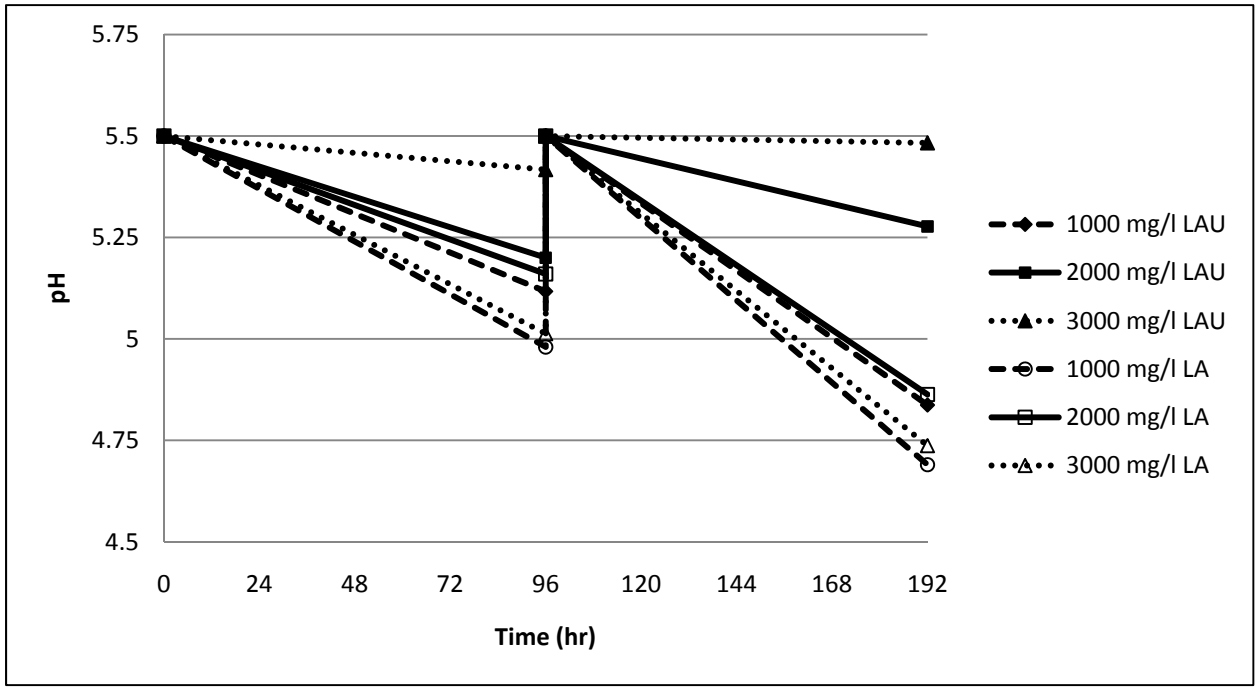


Figure 7.23: pH profile over the duration of 8-day experiment (Cultures 1-18)

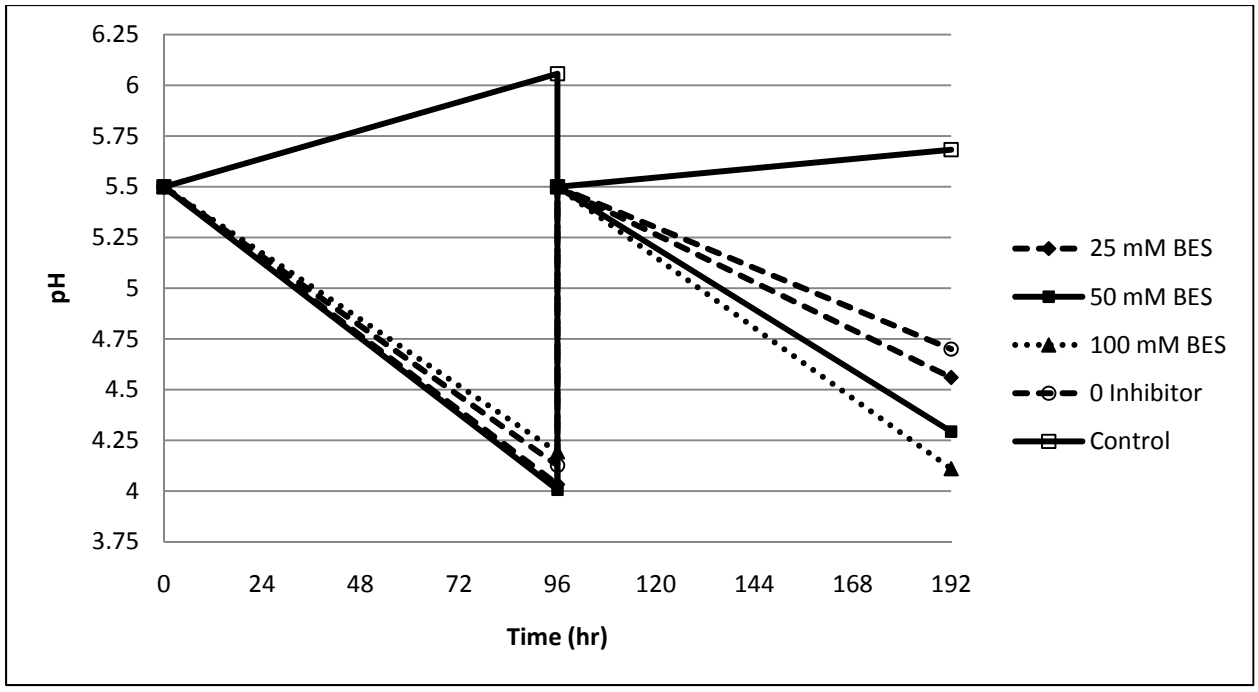


Figure 7.24: pH profile over the duration of 8-day experiment (Cultures 19-33)

7.6 Reactions Based on Electron Balance

Chemical equations are shown below for varying LA, LAU and BES levels and sampling periods. Notice in each chemical equation, biomass ($Y_{biomass/glucose}$) and H_2 ($Y_{H2/glucose}$) yields can be derived. For example, in the case of 1000 mg/l LAU at 96 hours the values for $Y_{biomass/glucose}$ and $Y_{H2/glucose}$ are 0.1199 mol/mol and 2.68 mol/mol, respectively. Notice the experimental H_2 yield in this case is 1.75 mol/mol.

1000 mg/l LAU at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.13875H_2O \rightarrow 0.11159H_2 + 0.005C_5H_7O_2N + 0.02849CH_3COH_2CH_3 + 0.01367CH_3CH_2OH + 0.11220HCO_3^- + 0.11720H^+$

1000 mg/l LAU at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.051809H_2O \rightarrow 0.02891H_2 + 0.005C_5H_7O_2N + 0.000565CH_3COO^- + 0.011588CH_3CH_2COO^- + 0.001189HCOO^- + 0.00041CH_3CH_2CH_2COO^- + 0.033945CH_3COH_2CH_3 + 0.002896CH_3CH_2OH + 0.00106CH_3CH_2CH_2OH + 0.075467HCO_3^- + 0.09422H^+$

2000 mg/l LAU at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.09120H_2O \rightarrow 0.082272H_2 + 0.005C_5H_7O_2N + 0.00667CH_3COO^- + 0.020471CH_3CH_2COO^- + 0.006562HCOO^- + 0.013415CH_3COH_2CH_3 + 0.008053CH_3CH_2OH + 0.002462CH_3CH_2CH_2OH + 0.079958HCO_3^- + 0.11866H^+$

2000 mg/l LAU at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.076613H_2O \rightarrow 0.037745H_2 + 0.005C_5H_7O_2N + 0.029682CH_3COH_2CH_3 + 0.024185CH_3CH_2OH + 0.087582HCO_3^- + 0.092582H^+$

3000 mg/l LAU at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.080312H_2O \rightarrow 0.070998H_2 + 0.005C_5H_7O_2N + 0.01368CH_3COO^- + 0.013813CH_3CH_2COO^- + 0.007776HCOO^- + 0.000775CH_3CH_2CH_2COO^- + 0.020853CH_3COH_2CH_3 + 0.002251CH_3CH_2OH + 0.001209CH_3CH_2CH_2OH + 0.074637HCO_3^- + 0.11568H^+$

3000 mg/l LAU at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.071554H_2O \rightarrow 0.03250H_2 + 0.005C_5H_7O_2N + 0.031062CH_3COH_2CH_3 + 0.02299CH_3CH_2OH + 0.085834HCO_3^- + 0.090834H^+$

1000 mg/l LA at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.04691H_2O \rightarrow 0.030934H_2 + 0.005C_5H_7O_2N + 0.007084CH_3COO^- + 0.015142CH_3CH_2COO^- + 0.004613HCOO^- + 0.001621CH_3CH_2CH_2COO^- + 0.023181CH_3COH_2CH_3 + 0.004955CH_3CH_2OH + 0.00284CH_3CH_2CH_2OH + 0.06634HCO_3^- + 0.099798H^+$

1000 mg/l LA at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.15278H_2O \rightarrow 0.12960H_2 + 0.005C_5H_7O_2N + 0.030441CH_3COH_2CH_3 + 0.007738CH_3CH_2OH + 0.118201HCO_3^- + 0.123201H^+$

2000 mg/l LA at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.04682H_2O \rightarrow 0.0468204H_2 + 0.00002CH_4 + 0.005C_5H_7O_2N + 0.005278CH_3COO^- + 0.015867CH_3CH_2COO^- + 0.004736HCOO^- + 0.001805CH_3CH_2CH_2COO^- + 0.020949CH_3COH_2CH_3 + 0.005386CH_3CH_2OH + 0.00303CH_3CH_2CH_2OH + 0.072156HCO_3^- + 0.104842H^+$

2000 mg/l LA at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.432313H_2O \rightarrow 0.44678H_2 + 0.005C_5H_7O_2N + 0.223925HCO_3^- + 0.228925H^+$

3000 mg/l LA at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.073988H_2O \rightarrow 0.05467H_2 + 0.000028CH_4 + 0.005C_5H_7O_2N +$
$+ 0.013609CH_3CH_2COO^- + 0.002508HCOO^- + 0.000554CH_3CH_2CH_2COO^- +$
$0.026263CH_3COH_2CH_3 + 0.005568CH_3CH_2OH + 0.002459CH_3CH_2CH_2OH + 0.08212HCO_3^- +$
$0.103789H^+$

3000 mg/l LA at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.363996H_2O \rightarrow 0.371447H_2 + 0.005C_5H_7O_2N +$
$0.003083CH_3CH_2COO^- + 0.001168CH_3CH_2CH_2COO^- + 0.007548CH_3CH_2OH + 0.19598HCO_3^-$
$+ 0.20523H^+$

25 mM BES at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.123428H_2O \rightarrow 0.102046H_2 + 0.001073CH_4 + 0.005C_5H_7O_2N$
$+ 0.00493125CH_3CH_2COO^- + 0.003086HCOO^- + 0.001174CH_3CH_2CH_2COO^- +$
$0.0186095CH_3COH_2CH_3 + 0.00785CH_3CH_2OH + 0.008879CH_3CH_2CH_2OH + 0.103245HCO_3^- +$
$0.117436H^+$

25 mM BES at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.11602H_2O \rightarrow 0.116985H_2 + CH_4 + 0.005C_5H_7O_2N +$
$CH_3CHOHCOO^- + 0.00371CH_3COO^- + 0.0292580CH_3CH_2COO^- + 0.003022HCOO^- +$
$0.002614CH_3CH_2CH_2COO^- + CH_3COH_2CH_3 + 0.014035CH_3CH_2OH + CH_3CH_2CH_2OH +$
$CH_3CH_2CHOHCH_3 + CH_3CH_2CH_2CH_2OH + 0.088259HCO_3^- + 0.131863H^+$

50 mM BES at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.126563H_2O \rightarrow 0.099522H_2 + 0.000556CH_4 + 0.005C_5H_7O_2N$
$+ 0.0038382CH_3CH_2COO^- + 0.005863HCOO^- + 0.0007CH_3CH_2CH_2COO^- +$
$0.0137457CH_3COH_2CH_3 + 0.021614CH_3CH_2OH + 0.006126CH_3CH_2CH_2OH + 0.101425HCO_3^-$
$+ 0.11683H^+$

50 mM BES at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.108174H_2O \rightarrow 0.115610H_2 + 0.000023CH_4 + 0.005C_5H_7O_2N$
$+ 0.02037CH_3COO^- + 0.025149CH_3CH_2COO^- + 0.002731HCOO^- + 0.00288CH_3CH_2CH_2COO^-$
$+ 0.007541CH_3CH_2OH + 0.079458HCO_3^- + 0.135588H^+$

100 mM BES at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.064101H_2O \rightarrow 0.033189H_2 + 0.000152CH_4 + 0.005C_5H_7O_2N$
$+ 0.0090577CH_3CH_2COO^- + 0.000682CH_3CH_2CH_2COO^- + 0.018630CH_3COH_2CH_3 +$
$0.021955CH_3CH_2OH + 0.005176CH_3CH_2CH_2OH + 0.07962HCO_3^- + 0.09436H^+$

100 mM BES at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.12313H_2O \rightarrow 0.134425H_2 + 0.005C_5H_7O_2N + 0.024655CH_3COO^- + 0.02227CH_3CH_2COO^- + 0.007045HCOO^- + 0.003178CH_3CH_2CH_2COO^- + 0.003705CH_3CH_2OH + 0.081709HCO_3^- + 0.14386H^+$

No Inhibitor at 96 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.098482H_2O \rightarrow 0.08021H_2 + 0.003913CH_4 + 0.005C_5H_7O_2N + 0.019346CH_3CH_2COO^- + 0.000175CH_3CH_2CH_2COO^- + 0.012392CH_3COH_2CH_3 + 0.01576CH_3CH_2OH + 0.001209CH_3CH_2CH_2OH + 0.090026HCO_3^- + 0.114548H^+$

No Inhibitor at 192 hours

$0.04167C_6H_{12}O_6 + 0.05NH_4^+ + 0.14879H_2O \rightarrow 0.15809H_2 + 0.000015CH_4 + 0.005C_5H_7O_2N + 0.008422CH_3COO^- + 0.023975CH_3CH_2COO^- + 0.007729HCOO^- + 0.003143CH_3CH_2CH_2COO^- + 0.005686CH_3COH_2CH_3 + 0.098855HCO_3^- + 0.147125H^+$

7.7 Discussion of Results

The cultures containing BES did not completely degrade the initial 5000 mg/l glucose which was injected at time 0. These culture sets observed very little gas production prior to time 48 hours. Based on Figure 7.2, the H_2 concentrations had not peaked at time 96. Glucose consumption was more rapid in cultures containing LCFAs. The majority of cultures contained only trace amounts of glucose at 96 hours and some observed none at all. The culture sets containing 1000 mg/l LAU, 2000 mg/l LA and 3000 mg/l LA showed increased H_2 accumulation from 72 to 96 hours. These concentrations at 96 hours may represent peak values; however, it is not possible to determine without continuing gas monitoring for another 24 hours. Taking these limitations into account, some comparisons can be made between LCFA and BES data samples at 96 hours (i.e. the end of the first glucose injection period). H_2 yields for all culture sets at 96 hours are shown in Table 7.4.

Table 7.4: Maximum H_2 yields for different inhibitors during the first glucose injection period

Inhibition Conditions	Yield at t=96 hrs (mol H_2/mol glucose)
1000 mg/l LAU	1.75
2000 mg/l LAU	2.02
3000 mg/l LAU	2.14
1000 mg/l LA	0.89
2000 mg/l LA	1.26
3000 mg/l LA	2.03
25 mM BES	1.33
50 mM BES	1.22
100 mM BES	0.32

A plot of H_2 yield (at time 96 hours) versus inhibitor concentration for both LAU and LA cultures is shown in Figure 7.25. Using the MS Word trendline function, an equation was generated relating H_2 yield (y-axis) and inhibitor concentration (x-axis). The respective yields of 25, 50 and 100 mM BES cultures at 96 hours can be inserted into these equations to determine the expected H_2 yield at a specific LCFA concentration.

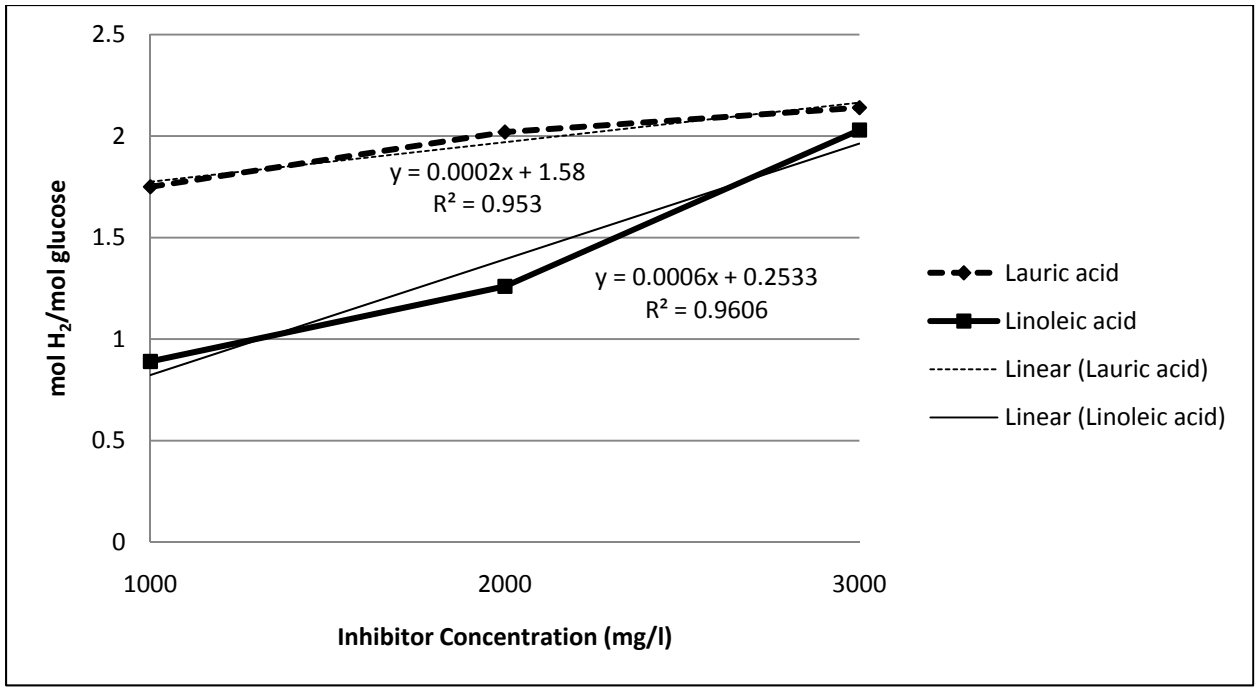


Figure 7.25: H_2 yield vs. LCFA inhibitor concentration (1st glucose injection period)

LAU: $x\,(\frac{mg}{l}) = \frac{y\,(\frac{mol \cdot H_2}{mol \cdot glucose}) - 1.58}{0.0002}$

LA: $x\,(\frac{mg}{l}) = \frac{y\,(\frac{mol \cdot H_2}{mol \cdot glucose}) - 0.2533}{0.0006}$

No comparison was made between LAU and BES cultures since no cultures containing BES had yields within the range of cultures containing LAU. The following equivalencies can be made between LA and BES: 25 mM BES = 1794.5 mg/l LA; 50 mM BES = 1611.2 mg/l LA; and, 100 mM BES = 111.2 mg/l LA.

The cultures containing BES produced substantially more H_2 during the second glucose injection period when compared to the first. Hydrogen was produced immediately after injecting glucose. All cultures, except the cultures containing 3000 mg/l LA, produced less H_2. The respective maximum yields for 25, 50 and 100 mM BES cultures were 2.85, 2.58 and 3.24 mol H_2/mol glucose at 144 hours. Whereas, the maximum yield for the cultures containing 3000 mg/l LA

was 3.22 mol H_2/mol glucose at 168 hours. From an analysis of the H_2 yields, 100 mM BES and 3000 mg/l LA are equivalent based on the equivalent amount of H_2 produced under the two inhibitor conditions. The cultures containing LAU did not produce enough H_2 during the second glucose injection period for a proper comparison with BES is prohibited.

An analysis such as the one made during the first glucose injection period can also be made for the second glucose injection. The maximum H_2 yields for each culture set during the second glucose injection period are shown in Table 7.5.

Table 7.5: Maximum H_2 yields for different inhibitors culture during the 2nd glucose injection period

Inhibition Conditions	Max yield 2nd Glucose Injection (mol H_2/mol glucose)
1000 mg/l LAU	1.38
2000 mg/l LAU	0.65
3000 mg/l LAU	0.51
1000 mg/l LA	1.57
2000 mg/l LA	1.34
3000 mg/l LA	3.22
25 mM BES	2.85
50 mM BES	2.58
100 mM BES	3.24

A plot of maximum H_2 yield (2nd glucose injection) versus inhibitor concentration for both LAU and LA is shown in Figure 7.24.

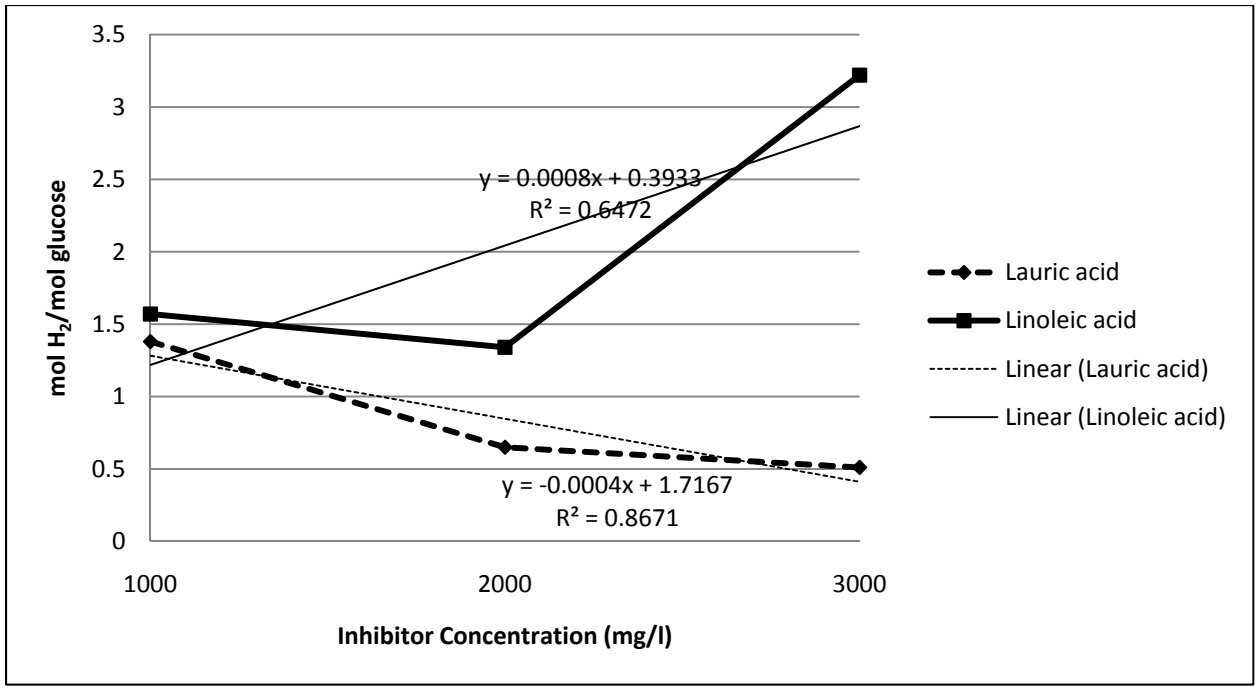


Figure 7.26: H_2 yield vs. LCFA inhibitor concentration (2nd glucose injection period)

LAU: $x\,(\frac{mg}{l}) = \frac{y\,(\frac{mol \cdot H_2}{mol \cdot glucose}) - 1.7167}{-0.0004}$

LA: $x\,(\frac{mg}{l}) = \frac{y\,(\frac{mol \cdot H_2}{mol \cdot glucose}) - 0.3933}{0.0008}$

No comparison can be made between LAU and BES cultures since none of the cultures containing LAU had H_2 yields within the range of cultures containing BES. The following equivalencies can be drawn between LA and BES: 25 mM BES = 3070 mg/l LA; 50 mM BES = 2730 mg/l LA; and, 100 mM BES = 3560 mg/l LA.

A comparison between LCFAs and BES in terms of methane reduction is not possible using the experimental data. BES, LAU and LA were proven to methanogenic inhibitors. The cultures containing LA and LAU showed no methane production over the entire 8-day experiment. Maximum methane concentrations in cultures containing BES were 23, 10 and 2 μmol/bottle for 25, 50 and 100 mM BES, respectively. In the culture set with no inhibitors, a maximum methane

concentration of 88.5 μmol/bottle was observed during the first glucose injection period. This indicates that a 100% reduction in methane production was observed in all cultures containing LCFA. In contrast, the culture sets containing 25, 50 and 100 mM BES observed 74%, 82%, and 98% methane reduction, respectively. Moreover, no methane was produced in the cultures containing no inhibitor during the second glucose injection period. This suggests the dependency of methane production on pH. The pH of 5.5, at which all cultures were initially set, was a more effective inhibitor than BES and LCFAs.

Chapter 8: Discussion of Results

Comparing BES, a synthetic inhibitor, to LCFAs, organic inhibitors, was the primary objective of this research work. BES and LCFAs have different modes of microbial inhibition. LCFAs are classified as 'non-specific' methanogenic inhibitors. LCFAs do not inhibit only methanogenic bacteria, even acidogens and acetogenic H_2 producing bacteria can be affected by their presence. Alternatively, BES is a classified as a 'specific' methanogenic inhibitor. The presence of BES, in theory, only disrupts the last stages of methane formation. 'In theory' is noted in the last sentence because it was observed that excess amounts of BES can be detrimental to H_2 formation. This was observed in both phase II and IV during the second glucose injection. In phase II, cultures with no BES were the strongest H_2 producers. These cultures reached a maximum yield of 3.32 mol H_2/mol glucose at 168 hours. In comparison, the highest yield in cultures containing 25 mM BES was 2.68 mol H_2/mol glucose at 72 hours. No methane was produced during the second glucose injection period, even in cultures without BES. This could have been due to the pH in the fermentation medium being less than 5.5. Essentially, the pH adjustment to low values is an excellent mode of inhibition. Based on the results of phase II, the presence of BES during the second glucose injection period was not needed. In phase IV, similar observations were noted. The results from this phase show that cultures containing no inhibitor produce more H_2 than cultures containing 25 and 50 mM BES. However, cultures fed 100 mM BES produced the most H_2 in this phase. With the current high cost of BES, it is difficult to justify its use considering how effective low pH was on methanogen inhibition.

The results from these experiments can be improved. All culture bottles were run in triplicates to account for any variability. However, there was no control at the mother reactor. Having triplicate mother reactors would provide an extra level of control. All three reactors would have

to be constructed at the same time, made up of the same culture sources and have identical maintenance schedules. Unfortunately, the resources required for the additional reactors were not available. The time required to maintain two additional reactors was also a detrimental factor. With three reactors used in experimental culture preparation, the results could be compared to changes in the microbial community in the mother reactor over time.

Variation in the results between separate experimental phases was striking. Population changes in the microbial culture over time played is a major factor responsible for variations in the experimental results. Many of the culture sets that were prepared in phase II were again prepared and analyzed in phase IV. Cultures containing BES in phase II showed no improvements in H_2 production during the second glucose injection period. However, in phase IV, the cultures containing BES inhibition, showed a remarkable increase in H_2 production during the second glucose injection when compared to the first injection. Theoretically, H_2 production should be greater during the second glucose injection period; especially considering that BES is a specific inhibitor. All H_2 producing bacteria should remain unaffected by the BES and be present in large quantities prior to the second glucose feeding. An issue with batch reactors is that the liquid products of the first glucose feeding period from 0 to 96 hours remain in the reactor culture. VFAs make up a great amount of the liquid byproducts. The presence of VFAs in high quantities can inhibit H_2 production. This is likely a factor reducing the H_2 yield during the second glucose injection period in Phase II. Continuous reactors do not have this disadvantage since the products (i.e. VFAs) are continually removed in the effluent.

In phase III, LA inhibition was demonstrated as a superior inhibition method to BES inhibition during the second glucose injection period from 96 to 192 hours. Hydrogen production did not significantly increase during the second glucose injection period in cultures containing BES. In

fact, H_2 production decreases in cultures containing 25 mM BES + 25 mM BES during the second glucose injection. This greatly contrasts the results of phase IV where all cultures containing BES showed increases in H_2 production during the second glucose injection period. Large scale anaerobic production facilities may wish to use inhibitors to direct the metabolic production of H_2 instead of methane. This work is a contribution to future work directed at improving H_2 production strategies using anaerobic mixed cultures. An improved H_2 yield during the second glucose injection is clearly an advantage when selecting inhibitors for large scale applications.

In phase IV, H_2 production in cultures fed LCFAs were contradictory. During the second glucose injection period, cultures containing 3000 mg/l LA were by far the best culture with H_2 producers. This result was unexpected considering cultures containing 1000 mg/l LA produced more H_2 than cultures containing 2000 mg/l LA during the second glucose injection period. Moreover, during the second glucose injection, cultures containing LAU produced H_2 in an inversely proportional relationship to LAU concentration (i.e. 1000 mg/l > LAU 2000 mg/l > LAU 3000 mg/l LAU). With the understanding that LCFAs are non-specific inhibitors, it appeared that their presence in large quantities had a negative effect on H_2 producing bacteria. This may account for a decrease in H_2 production with increasing LCFA concentration. Note this reasoning is not applicable to cultures containing 3000 mg/l LA. In the first glucose injection period, cultures with the higher concentration of LCFA produced the most H_2. In terms of H_2 yields, cultures fed LAU out produced those fed with LA. Theoretically, these results are logical for the first glucose injection. The greater the LCFA concentration resulted in decreasing methanogenesis. Note LCFA did not impair the activities of H_2 producing bacteria as is did for methanogens.

The maximum yield for cultures containing 3000 mg/l LA was 3.22 mol H_2/mol glucose at 168 hours. Moreover, the maximum H_2 yield was 3.24 mol H_2/mol glucose for cultures containing 100 mM BES at 144 hours. Consequently, 100 mM BES and 3000 mg/l LA were equivalent H_2 producers during the second glucose injection period. A comparison, based on H_2 production, was conducted using a correlation between H_2 yield and the inhibitor concentration. Unfortunately, no comparisons could be made between LAU and BES. In the first glucose injection period, H_2 production in cultures containing LAU was over the range of H_2 yields observed for cultures containing BES. The reverse was the case during the second glucose injection period. Hydrogen yields for BES and LA were compared and the following equivalences were established for the first glucose injection:

- 25 mM BES = 1800 mg/l LA;
- 50 mM BES = 1600 mg/l LA; and,
- 100 mM BES = 110 mg/l LA.

In the second glucose injection, the following equivalencies were established:

- 25 mM BES = 3070 mg/l LA;
- 50 mM BES = 2700 mg/l LA; and,
- 100 mM BES = 3560 mg/l LA.

One aspect that was consistent between experiments was the difference in how BES and LCFA initially produce H_2 during the first glucose injection period. In phase II and III, cultures containing BES produce small amounts of H_2 within 24 hours of glucose injection; however, from 24 to 72 hours, H_2 accumulated rapidly. In phase IV, the lag prior to H_2 accumulation was even longer. Hydrogen production was not initiated until 72 hours after glucose was injected.

Cultures containing BES exhibited an initial H_2 production lag during the first glucose injection. The glucose degradation rate was slow in cultures containing BES. Cultures fed LCFA behaved much differently during the first glucose injection. Notice in the presence of LCFAs, a long lag was not observed and H_2 accumulated quickly within 24 hours after glucose addition. Methane formation in cultures fed LCFAs and BES was compared when glucose was added during the first injection. No comparison was conducted for the second injection period because of the low methane yields. No methane was produced in phase IV in cultures fed LCFAs; however, methane was produced in phase III in cultures containing LA. Methane concentrations reached peak levels 12 hours after feeding glucose in a majority of cultures containing an inhibitor. The methane concentrations peaked at 48 hours in cultures containing no inhibitor. A summary of the methane yields at 12 and 48 hours for cultures containing the various inhibitors is shown in Table 8.1. When comparing BES and LCFAs in terms of their methane formation capabilities, different conclusions were reached depending on a particular the phase of study.

Table 8.1: Methane concentrations at 12 and 48 hours for culture sets with various inhibitor concentrations

Culture Set	Phase	Methane Concentration (µmol/culture) at 12 hours	Methane Concentration (µmol/culture) at 48 hours
0 inhibitor	II	203.82	454.47
10 mM BES	II	149.78	144.35
25 mM BES	II	139.70	123.24
50 mM BES	II	140.17	123.84
75 mM BES	II	130.99	120.79
2000 mg/l LA	III	95.59	80.83
1000 mg/l LA + 1000 mg/l LA	III	51.51	47.06
50 mM BES	III	87.82	79.17
25 mM BES + 25 mM BES	III	50.47	45.08
1000 mg/l LAU	IV	0	0
2000 mg/l LAU	IV	0	0
3000 mg/l LAU	IV	0	0
1000 mg/l LA	IV	0	0
2000 mg/l LA	IV	0	0
3000 mg/l LA	IV	0	0
25 mM BES	IV	21.94 (at 24 hrs)	21.94
50 mM BES	IV	8.88 (at 24 hrs)	10.40
100 mM BES	IV	0.19 (at 24 hrs)	0
0 Inhibitor	IV	81.27 (at 24 hrs)	86.15

Methane formation in cultures containing BES and LA inhibition was compared in phase III. Methane concentrations were very low in cultures containing 50 mM BES and 2000 mg/l LA at both 12 and 48 hours. A methane concentration difference of 8 and 2 μmol/bottle was observed between cultures containing 50 mM and 2000 mg/l LA at 12 and 48 hours, respectively. Therefore, the 50 mM BES and 2000 mg/l LA achieve an equivalent degree of methane inhibition. Methane formation was considerably lower in phase III and IV compared to phase II. A difference such as this could be attributed to changes in the microbial community dynamics. The methanogenic population appears to decrease over time. Consistent VSS concentrations were maintained throughout the course of all experiments. Changes in the microbial structure could be due to the reactor conditions (i.e. temperature and pH). The methanogenic populations in the reactor could have continued to decrease until the environmental conditions were adjusted. At a higher neutral pH, the methanogenic populations are expected to thrive, resulting in elevated methane levels. Ideally, a large number of methanogens should be present to ensure the removal of excess acetate and H_2. Regardless of the initial methanogenic activity, it is clear than increasing BES concentrations reduce methane formation.

8.1 Inhibitor Cost Breakdown

In order for a complete comparison between the different types of inhibitors, a cost comparison is essential. Cost and availability of each inhibitor are important factors to consider if full scale H_2 production will be considered an attractive energy supply option. The following cost breakdown was completed using the costs for inhibitors used in all experiments. The quantity of inhibitor needed will be significantly higher in large scale reactors and the bulk costs of each inhibitor would be different; however, the costs used here can provide a generalized perspective for comparison.

(100-litre reactor used as a basis for comparison)

3000 mg/l LA:

300,000 mg (or 300 g) of LA would be needed [100 litres × 3000 mg/l].

The density of LA is 0.9 g/ml.

$$Volume\ of\ LA = \frac{Mass\ of\ LA}{density\ of\ LA} = \frac{300\ \cancel{g}}{0.9\ \cancel{g}/ml} = 333.33\ ml$$

The cost of 500 ml of LA is $544 (Tokyo Chemical Industry Co. Ltd., 2011).

$$\frac{\$544}{500\ ml} = \$1.088/ml$$

$$\frac{\$1.088}{\cancel{ml}} \times 333.33\ \cancel{ml} = \$363$$

Therefore, a cost of $363 would be incurred to added 3000 mg/l LA to a 100-litre reactor

3000 mg/l Lauric acid:

300,000 mg (or 300 g) of OA would be needed [100 litres × 3000 mg/l].

The cost of 500 g of lauric acid is $17.40 (Tokyo Chemical Industry Co. Ltd., 2011).

$$\frac{\$17.40}{500\ g} = \$0.0348/g$$

$$\frac{\$0.0348}{\cancel{g}} \times 300\ \cancel{g} = \$10$$

Therefore, a cost of $10.44 would be incurred to added 3000 mg/l LAU to a 100-litre reactor

100 mM BES:

$$100\ mM = 0.1\ M = 0.1\frac{mol}{l}$$

$$BES(g/l) = 0.1\frac{\cancel{mol}}{l} \times \frac{211.0057g}{\cancel{mol}} = 21$$

2110.06 g of BES would be needed [100 litres × 21.10057 g/l].

The cost of 100 g of BES is $122 (Sigma-Aldrich, 2011).

$$\frac{\$122}{100\ g} = \$1.22/g$$

$$\frac{\$1.22}{\cancel{g}} \times 2110.06\ \cancel{g} = \$2600$$

Therefore, a cost of $2600 would be incurred if 100 mM BES is added to a 100-litre reactor. Table 8.2 summarizes the cost for each inhibitor for a 100-litre reactor. The results are remarkable because the cost to add one injection of 100 mM BES into a 100-litre reactor is approximately $2600. Clearly, in terms of economics, BES inhibition will lead to a high operational cost. LCFAs can be obtained from wastewater and food processing facilities effluents. Using these LCFA sources in large scale H_2 producing reactors could off-set the operational cost. Using naturally occurring LCFAs offers the development of a sustainable H_2 production process. LCFAs cost could be extremely low because most industrial sectors are willing to dispose of waste materials. Alternatively, the availability of BES is exclusively dependent on laboratory production. Based on this analysis, it is evident why most research is now focused on using LCFAs as methanogenic inhibitors.

Table 8.2: Cost to add inhibitor at specified concentration in a 100-litre reactor

Inhibitor type	Concentration	Cost
Linoleic acid	3000 mg/l	363
Lauric acid	3000 mg/l	10
BES	100 mM	2600

Chapter 9: Conclusions

The objectives of this research work were focused on improving fermentative H_2 production using mixed cultures. Using pure cultures in large scale production facilities would require extensive maintenance and operational costs. In addition, sterile feedstocks would have to be employed in order to avoid contamination. These disadvantages could impose severe problems and limit the use of pure cultures. For this reason, mixed cultures are more suitable in large scale production facilities.

Optimal H_2 yield can be achieved through the application of appropriate environmental conditions, engineering design and microbial inhibition. In these studies, the main objective was to evaluate two types of methanogen inhibitors on mixed cultures at an initial pH of 5.5 and a temperature of 37°C using glucose as a feedstock. Several reports have shown that BES is a specific methanogen inhibitor (Zinder, 1984; Danko et al., 2008). BES is a synthetic chemical produced in small quantities primarily for laboratory studies. Methanogen inhibition by BES has never been compared directly LCFAs. LCFAs have been studied extensively for their ability to inhibit methanogens. The reason for such high interest in LCFAs is due to the fact that they are organic compounds that are present in renewable chemicals which can be derived from many terrestrial and aquatic plants.

The primary inhibitory action of LCFAs is to disrupt nutrient transport across the cellular membranes. Another group of methanogenic inhibitors are the structural analogs of coenzyme M (Liu et al., 2011). Coenzyme M is necessary in the last step of methogenesis and blocking the action of the coenzyme results in the inhibition of methanogens. Analogs of coenzyme M include the following: 2-chloroethanesulfonate (CES), 2-mercaptoethanesulfonate (MES) and 2-

bromoethanesulfonate (BES). BES has received the most attention since it is the most structurally similar to coenzyme M (Liu et al., 2011).

A portion of this study addressed the outcome if BES and LA inhibitors were added in two incremental amounts. For direct comparison, one set of cultures received the full addition of inhibitor while another set received the same amount of inhibitor divided into two equal portions separated by 24 hours. The purpose of this study was to assess if additions of smaller quantities of inhibitor would result in efficient inactivation of H_2 consumers

The main conclusions from this study were as follows:

1. The maximum H_2 yield observed with LCFA inhibition was 3.22 mol H_2/mol glucose in cultures containing 3000 mg/l LA at an initial pH 5.5. This yield represents an 80% efficiency based a theoretical maximum yield of 4 mol H_2/mol glucose. The maximum yield observed with BES inhibition was 3.24 mol H_2/mol glucose in cultures containing 100 mM BES at an initial pH of 5.5. This yield also represents an 80% efficiency.
2. Increasing BES concentration fed to cultures leads to reduced methane production during the first glucose injection period. In phase II, compared to cultures with 0 BES, methane concentrations were reduced by 70% and 73% in cultures containing concentrations of 10 mM and 75 mM BES, respectively, at 48 hours after injecting glucose. In phase IV, the amount of methane produced was reduced by 70, 90 and 100%, in cultures containing 25, 50 and 100 mM BES, d respectively.
3. Dividing the inhibitor additions into two equal increments separated by 24 hours had no effect on improving H_2 yield. Cultures fed one addition of inhibitor versus those receiving two incremental additions produced higher H_2 yields. However, the incremental inhibitor addition had a significant effect on methane production. The maximum methane

concentrations in cultures containing 2000 mg/l LA and 1000 mg/l LA + 1000 mg/l LA were 96 and 50 μmol/bottle, respectively, at 12 hours. The maximum methane concentrations in cultures containing 50 mM BES versus those containing 25 mM BES + 25 mM BES were 88 and 50 μmol/bottle, respectively, at 12 hours. These differences account for an average methane reduction of 45% and 40% for LA and BES, respectively. Therefore, adding the inhibitors in two separate increments could lead to a further reduction in methanogenesis.

4. In phase II, the cultures containing 0 BES (no inhibitor) produced higher yields than cultures containing BES during the second glucose injection period. These cultures reached a maximum H_2 yield of 3.32 mol H_2/mol glucose. This was the highest yield recorded in all four experimental phases. Similarly, in phase IV, higher H_2 yields were observed in cultures containing no inhibitor compared to those containing 25 mM BES or 50 mM BES. Cultures containing 100 mM BES produced a larger H_2 yield compared to cultures containing no inhibitor. In all four phases, no methane production was observed during the second glucose injection period. At 96 hours, when the second glucose injection occurs, methanogens in the mixed culture were completely inhibited. The initial pH of 5.5 was likely responsible for reducing the methanogenic activity. Methanogens are pH sensitive and it is difficult to recommend the use of inhibitors, such as BES and LCFAs, when cultures containing no inhibitor produced roughly similar amount of H_2. However, their inhibitory effect is realized by decreasing methane production in the first glucose injection period. Moreover, if the experiments conducted were continued for a third and fourth glucose injection periods at pH 5.5, with no inhibitor addition, it is likely

that methanogens would become active again. Therefore, adding inhibitor becomes more beneficial after the second glucose injections period.

5. The highest H_2 yield in cultures fed with LCFA inhibition was observed in cultures containing 3000 mg/l LA. Cultures fed with LAU were better H_2 producers during the first glucose injection. The H_2 production data from phase IV of this study demonstrated the efficiency of LCFA inhibition compared to BES during the first injection. Cultures containing BES experience a lag period with no gas production after glucose addition. Using LCFAs could be a more economically attractive option when compared to BES. LAU could be a preferred inhibitor based on H_2 yields and cost.
6. Based on the results of phase III, a BES concentration of approximately 50 mM is required to ensure the same methane yield is achievable with 2000 mg/l LA. Based on the results of phase IV, the cultures containing 3000 mg/l LA and 100 mM BES produced equivalent maximum H_2 yields during the second glucose injection period.

Chapter 10: Engineering Significance and Future Recommendations

The reign of fossil fuels as the primary energy provider is projected to end in the near future. Society's dependency on fossil fuel sources to meet energy needs over the past century has caused an incalculable amount of damage to the environment as well as human health. Global warming is an ever growing fear and researchers are seeking methods to develop alternative energy sources that are sustainable and environmentally friendly. Hydrogen has been at the forefront of an abundant amount of research. Hydrogen combustion is clean and it could turn out to be the most energy rich fuel on the planet (Boyles, 1994). Biological methods of producing H_2 are sustainable. They rely on the ability of bacteria to degrade organic material into H_2. Dark fermentation is of significant interest and it has the potential of using low cost materials, such as, waste agricultural residues, solid waste, and wastewater effluents. Converting low cost materials into energy has a number of advantages. A primary obstacle preventing dark fermentation from becoming a major force in the energy market is low H_2 yields. Inhibiting methanogens by synthetic and organic inhibitors was the main focus of this research work.

Experiments conducted in batch reactors evaluated the inhibition potential of BES, a synthetic inhibitor, versus two LCFAs (LAU and LA). The results demonstrate that using LCFAs as microbial inhibitors may be advantageous compared to BES. This competitive edge is observed during the first glucose injection where H_2 formation and glucose degradation in cultures fed with BES was relatively slow. LCFAs not only outperformed BES in experimental results, when comparing them in terms of economics and environmental impacts, LCFAs are also superior. BES is very expensive and using it in the quantities required in large scale H_2 producing facilities could be uneconomical. LCFAs derived from edible oils are renewable chemicals which can be produced from terrestrial and aquatic plants. Lauric acid, a 12 carbon long, saturated, fatty acid

showed the greatest inhibitory effect during the first glucose injection. This acid demonstrated its methanogenic inhibitory capacity with the highest H_2 yield. The initial pH used in all experiments was 5.5. The use of low pH is a very effective methanogen inhibitor and control studies with no inhibitor at an initial pH 5.5 showed substantial H_2 production with little methane production. Using pH and LCFAs as forms of methanogenic inhibition may eliminate the need for other more costly and energy intensive forms of inhibition, such as, heat treatment or aeration.

Cellulosic materials are gaining widespread attention as feedstocks in fermentative H_2 producing reactors. Cellulosic materials initially degrade into hexose (glucose) and pentose (xylose) sugars. Feeding H_2 producing reactors with pure glucose is unrealistic from an economical perspective. This is practical for experimental small-scale laboratory experiments; however, in large-scale production facilities using a pure feedstock is uneconomical and does not take full advantage of the capabilities of fermentative bacteria. A more sustainable and economical approach in large scale production facilities would be to use feedstock chemicals derived from low value biomass such as corn stover and wheat stalks.

Batch reactor operations are unlikely to be feasible in large-scale H_2 production facilities. Batch reactors are extremely useful in a laboratory environment because of their inherent simplicity. However, a continuous H_2 producing reactor is much more practical when considering the amount of H_2 that would need to be produced to meet the energy demands of a small city.

Hydrogen is expected make its largest impact when considered as a fuel for automobiles. Technology exists in the form of the proton exchange membrane (PEM) fuel cell which can use H_2 to power automobiles. However, further advancements are required on the PEM fuel cell for

it to achieve the level of efficiency needed for commercial use (Johansson and Ahman, 2002). Perhaps, the more pressing issues are the lack of the needed infrastructure (i.e. the lack of H_2 filling stations) and the need for a safe on-vehicle H_2 storage tank. Improving the H_2 yield to a point warranting commercialization without also addressing these issues seems illogical.

The following are a list of recommendations for future research work:

1. Test the inhibition potential of other LCFAs. More data collection on LCFA inhibition could lead to a better understanding of the type of LCFAs which can be used in large scale production facilities. Preference should be given to LCFAs that are abundant in nature and are simple to obtain. The effect of LCFA mixtures should also be studied. This refers to several LCFA species being mixed together and then added to a anaerobic culture. For example, injecting 1000 mg/l LA + 1000 mg/l LAU + 1000 mg/l oleic acid (OA). Using pure LCFAs could be cost prohibitive and using unrefined mixtures in large scale reactors might be a more sustainable and economical approach.
2. The results of this study showed the importance of pH on H_2 production. The optimal pH of mixed cultures from different sources is different. A useful experiment would be to perform a pH optimization study on wide variety of pure and mixed cultures bacteria cultures. Microbial and genomic analysis can then be performed on the mixed culture to be used to determine microbial populations which are present in a H_2 producing culture. An optimized pH can be determined at for every mixed culture based on microbial population.
3. More research should be directed at LCFA inhibition on continuous reactors. Continuous reactors are more practical for commercialized H_2 production. The addition of pH buffers to help maintain constant pH should also be studied in continuous reactors.

4. Reduced methane production was observed when inhibitors were injected in two increments separated by 24 hours. Further research into incremental inhibitor injection could prove useful. Studies can assess optimal separation time of injections and increasing the number of increments.
5. The effect of adding products, specifically VFAs, at specific time intervals during the experiment could provide important knowledge. VFAs in specific concentrations have been shown to inhibit methane production. A substrate can also be added at specific time intervals during the experiment. A large scale continuous reactor would have materials added periodically during production; therefore, experiments where materials are added periodically in batch reactors can provide general understanding.
6. Assess the benefits of a secondary degradation process. VFAs and alcohols contain trapped electrons that can be diverted to H_2 or methane in a secondary fermentative process.

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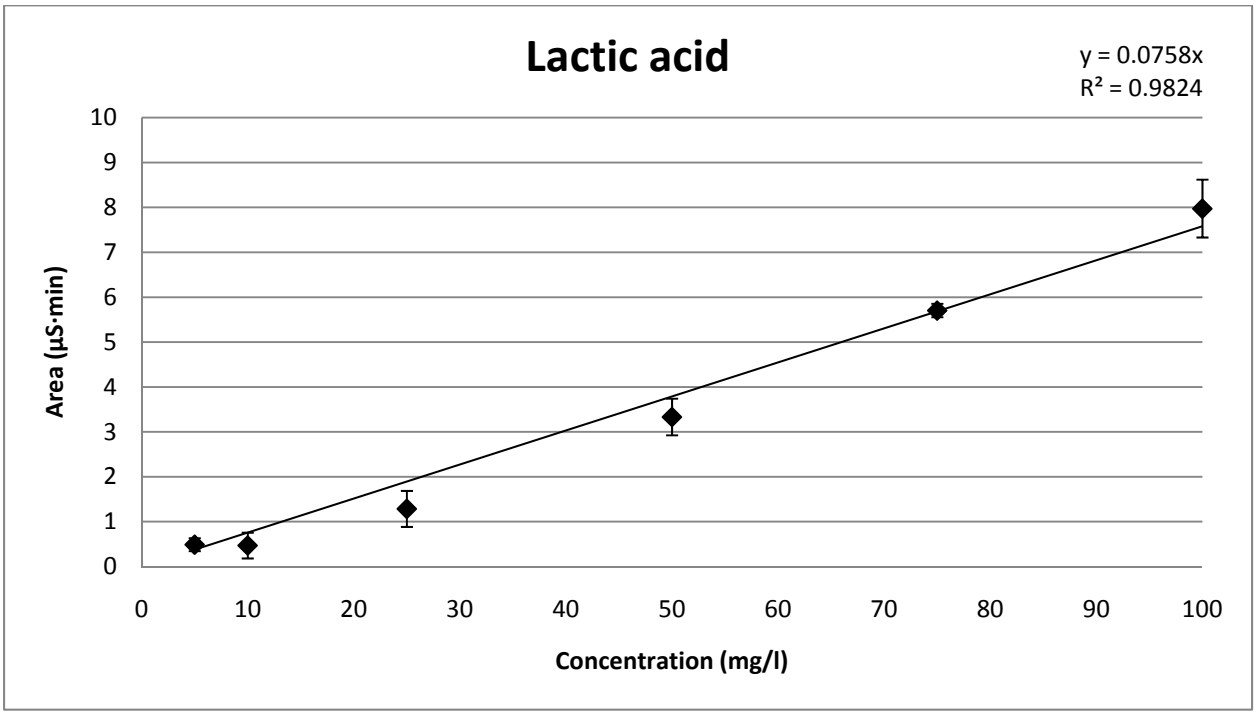
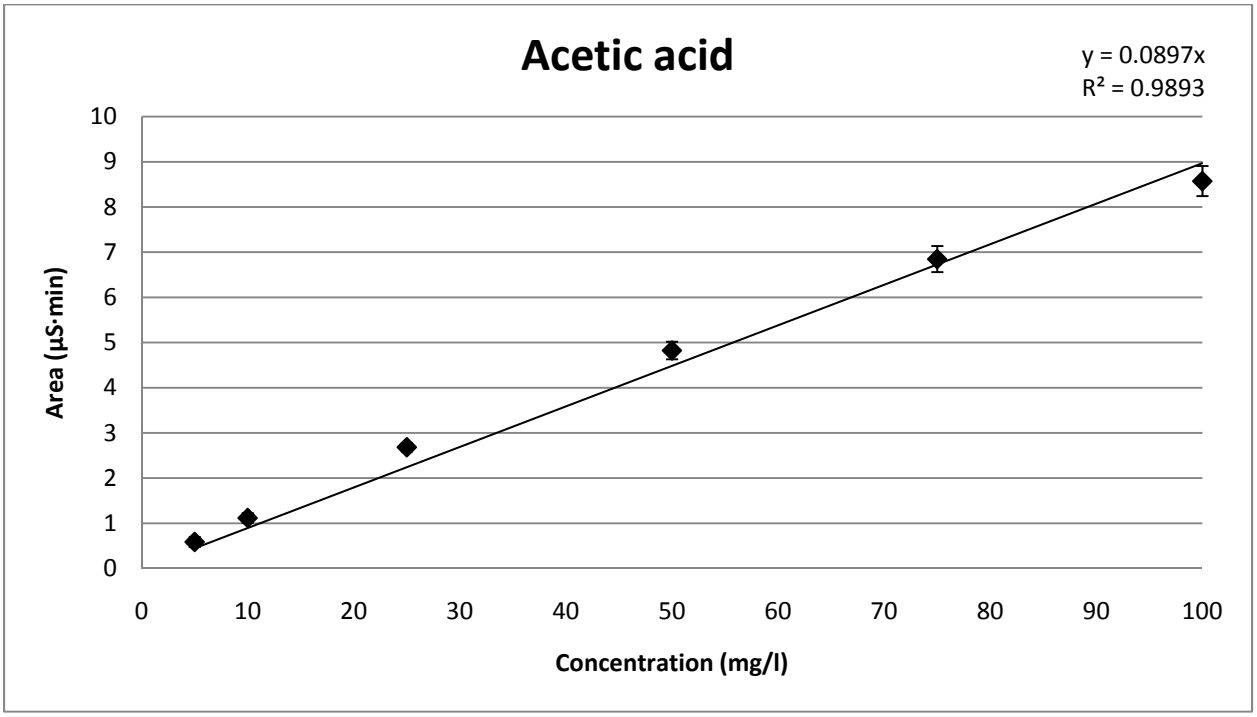
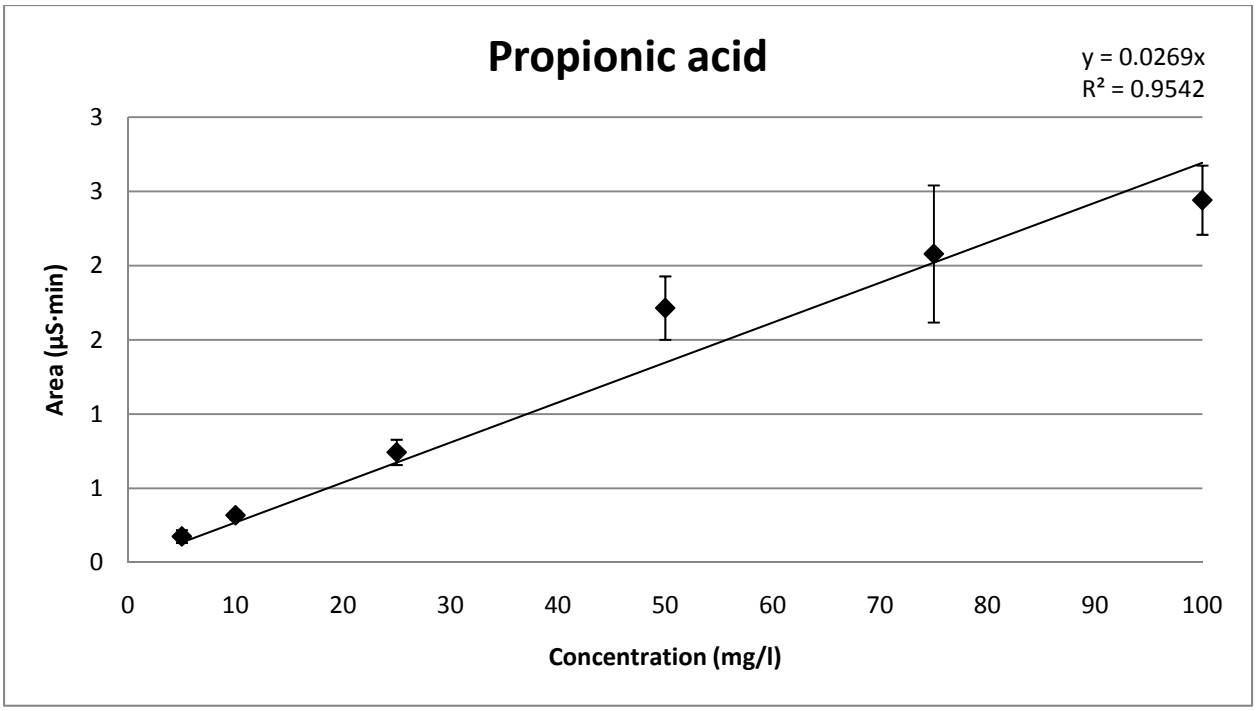
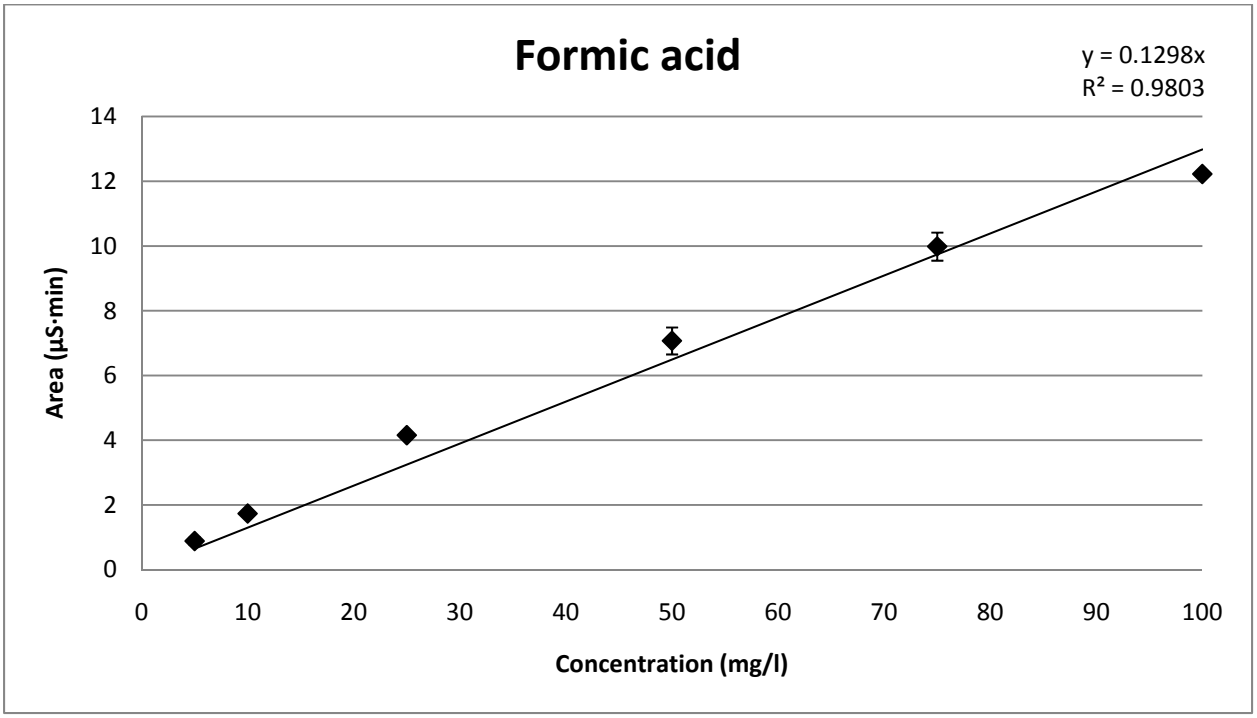
APPENDICES

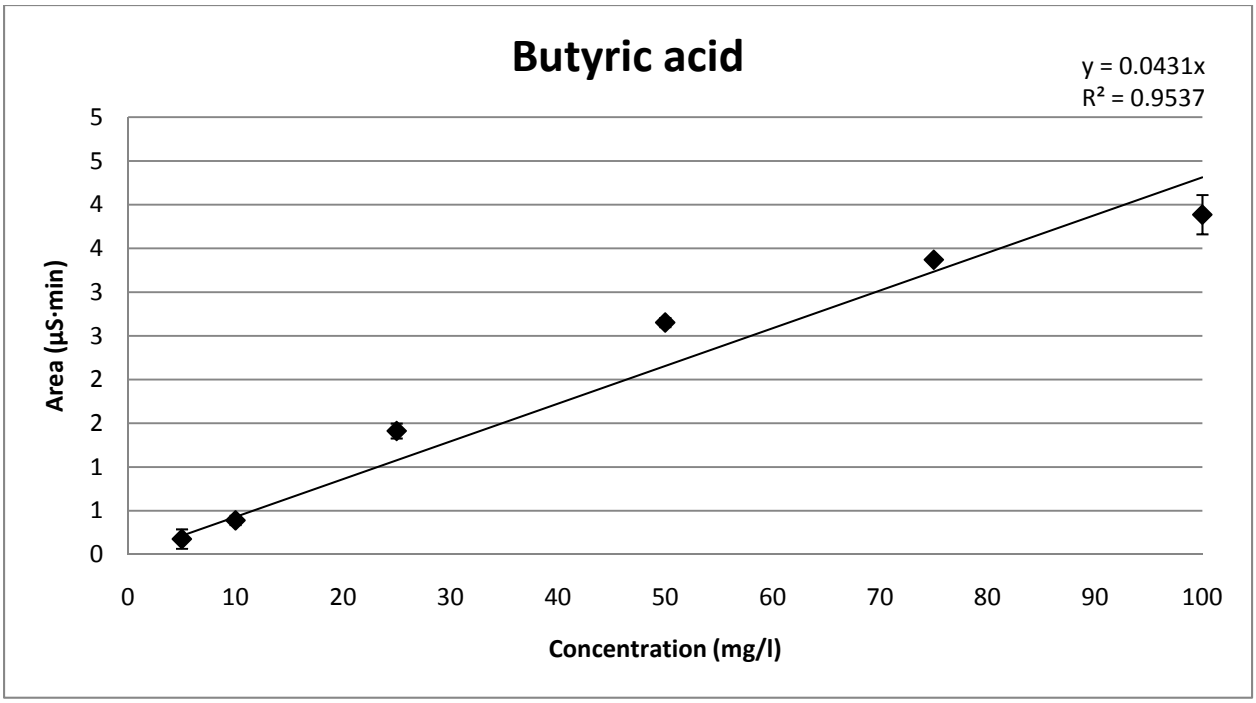
Appendix I: Volatile Fatty Acid Calibration Curves



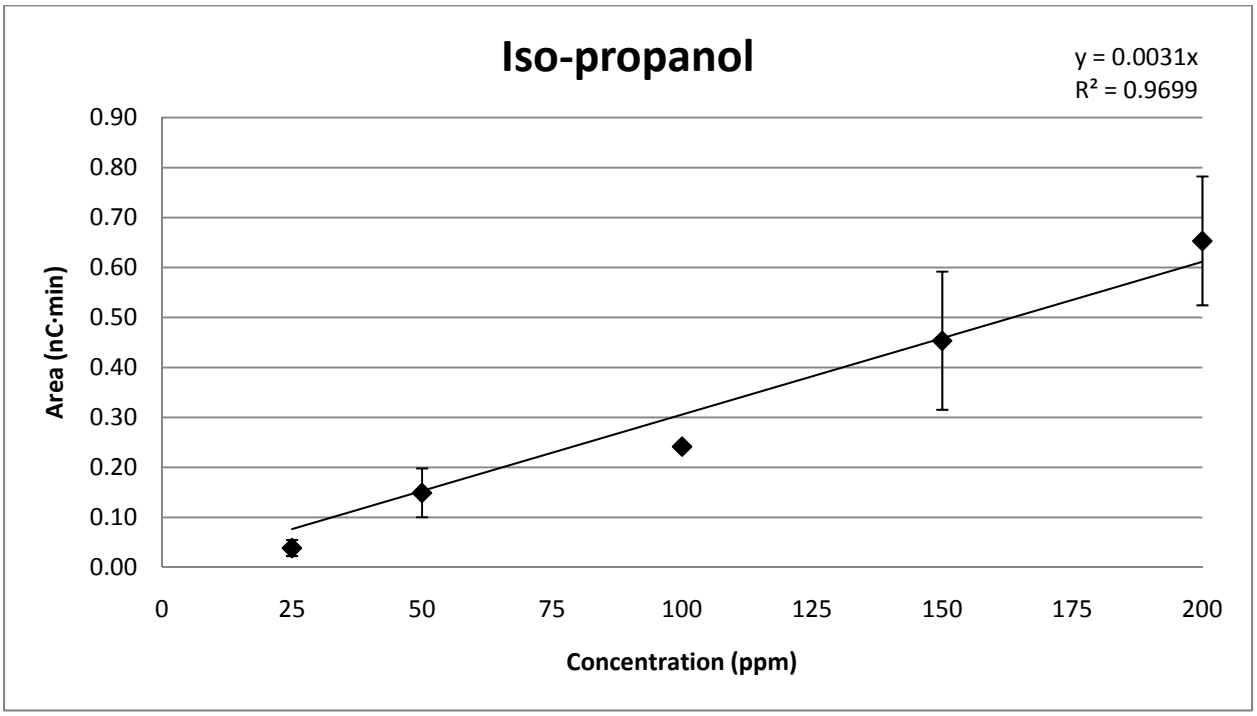


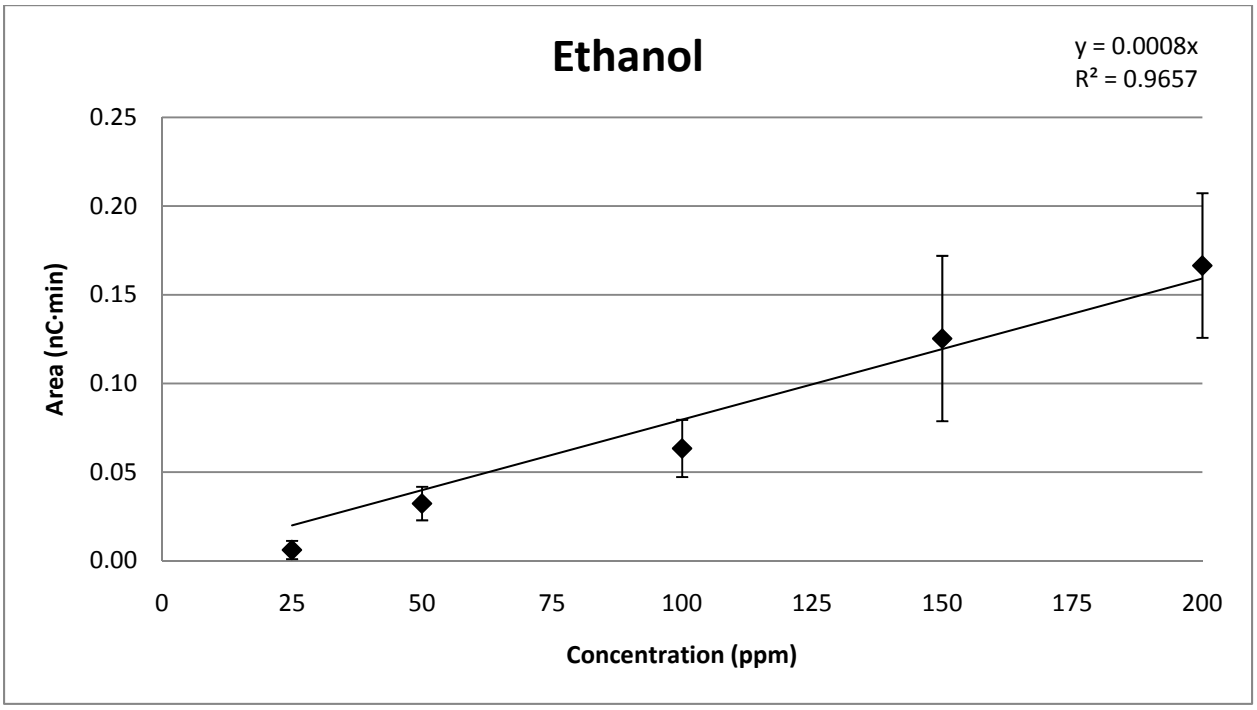

Propionic acid
y = 0.0269x
R² = 0.9542
Area (µS·min)
Concentration (mg/l)
0
1
1
2
2
3
3
0
10
20
30
40
50
60
70
80
90
100

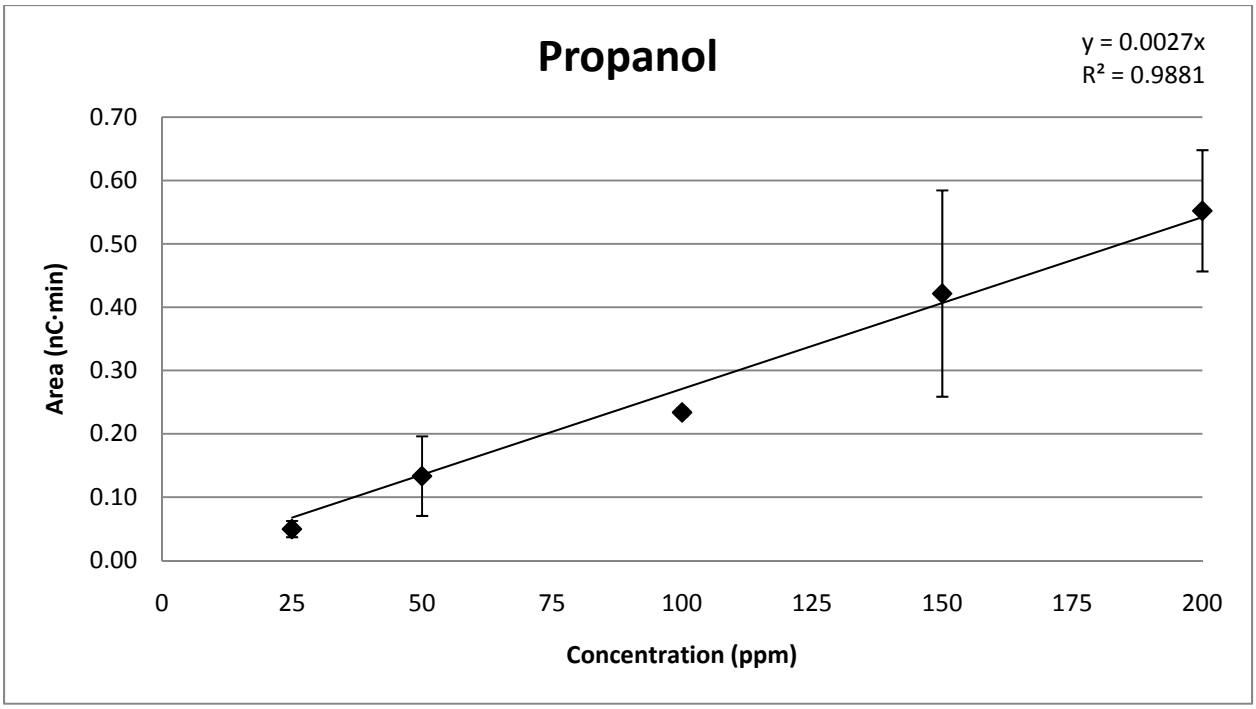

Formic acid
y = 0.1298x
R² = 0.9803
Area (µS·min)
Concentration (mg/l)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90
100

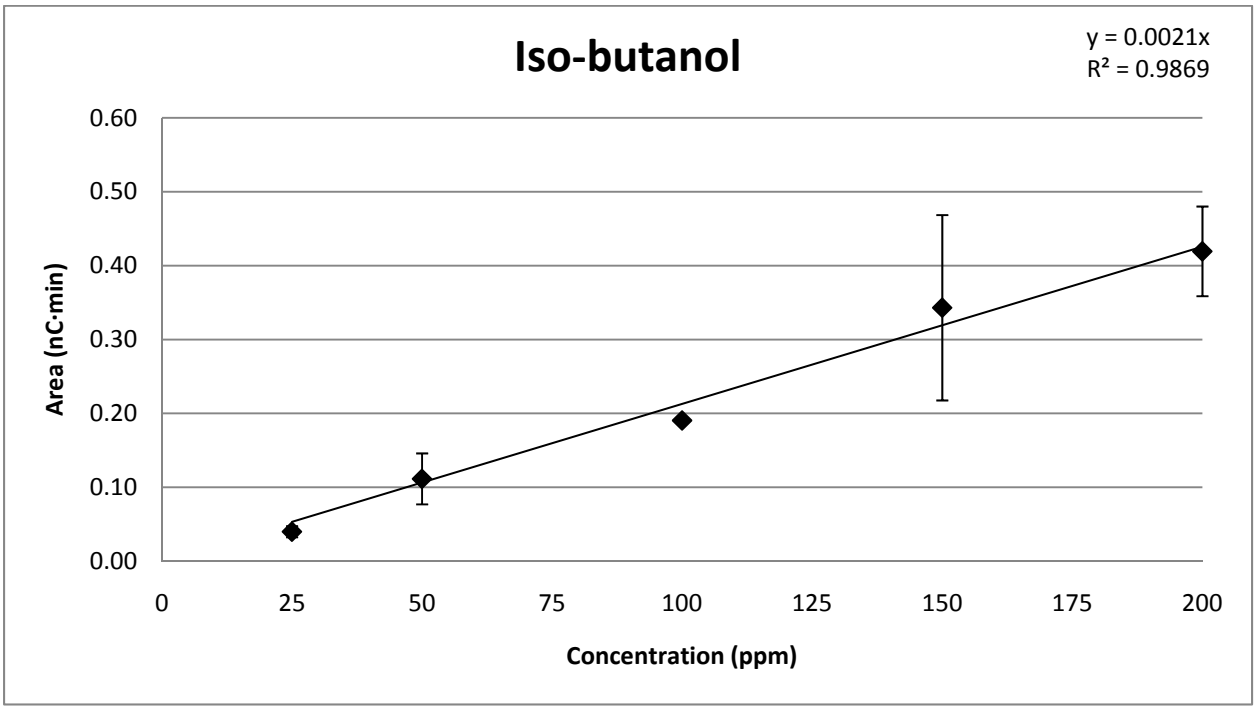

Butyric acid
y = 0.0431x
R² = 0.9537
Area (µS·min)
Concentration (mg/l)
0
1
1
2
2
3
3
4
4
5
5
0
10
20
30
40
50
60
70
80
90
100

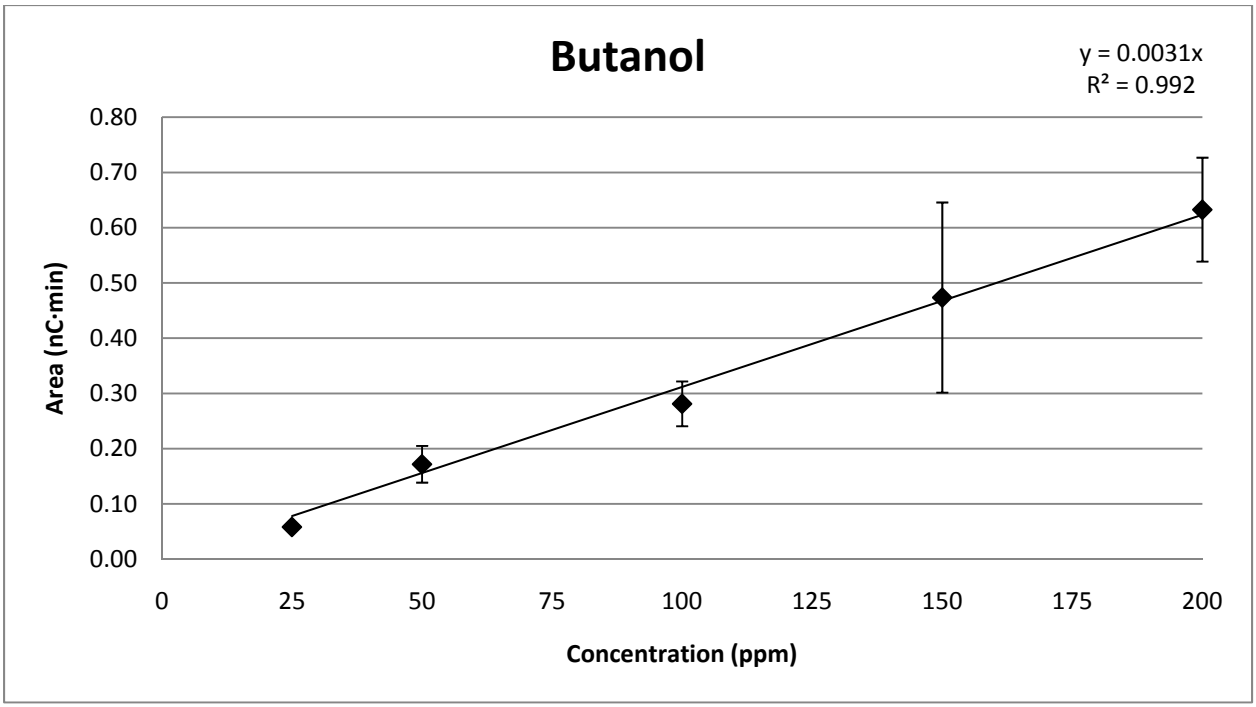
Appendix II: Alcohol Calibration Curves





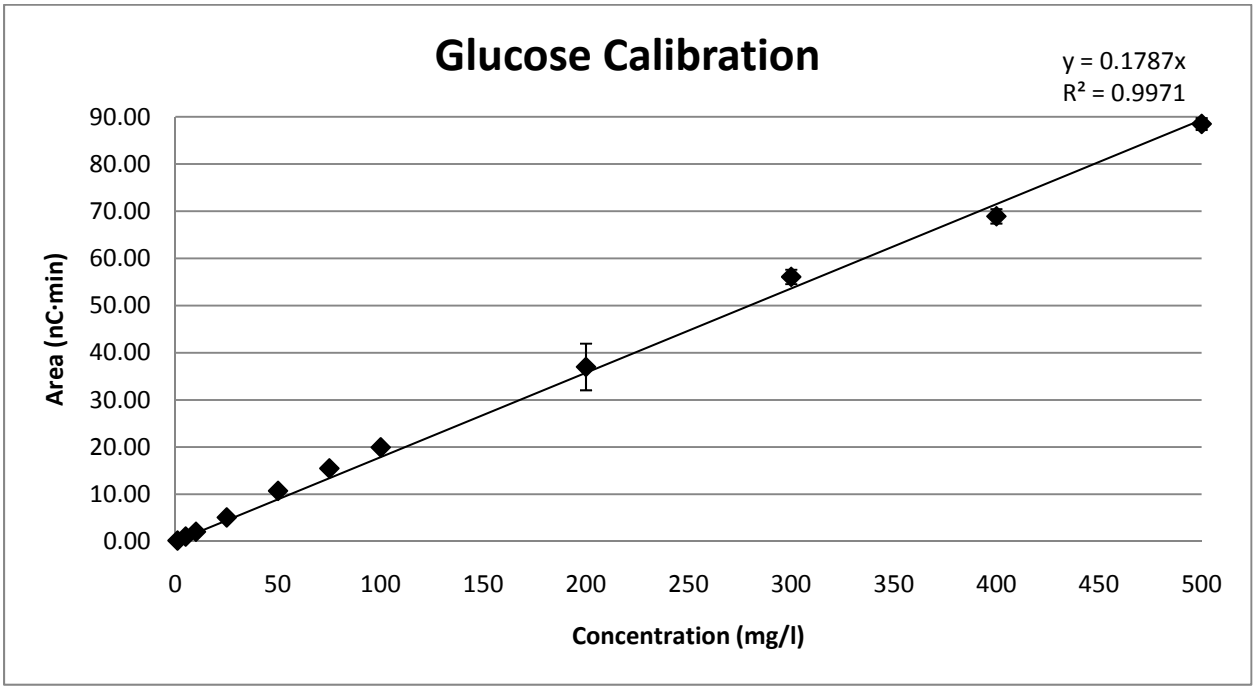

Propanol
y = 0.0027x
R² = 0.9881
Area (nC·min)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
25
50
75
100
125
150
175
200
Concentration (ppm)


Iso-butanol
y = 0.0021x
R² = 0.9869
Area (nC·min)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
25
50
75
100
125
150
175
200
Concentration (ppm)

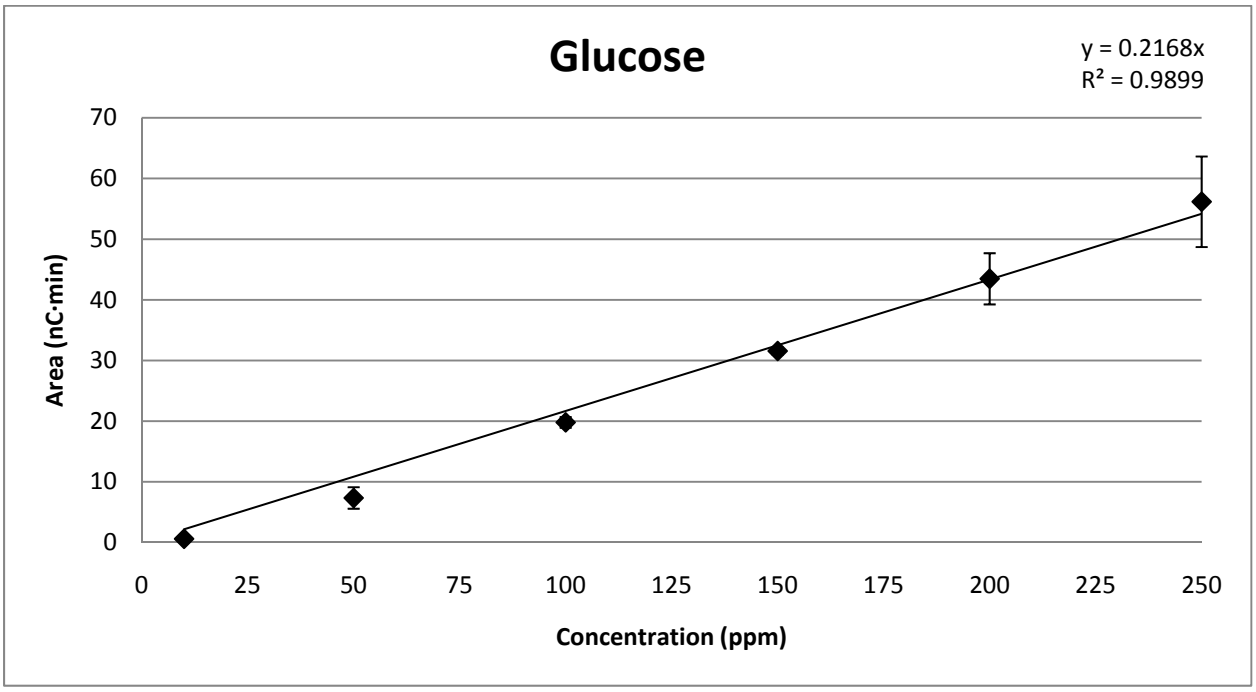

Butanol
y = 0.0031x
R² = 0.992
Area (nC·min)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
25
50
75
100
125
150
175
200
Concentration (ppm)

Appendix III: Glucose Calibration Curve

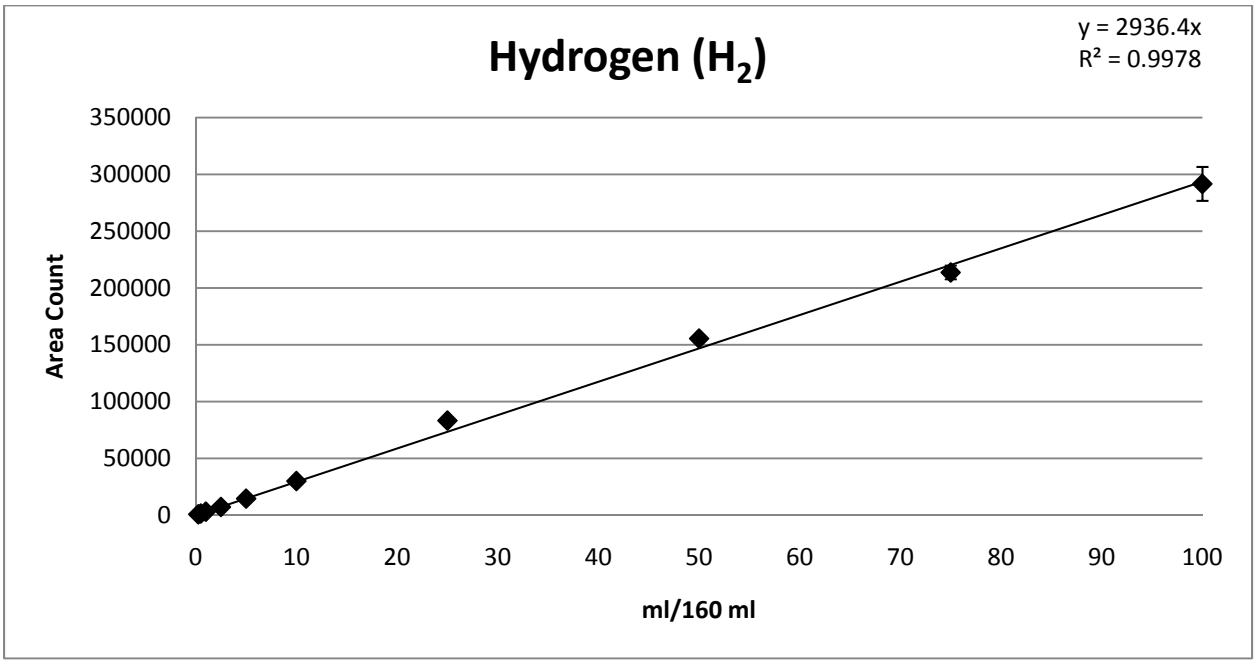
PA-20 Column (used in Phase I,II, III, IV)

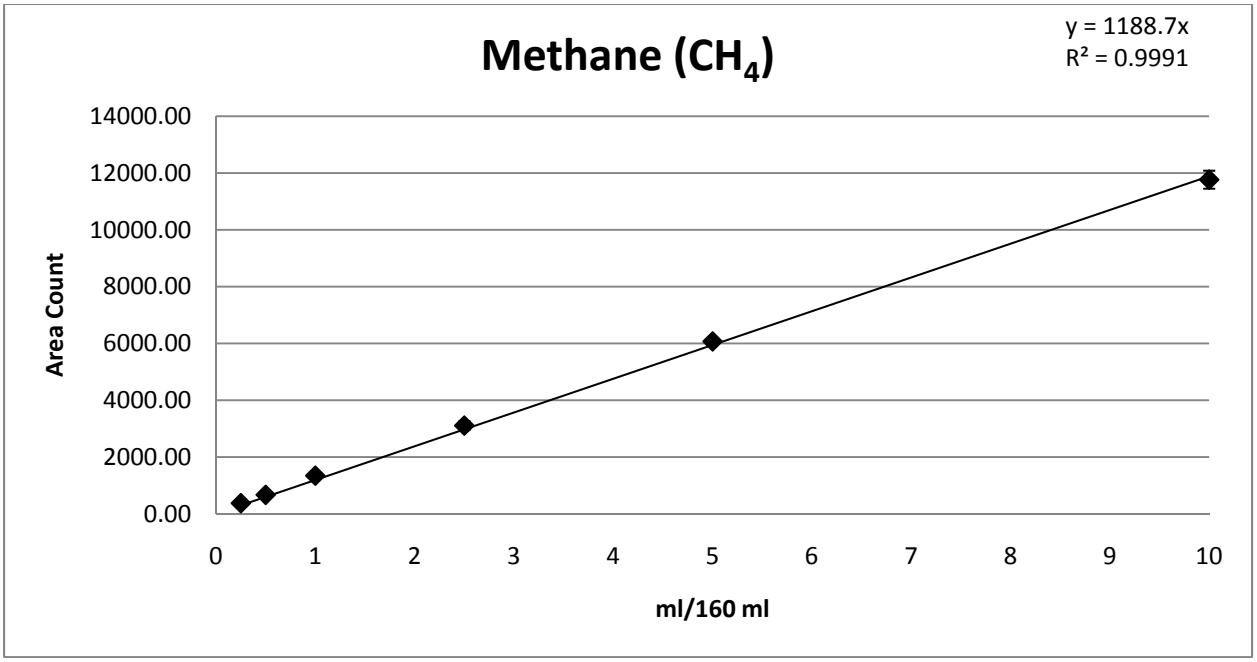


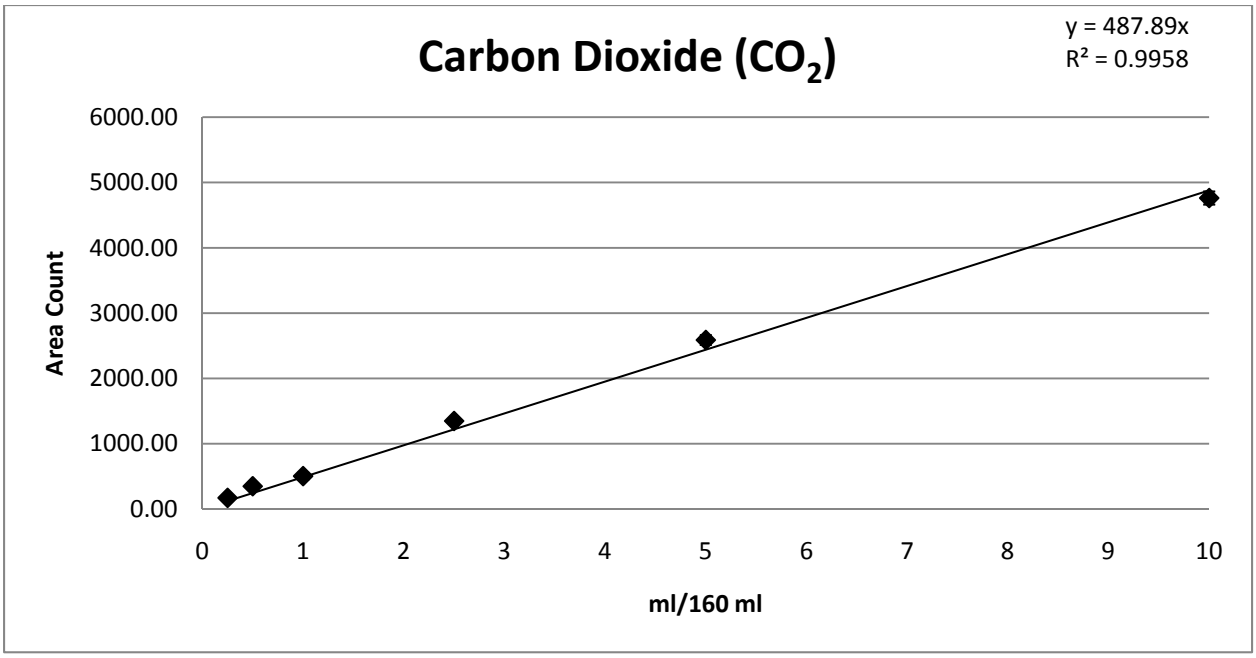
MA-1 Column (used in Phase V)



Appendix IV: Gas Calibration Curves






Carbon Dioxide (CO_2)
y = 487.89x
R² = 0.9958
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
Area Count
0
1
2
3
4
5
6
7
8
9
10
ml/160 ml

Appendix V: Sample Calculations

VSS/TSS Calculation:

$$\boldsymbol{Total\ Suspended\ Solids = TSS\ (\frac{mg}{l}) = \frac{mass\ 105°C\ (g) - \ empty\ mass\ (g)}{volume\ (ml)} \times 10^6}$$

$$\boldsymbol{Volatile\ Suspended\ Solids = VSS\ (\frac{mg}{l}) = \frac{mass\ 105°C\ (g) - \ mass\ 550°C\ (g)}{volume\ (ml)} \times 10^6}$$

Empty mass (g) = aluminum tin containing one piece of 0.45 µm glass fibre filter paper

Volume (ml) = amount of sample added to be filtered

Mass 105°C (g) = mass of aluminum tin + filter paper + sample after 1 hour in 105°C oven

Mass 550°C (g) = mass of aluminum tin + filter paper + sample after 1 hour in 505°C muffle furnace

Example:

Empty mass (g)	*Volume (ml)*	*Mass 105°C (g)*	*Mass 550°C (g)*
0.9002	4.5	0.9423	0.9166

$$\boldsymbol{TSS\ \left(\frac{mg}{l}\right) = \frac{0.9423 - \ 0.9002}{4.5} \times 10^6\ = 9355.56}$$

$$\boldsymbol{VSS\ \left(\frac{mg}{l}\right) = \frac{0.9423 - \ 0.9166}{4.5} \times 10^6 = 5711.11}$$

Note: All TSS and VSS measurements were made in triplicates

Stock Preparation:

1. Glucose

Glucose stock solution was prepared in quantities of 150 ml and in concentrations of 100,000 mg/l

$$100{,}000\frac{mg}{l} = \frac{x}{0.150\ l}$$

$$x = 100{,}000\frac{mg}{\cancel{l}} \times 0.150\ \cancel{l} = 15{,}000\ mg = 15\ g$$

Amount of Milli-Q water to be added:

$$150\ \cancel{ml} \times 0.9979 \frac{g}{\cancel{ml}} = 149.685\ g$$
$$149.685\ g = 15\ g\ gluose + x\ g\ MQ\ water$$
$$x = 149.685\ g - 15\ g = 134.685\ g\ MQ\ water$$

Note: assuming density of water to be 0.9979 g/ml at 22°C

Therefore, 15 grams of glucose was added to 134.685 g Milli-Q water to make 150 ml of 100,000 mg/l glucose stock.

2. BES

BES stock solution was prepared in quantities of 50 ml and in concentrations of 500 mM

$$500\ mM = 0.5\ M = 0.5 \frac{mol}{l}$$

$$0.5 \frac{mol}{l} = \frac{x}{0.05\ l}$$

$$x = 0.5 \frac{mol}{\cancel{l}} \times 0.05\ \cancel{l} = 0.025\ mol\ BESA$$

$$0.025\ \cancel{mol} \times 211.0057 \frac{g}{\cancel{mol}} = 5.275\ g\ BESA$$

Amount of Milli-Q water to be added:

$$50\ \cancel{ml} \times 0.9979 \frac{g}{\cancel{ml}} = 49.895\ g$$
$$49.895\ g = 5.275\ g\ BESA + x\ g\ MQ\ water$$
$$x = 49.895\ g - 5.275\ g = 44.62\ g\ MQ\ water$$

Note: assuming density of water to be 0.9979 g/ml at 22°C

Therefore, 5.275 grams of BES was added to 44.62 g Milli-Q water to make 50 ml of 500 mM BES stock.

3. LCFA (Linoleic acid, Lauric acid)

LCFA stock solutions were prepared in quantities of 20 ml and in concentrations of 50,000 mg/l

$$50{,}000 \frac{mg}{l} = \frac{x}{0.02\ l}$$

$$x = 0.02\ \cancel{l} \times 50{,}000 \frac{mg}{\cancel{l}} = 10{,}000\ mg = 1\ g$$

Amount of Milli-Q water to be added:

$$20\ \cancel{ml} \times 0.9979 \frac{g}{\cancel{ml}} = 19.958\ g$$
$$19.958\ g = 1\ g\ LCFA + y\ g\ NaOH + x\ g\ MQ\ water$$
$$x = 19.958\ g - 1\ g\ LCFA - 0.142\ g\ NaOH = 18.816\ g\ MQ\ water$$

Note: 0.142 g and 0.200 g NaOH are added for linoleic acid and lauric acid, respectively.

Note: assuming density of water to be 0.9979 g/ml at 22°C

Therefore, 1 gram of LA was added to 18.816 g Milli-Q water and 0.142 g NaOH to make 20 ml of 50,000 mg/l LA stock. Moreover, 1 gram of LAU was added to 18.758 g Milli-Q water and 0.200 g NaOH to make 20 ml of 50,000 mg/l LAU stock.

Culture Preparation Calculation:

The liquid volume was 50 ml in all bottles prepared. A VSS concentration of 2000 mg/l was attained in each bottle.

Example:

VSS of culture prior to preparation: 5711 mg/l

Amount of glucose to be added: 5 g/l = 5000 mg/l

Amount of Linoleic acid (LA) to be added: 2000 mg/l

*Glucose stock solution made to a concentration of 100,000 mg/l

*Linoleic stock solution made to a concentration of 50,000 mg/l

$$Amount\ of\ glucose\ to\ add\ (ml) = {Liquid\ volume} \Big/ \frac{[glucose\ stock]}{[desired\ glucose]}$$

$$Amount\ of\ glucose\ to\ add\ (ml) = {50\ ml} \Big/ \frac{1000000\ \cancel{mg/l}}{5000\ \cancel{mg/l}} = 2.5$$

$$Amount\ of\ LA\ to\ add\ (ml) = {Liquid\ volume} \Big/ \frac{[LA\ stock]}{[desired\ LA]}$$

$$Amount\ of\ glucose\ to\ add\ (ml) = {}^{50\ ml}\Big/_{\frac{50000\ \cancel{mg/l}}{2000\ \cancel{mg/l}}} = 2.0$$

$$Amount\ of\ culure\ to\ add\ (ml) = {}^{Liquid\ volume}\Big/_{\frac{[actual\ VSS]}{[desired\ VSS]}}$$

$$Amount\ of\ glucose\ to\ add\ (ml) = {}^{50\ ml}\Big/_{\frac{5711\ \cancel{mg/l}}{2000\ \cancel{mg/l}}} = 17.5$$

$$50\ ml\ liquid\ volume = Amount\ of\ glucose + amount\ of\ inhibitor\ (LA) + \ amount\ of\ culure + amount\ of\ basal\ media$$

Therefore, this bottle was prepared as follows:

$$17.5\ ml\ culture$$
$$+2.5\ ml\ glucose\ stock$$
$$+2.0\ ml\ LA\ stock$$
$$+\ 28\ ml\ basal\ media$$
$$=\ 50\ ml\ total\ volume$$

VFAs: Peak Area (µS·min) to concentration (mg/l)

Example for Acetic acid (peak area of 2.452 µS·min)

The calibration curve equation for acetic acid is: $y = 0.0897x$

$$y\ (\mu S \cdot min) = peak\ area$$
$$x\ \left(\frac{mg}{l}\right) = \ concentration$$

$$Concentration\ \left(\frac{mg}{l}\right) = \frac{peak\ area\ (\mu S \cdot min)}{slope\ of\ calibration\ curve} \times dilution\ factor$$

$$Dilution\ factor\ for\ VFAs = 20$$

$$Concentration\ \left(\frac{mg}{l}\right) = \frac{2.452}{0.0897} \times 20 = 546.71$$

Alcohols: Peak Area (nC·min) to concentration (mg/l)

Example for propanol (peak area of 1.054 nC·min)

The calibration curve equation for propanol is: $y = 0.0027x$

$$y\ (nC \cdot min) = peak\ area$$
$$x\ \left(\frac{mg}{l}\right) = \ concentration$$

$$Concentration\ \left(\frac{mg}{l}\right) = \frac{peak\ area\ (nC \cdot min)}{slope\ of\ calibration\ curve} \times dilution\ factor$$

$$Dilution\ factor\ for\ Alcohols = 5$$

$$Concentration\ \left(\frac{mg}{l}\right) = \frac{1.054}{0.0027} \times 5 = 1951.85$$

Glucose: Peak Area (nC·min) to concentration (mg/l)

Example for Glucose (peak area of 6.224 nC·min)

The calibration curve equation for glucose is: $y = 0.1787x$

$$y\ (nC \cdot min) = peak\ area$$
$$x\ \left(\frac{mg}{l}\right) = \ concentration$$

$$Concentration\ \left(\frac{mg}{l}\right) = \frac{peak\ area\ (nC \cdot min)}{slope\ of\ calibration\ curve} \times dilution\ factor$$

$$Dilution\ factor\ for\ Glucose = 10$$

$$Concentration\ \left(\frac{mg}{l}\right) = \frac{9.224}{0.1787} \times 10 = 516.17$$

Gases: Peak area count to number of mol

Example for H_2 (area count of 55,654 and pressure of 8.9 psi)

The calibration curve equation for H_2 is: $y = 2936.4x$

$$y = area\ count$$
$$x\ \left(\frac{ml}{160\ ml\ bottle}\right) = \ concentration$$

Note: Gas calibrations were conducted by injecting a known volume of gas into a 160 ml serum bottle filled with Nitrogen (N_2). The gas chromatography area count is a function of ml of gas per 160 ml bottle. Each bottle prepared contained a liquid volume of 50 ml in a 160 ml serum bottle. Therefore, there must be a correction for the headspace difference between experiments and calibrations.

$$Headspace\ correction = \frac{160\ ml\ bottle\ volume}{110\ ml\ headspace\ volume} = 1.454$$

$$Volume\ (l) = \frac{area\ count}{slope\ of\ calibration\ curve} \times headspace\ correction$$

$$Volume\ (ml) = \frac{55654}{2936.4} \times 1.454 = 27.56$$

$$Pressure\ (atm) = \frac{Pressure\ (\cancel{psi}) + 14.7\ \cancel{psi}}{14.7\ \cancel{psi}/atm}$$

Note: 14.7 psi (or atmospheric pressure) was added to the pressure readings taken in the bottle. The pressure meter is calibrated to zero at atmospheric pressure.

$$Pressure\ (atm) = \frac{8.9\ \cancel{psi} + 14.7\ \cancel{psi}}{14.7\ \cancel{psi}/atm} = 1.61$$

$$Number\ of\ moles = n = \frac{Pressure\ (\cancel{atm}) \times Volume\ (\cancel{l})}{R\ (\cancel{atm} \cdot \cancel{l} \cdot mol^{-1} \cdot \cancel{K^{-1}}) \times Temperature\ (\cancel{K})}$$

$$n\ (moles) = \frac{1.61 \times 0.02756}{0.082057 \times 310.15} = 0.001743 = 1743\ \mu mol$$

Yield Calculation:

5 g/l glucose is injected into each bottle. Each bottle has a liquid volume of 50 ml. Assuming 0.001743 mol of H_2 have been calculated.

$$amount\ of\ gluose\ (g) = volume\ (\cancel{l}) \times \left[glucose\ \left(\frac{g}{\cancel{l}}\right)\right] = 0.05 \times 5 = 0.25$$

$$moles\ of\ glucose = \frac{amount\ of\ glucose\ (\cancel{g})}{molecular\ weight\ of\ glucose(\frac{\cancel{g}}{mol})} = \frac{0.25}{180.16} = 0.001387655$$

Therefore, 0.001387655 mol of glucose were injected into each bottle

$$Hydrogen\ yield = \frac{moles\ of hydrogen}{moles\ of\ glucose} = \frac{0.001743}{0.001387655} = 1.256\ mol\ H_2 \cdot mol\ glucose^{-1}$$

Standard Deviation:

The standard deviation between triplicate data sets was calculated using the following formula:

$$Standard\ Deviation = \sqrt{\frac{\sum_n (x - mean)^2}{n}}$$

Example: 321.525, 245.838, 277.718

$$mean = \frac{321.525 + 245.838 + 277.718}{3} = 281.694$$

$$n = number\ of\ samples = 3$$

$$Standard\ Deviation$$

$$= \sqrt{\frac{\sum_3 (321.525 - 281.694)^2 + (245.838 - 281.694)^2 + (277.717 - 281.694)^2}{3}} = 37.9998$$

Electron Balance:

The electron balance was done on the following assumption:

$$\sum Substrate_0 = \sum Substrate_t + \sum Products_t$$

Sample calculation: 5000 mg/l glucose injection in the presence of 2000 mg/l lauric acid at an initial pH of 5.5 (Chapter 7)

Note: Experimental results have been modified. Results of alcohols and VFAs byproducts which are below 100 mg/l are not included since they are below the lower detection limit.

SUBTRACTED FROM experimental results are:

1. Concentration of specific byproduct at time = 0; and,
2. Concentration of specific byproduct at specific time in control studies with no glucose.

At time = 0,

$$\sum Substrate_0 = \frac{5000\ mg/l \times 0.05l}{180.16\ mg/mmol} \times 24 \frac{e^-}{mmol} = 33.304\ e^-$$

At time = 96 hours,

		FROM EXPERIMENTAL RESULTS			
Byproduct	**Molecular Weight (g/gmol)**	**Concentration (µmol/bottle or mg/l)**	**mmol**	**m_{eq}/mmol**	**m_{eq}**
H_2	1.0079	2806.75	2.807	2	5.614
Methane	16.0420	0	0.000	8	0.000
Lactate	89.0721	0	0.000	12	0.000
Acetate	59.0421	268.54	0.227	8	1.819
Propionate	73.0721	1020.61	0.698	14	9.777
Formate	45.0221	201.57	0.224	2	0.448
Butyrate	87.1021	0	0.000	20	0.000
Iso-propanol	60.1000	550.09	0.458	18	8.238
Ethanol	46.0700	506.29	0.549	6	3.297
n-Propanol	60.1000	100.96	0.084	18	1.512
Iso-butanol	74.1200	0	0.000	24	0.000
n-Butanol	74.1200	0	0.000	24	0.000
			5.047	**SUM→**	**30.70392**

$$\sum Substrate_t = 0$$

$$\sum Products_t = 30.70392\ e^-$$

$$\%recovery = \frac{30.70392}{33.304} \times 100\% = 92.2\%$$

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