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Hydrogen Production During the Dark Fermentation of Glycerol

By

Guochen Zhang

A Thesis

Submitted to the Faculty of Graduate Studies
through the Department of Civil and Environmental Engineering

in Partial Fulfillment of the Requirements for
the Degree of Master of Applied Science
at the University of Windsor

Windsor, Ontario, Canada

2021

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Hydrogen Production During the Dark Fermentation of Glycerol

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August 29th, 2021

DECLARATION OF PREVIOUS PUBLICATION

I. Previous publication

This thesis includes two original manuscripts that will be published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication Title/Full Citation	Publication Status
Chapter 5	*Effects of long-chain fatty acids (LCFAs) on* H_2 *production from glycerol in mixed anaerobic cultures*	*In Preparation*
Chapter 6	*Using a statistical approach to optimize* H_2 *production from glycerol by mixed anaerobic cultures*	*In Preparation*

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ABSTRACT

The focus of this thesis was on the production of H_2 from glycerol by mixed anaerobic cultures through dark fermentation. Laboratory scale experiments were conducted to demonstrate H_2 production from glycerol. The impact of various factors was evaluated using different analytical and statistical methods. Three pH levels (5.5, 6.5, and 7.5) were examined to determine the effects of the initial pH on H_2 production from glycerol. A hydrogen yield of 0.33 ± 0.03 mol H_2 mol^{-1} glycerol was observed in cultures with the initial pH set at 5.5.

Further experiments were focused on increasing the H_2 yield using long chain fatty acids (LCFAs) as inhibitors together with glycerol in mixed anaerobic cultures with an initial pH of 5.5. Six LCFAs including lauric acid (LUA), myristic acid (MA), palmitic acid (PA), stearic acid (SA), oleic (OA), and linoleic acid (LA) were examined in this study. Higher H_2 yields were observed in cultures fed PA, OA, or LA when compared to cultures fed with only glycerol. The H_2 yield for the OA and LA treated cultures were 0.42 ± 0.01 and 0.46 ± 0.03 mol H_2 mol^{-1} glycerol, respectively. In the LA and glycerol fed cultures, the H_2 yield was 29% larger when compared to the glycerol control. Based on the electron balance, ethanol (EtOH) (approximately 23.1% of the total electron equivalents) and 1,3-propanediol (1,3 PDO) (approximately 50.0% of the total electron equivalents) were the major metabolites in the LA treated cultures, while approximately 6.5% and 7.9% electron equivalents were directed to H_2 and acetate (Ac^-) formation, respectively.

A three-factor and three-level BBD model was conducted to maximize the H_2 yield in cultures fed glycerol and LA. The initial pH levels (5.5, 6.5, 7.5), glycerol concentrations (1,300, 2,600, 5,110 mg L^{-1}), and operational temperatures (22, 37, 52 ºC) were three factors selected in this study. The highest H_2 yield was 0.86 ± 0.02 mol H_2 mol^{-1} glycerol at 55 ºC, a pH of 5.5, and a glycerol concentration of 2,600 mg L^{-1}. The predicted result was 0.84 mol H_2 mol^{-1} glycerol at 55 ºC, a pH of 5.5, and a glycerol concentration of 2,710 mg L^{-1} using the D-optimality analysis. Based on the designed BBD model, the optimum levels of three factors were significant when predicting the highest H_2 yield by the D-optimality analysis.

DEDICATION

To my parents for their supports and love.

To my wife and my lovely kids.

To my friends for their encouragement and help.

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First, I am highly thankful to my advisor Dr. Jerald A. Lalman for his technical support, academic guidance, and continuous feedback throughout the duration of my study. His patience and immense knowledge highly supported me to complete this work. I express my deepest appreciation for the opportunity to advance my education under Dr. Lalman's supervision.

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NOMENCLATURE

ANOVA	Analysis of variance
AD	Anderson-Darling
BBD	Box-Behnken design
BESA	Bromoethanesulphonic acid
Bio-H_2	Biohydrogen
ButOH	Butanol
CH_4	Methane
C:N	Carbon and nitrogen ratio
C:N:P	Carbon:nitrogen:phosphorus ratio
CO_2	Carbon dioxide
CSTR	Completely stirred tank reactor
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
DF	Degrees of freedom
DO	Dissolved oxygen
EtOH	Ethanol
Fd	Ferredoxin
Fd^{red}	Ferredoxin (reduced)
Fd^{ox}	Ferredoxin (oxidized)
F/M	Food-to-microorganism ratio
GC	Gas chromatograph

$\Delta G^{0\prime}$	Gibbs free energy (standard conditions and pH = 7)
H_2	Hydrogen
H^+	Proton
Ac^-	Acetate
But^-	Butyrate
For^-	Formate
La^-	Lactate
Pr^-	Propionate
$Succ^-$	Succinate
HHV	High heating value
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
i-PrOH	iso-Propanol
LA	Linoleic acid
LCFAs	Long chain fatty acids
LHV	Low heating value
LUA	Lauric acid
MA	Myristic acid
MEC	Microbial electrolysis cell
m_{eq}	Mole electron equivalents
MS	Mean square
MQ	Milli-Q® water

MTBE	Methyl tertiary butyl ether
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide (reduced)
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
OA	Oleic acid
OLR	Organic loading rate
PA	Palmitic acid
PrOH	n-Propanol
1,3-PDO	1,3-propanediol
RSM	Response surface methodology
SA	Stearic acid
SS	Sum of squares
TCD	Thermal conductivity detector
TSS	Total suspended solids
UV	Ultraviolet
VFAs	Volatile fatty acids
VSS	Volatile suspended solids

Chapter 1: INTRODUCTION

1.1 Background

The increasing consumption of non-renewable resources such as coal, petroleum, and natural gas has caused numerous global environmental issues. As of 2017, these resources, commonly referred to as fossil fuels, accounted for approximately 80-90% of the global primary energy consumption (BP, 2017). These energy sources are related to environmental, social, and economic issues including air pollution, greenhouse gas emissions, and global warming (Kampa and Castanas, 2008; Wuebbles and Jain, 2001). According to Fulton (2009), trends in energy consumption and carbon dioxide (CO_2) emissions are anticipated to increase from 50% in 2030 to 80% by 2050. In addition, increased consumption will exhaust global fossil fuel resources and drive the need to develop clean energy alternatives. Renewable energy sources currently utilized include solar, wind, and biomass energy; however, current limitations on storage, transmission, and utilization make these technologies problematic for fulfilling the global energy demand.

Biohydrogen (bio-H_2), a carbon neutral fuel, is a clean energy alternative which can assist with reducing the negative effects of fossil fuels combustion. Hydrogen produced from renewable energy such as wind or solar and renewable agriculture residues is advantageous when compared to using fossil fuels since, renewable energy sources are carbon neutral. When compared to other commonly used fuels (Table 1.1), renewable H_2 is an energy alternative because of its higher energy content. Hydrogen has the largest high heating value (HHV) and low heating value (LHV) (Dincer, 2012). Since the heating value of a fuel represents the amount of heat released during combustion, the HHV and LLV values indicate the energy content of the fuel. For example, at standard temperature and pressure conditions (298K and 101.325 KPa), the heat of combustion for H_2 is 119.9 $MJ \cdot kg^{-1}$ (LHV), while for gasoline this value is 44.5 $MJ \cdot kg^{-1}$ (LHV). When compared to methane (CH_4) and other gaseous fuels, H_2 has several advantages. Hydrogen can be regarded as the cleanest energy alternative since, no carbon based by products or pollutants are produced during combustion. The only by-product from the H_2

combustion reaction is water.

Table 1.1: High and low heating values for different fuels

Fuel	State at ambient temperature and pressure	HHV ($MJ \cdot kg^{-1}$)	LHV ($MJ \cdot kg^{-1}$)
Hydrogen	Gas	141.9	119.9
Methane	Gas	55.5	50
Ethane	Gas	51.9	47.8
Gasoline	Liquid	47.5	44.5
Diesel	Liquid	44.8	42.5
Methanol	Liquid	20	18.1

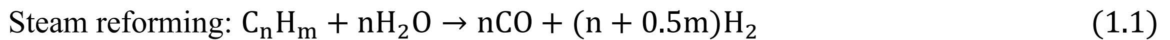
Adapted from Dincer(2012)

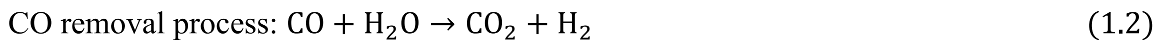
According to Nikolaidis and Poullikkas (2017), based on raw material consumption, fossil fuels and renewable sources are two primary methods for producing H_2. Until 2001, approximately 90% of the total global H_2 production originated from fossil fuel sources (Das and Veziroğlu, 2001). The three main sources of H_2 from fossil fuels which include natural gas, heavy oils, and coal account for 48%, 30%, and 18% of the total production, respectively (Kothari et al., 2008). According to Nikolaidis and Poullikkas (2017), hydrocarbon reforming and hydrocarbon pyrolysis are the two leading processes employed to produce H_2 from fossil fuels. Although H_2 production from fossil fuels is dominant, these processes are limited because of the increasing cost due to dwindling fossil fuel supplies. Hence, another platform feedstock alternative such as biomass can be utilized to produce H_2. Currently, H_2 is produced by natural gas reforming, the partial oxidation of CH_4, coal gasification, and electrolysis (Dincer and Acar, 2015; Kalamaras and Efstathiou, 2013). Other processes under consideration include electrolysis driven by solar and wind energy, biomass gasification, photoelectrochemical and photobiological water splitting, as well as bacteria and algae processes. (U.S. Department of Energy, 2020; Nikolaidis and Poullikkas, 2017; Dincer and Acar, 2015).

1.1.1 Hydrocarbon reforming

During hydrocarbon reforming, H_2 is produced by the degradation of hydrocarbons. Hydrocarbon reforming processes are categorized as steam reforming,

auto-thermal reforming, or partial oxidation. Steam reforming is employed to convert hydrocarbons into H_2 and CO_2 (reactions 1.1, 1.2 and 1.3) by employing a catalytic reaction in which steam at elevated temperatures and pressures are applied at a particular steam to carbon ratio. According to Ersöz (2008), the catalytic reforming reaction operating conditions are at approximately 850 ºC and pressures up to 3.5 MPa with a steam to carbon ratio of 3.5. Producing a higher purity H_2 product is accomplished by reacting the CO byproduct (reaction 1.2) with steam to produce CO_2 additional H_2 (Steinberg and Cheng, 1989). The CO_2 byproduct is separated in injected underground (Damen et al., 2006). The main chemical reactions for steam reforming and H_2 purification are shown in equations 1.1 and 1.2. The steam CH_4 reforming process is a full-scale production method with a conversion efficiency range between 74-85% (Nikolaidis and Poullikkas, 2017). Methanation can be applied as a purification process to remove CO without generating CO_2 (Equation 1.3).

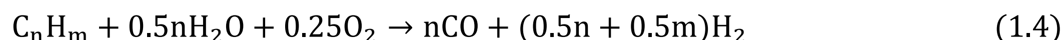

Steam reforming: $C_nH_m + nH_2O \rightarrow nCO + (n + 0.5m)H_2$ (1.1)


CO removal process: $CO + H_2O \rightarrow CO_2 + H_2$ (1.2)

Methanation: $CO + 3H_2 \rightarrow CH_4 + H_2O$ (1.3)

When compared to steam reforming, the auto-thermal reforming reaction produces H_2 and various oxides of carbon from hydrocarbons feedstocks. Based on Equation 1.4, the hydrocarbon is combined with steam and oxygen or air to produce H_2 and carbon monoxide. The auto-thermal reforming reaction combines the steam reforming and oxidation reactions into a single process when compared to the steam-CH_4 reforming process. According to Voitlc et al. (2018), the enthalpy of the auto-thermal reforming reaction is close to zero. In this process, an initial partial oxidation zone supplies the process heat for the subsequent endothermic steam reforming step (Voitlc et al., 2018; Chen et al., 2008). The costs of auto-thermal reforming are approximately 15-25% lower than steam reforming when CH_4 is the feed hydrocarbon (Nikolaidis and Poullikkas, 2017).

Thermal reforming:



$$C_nH_m + 0.5nH_2O + 0.25O_2 \rightarrow nCO + (0.5n + 0.5m)H_2 \quad (1.4)$$

In the partial oxidation process, oxygen, steam, and hydrocarbon are converted into H_2 through catalytic and non-catalytic processing under different temperature conditions. Typically, a Ni catalyst is used in the CH_4 to syngas (a combination of CO, H_2, CO_2, and other short chain carbon gases) processes (Keiski et al., 2011). When CH_4 or other hydrocarbons reacts with close to less than a stoichiometric amount of oxygen, CO is produced together with heat (Equation 1.5). In a subsequent water gas shift reaction, H_2 is produced by CO reacting with H_2O (Equation 1.6). When compared to steam reforming, the partial oxidation process is more efficient (Khila et al., 2013).

Partial Oxidation:

$$CH_4 + 0.5O_2 \rightarrow CO + 2H_2 \quad (1.5)$$

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (1.6)$$

1.1.2 Hydrocarbon pyrolysis

Hydrocarbon pyrolysis is a process employed to produce H_2 from hydrocarbons. Hydrocarbon pyrolysis involves a two-step cracking reaction which includes hydrogasification and the cracking of CH_4 (Equation 1.7 and 1.8).

Hydrogasification: $CH_{1.6} + 1.2H_2 \rightarrow CH_4$ (1.7)

Methane cracking: $CH_4 \rightarrow C + 2H_2$ (1.8)

1.1.3 Thermochemical Processes

Thermochemical processes can utilize various feedstocks such as natural gas, coal, and biomass, to produce H_2. Biomass gasification is a thermochemical process which is used to convert biomass into H_2 rich gases (Wang et al., 2015). The process is carbon neutral because biomass is utilized to produce a fuel with no net CO_2 production (Fremaux et al., 2015). According to Iribarren et al. (2014), the biomass gasification process involves oxygen or oxygen-rich air and temperatures of 500 to 1400 ºC, and pressures up to 3.3 MPa. The transformation of biomass into H_2 rich gases is shown in equation 1.9. Additional gas purification process can be employed after the gasification to

remove CH_4 and CO.

$$\mathrm{Biomass} + O_2 \rightarrow H_2 + CO + CO_2 + N_2 + CH_4 + \text{other hydrocarbons} \quad (1.9)$$

1.1.4 Water splitting

The water splitting process can be employed to produce H_2. The splitting reaction can be mediated by using electrolysis, thermolysis, and photo-electrolysis, wind electrolysis (U.S. Department of Energy, 2020; Steinfeld, 2005). Electrolysis is an effective method for producing H_2 from water. During this process, water is converted in H_2 and O_2 using electricity. However, the reaction is endothermic and requires an energy input (Equation 1.0). In an electrolytic cell, water is converted to oxygen and protons at the anode while H_2 is produced from protons (H^+) and electrons at the cathode.

Water splitting: $2H_2O \rightarrow O_2 + 2H_2$ (1.10)

At a high temperature, the water thermolysis reaction results in the production of H_2 and oxygen. This reaction is mediated in a single-stage decomposition process at temperatures greater than 2500 ºC (Steinfeld, 2005). Reducing the operational temperatures and improving the overall efficiency can be accomplished by employing multi-stage water splitting cycles (Abanades et al., 2008; Orhan et al., 2008). Orhan et al. (2008) reported a copper chlorine water splitting cycle to produce H_2 without producing CO_2 (Equations 1.11-1.14). This multi-stage cycle is operated under 550 ºC using nuclear energy as a heat source. .

$$2CuCl_2 + H_2O \rightarrow CuO * CuCl_2 + 2HCl \quad (1.11)$$

$$CuO * CuCl_2 \rightarrow 2CuCl + 0.5O_2 \quad (1.12)$$

$$4CuCl + H_2O \rightarrow 2CuCl_2 + 2Cu \quad (1.13)$$

$$2Cu + 2HCl \rightarrow 2CuCl + H_2 \quad (1.14)$$

The photo-electrolysis method is similar to electrolysis. However, the required temperature is derived from solar energy. Solar energy is absorbed by semiconductor materials to produce the temperature required for decomposing water. According to Wijayantha and Auty (2011), semiconductor materials can be used as the photoanode to

produce oxygen, while H_2 is produced at the cathode. Kothari et al. (2008) reported that the efficiency of photo-electrolysis process could be improved by using photocatalysts comprised of salts, semi-conductors, and dyes. The overall reaction is shown in Equation 1.15.

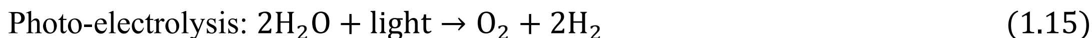


Photo-electrolysis: $2H_2O + light \rightarrow O_2 + 2H_2$ (1.15)

The H_2 yield can be improved; however, the cost is an issue which must be considered for developing this method. For example, for the thermochemical processes, the cost of biomass pyrolysis ranges from 1.25 US$·kg^{-1} to 2.20 US$·kg^{-1} (Ni et al., 2006). Further gasification steps can significantly increase the cost and prevent the production of gaseous by-products such as CO and hydrocarbons which can be processed to increase the H_2 yield (Nikolaidis and Poullikkas, 2017). Moreover, a benefit of photo-electrolysis water splitting is that pure H_2 gas can be produced from water (Steinfeld, 2005). However, the substantial input energy affects the economic feasibility and as a result, the process cannot compete with other large-scale H_2 production technologies.

1.2 Bio-hydrogen production

Over the past two decades, a substantial quantity of research has examined bio-H_2 production. The major advantage of bio-H_2 production is the utilization of renewable crops and waste biomass (Das and Veziroğlu, 2001). Biological H_2 production can be divided into three main methods: bio-photolysis (direct and indirect), photo-fermentation, and dark fermentation (Veeravalli et al., 2019; Kapdan, and Kargi, 2006).

1.2.1 Bio-photolysis

The bio-photolysis process uses similar principles as those utilized during photosynthesis in the green plants and algae to generate H_2. Green or blue algae can produce H_2 by splitting water molecules (Equation 1.16) (Veeravalli et al., 2019; Kapdan and Kargi, 2006). The hydrogenase enzyme used in this process is oxygen sensitive (Ni et al., 2006). According to Das and Veziroglu (2008), direct bio-photolysis with oxygen uptake employs a two-stage process to split water into H_2 and oxygen. Indirectly, the hydrogenase and nitrogenase enzymes are the two primary enzymes for H_2 production

(Equation 1.17-1.18) (Veeravalli et al., 2019). Indirect bio-photolysis is considered as an economical and environmentally friendly process which consumes water and carbon dioxide (Nikolaidis and Poullikkas, 2017). However, this method cannot utilize waste as the feedstock and the large area requirement for cultivating is a limiting factor (Holladay et al., 2009).

Direct bio-photolysis: $2H_2O + Light \rightarrow O_2 + 2H_2$ (1.16)

Indirect bio-photolysis: $12H_2O + 6CO_2 + Light \rightarrow C_6H_{12}O_6 + 6O_2$ (1.17)

$12H_2O + C_6H_{12}O_6 + Light \rightarrow 6CO_2 + 12H_2$ (1.18)

1.2.2 Photo-fermentation

The second method is the employing photo-fermentation to produce H_2 utilizing solar energy and organic acids. For example, when acetic acid is the electron donor, photosynthetic bacteria produce H_2 in the presence of the nitrogenase enzyme (Equation 1.19) (Das and Veziroglu, 2008). Although H_2 production utilizes water and CO_2, the low H_2 yield, low conversion rate, high lighting requirement, and large surface area are important factors affecting the development of this method (Holladay et al., 2009; Kapdan and Kargi, 2006).

Photo-fermentation: $CH_3COOH + 2H_2O + Light \rightarrow 2CO_2 + 4H_2$ (1.19)

1.2.3 Dark fermentation

In the dark fermentation process, a variety of carbohydrates are used as feedstock chemicals. Carbohydrate containing substrates can be obtained from industrial effluents, agriculture wastes, and municipal wastes (Azwar et al., 2014). When glucose is the model substrate and acetic acid as the end-product, the theoretical H_2 yield by the dark fermentation process is 4 mol H_2 mol^{-1} glucose (Equation 1.20). Other advantages include faster microorganism growth rate and high H_2 production rates when compared to the light-dependent methods (Nath and Das, 2004; Tanisho and Ishiwata, 1995). According to Holladay et al. (2009), the H_2 synthesis rate for dark fermentation is larger when compared to other processes (see Table 1.2).

Table 1.2: H_2 synthesis rate for biohydrogen production methods

Methods	H_2 production rate (mmol H_2 (l h)$^{-1}$)
Direct bio-photolysis	0.07
Indirect bio-photolysis	0.355
Dark fermentation	8.2-12.1
Light fermentation	0.16

Holladay et al. (2009)

When glucose ($C_6H_{12}O_6$) is the preferred substrate, the fernentation process is uneconomical and cannot be utilized for large-scale production. Therefore, an alternative substrate is critical to improving bio-H_2 production using the dark fermentation process.

Dark fermentation: $2H_2O + C_6H_{12}O_6 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (1.20)

1.3 Glycerol as a feedstock for biohydrogen production

Glycerol ($C_3H_8O_3$) is waste byproduct from biodiesel production. During the biodiesel production process, vegetable oil or animal fats are combined with EtOH or methanol to biodiesel and glycerol via a transesterification rection catalyzed by NaOH or KOH (Mu et al., 2009). Glycerol is the main by-product from biodiesel production and approximately a ten-fold on a volume basis of biodiesel produces one volume of glycerol (Selembo et al., 2009). Glycerol cannot be directly released or disposed into the environment after the biodiesel production because of the pollution impact. From 2015 to 2016, biodiesel production increased 20%, from approximately 4,800 million liters to 5,950 million liters, in the United States of America (EIA-22M, 2017). The global glycerol production increased from approximately 1.0 to 1.5 million metric tons from 2007 to 2011 (Quispe et al., 2013). Also, the cost of crude glycerol was 0.17 US$·kg^{-1} in 2019 (da Silva Ruy et al., 2020).

According to Sarma et al. (2012), many microbes such as anaerobes can utilize glycerol for H_2 production. When compared to cellulosic waste materials, pure glycerol is not pretreated before using as a feedstock for anaerobic H_2-producing microorganism. Glycerol is a renewable carbon source that is primarily produced from biodiesel

production (Trchounian and Trchounian, 2015; Khanna et al., 2012). Theoretically, one mol of glycerol can produce 7 mol H_2 (Equation 1.21). When acetate acid is the only by-product, the yield is reduced to 3 mol H_2 mol^{-1} glycerol (Equation 1.22). Glycerol can also produce other valuable byproducts such as EtOH and 1,3-propanediol (1,3-PDO) when utilizing pure cultures (Biebl et al., 1999). Glycerol is a low-cost feedstock and can be an economical and competitive substrate for bio-H_2 production by dark fermentation.

$$C_3H_8O_3 + 3H_2O \rightarrow 7H_2 + 3CO_2 \quad (1.21)$$

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \quad (1.22)$$

1.4 Objectives

The objective of this study is to determine the conditions for maximizing H_2 production from glycerol degradation by mixed anaerobic cultures. To accomplish the primary objective, this study is divided into the following sub-objectives:

1) The objective for the work in chapter 4 was to investigate the effect of initial pH on H_2 production as well as the effects on metabolites during the dark fermentation of glycerol in anaerobic mixed cultures.

2) The objective for the work in chapter 5 was to compare the effects of adding inhibitors on H_2 production from glycerol degradation via dark fermentation in mixed cultures at 37 ºC and with the optimum pH obtained from objective 1.

3) The objective for the work in chapter 6 was to employ a Box-Behnken design (BBD) to optimize the H_2 yield from glycerol degradation using inhibited anaerobic microbial cultures.

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Chapter 2: LITERATURE REVIEW

Increasing population growth, urbanization, and energy demand are global economic development factors. Using fossil fuels to drive economic development has caused serious environmental problems such as global warming because of CO_2 emissions. According to O'Neill et al. (2010), by 2050, CO_2 emissions must be reduced by approximately 16-29% as a means of avoiding climate change. Climate change has become a global issue and researchers are investigating the development of energy producing technologies from renewable energy sources.

Unlike non-renewable energy sources, biohydrogen (bio-H_2) is carbon neutral with water as the final by-product during combustion (Meher Kotay, and Das, 2008). Hydrogen production through dark fermentation has been widely studied as a reliable technology with benefits such as easy operation, less expensive, and abundant sources of biomass feedstocks (Hallenbeck et al., 2009).

Utilizing biomass to produce fuels is classified into different categories. First-generation fuel crops include sugar cane, corn, and sugar beets, while second-generation biomass includes agricultural wastes as well as industrial wastewaters (Das and Veziroglu, 2008). Algae, a third-generation feedstock, can be utilized to produce fuels such as biogas, bio-oils, EtOH, H_2, and biodiesel (Behera et al., 2015).

Glycerol, a low-value byproduct, is produced from biodiesel manufacture. This chemical can be employed to produce H_2 and hence, reduce the cost of H_2 production. However, many factors affecting H_2 production by the dark fermentative process can impact the theoretical yield. These factors include inoculum type, substrates, feedstock pre-treatment methods, inoculum pretreatment, environmental conditions, and microbial inhibitors (Toledo-Alarcón et al., 2018; Bundhoo et al., 2015; Wang and Wan, 2009; Li and Fang, 2007).

2.1 Microbial anaerobic degradation

Anaerobic degradation of carbohydrates, proteins and fats is a complex process which is mediated by many different groups of bacteria in the absence of oxygen. In the

final methanogenic stage of the anaerobic degradation process, the electron equivalences are diverted to methane (CH_4) production. The anaerobic degradation process includes the following stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 2.1).

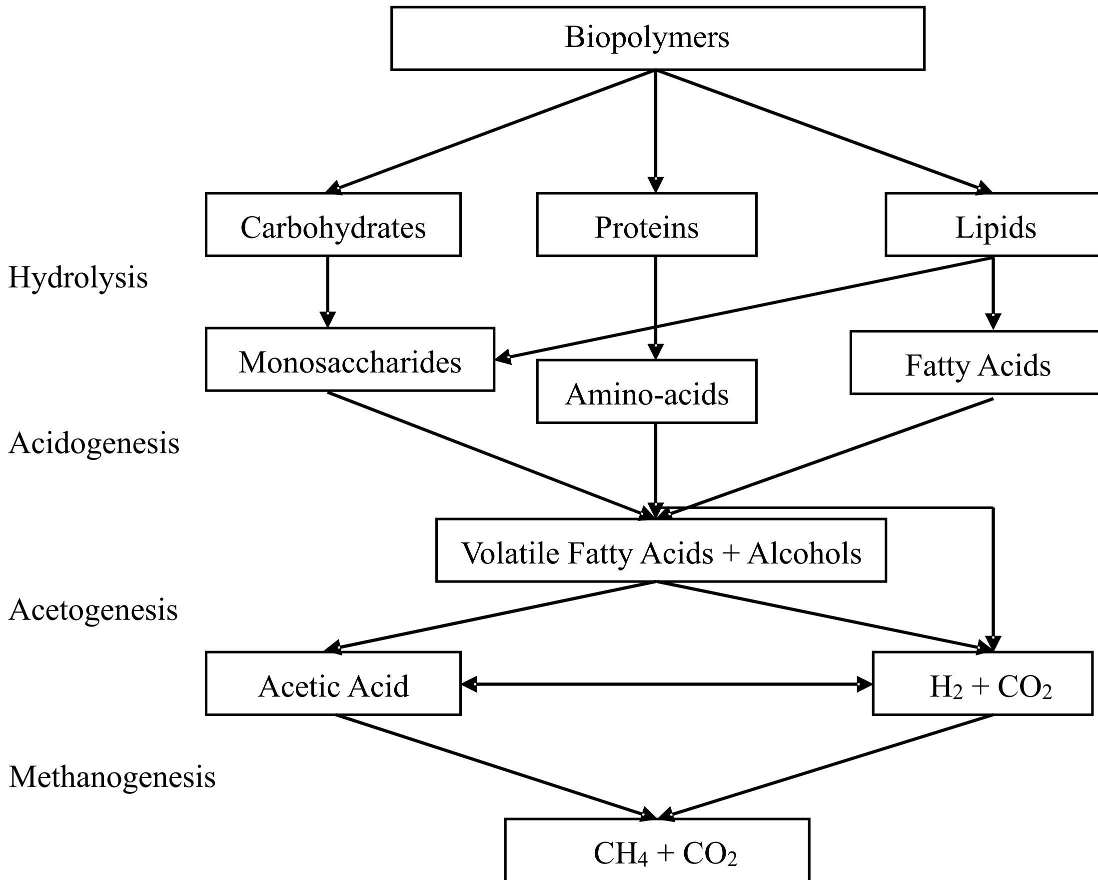


Figure 2.1: Schematic illustration of the anaerobic degradation stages and pathways (Peiris et al., 2006)

2.1.1 Hydrolysis

During the hydrolysis stage, complex polymers are converted into monomers. During hydrolysis, water is used to cleave the ether linkage between two sugar molecules. According to Jordan and Mullen (2007), the hydrolysis rate is dependent on factors such as the hydrophobic components, the particle size, the pH, the temperature, and the composition of the enzymes (hydrolases, amylases, proteases, and lipases). The reaction is mediated by obligate or facultative anaerobes such as *Enterobacterium*

(Gerardi, 2003).

2.1.2 Acidogenesis

During acidogenesis, acidifying bacteria convert the products of hydrolysis, such as sugars, amino acids, and fatty acids, into volatile fatty acids (VFAs), alcohols, and CO_2 as well as H_2. Typical VFAs produce in this stage shown in Table 2.1. Acidogenesis is mediated by common anaerobes such as *Clostridium*, *Pseudomonas*, *Bacillus*, *Enterobacterium*, *Micrococcus* and *Flavobacterium* (Ziemiński and Frąc, 2012).

Table 2.1: Major VFAs produced through the acidogenesis process

Name	Formula
Acetic$^-$ (Ac^-)	CH_3COO^-
Butyric$^-$ (Bu^-)	$CH_3(CH_2)_2CH_2O^-$
Formic$^-$ (For^-)	$HCOO^-$
Lactic$^-$ (La^-)	$CH_3CHOHCOO^-$
Propionic$^-$ (Pr^-)	$CH_3CH_2COO^-$

Gerardi (2003)

2.1.3 Acetogenesis

In the acetogenesis stage, bacteria convert organic acids from the acidification phase into acetate (Ac^-), H_2, and CO_2. Acetate can be produced not only by organic molecules having more than two carbons but also by the reduction of CO_2 with H_2 (Gerardi, 2003). The accumulation of Ac^- lowers the pH level and enhances the H_2 yield (Denac, Miguel and Dunn, 1988). However, increasing the H_2 partial pressure or lowering the pH level can inhibit H_2 production and subsequently, lead to the production of alcohols (Kim et al., 2004; Mara and Horan, 2003). The syntrophic association between H_2-consuming bacteria and H_2-producing bacteria can maintain low H_2 partial pressures (< 10 Pa) and hence, create thermodynamically favorable conditions for acetogenic reactions (Schink, 1997).

2.1.4 Methanogenesis

During the final stage of anaerobic digestion, methanogenic bacteria produce CH_4 from Ac^- and from the reduction of CO_2 by H_2. Hydrogenotrophic methanogens and acetoclastic methanogens are two bacteria responsible for producing CH_4. Aceticlastic

methanogens are capable of producing CH_4 from acetic acid (Demirel and Scherer, 2008). According to Ziemiński and Frąc (2012), approximately 30% of the CH_4 is produced from the consumption of CO_2 and H_2 by hydrogenotrophic methanogens (Figure 2.1).

2.2 H_2 production through dark fermentation

Fermentative bio-H_2 production is attractive because this method can sequentially degrade complex organic wastes into H_2 and volatile fatty acids (VFAs) plus short chain alcohols (Lee et al., 2009). The mechanism for fermentative bio-H_2 production was developed from pure-culture studies. A simplified illustration of glucose metabolism is shown in Figure 2.2.

A metabolic network that includes the conversion of a sugar to H_2, CO_2, fatty acids, and solvents is shown in Figure 2.2. Hydrogen is produced from pyruvate ($C_3H_4O_3$) decarboxylation and formate (CH_2O_2) (For^-) cleavage. During glycolysis, pyruvate is produced from the degradation of hexose sugars such as glucose. One mole of glucose produces two moles of pyruvate during glycolysis. Glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate are produced during glycolysis (not shown in Figure 2.2). During the conversion of pyruvate decarboxylation to acetyl-CoA, electrons are transferred from pyruvate to ferredoxin (Fd) and then protons (H^+) are reduced to produce H_2 gas. In this step, ferredoxin is an important electron carrier with two different valences. Reduced ferredoxin (Fd^{red}) transfers electrons to the hydrogenase enzyme which uses protons as an electron acceptor. This process releases the re-oxidized ferredoxin (Fd^{ox}) and H_2 (Saint-Amans et al., 2001). Reduced nicotinamide adenine dinucleotide (NADH) also contributes to the formation of H_2. This process results in releasing the oxidized form of NAD^+ by the catalysis of NADH-ferredoxin oxidoreductase. Equations 2.1, 2.2, and 2.3 show that the NADH-ferredoxin oxidoreductase plays a key role in the equilibration of electrons between NAD^+ and Fd^{ox} (Hallenbeck and Benemann, 2002). In the second H_2 production route, pyruvate is degraded to For^- by the pyruvate formate lyase enzyme (Equation 2.4). In a subsequent reaction, For^- is split into H_2 and CO_2 by formate hydrogenase (Equation 2.5).

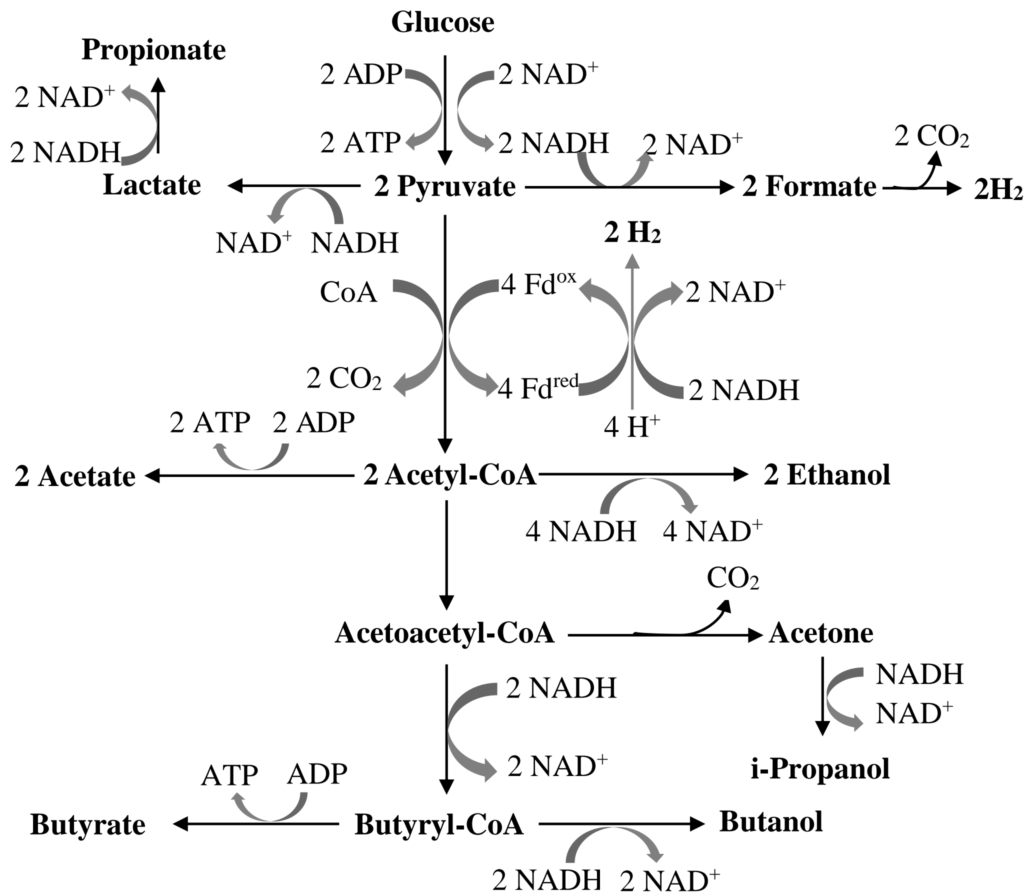


Figure 2.2: Metabolic pathway in glucose fermentation (adapted and modified from Jones and Woods, 1986; Ray et al., 2010)

$$\text{Pyruvate} + 2\text{Fd}^{\text{ox}} + \text{CoA} \leftrightarrow \text{Acetyl} - \text{CoA} + \text{CO}_2 + 2\text{Fd}^{\text{red}} \quad (2.1)$$

$$2\text{NADH} \leftrightarrow 2\text{NAD}^+ + 2\text{H}^+ + 4\text{e}^- \quad (2.2)$$

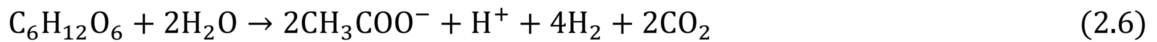
$$2\text{Fd}^{\text{red}} + 2\text{H}^+ \leftrightarrow 2\text{Fd}^{\text{ox}} + \text{H}_2 \quad (2.3)$$

$$\text{Pyruvate} + \text{CoA} \leftrightarrow \text{Acetyl} - \text{CoA} + \text{Formate} \quad (2.4)$$

$$\text{Formate} + \text{H}^+ \leftrightarrow \text{H}_2 + \text{CO}_2 \quad (2.5)$$

Various gaseous metabolites include H_2, CO_2, and CH_4, while the soluble metabolites include acetate (Ac^-), butyrate (But^-), lactate (La^-), propionate (Pr^-), EtOH, and butanol (ButOH) (Tao et al., 2007). The stoichiometric reactions shown in Equations 2.6 and 2.7 indicate the theoretical maximum H_2 yield from glucose metabolism with different reduced carbon byproducts. When Ac^- is the only by-product, the maximum H_2

yield is 4 mol H_2 mol^{-1} glucose; however, 2 mol H_2 per mol of glucose is produced when But^- is the only end product. Although the maximum yield of 4 mol H_2 is theoretical, the dark fermentation process does not produce only one acid or alcohol. According to Van Ginkel and Logan (2005), a high H_2 yield of 2.8 mol H_2 mol^{-1} glucose can be attained when Ac^- and But^- are the main metabolites. Ray et al. (2010) reported a higher yield of 3.38 mol H_2 mol^{-1} glucose in the presence of linoleic acid (LA). However, the low H_2 yield is related to the production of other end products, such as EtOH, ButOH, La^-, and Pr^-. The diversion of electron equivalents from the substrate to the various reduced metabolites is dependent on factors such as culture type, pH, temperature, and inhibitors.

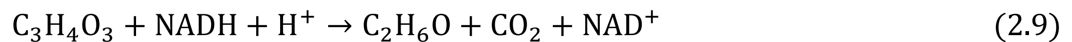


$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 4H_2 + 2CO_2 \quad (2.6)$$

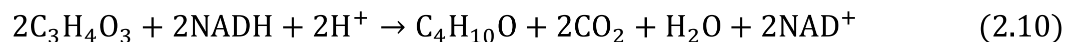
$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + H^+ + 2H_2 + 2CO_2 \quad (2.7)$$

The electron equivalences generated from the regeneration of NAD^+ are transferred into non-H_2 producing end products, such as HLa ($C_3H_6O_3$), EtOH (C_2H_6O), and ButOH ($C_4H_{10}O$) (Equations 2.8 to 2.10). As shown in Figure 2.2, non-H_2 producing end products are essential for consuming acetyl-CoA and balancing the NADH produced through glucose glycolysis. The concentration of acetyl-CoA and NADH can influence the reduction of H^+ ions into H_2 (Lee et al., 2011). Theoretically, several non-H_2 producing end products can be converted into Ac^- to elevate the H_2 yield during the acetogenesis stage if the fermentation process is controlled by a low H_2 partial pressure. According to Junghare et al. (2012), H_2 production increased from 26.66 to 69.65 mmol L^{-1} when H_2 partial pressure decreased from 33.90 to 10.12 KPa. The metabolites distribution and end-product conversion are dependent on many factors which will be discussed in Section 2.6.

$$C_3H_3O_3^- + NADH + H^+ \rightarrow C_3H_5O_3^- + NAD^+ \quad (2.8)$$



$$C_3H_4O_3 + NADH + H^+ \rightarrow C_2H_6O + CO_2 + NAD^+ \quad (2.9)$$



$$2C_3H_4O_3 + 2NADH + 2H^+ \rightarrow C_4H_{10}O + 2CO_2 + H_2O + 2NAD^+ \quad (2.10)$$

2.3 H_2 production from glycerol degradation through dark fermentation

The conversion of glycerol to H_2 by dark fermentation is attractive because this process produces not only H_2 but also other valuable by-products such as EtOH and 1,3-PDO (Nakashimada et al., 2009). The fermentative metabolic mechanism of H_2 production from glycerol degradation has been studied with pure cultures using *Escherichia coli* (*E. coli*) BW25113 *frd*C (Hu and Wood, 2010), wide-type *E. coli* strains (Murarka et al., 2008), *Enterobacter aerogenes* (Ito et al., 2005), *Klebsiella pneumoniae* (Liu and Fang, 2007), and *Thermotoga maritima* (Maru et al., 2012). Mixed cultures are easier to obtain, they can utilize non-sterile feedstocks and the low cost to prepare and maintain are major advantages when compared to pure cultures. The optimum reaction condition is variable for the different cultures because of the variable composition of mixed anaerobic microbial cultures. Selembo et al. (2009) reported that fermentative H_2 production from glycerol indicated the H_2 yield was lower than the yield from glucose fermentation when using the same mixed cultures. Theoretically, glycerol can produce more H_2 than glucose if glycerol fermentation has the same initial intermediate (glyceraldehyde-3-phosphate) and end products, such as Ac^- and For^- (Selembo et al., 2009). An overview of metabolic pathways from the glycerol fermentation to the H_2 is shown in Figure 2.3.

Zhu et al. (2002) reported that glycerol fermentation proceeds by the oxidative and reductive degradation by *Klebsiella*, *Citrobacter*, *Clostridium* and *Enterobacter*. As shown in Figure 2.3, glycerol is oxidized to dihydroxyacetone (DHA) through the catalysis caused by a NAD^+-dependent enzyme (glycerol dehydrogenase). Dihydroxyacetone kinase phosphorylates DHA to dihydroxyacetone-phosphate (DHAP), and the latter product proceeds to further degradation. In the reduction pathway, glycerol is converted to 3-hydroxypropionaldehyde and subsequently to 1,3-PDO by the catalysis of the B_{12}-dependent coenzyme (glycerol dehydratase) and the NADH-dependent enzyme (1,3-propanediol dehydrogenase) (Seifert et al., 2001; Forage and Lin, 1982; Toraya et al., 1980). Wang et al. (2001) reported that glycerol could be converted to either glycerol-3-phosphate or DHA intermediates in *S. cerevisiae* and yeasts. In subsequent steps, phosphoenolpyruvate or pyruvate degrades via different pathways (Figure 2.3).

When succinic acid is the end-product, phosphoenolpyruvate is degraded by consuming NADH and CO_2. After DHAP is produced, the pathway proceeds to pyruvate which degrades to one or more metabolites, such as Ac^-, But^-, For^-, and EtOH. As shown in Figure 2.3, the pathways for glycerol fermentation are similar to the pathways of glucose fermentation. The NADH-ferredoxin oxidoreduction system and formic acid (HFor) cleavage are the two major H_2 production processes. Therefore, understanding the glycerol dark fermentation pathways can aid researchers to improve the H_2 yield by inhibiting different pathways.

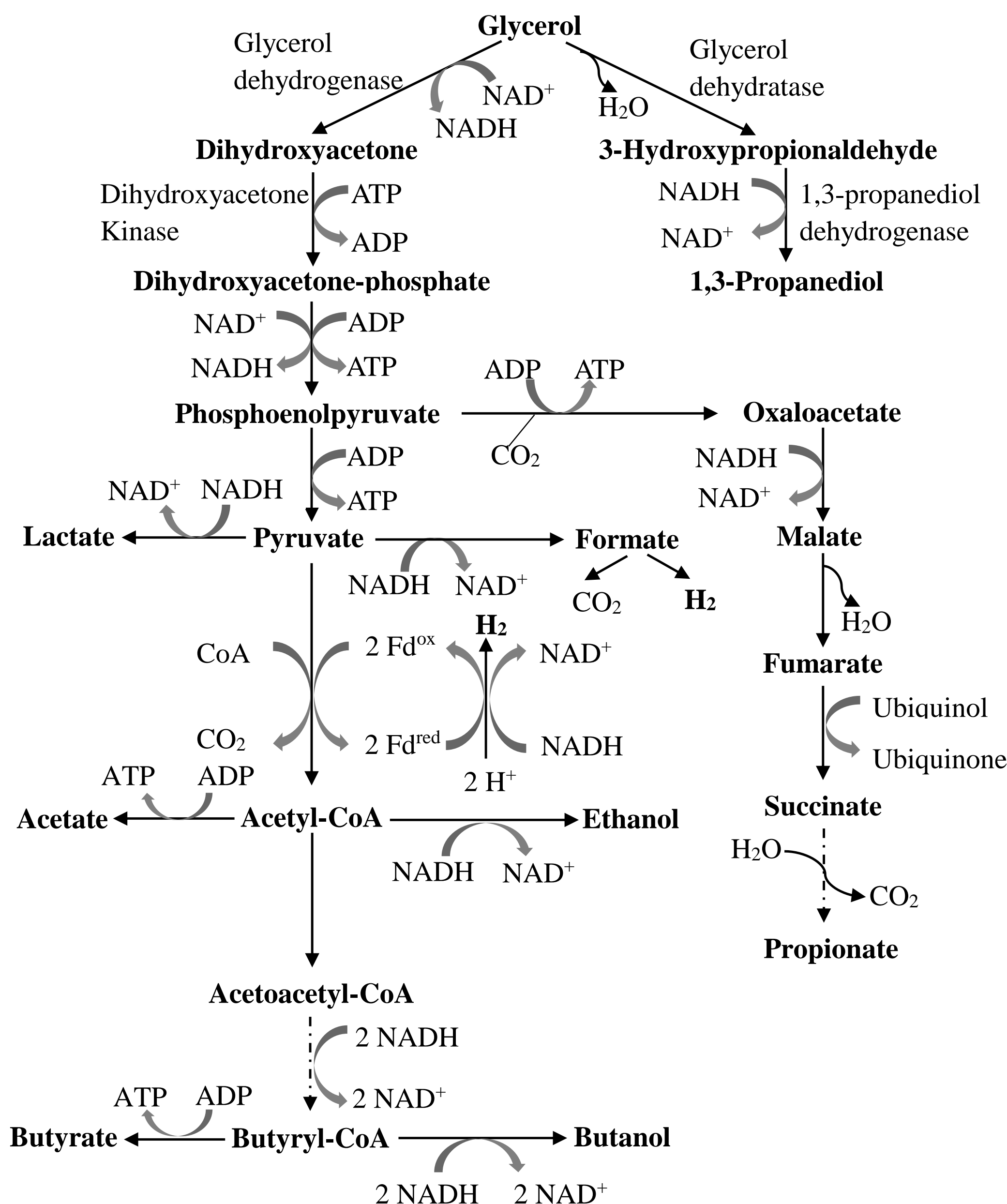


Figure 2.3: Overview of possible metabolic pathways and end products in mixed cultures through glycerol fermentation (Maru et al., 2016; Silva et al., 2009; Bouvet et al., 1995)

2.4 Inocula source

2.4.1 H_2 producing microorganism

Bio-H_2 can be produced by anaerobes, facultative anaerobes, obligate aerobes, methylotrophs, and photosynthetic bacteria. A variety of bacteria species belonging to obligate anaerobe or facultative anaerobes mediate pathways in fermentative bio-H_2

production. *Clostridiums* are strict anaerobes which can utilize many carbohydrates, such as arabinose, fructose, xylose, and glucose (Nandi and Sengupta, 1998). Hawkes et al. (2002) reported that *Clostridium* could survive and adapt to various environmental conditions, even in high temperatures or acidic conditions. *Clostridiaceae*, among which *Clostridium butyricum*, *Clostridium pasteurianum*, and *Clostridium beijerinckii* are the most active species employed for fermentative bio-H_2 production (Pugazhendhi et al., 2019). Hydrogen yields ranging from 1.1 to 2.6 mol H_2 mol^{-1} hexose is dependent on the various conditions (Lee et al., 2011). *Clostridium butyricum* is able to produce 1,3-PDO without producing H_2 by utilizing glycerol (Saint-Amans et all., 1994). *Methylotrophs*, such as *Methylomonas albus* (*M. albus*) can produce H_2 under anaerobic conditions with different substrates such as methanol, For^-, and pyruvate (Kawamura et al., 1983).

Facultative anaerobes such as *E. coli* and *Enterobacter* have been studied for producing H_2 (Nandi and Sengupta, 1998). When facultative anaerobes are exposed to low oxygen levels, they can consume oxygen and then produce H_2 (Nath and Das, 2004). *E. coli* is a commonly used host organism for metabolic engineering and can be genetically modified to improve H_2 yields (Maeda et al., 2007). *E. coli* is able to decompose carbohydrates (glucose) with a high H_2 yield via dark fermentative, but the low growth rate and H_2 yield have been reported when glycerol is the feedstock (Maru et al., 2016). *Enterobacter* is able to produce from 20% to 80% of the theoretical H_2 yield by degrading substrates such as glucose, sucrose, and cellobiose. A maximum yield of 2.2 mol H_2 mol^{-1} glucose was reported when the initial pH was 6.0 and temperature was 36℃ (Kumar and Das, 2000). However, according to Maru et al. (2016), the H_2 yield was 0.61 and 0.37 mole per mole of glycerol was consumed for *E. coli* and *Enterobacter cloacae*, respectively, at a pH level of 6.7 and a temperature of 120℃. Understanding strict anaerobes and facultative microorganisms can aid in selecting microbial cultures for bio-H_2 production. Also, understanding the dominant by-products from mixed cultures through glycerol fermentation can aid in explaining the microbial pathway.

2.4.2 Mixed cultures versus pure H_2-producing bacteria

Mixed cultures contain different bacteria species. Mixed cultures can also be

produced by combining two or more pure cultures. Bio-H_2 production using mixed cultures, and the analysis of diverting electron equivalencies from a hexose feedstock to H_2 has been reported by Chaganti et al. (2011) and Ray et al. (2010). Although selected pure strains can generate higher H_2 yields when compared to mixed cultures, mixed cultures are easy to maintain because feedstock sterilization is not necessary (Das, 2009). Another advantage of using mixed cultures is that facultative anaerobes can consume dissolved oxygen to create anaerobic conditions for strict anaerobes. Also, various organic wastes can be used as feedstocks for mixed anaerobic cultures (Antonopoulou et al., 2007). In mixed cultures, interaction between different species can increase the efficiency of degrading complex substrates to H_2 (Hung et al., 2011). When compared to facultative H_2 producers (*E. coli* or *Enterobacter*), Maru et al. (2016) reported that the H_2 yield increased from 0.61 to 1.26 mol H_2 mol^{-1} glycerol when using a co-culture of *E. coli* and *Enterobacter*. Furthermore, Selembo et al. (2009) reported that 0.28 mol H_2 mol^{-1} glycerol and 0.69 mol 1,3-PDO mol $glycerol^{-1}$ were produced from a mixture of four different microorganisms via the dark fermentation process.

The main disadvantage of using mixed cultures is that the mixed cultures contain H_2 consuming populations such as hydrogenotrophic methanogens, sulfate-reducing bacteria, and homoacetogens (Cord-Ruwisch et al., 1988). Including H_2 consumers in the mixed cultures (equation 2.11 to 2.13) lead to a reduction in H_2 production (Hung et al., 2011; Ray et al., 2008). In addition, other H_2 consuming bacteria such as propionate-producing or lactate-producing bacteria leads to a decrease in the H_2 yield.

$$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O \quad (2.11)$$

$$SO_4^{-2} + H^+ + 4H_2 \rightarrow HS^- + 4H_2O \quad (2.12)$$

$$2HCO_3^- + 2H^+ + 4H_2 \rightarrow CH_3COOH + 4H_2O \quad (2.13)$$

2.5 Microorganism enrichment

In order to enhance the H_2 yield, numerous pretreatment methods have been employed to eliminate H_2 consumers in mixed cultures. Pretreatment studies by Wang and Yin (2017) include heat treatment, acid or base treatment, and chemical inhibition, as

well as other treatments methods such as UV radiation, microwave, ultrasound, and freezing. The different treatment methods are described in the following sections.

2.5.1 Heat treatment

Heat treatment is a widely applied method used to improve the H_2 yield when employing mixed cultures. Mixed cultures can be classified into the following categories: ambient-temperature (20-25°C), mesophilic (32-40°C), thermophilic (40-65°C), and hyper-thermophilic (>70°C) (Li and Fang, 2007). Inocula used for fermentative H_2 production are heat treated to inhibit the H_2 consuming bacterial populations (Ginkel et al., 2001). Depending on the bacteria, high temperatures destroy the cell walls and membranes leading to the inactivation of the bacteria (Appels et al., 2008). However, some bacteria can survive high-temperature conditions because they can form spores to resist heat treatment. Hence, heat treatment can be used to destroy non-spore-forming bacteria, and the remaining spore forming H_2 producer contributes to H_2 production. Oh et al. (2003) noted that methanogens, a non-spore forming microorganism, was killed by heat treatment. Nevertheless, non-spore-forming bacteria are not equivalent to all H_2 consumers. For example, H_2 consumers, including homo-acetogens and propionate-producing bacteria can survive heat treatment (Hussy et al., 2003; Oh et al., 2003). Moreover, heat treatment also inhibits H_2 producing microorganisms such as *Enterobacter*, *Bacillus*, and cellulose-degrading microbes (Wang and Yin, 2017).

Temperature and heating time are two major parameters employed to optimize heat treatment in mixed cultures. Wang and Yin (2017) concluded that temperatures ranging from 65 to 121 °C and the heating duration varying from 10 min to 10 hours are effective in inhibiting anaerobic cultures. These researchers reported that 100 °C and 60 min were commonly used to treat mixed cultures. According to Lay et al. (2011), heat treatment was employed at various temperatures (60-97°C) and treatment durations (20-60 min). These researchers also reported a maximum H_2 production rate was obtained when mixed cultures treated at 70°C for 50 min. A H_2 yield of 2.19 mol H_2 mol^{-1} hexose was reported by preheating an anaerobic mixed sludge at 100 °C for 60 min (De Sá et al., 2013).

Although heat treatment is a simple and widely used method to improve the H_2 yield, there are several disadvantages for enriching anaerobic microbial cultures. The main disadvantage is that treatment not only destroys H_2 consuming bacteria, but also eliminate H_2 producing bacteria, which are unable to form spore. Duangmanee et al. (2007) reported that a lag phase was observed in the initiation of H_2 production by using a continuous flow reactor. Continuously heating treatment is not a cost-effective method for a large-scale system because of the required energy to maintain the temperature of the bioreactor and feedstock sterilization.

2.5.2 Acid and alkali treatment

Acidic condition, acid or alkali treatments are widely used pretreatment methods employed to inhibit H_2 consumers (Fang and Liu, 2002). The growth rate of bacteria is affected with changing pH levels. Most bacteria, such as *E. coli*, are neutrophil, which means the optimal growth pH level is neutral. In contrast, acidophiles and alkaliphiles are microorganisms that grow optimally when pH level is less than 5.5 or greater than 8.0, respectively. For acid treatment, the pH range is from 2 to 4 and a treatment time from 30 min to 24 hours. In comparison, pH levels between 10 and 12 are employed for alkali treatment (Wang and Yin, 2017). According to Fang and Liu (2002), increasing the glucose degradation rate is related to low pH levels from 4.0 to 5.5. Chang et al. (2011) observed a high H_2 yield with mixed cultures using acid treatment; however, Demirel et al. (2010) and Yin et al. (2014) reported that increasing H_2 production was reported with alkaline treatment. This difference in results could be due to the different compositions in mixed cultures. Also, this is the main concern when using acid or alkaline treatment for the enrichment of microorganisms to enhance H_2 production.

2.5.3 Chemical inhibition

Chemical treatment is process which can selectively inhibit H_2 consumers. For example, bromoethane sulphonic acid (BESA), chloroform, and iodopropane are methanogenic inhibitors (Chidthaisong and Conrad, 2000; Mohan et al., 2008; Zhu and Béland, 2006). Long-chain fatty acids (LCFAs) are inhibitors which can be anaerobically degraded to shorter chain LCFA (Lalman and Bagley, 2001). Chloroform or methyl

chlorinated compounds can inhibit methanogens (Hu and Chen, 2007; Oremland and Capone, 1988). Chloroform blocks corrinoid enzymes, which control CH_4 formation (Wang et al., 2017; Oremland and Capone, 1988). Zhu and Béland (2006) observed a H_2 yield of 2.82 mol H_2 mol^{-1} hexose by employing iodopropane. BESA is a well-known inhibitor which binds to the methyl coenzyme, a critical enzyme for CH_4 formation through methanogenesis (Ermler et al., 1997). As shown in Figure 2.4, CH_4 is produced by the CO_2 reduction or the aceticlastic pathways.

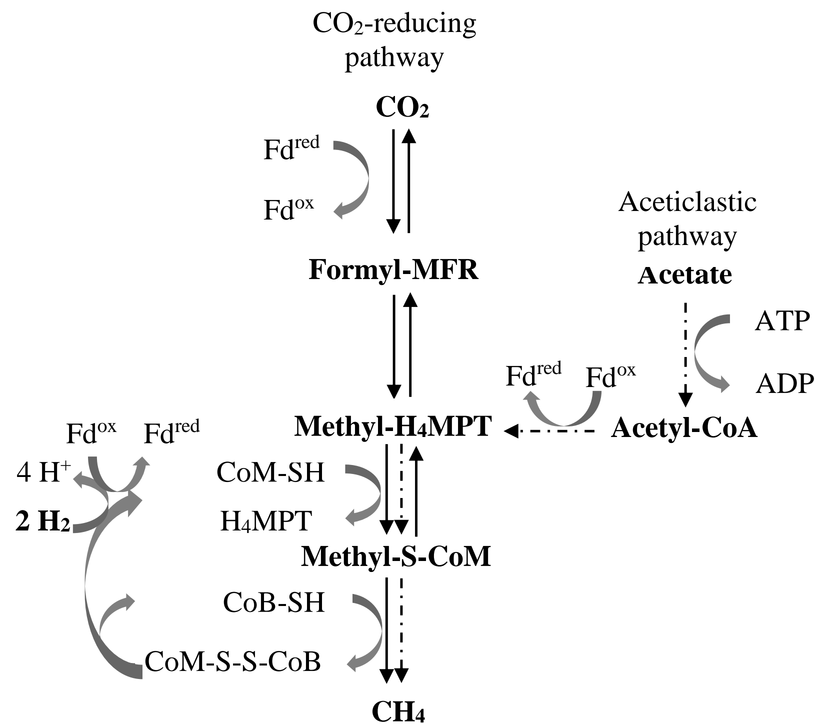


Figure 2.4: Two enzymatic pathways of methanogenesis (adapted and modified from Lyu et al., 2018).

Note: The solid line indicates CO_2-reducing pathway and the dash-dot line indicates aceticlastic pathway; (Abbreviations: MFR = methanofuran; H_4MPT = tetrahydromethanopterin; CoM = coenzyme M; CoM-S-S-CoB = heterodisulfide coenzyme M with coenzyme B; Fd^{red} = reduced form of ferredoxin; Fd^{ox} = oxidized form of ferredoxin)

According to Zhu and Béland (2006), 1 mmol of BESA can inhibit the aceticlastic pathway and 50 mmol of BESA can prevent the H_2 reduction and consumption. However, BESA has been reported as a toxic inhibitor to H_2 producing bacteria (Kotsopoulos et al., 2006). This inhibitor can lead to contamination when the effluents from anaerobic

bioreactors are discharged to receiving water bodies (Cheong and Hansen, 2006).

Long-chain fatty acids (LCFAs) are classified as saturated and unsaturated fatty acids contain both hydrophobic and hydrophilic groups. Unsaturated LCFAs contain one or more carbon-carbon unsaturated double bonds. In comparison, saturated LCFAs contain only carbon-carbon single bonds. Unsaturated fatty acids with one double bond are mono-unsaturated fatty acids, while poly-unsaturated fatty acids contain two or more double bonds.

LCFAs which are available include lauric acid (LUA, C12:0), myristic acid (MA, C14:0), palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1), and linoleic acid (LA, C18:2). Currently, most fatty acids are found in animal lipids and vegetable or seed oils. According to Nieman (1954), gram-positive bacteria, such as methanogens, homo-acetogens, clostridium, and sulfate-reducing bacteria, can be inhibited by unsaturated fatty acids. This study concluded that the increasing number of double bonds resulted in increasing inhibition. For example, linoleic acid (LA) with two double bonds, has a much larger inhibitory impact on different anaerobic microorganisms when compared to SA (Ray et al., 2008; Lalman and Bagley, 2000).

LCFAs are biodegradable inhibitors which are converted into shorter chain LCFAs, acetic acid, and H_2. The degradation of LCFAs proceeds by α-oxidation, β-oxidation, and ω-oxidation; however, β-oxidation is the main degradation mechanism (Batstone, 2000). Note α-oxidation proceeds under aerobic conditions (Nieman, 1954).

According to Lalman and Bagley (2000) and Saady (2011), LCFA toxicity is dependent on the concentration and the LCFA chemical structure. Also, a combination of various LCFAs is more toxic than a single LCFA. Koster and Cramer (1987) reported that a mixture of myristic, capric, and lauric acid was more toxic than each of the individual acid. LCFA can cause two negative effects during the operation of an anaerobic bioreactor. The first is that using LCFAs as an inhibitor can delay the substrate degradation, while the other is that the LCFAs inhibition can lead to biomass flotation. Angelidaki and Ahring (1992) observed a lag phase during VFAs degradation by adding LCFAs. Cho et al. (2013) reported a four-day lag when CH_4 was produced in the presence

of LCFA. In comparison, a three-day lag was observed by using a lipid-free food waste. Alosta et al. (2004) reported that the glucose degradation rate and the byproducts production rate were decreased when the concentrations of LCFAs were greater than 300 mg L^{-1}. In work by Alosta et al. (2004), Bu^- was not observed when using OA and SA; however, Bu^- was observed when anaerobic mixed cultures were inhibited by LA with a concentration greater than 300 mg L^{-1}.

Although using LCFAs as inhibitors in fermentative H_2 production has negative impacts, the advantages of the LCFA inhibition are significant. LCFAs can be obtained from economical sources when compared to synthetic microbial inhibitors. Animal fats, vegetable oils, fish oils, and seed oils are commonly available sources for LCFAs production. For example, safflower oil and corn oil are LA rich raw materials from fatty acids extraction (Sonntag, 1979). This advantage highlights the potential benefits of using LCFAs in large-scale systems. Additionally, LCFAs have been studied as the methanogenic inhibitor in H_2 production with less environmental pollution because of their biodegradability. According to Ray et al. (2010), experiments conducted with LA as a methanogenic inhibitor diverted electron fluxes from CH_4 production to H_2 production. A maximum H_2 yield of 1.3 mol and 2.4 mol H_2 mol^{-1} glucose was observed when pH values were 7.8 and 5.0 respectively (Ray et al., 2008). Furthermore, Chaganti et al. (2013) reported a maximum H_2 yield of 2.89 mol H_2 mol^{-1} glucose which was 73% of the theoretical maximum H_2 yield when HAc was an assumed end product through dark fermentation.

2.5.4 Other enrichment treatments

Aeration can be used as an inhibition treatment for methanogens (strict anaerobes) because oxygen leads to the inactivity of methanogens, while H_2 producing bacteria such as *Enterobacter* are facultative bacteria. Hence, aeration treatment can assist in enriching H_2 producers. However, the growth of strict anaerobic H_2 producers such as *Clostridium* are suppressed by aeration treatment. Ren et al. (2008) used dissolved oxygen (DO) to repetitively aerate mixed cultures to suppress methanogenic activity. These researchers reported a H_2 yield of 1.96 mol mol^{-1} glucose.

Ultrasonication is a mechanical enrichment pretreatment which causes physical damage to the cell structure of microorganisms. The microbubbles formation, shear forces generation, high localized temperature, high pressure, and radical formation are major factors which can destroy the cell structure (Wang and Yin, 2017). Elbeshbishy et al. (2010) adopted ultrasonication as a pretreatment method for H_2 production and obtained a yield of 1.55 mol H_2 mol^{-1} glucose from sonicated sludge combined with heat and acidic pretreatments.

Microwave microbial treatment utilizes electromagnetic radiation with frequencies between 300 MHz and 300 GHz (Kumar and Shukla, 2014). According to Hong et al. (2006), the highest average log reduction of fecal coliforms was attained using microwave-pretreated sludge from an anaerobic digester. When compared to common thermal pretreatment, microwave treatment creates a high temperature environment; however, extreme temperatures are reported to deactivate microorganisms (Kuglarz et al., 2013). Since microwaves are not widely used for pretreatment, there is not enough evidence for the inhibition of H_2 consumers using this method.

Ultraviolet (UV) radiation is a method used to destroy the DNA structure of bacteria. Keyser et al. (2008) reported that the number of spoilage bacteria, pathogenic bacteria, and yeasts could be reduced by applying UV light in the fruit juice. Likewise, Hou et al. (2014) proposed that microbial electrolysis cell (MEC) with UV irradiation can improve H_2 production by the inhibition of methanogenesis. In that study, a high concentration of H_2 was achieved in the MEC with the UV treatment, while the MEC without the UV treatment only accumulated CH_4.

2.5.5 Combined enrichment treatments

Since mixed cultures are a mixture of many different microorganisms, combined treatments can achieve an enhanced inhibitory effect on H_2 consumers. Ren et al. (2008) reported that a higher H_2 yield was achieved by heat treatment combined with acidic treatment; however, the inhibition on H_2 production was observed when heat treatment was combined with a chemical treatment. Elbeshbishy et al. (2010) reported that when experiments used ultrasonication treatment combined with the heat and acidic treatment,

the H_2 yield was higher when compared to the H_2 yield obtained from single treatment. In comparison, heat treatment, chloroform inhibition, and two combined treatments were analyzed using an anaerobic sludge for bio-H_2 production. The single heat treatment method was the most effective process in improving H_2 production (Argun and Kargi, 2009).

In conclusion, the different microbial culture treatment methods need to be considered to inhibit H_2 consumers and subsequently, enhancing the H_2 producers producing population.

2.6 Other factors affecting fermentative bio-H_2 production

Factors, such as nutrients, temperature, pH level, hydraulic retention time (HRT), H_2 partial pressure, and substrates, can affect the conditions for optimizing H_2 production. These effects are described in the following sections.

2.6.1 Nutrients

Bacteria growth is affected by macro- and micronutrients. Feeding substrates, such as glucose and glycerol, are usually carbon-rich sources; however, reduced carbon is not the only major nutrient required for bacterial growth and metabolism. Nitrogen and phosphorous are essential macro-nutrients for the bacterial growth. Nitrogen is a necessary component in the synthesis of protein, amino acids, and nucleic acids. A suitable carbon, nitrogen, and phosphorus (C:N:P) ratio (100:0.5:0.1) (weight %) is related to the performance of anaerobic bacteria growth and H_2 yield (Argun et al., 2008b). H_2-producing bacteria are inhibited by threshold levels of ammonia (Salerno et al., 2006). According to Kalil et al. (2008), an optimum C:N ratio of 70:1 (weight %) has been reported when the dark fermentation process utilize glucose in a pure culture in a batch reactor at 30 ºC. An optimum C:N ratio of 47:1 (weight %) was reported for anaerobic sewage sludge fed sucrose at 35 ºC in a dark fermentative batch reactor (O-Thong et al., 2008). Employing different C:N ratios may be due to different operating conditions, microorganisms, and feeding substrates to an anaerobic reactor. Phosphorous is important for energy storage, DNA synthesis, and buffering capacity (Argun et al.,

2008b; Lin and Lay, 2005). Intanoo et al. (2012) used a COD:N:P ratio of 100:6:0.5 (weight %) to optimize H_2 production under thermophilic conditions.

Based on the bio-H_2 production pathway described in section 2.2, oxidation of reduced ferredoxin is an essential step for H_2 production. Hence, iron (Fe^{2+}) is another important inorganic nutrient. A maximum H_2 yield of 2.84 mol H_2 mol^{-1} glucose was obtained when N:C, P:C, and Fe^{2+}:C ratios were 0.02:1, 0.008:1, and 0.015:1 (weight %), respectively (Oztekin et al., 2008). Other nutrients, for example, yeast extract, minerals, buffering agents, and vitamins, are also necessary for the growth of anaerobic microorganisms.

2.6.2 Substrate

Hydrogen production is affected by the substrate concentration and the substrate type. In batch studies, the food-to-microorganism (F/M) ratio is considered a factor which controls the operation of a bioreactor. A suitable F/M ratio is essential to avoid substrate inhibition. This leads to the accumulation of VFAs with a subsequently decrease in the pH, which eventually influences the H_2 production yield. In a continuous flow reactor, the organic loading rate (OLR) is controlled by the substrate concentration and the hydraulic loading rate (HRT). Numerous of substrates have been employed for fermentative bio-H_2 production, such as lignocellulosic substrates, starch, organic wastes, and wastewaters as well as pure sugars such as glucose, xylose, sucrose (Veeravalli, 2014; Cheng et al., 2011; Argun et al., 2008a; Wu and Lin, 2004). However, feeding a pure sugar into a large-scale system is not cost effective and sugars are essential consumer products used in the food industry. Ghimire et al. (2016) examined varing F/M ratios (0.5, 1.0 and 1.5) and reported that the lower F/M ratio was favorable for H_2 production at an initial pH value of 5.0. Wang and Wan (2008a) analyzed a wide range of substrate concentration from 0 to 300 g L^{-1}, and a maximum H_2 production was reported at a glucose concentration of 25 g L^{-1}.

Glycerol is a waste by-product of biodiesel production. Crude glycerol is obtained during biodiesel production with additional chemicals such as metals and hence, pretreatment is required before using this chemical as a feed for H_2 production. Selembo

et al., (2009) compared the effect of initial substrate concentration for fermentative bio-H_2 production using glucose and glycerol. These authors reported that the gas production rates were reduced when substrate concentration increased. Especially, when the substrate concentration increased from 1 g L^{-1} to 6 g L^{-1}, the yield from glucose degradation was 2.6 times larger than the yield from glycerol degradation.

2.6.3 pH

Of all the sensitive operating parameters affecting H_2 production through dark fermentation, pH is one of the most important factors. pH can affect the microbial community structure, the production of metabolites, and the intracellular metabolic enzymatic activities (Temudo et al., 2008; Ginkel, et al., 2001; Dabrock et al., 1992). The pH level has a strong effect on suppressing H_2-consuming bacteria. For example, methanogenesis is suppressed at a pH lower than 6.0, and a pH level of 5.5 is the preferred condition to inhibit CH_4 production (Liu et al., 2008). According to Fang and Liu (2002), a decrease in the diversity of microbial communities coincided with a decrease in pH level, and the CH_4 production increased with increasing the pH from 6.0 to 7.0. When the initial pH was 3.0, methanogenic bacteria were suppressed and were not detected after 35 days (Park et al., 2005).

The metabolic products and metabolism pathways are also affected by the pH conditions because enzymes have different optimal activities at different pH conditions. The acidic pH level responds to the accumulation of Ac^- and But^-, while Pr^- and EtOH formation is increased when pH is larger than 7 (Lee et al., 2009; Lay et al., 2010). As discussed in Section 2.2, the pathway for Pr^- formation decreases the H_2 yield. Hence, maintaining an optimum pH condition for H_2 production is important because the pH depends on the byproduct distribution.

In batch studies, the pH level is adjusted to an initial value; however, after fermentation, the pH level changes because of the accumulation of metabolites. Changes in external pH conditions result in the variation of the internal pH of the microorganisms. Katharina and Colman (1985) reported that the internal pH of the acid-tolerant green alga was maintained at approximately pH 7.3 when the external pH changed from 5.0 to 7.5.

Furthermore, the internal pH gradually decreased to 6.4 when the external pH ranged from 3.0 to 5.0. The green alga was analyzed at different external pH conditions, and the shifted formation from acids to alcohols was obtained when the internal pH was greater than 5.5 (Gottwald and Gottschalk, 1985).

The impact of different initial pH conditions on H_2 production from various batch studies are summarized in Table 2.2. A wide range of pH conditions was analyzed and most of the studies reported that acidic pH condition was optimal for achieving higher H_2 yields. In contrast, Lee et al. (2002) reported an optimum H_2 yield of 0.73 mol mol^{-1} at a pH level of 9.0 for a sucrose feedstock.

Table 2.2: Summary of pH impact on bio-H_2 production

Substrates	pH		Temperature (ºC)	Yield (mol H_2 mol^{-1} substrate)	Reference
	Range	Optimum			
Glucose	4-11	4.0	37.5	2	Lee et al. (2008)
Sucrose	3-12	9.0	37.0	0.73	Lee et al. (2002)
Sucrose	5.5-6.5	5.5	37.0	2.78	Chen et al. (2005)
Starch	5-7	5.5	35.0	1.1	Lin et al. (2008)
Xylose	5-8	6.5	35.0	1.3	Lin et al. (2006)

2.6.4 Temperature

The operating temperature is an important factor affecting bio-H_2 production. Generally, several components can be influenced by temperature, including the degradation rate, the activity of enzymes such as hydrogenases, the distribution of metabolic products, and the composition of bacterial communities (Kothari et al., 2017). According to Dinamarca and Bakke (2011), the activity of enzymes increased 2-fold with a 10ºC temperature rise. In other words, increasing enzymatic activities such as hydrogenases have resulted in the improvement of H_2 yields. Although higher temperatures can improve H_2 yields, the cell densities can be decreased with increasing temperatures (Hallenbeck, 2005).

Mesophilic (32-40ºC), thermophilic (40-65°C), and hyper-thermophilic (>70°C) are three ranges used to conduct bio-H_2 production studies. Shifting the distribution of metabolic products towards the expected end products can be achieved by optimizing the

temperature. The optimum temperatures with different temperature ranges and the highest H_2 yields for different studies are summarized in Table 2.3. In these studies, the optimum temperature from 37 to 60°C for maximum H_2 production is related to factors such as culture type and substrates (Table 2.3).

Table 2.3: Summary of temperature impacts on bio-H_2 production

Substrate	Temperature (°C)		Maximum H_2 yields	Reference
	Range	Optimum		
Glucose	20-55	40	275.1 ml H_2 g^{-1} glucose	Wang and Wan (2008b)
Sucrose	30-45	40	3.88 mol H_2 mol^{-1} sucrose	Lee et al. (2006)
Xylose	30-55	50	1.4 mol H_2 mol^{-1} xylose	Lin et al. (2008)
Starch	37-55	37	9.47 mmol H_2 g^{-1} starch	Lee et al. (2008)
Waste	37-85	60	392 ml H_2 L^{-1} cow-waste	Yokoyama et al. (2007)

2.6.5 H_2 partial pressure

The accumulation of H_2 in the liquid phase causes a shift in the metabolic byproduct distribution (Bundhoo and Mohee, 2016; Angenent et al., 2004). As illustrated in Section 2.2 and Fig 2.2, the NADH-ferredoxin oxidoreduction process is one pathway used to reduce protons and then release H_2. Reducing protons by using NADH and reduced ferredoxin (Fd_{red}) depend on the redox potential of the reactions. Stams (1994) calculated the Gibbs free energy changes ($\Delta G^{0\prime}$ values) for different redox reactions (Table 2.4). When the $\Delta G^{0\prime}$ values are negative, the reactions are thermodynamically favorable. The $\Delta G^{0\prime}$ values are based on the following standard conditions: 298 K, a pH of 7, 1 M, and 1 atm.

In bio-H_2 producing bioreactors, when the H_2 and For- concentrations are significantly low, H_2 production become favorable. According to Stams (1994), the ferredoxin reaction is more likely to produce H_2 than the NADH reaction under a low H_2 partial pressure. Additionally, the NADH oxidation reaction was able to produce other byproducts, such as But^- and EtOH, instead of H_2 at a H_2 partial pressure greater than 10 Pa. Angenent et al. (2004) reported that the ferredoxin and NADH oxidoreduction reactions are able to produce H_2 when the partial pressure is less than 60 Pa. In comparison, when the partial pressure is greater than 60 Pa, NADH is oxidized to produce other metabolites such as La^-, EtOH, ButOH, But^-, and acetone (Angenent et al.,

2004; van Niel et al., 2003).

Table 2.4: $\Delta G^{0\prime}$ values for redox reactions

Redox reactions	$\Delta G^{0\prime}$ (kJ mol^{-1})
$2Fd^{red} + 2H^+ \rightarrow 2Fd^{ox} + H_2$	+3.1
$2NAHD + 2H^+ \rightarrow 2NAD^+ + H_2$	+18.1
$NADH + HCO_3^- + H^+ \rightarrow NAD^+ + Formate^- + H_2O$	+16.8

Adapted from Stams (1994)

Several studies have reported methods to reduce the H_2 partial pressure and hence, increase the H_2 yield. Foglia et al. (2011) reported using a vacuum stripping system to reduce the H_2 partial pressure. Increasing the headspace volume can reduce H_2 partial pressure. According to Nguyen et al. (2010), the optimum headspace volume for the maximum H_2 production is approximately 2/3 volume of the reactor. Nitrogen (N_2) and CO_2 are inert gases usually used to strip H_2 and hence, reduce the H_2 partial pressure. Nitrogen gas used to sparge biogas from a continuously bioreactor was able to reduce the H_2 partial pressure and increase the H_2 yield (Hussy et al., 2003). Kim et al. (2006) reported that sparging inert gases such as N_2 and CO_2 into a completely stirred-tank reactor (CSTR) increased the H_2 yield.

2.6.6 Hydraulic retention time (HRT)

The hydraulic retention time (HRT) is an important factor for the design of bioreactors. The HRT refers to the average time of a volume element resides in a reactor. In a batch reactor, the inoculum, media, substrate, and other chemicals are added at the same time. Although batch reactors have advantages, such as the easy control and simplified reactor design, these reactors have limited use in large scale production systems such as waste treatment and producing energy chemicals such as ethanol and H_2.

The microbial population and degradation products distribution are affected by the HRT (Santiago et al., 2019). Jo et al., (2008) reported a maximum H_2 production rate at a 2h HRT. Low HRTs are favorable towards H_2 production because H_2 consumers are washed out from the bioreactor (Jo et al., 2008; Zhang et al., 2007). In contrast, high HRTs increase the contact time between substrates and H_2 consumers. According to Li

and Fang (2007), the optimal HRT range of 3-8h was employed to produce H_2 from glucose and sucrose in mixed anaerobic cultures. Additionally, Kim et al. (2008) used 0.5h as the optimum HRT for continuous H_2 production from food wastes.

2.7 References

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Chapter 3: MATERIALS AND METHODS

3.1 Chemicals

All of chemicals used for the experiments are summarized in Table 3.1.

Table 3.1: Chemicals used in the experiments

Chemical	Purity	Vendor	Location
$C_6H_{12}O_6$	99.5%	Bedessee Imports LTD	Ontario, Canada
$C_3H_8O_3$	99.0%	Sigma-Aldrich Co	Ontario, Canada
		Basal medium	
$NaHCO_3$	99.7%	BDH® VWR ANALYTICAL	Ontario, Canada
NH_4HCO_3	99.0%	Sigma-Aldrich Co	Ontario, Canada
KCl	99.0%	ACP Chemicals	Quebec, Canada
K_2HPO_4	97.5%	BDH® VWR ANALYTICAL	Ontario, Canada
Yeast extract	N/A	Bio Basic Inc.	Ontario, Canada
$(NH_4)_2SO_4$	99.0%	EM® Science	USA
$MgCl_2 \cdot 4H_2O$	98.0%	BDH® VWR ANALYTICAL	Ontario, Canada
EDTA	Pure	Bio Basic Inc.	Ontario, Canada
$FeCl_2 \cdot 4H_2O$	98.0%	ACP Chemicals	Quebec, Canada
$MnCl_2 \cdot 4H_2O$	99.4%	Mallinckrodt Baker Inc.	New Jersey, USA
$CoCl_2 \cdot 6H_2O$	98.0%	EM® Science	USA
Na_2SeO_3	99.0%	Alfa Aesar	Massachusetts, USA
$(NH_4)_6MoO_7 \cdot 4H_2O$	83.0%	EM® Science	USA
$ZnCl_3$	97.0%	Alfa Aesar	Massachusetts, USA
H_3BO_3	99.5%	EM® Science	USA
$NiCl_2 \cdot 6H_2O$	98.0%	ACP Chemicals	Quebec, Canada
$CuCl_2 \cdot 2H_2O$	98.0%	ACP Chemicals	Quebec, Canada
		Long chain fatty acids (LCFAs)	
Lauric acid	98.0%	TCI Chemical Industry Co., Ltd	Portland, USA
Myristic acid	99.0%	TCI Chemical Industry Co., Ltd	Portland, USA
Palmitic acid	95.0%	Lancaster Synthesis	New Hampshire, USA
Stearic acid	95.0%	Sigma-Aldrich Co	Ontario, Canada
Oleic acid	99.0%	TCI Chemical Industry Co., Ltd	Portland, USA
Linoleic acid	95.0%	TCI Chemical Industry Co., Ltd	Portland, USA
		Volatile fatty acids (VFAs) and Alcohols	
Lactic acid	90.0%	BDH® VWR ANALYTICAL	Ontario, Canada
Formic acid	95.0%	ACP Chemicals	Quebec, Canada
Acetic acid	99.7%	EM® Science	USA
Propionic acid	99.0%	ACP Chemicals	Quebec, Canada
Butyric acid	99.0%	ACP Chemicals	Quebec, Canada
Ethanol	95.0%	Sigma-Aldrich Co	Ontario, Canada
n-Propanol	99.0%	ACP Chemicals	Quebec, Canada
i-Propanol	99.9%	Sigma-Aldrich Co	Ontario, Canada
Butanol	99.0%	ACP Chemicals	Quebec, Canada

1,3-Propanediol	85.0%	Sigma-Aldrich Co	Ontario, Canada
	Others		
NaOH	98.0%	Sigma-Aldrich Co	Ontario, Canada
H_2	99.9%	CCC of University of Windsor	Ontario, Canada
CH_4	99.9%	CCC of University of Windsor	Ontario, Canada
CO_2	99.9%	CCC of University of Windsor	Ontario, Canada
N_2	99.9%	CCC of University of Windsor	Ontario, Canada

3.2 Inoculum source

The anaerobic iocula used in the experiments were obtained from anaerobic bioreactors located at an EtOH producing facility and a municipal wastewater treatment facility in Chatham, Ontario, Canada. The cultures were mixed (1:1 ratio) and maintained in a 5-L (4 L liquid and 1 L gas space) mother reactor. The semi-continuous mother reactor was maintained at 37±1℃. Aluminum foil was used to cover the reactor to prevent photosynthetic activity. The mother reactor was placed on a stir plate set to continuously stir at 200 rpm. The reactor was sealed using a rubber stopper with two gas lines connected to a nitrogen (N_2) gas tank and a gas counter. The mother reactor set-up is shown in Figure 3.1. The reactor was fed 5,000 mg L^{-1} glucose (Bedessee Imports Ltd, Ontario, Canada) every 7 days, and the volatile suspended solids (VSS) of the reactor was maintained at 20,000 mg L^{-1}. The reactor was purged with N_2 gas for 2 mins to remove the gas by-products and maintain anaerobic conditions after the weekly glucose feeding. Each week, the gas counter reading was recorded before feeding and reset to zero after gas purging.

The cultures were characterized using the quantity of glucose removed, the VSS/TSS content, the VFA concentration and gas production (Chowdhury et al., 2007). The characterization analysis was conducted in 160 mL serum bottle reactors. The 5 L mother reactor cultures were transferred into serum bottle reactors and diluted to achieve a culture concentration of 2,000 mg L^{-1} VSS. Diluting the culture from the mother reactor and into the serum bottles was performed in an anaerobic glove box (4% H_2, 20% CO_2, and 76% N_2) (COY Laboratory Products Inc., Grass Lake, MI). The serum bottles were fed a 5,000 mg L^{-1} glucose at the initial pH 7.6. The serum bottle reactors were prepared according to the description provided in Section 3.2. After the characterization results

confirmed the cultures degraded 5,000 mg L^{-1} glucose into CH_4 and CO_2 within 7 days, the cultures in the mother reactor were used for further experiments.

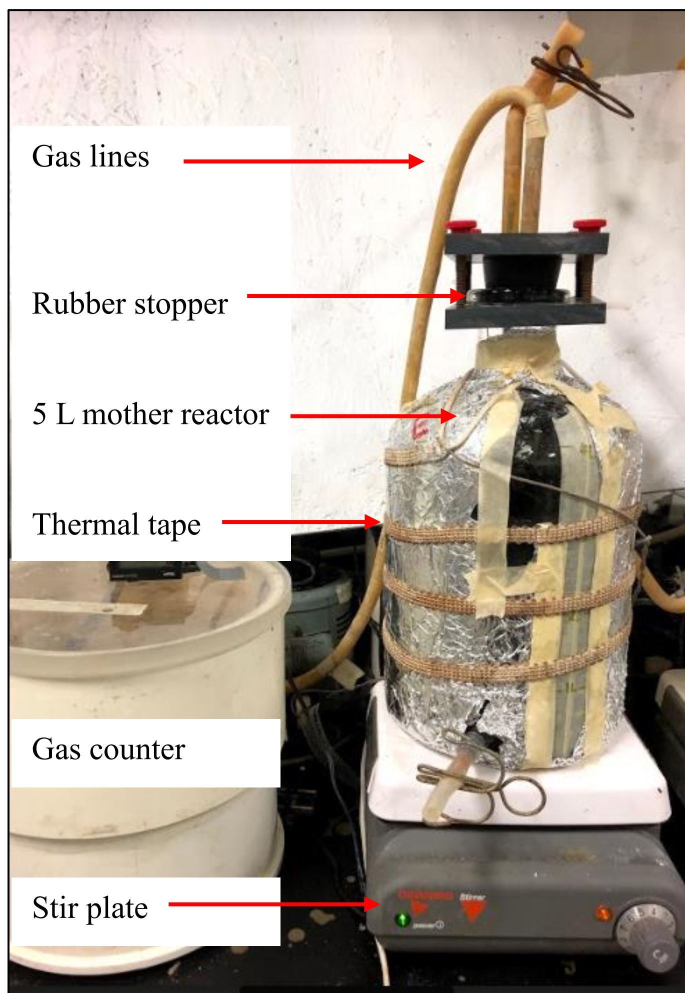

Figure 3.1: Mother reactor configuration.

3.3 Batch studies

All the batch studies used experimental methods that were adapted from previous studies reported by Lalman and Bagley (2004). Each experimental condition was examined in triplicates. Triplicate serum bottles (160 mL) were wrapped in aluminum foil. Preparation of the serum bottles was conducted in an anaerobic glove box (COY Laboratory Products Inc., Grass Lake, MI) with an atmosphere containing 20% CO_2 and 80% N_2. Anaerobic condition in the glove box was monitored using a solution containing approximately 100 ppm resazurin. Change of the resazurin solution from a faint purple

to pink indicated an aerobic atmosphere. Each serum bottle was filled with a predetermined quantity of basal medium and a calculated amount of culture was added to the basal media to achieve a VSS concentration of 2,000 mg L^{-1}. The basal medium solution composition is shown in Table 3.2 (Wiegant and Lettinga, 1985). The basal medium solution was prepared in Milli-Q (Millipore, Barnstead, USA) water.

Table 3.2: Basal medium composition

Chemical	Concentration (mg L^{-1})	Chemical	Concentration (mg L^{-1})
$NaHCO_3$	6000	$CoCl_2 \cdot 6H_2O$	0.15
NH_4HCO_3	70	Na_2SeO_3	0.1
KCl	25	$(NH_4)_6MoO_7 \cdot 4H_2O$	0.09
K_2HPO_4	14	$ZnCl_3$	0.05
$(NH_4)_2SO_4$	10	H_3BO_3	0.05
Yeast extract	10	$NiCl_2 \cdot 6H_2O$	0.05
$MgCl_2 \cdot 4H_2O$	9	$CuCl_2 \cdot 2H_2O$	0.03
Resazurin	1	EDTA	1
$FeCl_2 \cdot 4H_2O$	2	$MnCl_2 \cdot 4H_2O$	0.5

Wiegant and Lettinga (1985)

Inhibition of the cultures was accomplished using LCFAs. Different LCFAs stock solution was injected into the serum bottles (Table 3.3). After adding the cultures, basal medium, and the inhibitor into each serum bottle, the initial pH was adjusted to a predetermined level using 1 M HCl or 1 M NaOH. The bottles were sealed with Teflon®-lined silicone rubber septa (P.J. Cobert Associates, Inc. St. Louis, Missouri, USA) and capped with aluminum caps (Chromatographic Specialties, Inc. Brockville, Ontario, Canada). Next, all the bottles were pressurized with 20 mL of the glove-box gas atmosphere to avoid the formation of a negative pressure due to sampling of the liquid and headspace. Before receiving the substrate (glucose or glycerol), all the bottles were removed from the anaerobic glove box and placed in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, USA). The shaker was set at 200 rpm and 37±1ºC. Any residual H_2 injected into the headspace from the glove box atmosphere was removed by mixing serum bottles for 24 hours. After 24 hours, the headspace gas content was determined and each set of serum bottles received the specified amount of glucose or glycerol. When the substrate was injected, the injection date was considered as day 0. The total liquid volume for each serum bottle was 50 mL on day 0. Gas and liquid

samples were removed and analyzed daily to determine the gas, VFAs, and alcohols concentration in the serum bottles. Figure 3.2 shows the experiment process and related instruments.

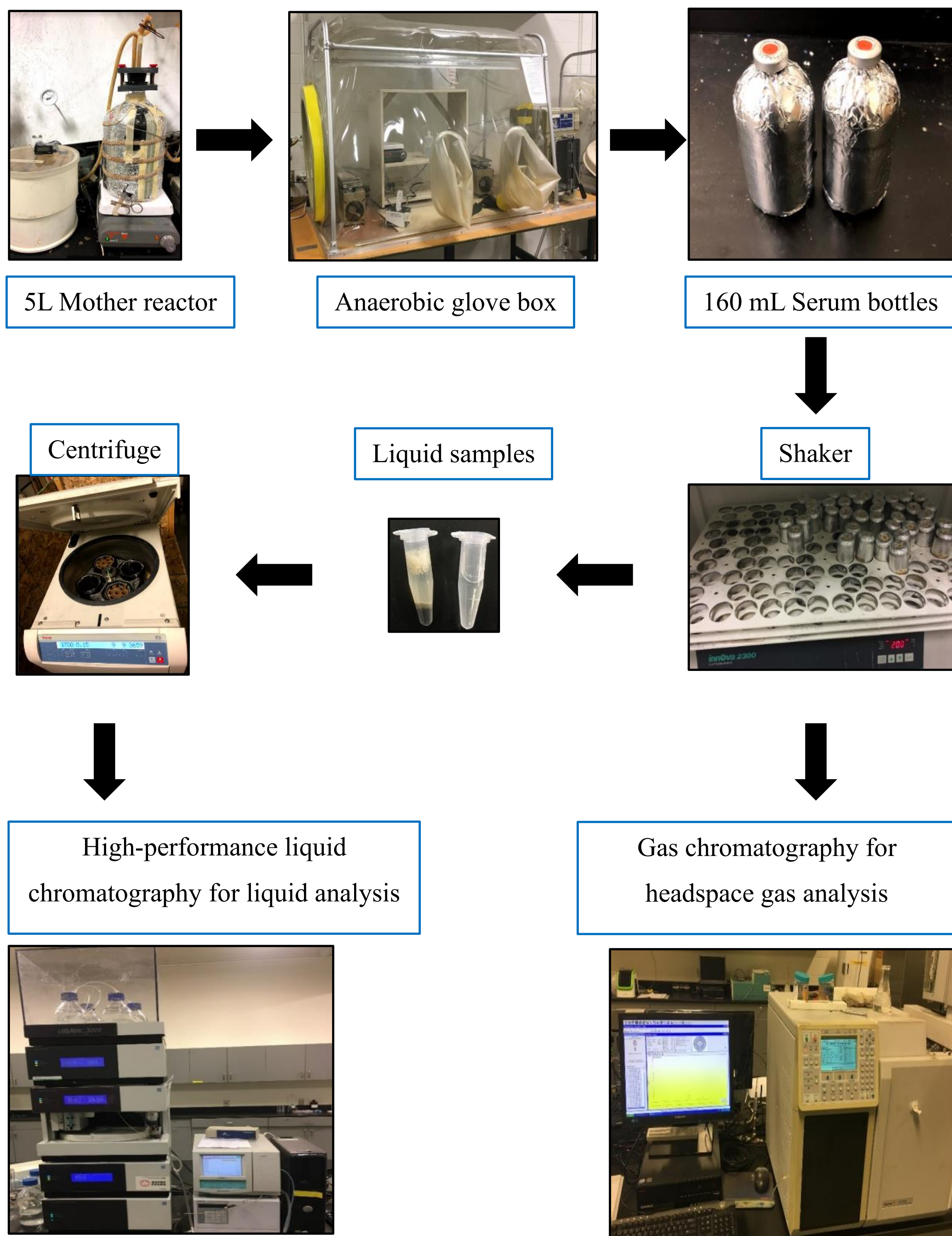

Figure 3.2: Experiment process and related instruments.

The LCFAs used in the experiments are shown in Table 3.3. All LCFAs stock solutions were prepared using the delivery method reported by Rinzema et al. (1994). Details of the preparation procedure are described in Section 3.5.5. The LCFA stock solutions were injected into the serum bottles 24 hours before adding substrates (glucose or glycerol) to allow the LCFA to attain equilibrium with the microbial culture.

Table 3.3: LCFAs used in the experiments

LCFA	Carbon number	Abbreviation	Solubility in water* (g LCFA per 100 g H_2O)
Lauric acid	12	$C_{12:0}$	0.0055 to 0.0087
Myristic acid	14	$C_{14:0}$	0.0020 to 0.0034
Palmitic acid	16	$C_{16:0}$	0.00072 to 0.0012
Stearic acid	18	$C_{18:0}$	0.00029 to 0.00050
Oleic acid	18	$C_{18:1}$	
Linoleic acid	18	$C_{18:2}$	

Note: * Solubilities of each LCFA at 20 and 60 ºC (Ralston and Hoerr, 1942)

3.4 Experimental plan

3.4.1 Phase I – Impact of initial pH on dark fermentative hydrogen production from glycerol using mixed anaerobic cultures

Metabolic pathways are affected by the pH and hence, this factor is a significant parameter affecting the optimization conditions for H_2-producers. Ray et al. (2008) reported that acidic conditions were selected as the optimum initial pH such that a maximum H_2 yield was achievable when feeding glucose. Phase I experiments were designed as shown in Table 3.4 to investigate the effects of the initial pH on the H_2 yield and glycerol degradation. The optimal pH condition from these experiments was selected as the initial pH level for phase II experiments.

The mixed culture used in phase I was obtained from the mother reactor. The serum bottle reactors were prepared using the procedure described in Section 3.3. The glycerol concentration of 5,110 mg L^{-1} was equivalent on a percent carbon basis to a glucose concentration of 5,000 mg L^{-1}. The production of metabolites and degradation of substrates were monitored for 4 days.

Table 3.4: Phase I – Impact of initial pH on dark fermentative hydrogen production from glycerol using mixed cultures

Substrate	pH	Substrate concentration (mg L^{-1})	Incubation period (days)
Glucose (control)	5.5	5,000	4
	6.5	5,000	4
	7.5	5,000	4
Glycerol	5.5	5,110	4
	6.5	5,110	4
	7.5	5,110	4

3.4.2 Phase II – Effects of long-chain fatty acids (LCFAs) on H_2 production from glycerol in mixed cultures through dark fermentation

Phase II was designed as shown in Table 3.5 to evaluate the effects of LUA, MA, PA, SA, OA, and LA on H_2 production from glycerol degradation at 37ºC and an initial pH of 5.5. LCFAs were reported to inhibit acetoclastic methanogen and hydrogenotrophic methanogen (Hanaki et al., 1981). An LA concentration of 2,000 mg L^{-1} was reported as the most effective concentration for inhibiting methanogens (Reaume, 2009). Based on the work by Reaume (2009), a concentration of 2,000 mg L^{-1} was selected for six LCFAs used in this phase. The objectives of phase II were to determine the effects of LCFAs at different carbon-chain lengths on H_2 production, to determine the effects of LCFAs at different bond saturation on H_2 production, and to explain the dominant metabolites. Cultures without glycerol were designated as control samples to estimate the degradation of LCFAs within the incubation period. Also, serum bottle reactors, which were prepared with no inhibitor and only glycerol, were designated as controls.

LCFAs stock solutions were prepared using the protocol described in Section 3.5.5. Preparing the serum bottle reactors followed the instructions outlined in Section 3.3. The analytical methods described in Section 3.5 were used to determine the concentration of glycerol, VFAs, and alcohols in the liquid samples, and H_2, CH_4, and CO_2 in the gas samples. Before the second glycerol injection (day 4), the serum bottle reactors were opened and purged with N_2 gas (99.99%) for 3 minutes. Next, the pH of each bottle was adjusted to 5.5. The reactors were sealed with Teflon® lined silicone rubber septa inserted into aluminum caps and subsequently, capped with a crimper. After all the samples were

withdrawn daily from the serum bottles, the reactors were opened on day 8 in the anaerobic glove box to determine the pH.

The performance of the mixed cultures after the second feeding was examined after the second substrate injection for further substrate degradation. After sampling on day 8, the cultures in the serum bottles were transferred into centrifuge tubes to separate the solids from the liquid. The centrifuged cultures were placed into new serum bottles to repeat the preparation procedure as described in Section 3.3, and continuously analyzed for an additional 4 days. The related results are shown in Appendix A.

Table 3.5: Phase II – Effects of LCFAs on H_2 production by feeding glycerol

Substrate	Inhibitor	1st Glycerol feeding (day 0) (mg L^{-1})	2nd Glycerol feeding (day 4) (mg L^{-1})	Incubation period (days)
Glycerol	None	5,110	5,110	8
	LUA(C12:0)	5,110	5,110	8
	MA (C14:0)	5,110	5,110	8
	PA (C16:0)	5,110	5,110	8
	SA (C18:0)	5,110	5,110	8
	OA (C18:1)	5,110	5,110	8
	LA (C18:2)	5,110	5,110	8

Note: 1. The inhibitor was injected one day before the first substrate feeding
2. Controls injected with only glycerol or inhibitor are not shown in the table

3.4.3 Phase III – Using a statistic approach to optimize H_2 production from glycerol by mixed anaerobic cultures

Phase III was designed to determine the optimal conditions for maximizing H_2 production using a three-factor and three-level Box-Behnken Design (BBD) with three replicates. The design factors and levels are shown in Table 3.6. The three levels for each factor were selected based on a literature review and the results from screening experiments.

All the samples analyzed from the serum bottle reactors in this phase were prepared based on Section 3.3. All the bottles received 2,000 mg L^{-1} LA at 24 hours before feeding glycerol on day 0. The incubation period was 4 days. Headspace gas samples were removed daily to monitor H_2 production. Table 3.7 shows the design matrix

for the BBD experiments. Experiments conducted under the same conditions are the central points to assess the statistic error in the BBD model.

Table 3.6: Levels and factors selected for the experimental design

Levels	Factors		
	A	B	C
	Initial pH	Glycerol (mg L^{-1})	Temperature (ºC)
-1	5.5	1,300	22
0	6.5	2,600	37
+1	7.5	5,110	52

Table 3.7: Design matrix for selected factors at different factor levels

	Factors		
	Initial pH	Glycerol (mg L^{-1})	Temperature (ºC)
Expt.#	X_1	X_2	X_3
1	5.5	2,600	22
2	5.5	2,600	52
3	6.5	1,300	22
4	6.5	2,600	37
5	6.5	1,300	52
6	7.5	5,110	37
7	5.5	1,300	37
8	7.5	1,300	37
9	6.5	5,110	22
10	5.5	5,110	37
11	6.5	2,600	37
12	7.5	2,600	52
13	7.5	2,600	22
14	6.5	5,110	52
15	6.5	2,600	37

3.5 Analytical methods

All the liquid samples were purified before the analysis. The purification procedures used for sampling of the VFAs, alcohols, and substrates were the same throughout all of the experiments.

At the predetermined intervals, liquid samples were withdrawn depending on the

experimental plan. Disposable syringes (Becton Dickinson, NJ, USA) were used to transfer 1 mL samples from serum bottles into 1.5 mL microcentrifuge tubes (VWR® International, Ontario, Canada). Next, the tubes were centrifuged at 1750 × g for 15 minutes to separate the liquid and solid phases in the centrifuge (Model No. ST16) (Thermo Scientific, Waltham, MA, USA). The supernatant was withdrawn using a 5 mL syringe and filtered through a two-stage filter to remove suspended solids and heavy metals. The first filter used was a 25 mm diameter plastic syringe filter holder fitted with a 25 mm diameter 0.45 µm hydrophilic supported nylon membrane (GE Osmonics, MN). The second filter used was a 1 mL polypropylene cartridge tube consisted of a pair of 20 µm polyethylene (PE) frits (Supelco, PA, USA) and filled with the Chelex® 100 resin (Biotechnology Grade) (Bio-Rad Laboratories Inc., California, USA). The purified samples were injected into the 2 mL vials (Chromatographic Specialties Inc. Brockville, Ontario, Canada) and stored at 4 ºC before analysis using a high-performance liquid chromatograph (HPLC).

3.5.1 VFAs analysis

VFAs were analyzed using an UltiMate 3000 HPLC (Dionex, Sunnyvale, USA) equipped with a photodiode array detector. The HPLC was configured with a HiPlex H, 300 × 7.7 mm diameter Column (Agilent, Santa Clara, California, USA). The temperature in the column compartment was set at 65 ºC. The eluent, 5 mM H_2SO_4, flow rate was set at 0.6 mL min^{-1}. The injection volume was 25 µL and the wavelengths of the photodiode array detector were selected at 205, 210, and 215 nm. The detection limit for the VFAs was 2.0 mg L^{-1}.

Stock VFAs solutions (5000 mg L^{-1}) were prepared using HLa, HFor, HAc, HPr, and HBu. The VFA calibration curves were prepared in triplicate standards containing 10, 50, 100, 200, 500, 1,000, 2,000 mg L^{-1}. Milli-Q water was used as a blank. The VFAs calibration curves were generated by analyzing triplicate standards. The VFAs calibration curves are shown in Appendix B.

3.5.2 *Glucose, glycerol, and alcohols analysis*

Glucose, glycerol, and alcohols were analyzed using an UltiMate 3000 HPLC configured with a HiPlex H, 300 × 7.7 mm diameter column and a refractive index detector (RI-101, Shodex, Tokyo, Japan). The column temperature was set at 65 ºC. The eluent was Milli-Q water, and the eluent flow rate was set at 0.6 mL min^{-1} and the injection volume was 25 µL.

The substrate calibration curves were prepared for glucose and glycerol. Each calibration curve was generated using triplicates containing 50, 100, 200, 400, 800, 1,000, 2,000, 5,000 mg L^{-1} of each analyte. A blank was prepared using Milli-Q water. The standards were prepared and diluted from a 100,000 mg L^{-1} stock solution. The glucose and glycerol calibration curves are shown in Appendix B. The detection limits were 1.0 mg L^{-1} for glucose and 2.0 mg L^{-1} for glycerol.

EtOH, n-propanol (PrOH), i-propanol (i-PrOH), ButOH, and 1,3-PDO were selected to prepare the calibration curves. The alcohol calibration curves were prepared from triplicate standards containing 10, 50, 100, 200, 500, 1,000, 2,000 mg L^{-1} of each analyte. Blanks were prepared with Milli-Q water. The alcohol calibration curves are shown in Appendix B. The detection limit for each alcohol was 5.0 mg L^{-1}.

3.5.3 *Headspace gas analysis*

The serum bottles headspace gas samples were analyzed using a Varian 3800 gas chromatograph (GC) (Varian Inc., Palo Alto, USA). This GC was equipped with a thermal conductivity detector (TCD) and a 2 m × 3.175 mm diameter ShinCarbon ST (RESTEK, USA) Packed column. The operating temperatures of the TCD, injector, and column oven were set at 200 ºC, 150 ºC, and 200 ºC, respectively. Nitrogen gas (99.99%, Praxair, ON) was used as the carrier gas with a flow rate of 15 mL min^{-1}. The headspace gas samples (25 µL) were withdrawn from the serum bottles using a 50 µL Hamilton Gastight syringe (Chromatographic Specialties Inc., CA). Next, the gas sample was injected into the GC. The total analysis time was 3 minutes. The pressure of headspace gas in the serum bottles was measured using a digital pressure meter (DPGA-12, Dwyer Instruments Inc., Michigan City, USA). The pressure data was used to convert the

volumes of gas to the moles of gas using the ideal gas law equation. The detection limits were 0.1 mL per 160 mL for H_2 and 0.2 mL per 160 mL for CH_4.

The serum bottles (160 mL) were used to prepare the calibration standards for the GC. The bottles were purged with N_2 (99.99%) gas for 3 minutes and then sealed with Teflon™-lined septa and aluminum caps. The quantity of CH_4 and CO_2 standards added in each 160 ml serum bottle was 0.5, 1, 2, 3, 5, 10, 20, and 50 mL. The quantity of H_2 standard added in each serum bottle was 0.5, 1, 2, 3, 5, 10, 20, 50, and 100 mL. The gas calibration curves are shown in Appendix B.

3.5.4 VSS/TSS and pH measurements

The volatile suspended solids (VSS) and total suspended solids (TSS) concentration in the mother reactor were determined at the beginning of each experiment to determine the dilution ratio required for the serum bottle reactors to ensure a VSS concentration of 2,000 mg L^{-1}. The VSS and TSS concentrations were analyzed regularly to assess the microorganism levels in the mother reactor. These measurements were conducted in triplicates using 5 mL liquid samples and filtered using glass microfiber filters (VWR, Radnor, USA), with a 0.45 μm pore size. The TSS and VSS concentrations were determined using Standard Methods (APHA et al., 1999).

The pH of the microbial and chemical mixture in the serum bottle reactor was measured using a pH meter (Orion model 320 PerpHecT® LogR® Meter, Thermo Fisher Scientific, USA). The pH value of liquid in the mother reactor was analyzed periodically to ensure the inoculum was between a pH of 7.0 and 7.5. The pH meter was calibrated using standard buffer solutions (pH 4 and 7).

3.5.5 LCFAs preparation

LCFAs are insoluble in water and hence, LCFA were saponified to increase their aqueous solubility (Rinzema et al., 1994). Stock solutions (100,000 mg L^{-1}) were prepared by adding known amounts of each LCFA plus NaOH into a 20 mL serum vial (Table 3.8). Milli-Q water were added into 20 mL vials. The vial was shaken and placed

in a water bath maintained at 60 ºC. After vigorous shaking, the stock LCFA solution was used for the serum bottle experiments.

Table 3.8: Quantity of sodium hydroxide used to prepare LCFA stock solutions

LCFAs	NaOH (g g^{-1} of LCFA)
LUA (C12:0)	0.200
MA (C14:0)	0.175
PA (C16:0)	0.156
SA (C18:0)	0.141
OA (C18:1)	0.142
LA (C18:2)	0.143

3.6 Electron balance

An electron balance was used to explain the distribution of electron equivalents for phase II of experiments. Gas, liquid reactants, and by-products concentrations were converted into the units of mmol. Next, the mole of electron equivalents per mmole of the reactant and byproducts (m_{eq} $mmol^{-1}$) were determined from their half-reactions. In phase II, glycerol, the electron donor, was converted into various byproducts. The m_{eq} of the electron donor was divided by the m_{eq} for the electron acceptor to generate the percent electron equivalent for the different acceptor reactions. The sum of all the percentages of m_{eq} is 100%. A variation (±10 %) of the sum of percentages was acceptable because of cell synthesis. An example of these calculations is shown in Appendix C.

3.7 Statistical analysis

Response surface methodology (RSM) is a valuable statistical tool used to optimize a process. RSM is employed to analyze the impact of independent factors on a response variable and to determine conditions which optimize the factors leading to a maximum response (Varrone et al., 2012). RSM methods include Box-Behnken design (BBD) and Taguchi. The BBD is commonly used for optimization analysis and was employed in this study.

Based on the BBD method, the selected factors and three levels are shown in Table 3.5. The 15 experimental conditions with three replicates are shown in Table 3.6. The objective for using BBD is to model maximization of the H_2 yield (the response variable). The selected factors and responses were modelled using Minitab 15 (Minitab

Inc., State College, PA) to generate a quadratic polynomial equation. An analysis of variance (ANOVA) was used to analyze the significance of the full quadratic model. The D-optimality analysis was utilized to generate the optimal conditions when the maximum H_2 yield is achieved. According to the BBD model, interactive effects among the selected factors and the responses can be explained by the different plots. Details of selecting the various independent factors and modeling are discussed in Section 6.2.

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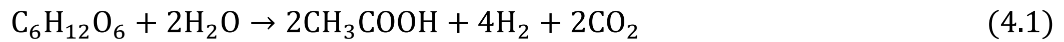
Chapter 4: IMPACT OF INITIAL pH ON DARK FERMENTATIVE HYDROGEN PRODUCTION FROM GLYCEROL USING MIXED ANAEROBIC CULTURES

4.1 Introduction

Fossil fuels have been a primary source of global energy supplies for over the past century. However, the negative effects of using fossil fuels are raising global public awareness. Air pollution, greenhouse gas emissions, and global warming are environmental issues accelerated by using fossil fuels. In addition, elevated consumption of fossil fuels is rapidly depleting the supply of known fossil fuel reserves worldwide. Hence, developing an alternative and renewable supply of energy is essential to fulfill the global energy demand. Hydrogen (H_2) is considered as a potential alternative energy carrier because it is carbon free, has a high energy yield, and yields non-polluting combustion byproducts (Dincer, 2012). Historically, approximately 90% of H_2 supplies are produced using fossil fuels including natural gas, heavy oils, and coal (Das and Veziroğlu, 2001). Hydrogen can be also produced from processes such as thermochemical, biological, and the water-splitting. Within the array of different biological H_2 production methods, dark fermentation has several advantages. Dark fermentation can use a wide range of carbohydrates including short chain organic chemicals or organic wastes. Additionally, there is no energy input required when compared to the light dependent fermentation process. In addition, dark fermentation has a higher growth rate of microorganisms and the highest H_2 synthesis rate when compared to other biological H_2 production processes (Holladay et al., 2009; Nath and Das, 2004). Biological H_2 production via dark fermentation using mixed anaerobic cultures is dependent on several factors which can be optimized to ensure a maximum yield.

Different bacterial species such as H_2 consumers and H_2 producers in mixed anaerobic cultures can affect the H_2 yield. Traditionally, glucose has been used as a model substrate for dark fermentative H_2 production. When glucose is the substrate and acetate is the only by-product, the theoretical H_2 yield is 4 mole H_2 per mole of glucose (Equation 4.1). However, the actual H_2 yield is less than the theoretical yield because of the presence of H_2-consuming microorganisms and the production of short chain fatty

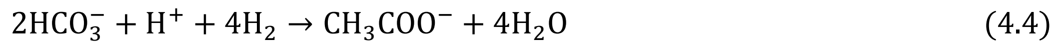
acids and short chain alcohols. Hydrogen consumers such as hydrogenotrophic methanogens, sulfate-reducing bacteria, and homoacetogens can affect the H_2 yield (Stams et al., 2005). Hydrogen consuming reactions are shown in Equation 4.2 – 4.4. Minimizing the H_2 utilization can be achieved by reducing the growth of these populations.



$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (4.1)$$

$$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O \quad (4.2)$$

$$SO_4^{-2} + H^+ + 4H_2 \rightarrow HS^- + 4H_2O \quad (4.3)$$



$$2HCO_3^- + H^+ + 4H_2 \rightarrow CH_3COO^- + 4H_2O \quad (4.4)$$

The pH is a critical operating parameter that affects the H_2 consuming populations in mixed anaerobic cultures. Varying the pH condition can affect the microbial community composition, the production of metabolites, and the enzymatic activity (Silva-Illanes et al., 2017; Ginkel et al., 2001). According to Liu et al. (2008), methanogenesis was suppressed at a pH level less than 6.0. These researchers also reported a pH of 5.5 was the preferred condition to inhibit methanogens and subsequently increase H_2 production. Moreover, pH values between 5.2 and 6.8 are optimal for H_2 production (Fang et al., 2004). According to Park et al. (2005), when the initial pH was set to 3.0, the growth of methanogenic bacteria was restricted and not detected after 22 days. In comparison, Huang et al. (2003) reported acetogens can survive at a pH condition less than 6.5.

Evidence from previous studies has shown that pure sugars, such as glucose, xylose, and sucrose, have been widely utilized for dark fermentative H_2 production (Ray et al., 2008; Lin et al., 2006; Wu and Lin, 2004). However, pure sugars are valuable chemicals for the production of consumer food products and utilizing pure sugars for dark fermentation H_2 production is not cost effective for large-scale H_2 production systems (Ren et al., 2011). Glycerol ($C_3H_8O_3$) is a waste by-product from the biodiesel production process and cannot be disposed into the environment. Growth of the biodiesel industry has caused the price of crude glycerol to decrease by 0.3 US$$\cdot$L^{-1} and refined

glycerol costs 0.9-1.1 US$·$L^{-1}$ in 2009 (Ahmed and Papadias, 2010). In addition, microorganisms can utilize crude glycerol as a substrate via the dark fermentative process (Sarma et al., 2012). The theoretical H_2 yield from 1 mole of glycerol is 7 moles (Equation 4.5). Moreover, if Ac^- is the only by-product, the theoretical yield is 3 mole H_2 per mole of glycerol (Equation 4.6). The objective of this study was to investigate the effect of the initial pH on H_2 production and the carbon byproducts distribution during the dark fermentation of glycerol.

$$C_3H_8O_3 + 3H_2O \rightarrow 7H_2 + 3CO_2 \quad (4.5)$$

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \quad (4.6)$$

4.2 Materials and methods

4.2.1 Chemicals

Glucose (≥ 99.5%, Bedessee Imports LTD, Ontario, Canada) and Glycerol (≥ 99.0%, Sigma-Aldrich, Co., Oakville, Ontario, Canada) were feedstocks used in this study. Chemicals used to prepare basal medium solution were listed in Section 3.1.

4.2.2 Inoculum source

The anaerobic inocula used in this study were obtained from anaerobic bioreactors at an ethanol producing facility and a municipal wastewater treatment facility located in Chatham, Ontario. The cultures were mixed (1:1 ratio) and maintained in a 5-L (4 L liquid and 1 L gas space) anaerobic mother reactor. The preparation and maintenance procedures of the mother reactor were described in Section 3.2. Cultures from the mother reactor were diluted with basal medium to achieve a concentration of 2,000 mg L^{-1} VSS in 160 mL serum bottles. The basal medium used for the inoculum dilution was adapted from Wiegant and Lettinga (1985). The chemical composition of the basal medium was provided in section 3.3.

4.2.3 Experimental design

Experiments were designed to estimate H_2 production from glycerol by receiving initial pH adjustments of 5.5, 6.5, and 7.5 at 37±1ºC. The experimental methods used in

this study were adapted from Lalman and Bagley (2000). The 2,000 mg L^{-1} VSS cultures were fed with 5,110 mg L^{-1} glycerol on day 0. Control cultures were fed 5,000 mg L^{-1} glucose on day 0. Preparation of serum bottle reactors was outlined in Section 3.3.

The substrate injection date was considered as day 0. The duration of this study was 4 days. After the substrate injection, the total liquid volume of each serum bottle was 50 mL on day 0. Headspace samples and liquid samples were withdrawn every 24 hours.

4.2.4 Analytical methods

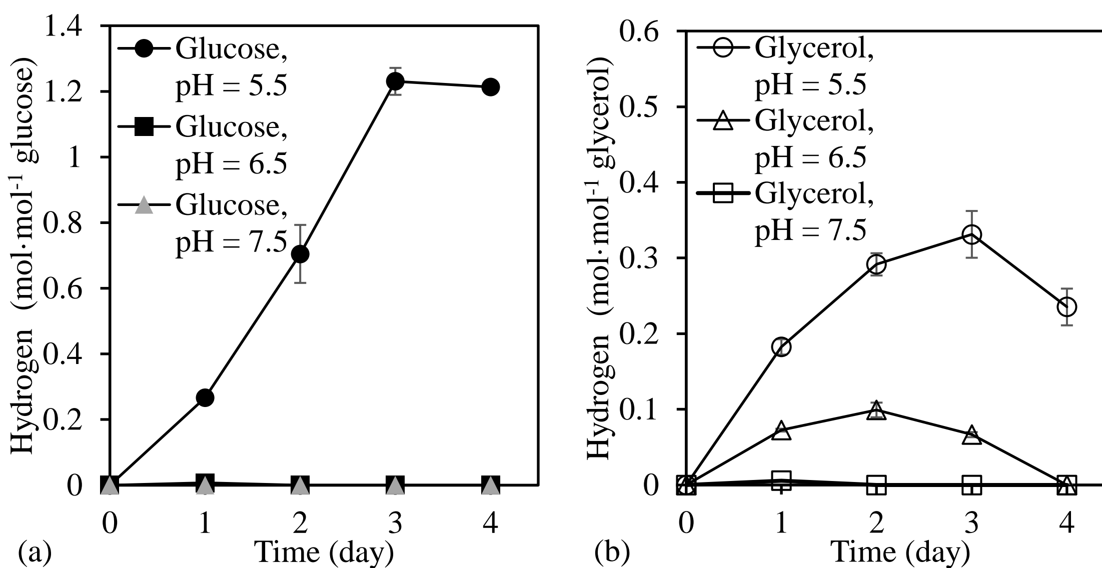
Headspace gas samples (25 μL) were analyzed using a Varian 3800 gas chromatograph (GC) (Varian Inc., Palo Alto, USA) as outlined in Section 3.5.3. The sampling procedure for removing liquid samples was discussed in Section 3.5. VFAs were analyzed using an UltiMate 3000 high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, USA) configured with a HiPlex H, 300 × 7.7 mm diameter Column (Agilent, Santa Clara, California, USA). Details of VFAs analysis were provided in Section 3.5.1. Glucose, glycerol, and alcohols were analyzed using the UltiMate 3000 HPLC equipped with a refractive index detector (RI-101, Shodex, Tokyo, Japan) and the analytical column as outlined in Section 3.5.2.

4.3 Results

4.3.1 Hydrogen and methane production

Hydrogen production was detected only when the initial pH was adjusted to 5.5 in cultures fed with glucose. Hydrogen was detected from day 1 to day 4 (Figure 4.1 (a)). The highest yield of 1.23 ± 0.04 mol H_2 mol^{-1} glucose was observed on day 3. No measurable level of H_2 was produced by glucose samples at initial pH values of 6.5 and 7.5. In cultures receiving glycerol, H_2 production was detected at initial pH values of 5.5 and 6.5 (Figure 4.1 (b)). When the initial pH was adjusted to 5.5, the highest yield was 0.33 ± 0.03 mol H_2 mol^{-1} glycerol. The H_2 yield was less than 0.1 mol H_2 mol^{-1} glycerol at an initial pH of 6.5. From day 3 to 4, H_2 consumption was observed in glycerol samples at initial pH values of 5.5 and 6.5. There was no H_2 produced at an initial pH of 7.5. For the different substrates, the pH value of 5.5 was favourable for H_2 production.

At elevated initial pH conditions, increased quantities of CH_4 were produced in cultures fed glucose (Figure 4.1 (c)). On day 4, the highest CH_4 yield (approximately 0.76 mol CH_4 mol^{-1} glucose) was observed in glucose controls at an initial pH of 7.5. In comparison, at the initial pH of 5.5, there was no CH_4 produced in cultures fed glucose. In cultures receiving glycerol, a similar trend demonstrated that increasing quantities of CH_4 was produced with increasing the initial pH. A maximum amount of CH_4 (approximately 0.52 mol CH_4 mol^{-1} glycerol) was observed at the initial pH value of 7.5 and a lower amount of CH_4 was obtained when the pH value was 6.5. At the initial pH of 5.5, a small amount of CH_4 was observed in glycerol samples on day 4 (Figure 4.1 (d)). From day 3 to day 4, H_2 consumption was observed in glycerol fed cultures at initial pH values of 5.5 and 6.5 (Figure 4.1 (b)). On day 4 for glycerol fed cultures at pH 5.5, H_2 consumption caused a decrease in the H_2 yield.



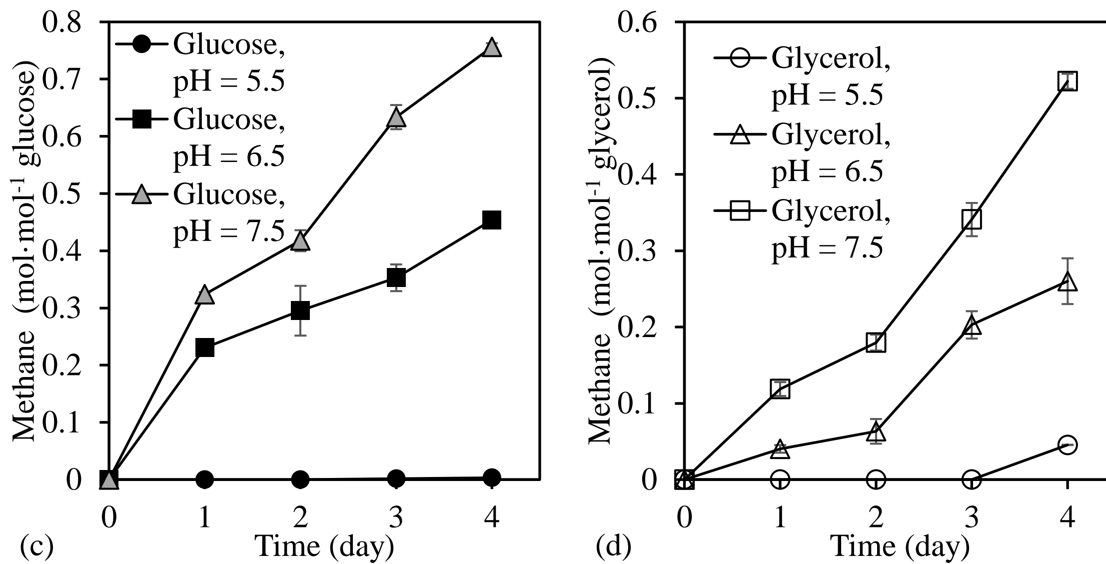


Figure 4.1: Hydrogen and Methane production profiles for mixed cultures receiving 5,000 mg L^{-1} glucose or 5,110 mg L^{-1} glycerol (Hydrogen production is shown in a and b; Methane production is shown in c and d).

Note: The average and SD for triplicate samples are shown in the figures.

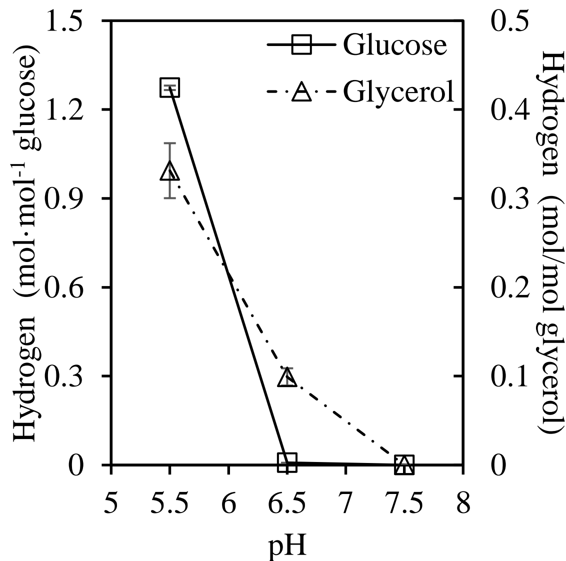


Figure 4.2: The highest hydrogen yields versus varying initial pH values for the mixed cultures receiving 5,000 mg L^{-1} glucose or 5,110 mg L^{-1} glycerol.

Note: The average and SD for triplicate samples are shown in the figure.

4.3.2 VFA and alcohol production

The VFAs produced in the glucose controls included Ac^-, Pr^-, La^-, and But^- (Figure 4.3). At an initial pH of 5.5, La^- accumulated and reached a peak level at approximately 420 mg L^{-1} on day 2. In comparison, La^- was not detected at the other pH conditions (Figure 4.3 (a)). The Ac^- levels increased with increasing the initial pH values. The Ac^- concentration reached a maximum of approximately 730 mg L^{-1} at an initial pH of 7.5. Under all the initial pH conditions, the Ac^- level was substantially elevated on day 1 and gradually increased over the duration of the study (Figure 4.3 (b)). The formation of Pr^- was dominant at initial pH values of 6.5 and 7.5, but no Pr^- was detected at a pH of 5.5 (Figure 4.3 (c)). On day 3, the But^- concentration (approximately 920 mg L^{-1}) was significantly higher in cultures with an initial pH of 5.5 when compared to cultures with the other initial pH values (Figure 4.3 (d)). At the initial pH of 7.5, the But^- concentration decreased from day 2 to day 4.

The initial pH level also influenced the alcohol production profile. EtOH and i-PrOH were the primary alcohols detected in mixed cultures fed with glucose. EtOH accumulated and reached a maximum concentration (approximately 380 mg L^{-1}) at a pH of 5.5 on day 3. At the initial pH of 6.5, a maximum concentration of 390 mg L^{-1} was observed on day 1 and then the EtOH concentration decreased to 76 mg L^{-1} on day 3. The EtOH concentration did not surpass 200 mg L^{-1} at an initial pH of 7.5 (Figure 4.4 (a)). The i-PrOH concentration increased steadily and reached a maximum concentration of 388 mg L^{-1} at an initial pH of 5.5. When the initial pH was 6.5, the i-PrOH concentration significantly increased to approximately 700 mg L^{-1} and then sharply decreased to 305 mg L^{-1} on day 2. At a pH of 7.5, the i-PrOH concentration increased to 255 mg L^{-1} on day 1 and then reduced to 15 mg L^{-1} on day 4 (Figure 4.4 (b)).

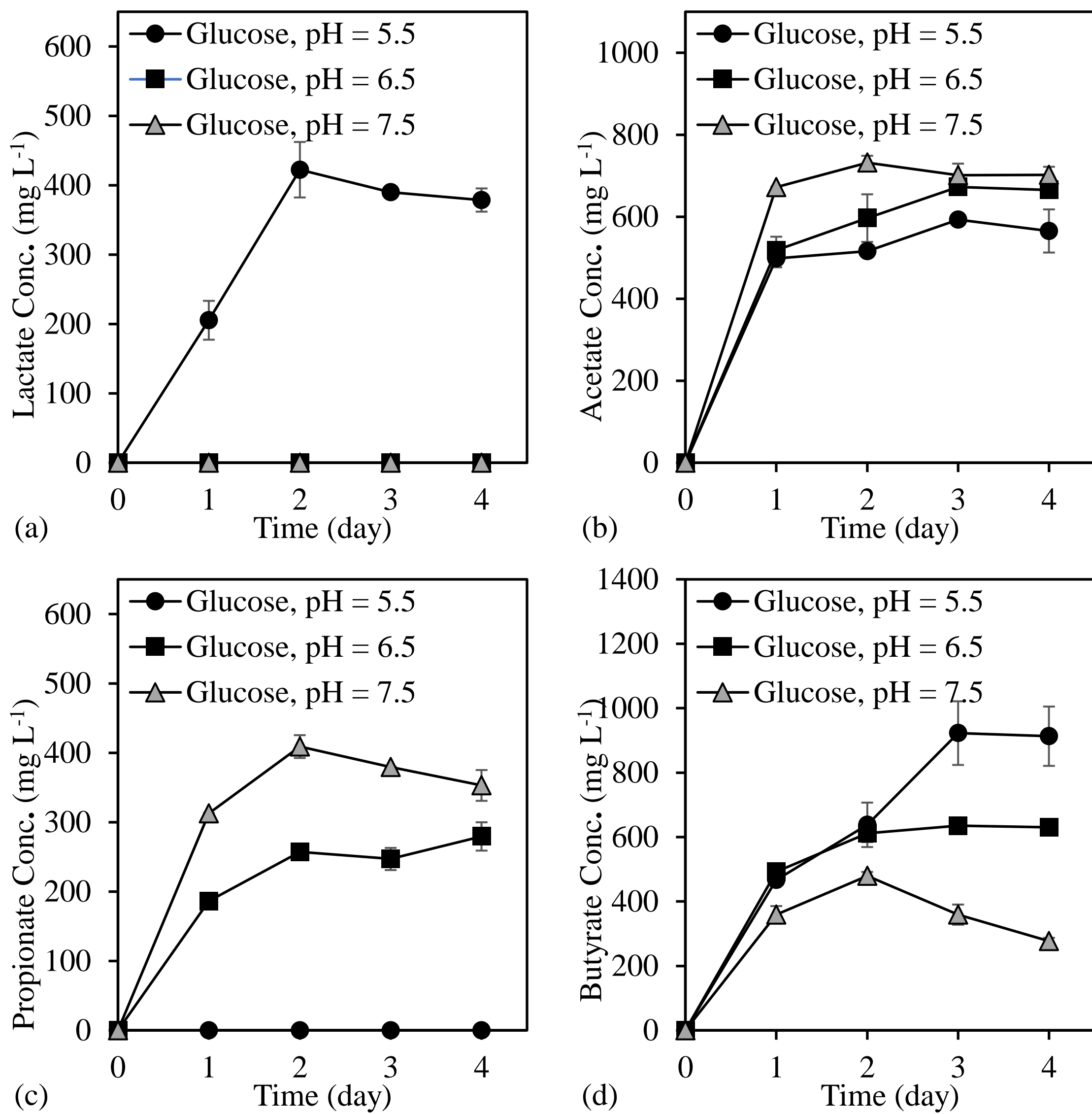


Figure 4.3: VFA production at different initial pH conditions for mixed cultures fed with 5,000 mg L^{-1} glucose (Lactate production is shown in a; Acetate production is shown in b; Propionate production is shown in c; Butyrate production is shown in d).

Note: The average and SD for triplicate samples are shown in the figures.

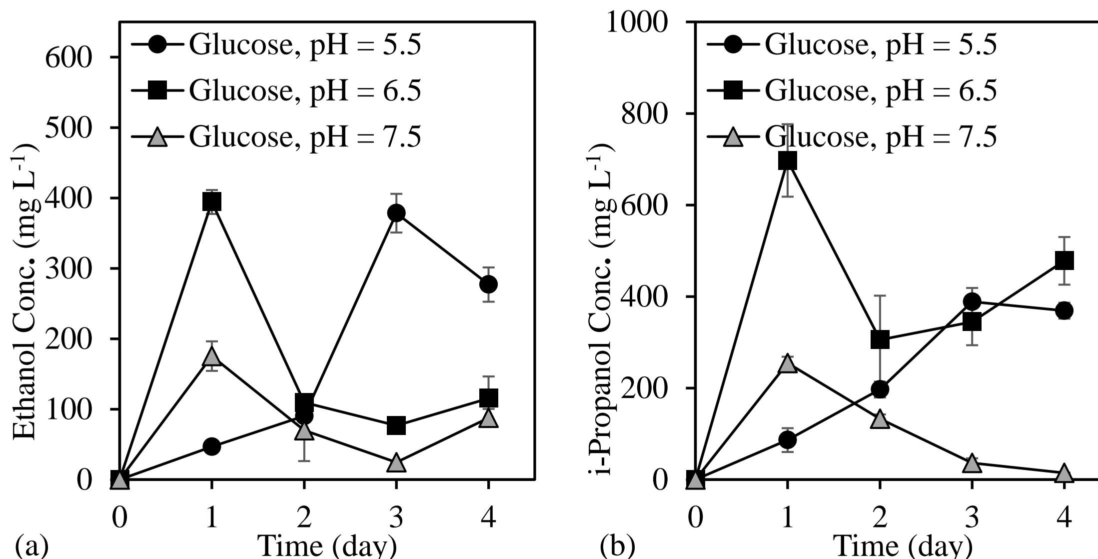


Figure 4.4: Alcohol production at different initial pH conditions for mixed cultures fed with 5,000 mg L^{-1} glucose (Ethanol production is shown in a; iso-Propanol production is shown in b).

Note: The average and SD for triplicate samples are shown in the figures.

When the cultures were fed glycerol, the major VFAs produced were different when compared to the glucose controls at the various initial pH conditions. Lactate formation was dominant at an initial pH of 6.5 with a maximum concentration of approximately 300 mg L^{-1} on day 2 (Figure 4.5 (a)). However, the La^- concentration was low at an initial pH of 5.5 and was not detected at a pH of 7.5. Acetate was observed under all the three pH conditions with the level increasing to approximately 800 mg L^{-1} at the initial pH of 7.5 (Figure 4.5 (b)). In comparison, the Ac^- level was approximately 370 mg L^{-1} and maintained a similar level until day 4 when the initial pH values were 5.5 and 6.5. Propionate production was not detected in cultures with initial pH values at 5.5 and 6.5. However, at the initial pH of 7.5, Pr^- accumulated and peaked at approximately 230 mg L^{-1} on day 3 (Figure 4.5 (b)). From day 0 to day 1, a lag VFAs production period was observed in cultures fed with glycerol at all pH conditions.

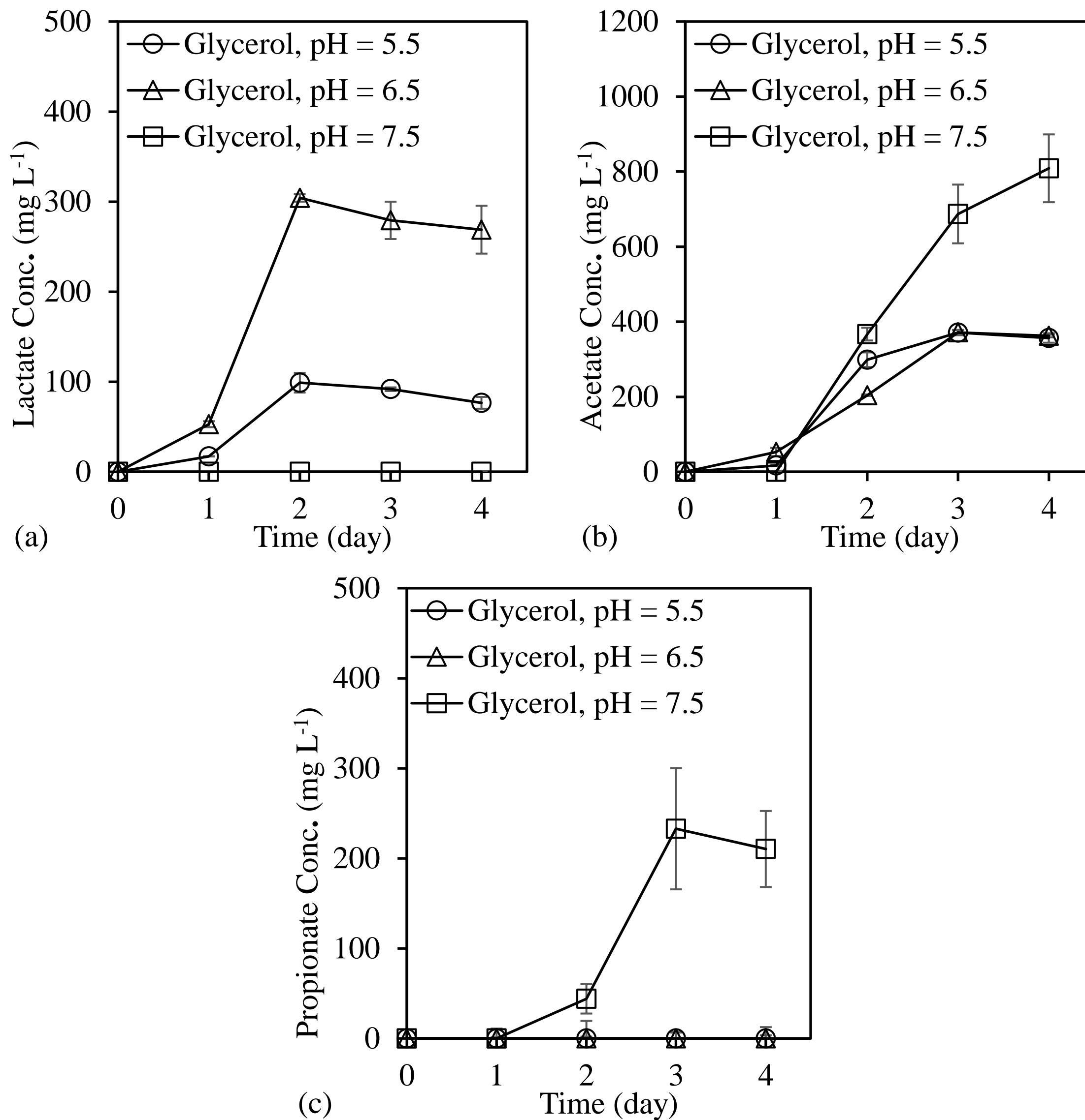


Figure 4.5: VFA production at different initial pH conditions for mixed cultures fed with 5,110 mg L^{-1} glycerol (Lactate production is shown in a; Acetate production is shown in b; Propionate production is shown in c).

Note: The average and SD for triplicate samples are shown in the figures.

In the cultures fed with glycerol, the alcohols produced were not the same as cultures receiving glucose. EtOH and 1,3-PDO were two major alcohols observed in the glycerol fed samples. 1,3-PDO production trends were similar when compared to the glycerol samples at all pH conditions. The 1,3-PDO level increased to a maximum concentration of 1,180 mg L^{-1} and 1,300 mg L^{-1} at initial pH values of 5.5 and 7.5,

respectively (Figure 4.6 (a)). At the initial pH of 6.5, lesser amounts of 1,3-PDO were detected from day 2 to day 4. The EtOH production reached a plateau (approximately 710 mg L^{-1}) on day 3 for cultures receiving glycerol and with an initial pH value of 6.5. When the initial pH was 5.5, the EtOH concentration increased to approximately 650 mg L^{-1} on day 4. A low EtOH level was detected in cultures at an initial pH value of 7.5 (Figure 4.6 (b)).

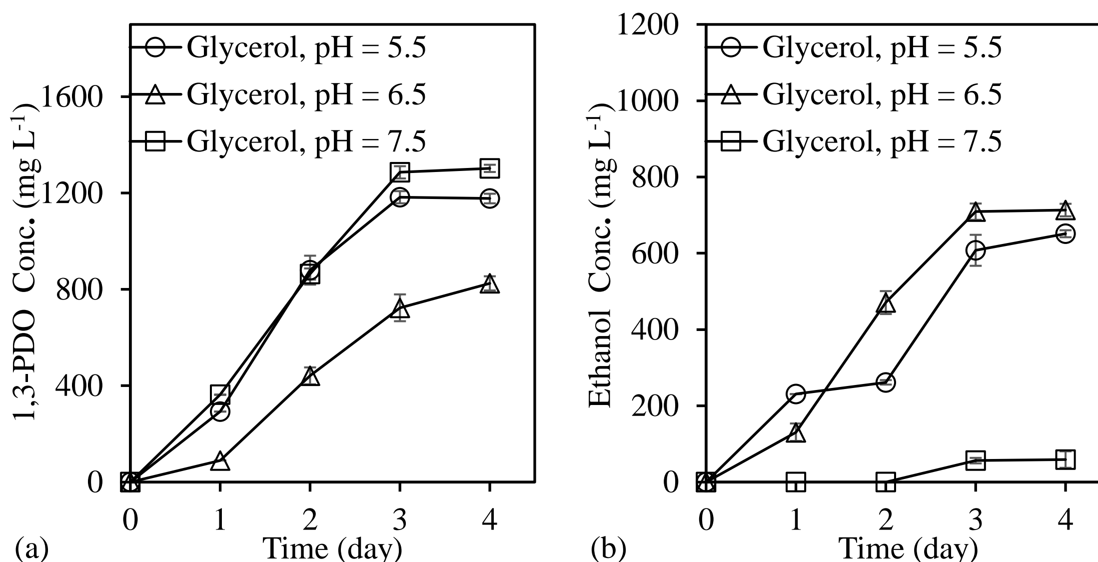


Figure 4.6: Alcohol production at different initial pH conditions for mixed cultures fed with 5,110 mg L^{-1} glycerol (1,3 propanediol production is shown in a; Ethanol production is shown in b).

Note: The average and SD for triplicate samples are shown in the figures.

4.3.3 Glucose and glycerol degradation

Changing the pH affected the glucose degradation rate. The largest degradation rate (5.2 µg $mgVSS^{-1}$ min^{-1}) was observed for the glucose controls at the initial pH of 7.5 (Figure 4.7 (c)). Within approximately 8 hours, the glucose concentration decreased from 5,000 mg L^{-1} to 0 mg L^{-1} (Figure 4.7 (a)) at pH 7.5. When the initial pH level was adjusted to 5.5, the degradation time increased, and the glucose degradation rate decreased. All the glucose was consumed within approximately 36 hours when the initial pH was 5.5. For mixed cultures fed glycerol, the substrate degradation rates (from 0.76 to

0.83 µg mgVSS^{-1} min^{-1}) was not significantly affected by the different initial pH conditions (Figure 4.7 (c)). The glycerol degradation rate was insignificant during the first 12 hours when compared to the trend for the 12 to 24 hours and 24 to 48 hours periods. Glycerol was fully degraded within approximately 72 hours for all glycerol samples adjusted for the different initial pH values (Figure 4.7 (b)). The lowest substrate degradation rates for glucose and glycerol samples were observed at an initial pH of 5.5.

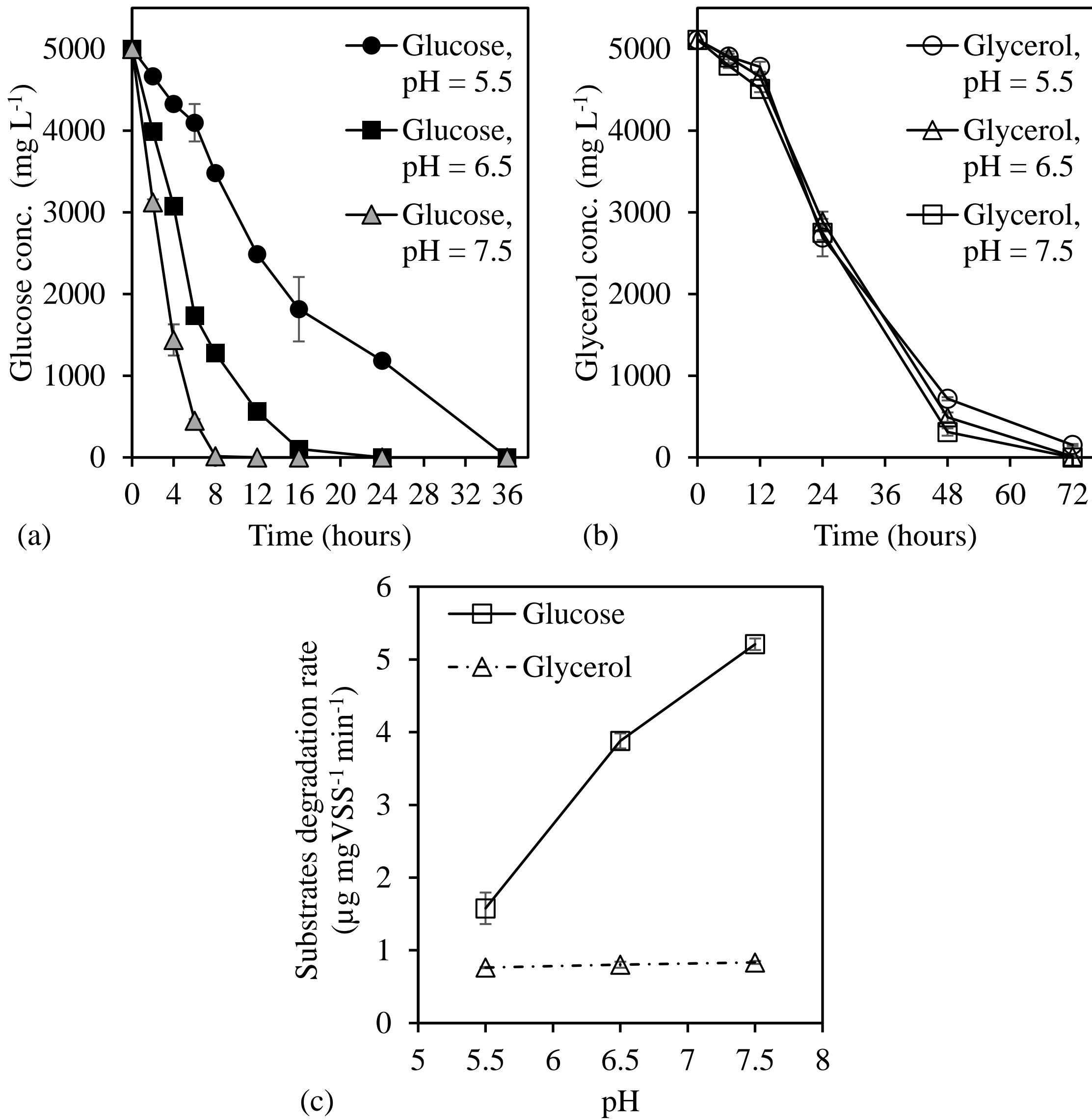


Figure 4.7: Substrate degradation and degradation rates at different initial pH conditions in the mixed cultures fed with 5,000 mg L^{-1} glucose or 5,110 mg L^{-1} glycerol (Glucose degradation is shown in a; Glycerol degradation is shown in b; Degradation rate of two substrates is shown in c).

Note: The average and SD for triplicate samples are shown in the figures.

4.4 Discussion

Many studies have reported using glucose as a model substrate for dark fermentative hydrogen production (Ray et al., 2008; Fang and Liu, 2002; Das and Veziroğlu, 2001). In the glucose controls, a maximum H_2 yield of 1.23 ± 0.04 mol H_2 mol^{-1}glucose was observed when the initial pH was 5.5. The pH of 5.5 has been reported as an optimum to produce H_2 from glucose (Tapia-Venegas et al., 2013; Rat et al., 2008; Khanal et al., 2004; Fang and Liu, 2002). In this study, when the initial pH was adjusted to 6.5 and 7.5, H_2 production was not detected. Fang and Liu (2002) also demonstrated that the H_2 yield was extremely diminished when the pH was higher than 7.5. When the initial pH value was changed from 5.5 to 7.5, the gas production shifted from H_2 to CH_4 with the observed maximum CH_4 yield at the initial pH of 7.5. Ray et al. (2008) reported that the largest amount of CH_4 production was observed when the initial pH was adjusted to 7.6 in the mixed anaerobic cultures fed with glucose.

In this study, the pH affected H_2 production from glycerol degradation. The maximum H_2 yield (0.33 ± 0.03 mol H_2 mol^{-1} glycerol) was observed at an initial pH of 5.5. Tapia-Venegas et al. (2015) reported that a H_2 yield of 0.4 mol H_2 mol^{-1}glycerol was achieved in the mixed cultures at pH 5.5. Similar work by Silva-Illanes et al. (2017) reported that an optimum quantity of 0.58 mol H_2 mol^{-1}glycerol was produced from glycerol at a pH value of 5.5 by mixed anaerobic cultures.

Hydrogen consumption was observed in cultures fed glycerol at pH of 5.5 and 6.5, and this resulted in CH_4 production. Saady (2013) and Ray et al. (2008) reported preventing H_2 consumption and hence, increasing the H_2 yield can be accomplished by including an additional culture treatment method to inhibit the H_2-consumers. Saady (2013) and Ray et al. (2008) reported employing LCFAs to inhibit H_2-consumers with a subsequent increase in H_2 production. Other pH conditions from 6.5 to 8 were examined for producing H_2 from glycerol (Varrone et al., 2013; Seifert et al., 2009). Although different pH conditions were considered and analyzed, pure cultures were employed to produce H_2 in studies reported by Varrone et al. (2013) and Seifert et al. (2009).

Short-chain carboxylic acids (Ac^-, Pr^-, and But^-) were the main VFAs produced in the glucose controls. The metabolic pathway for producing Pr^- was influenced by varying the initial pH levels in the glucose fed cultures. The acidic pH condition indicates the inhibitive effect for Pr^- producing bacteria (Inanc et al., 1996). In this study, Pr^- was not detected in cultures with an adjusted initial pH of 5.5. The highest H_2 yield was associated with the maximum But^- production at a pH of 5.5 in cultures fed glucose. Levin et al. (2004) reported that when the end fermentation product was Ac^- or But^-, the associated H_2 yields were high; however, the Pr^- production caused lower H_2 yields. In a glucose fed *Clostridium* culture, the alcohols produced included EtOH, i-Prop and ButOH (Sung et al., 2002). The accumulation of EtOH and i-Prop in the glucose fed cultures was observed at an initial pH of 5.5; however, lower levels of these alcohols was detected at pH values of 6.5 and 7.5. Similarly, Ray et al. (2008) reported that EtOH and i-Prop were produced under acidic conditions.

In glycerol fed cultures, Ac^-, EtOH, and 1,3-PDO were the major fermentation by-products. The results are consistent with other studies (Akutsu et al., 2009; Moscoviz, Trably, and Bernet, 2016; Temudo et al., 2008). 1,3-PDO production was observed for pH values from 5 to 9 and the production increased at pH levels greater than a pH of 7 (Moscoviz et al., 2016). Based on the work by Moscoviz et al. (2016), the formation of 1,3-PDO was correlated with Ac^- production but negatively correlated with EtOH and La^- formation. In this study, the highest 1,3-PDO production was associated with the highest Ac^- production and the lowest EtOH production at the initial pH value of 7.5. In addition, 1,3-PDO was produced under all the pH conditions under consideration. 1,3-PDO is produced via the reduced metabolic pathway for glycerol degradation which is competitive with the oxidative pathway in which H_2 is produced (Silva-Illanes et al., 2017). Tapia-Venegas et al. (2015) indicated that the higher H_2 yield of 0.4 mol mol^{-1} glycerol was observed when the major metabolites were Ac^- and EtOH. Other studies have also reported the H_2 yield was 0.82 to 1.05 mol H_2 mol^{-1} glycerol when EtOH was the main reduced metabolite using pure cultures at a pH of approximately 6.5 (Ito et al., 2005; Nakashimada et al., 2002). Similarly, in this study, EtOH production was

substantially higher under an acidic pH when compared to the byproducts observed at a pH of 7.5.

In the glucose controls, the varying pH demonstrated an effect on the substrate degradation rates. The lowest degradation rate with the highest H_2 yield was detected at an initial pH of 5.5. Viana et al. (2014) reported the activity of methanogens was suppressed at a pH range of 4.5 to 5.7 and H_2-producing bacteria adapted in this range. In this study, the substrate degradation rates increased when the initial pH increased from 5.5 to 7.5. Glycerol and glucose were almost completely degraded over 72 hours and 36 hours, respectively. The degradation rate of glycerol was low during the first 12 hours and lag phases were detected during the formation of VFAs. According to Tapia-Venegas et al. (2015), when the glucose fed cultures gradually changed the feedstock to glycerol at a pH of 5.5, the biomass yield decreased, and this resulted in lower the substrate degradation rate and biogas production.

4.5 Conclusion

In this study, glucose and glycerol were examined for H_2 production in mixed anaerobic cultures. The initial pH condition affected the H_2 yield, the production of metabolites, and the substrate degradation rate. The largest H_2 yields for both glucose controls and glycerol fed cultures were observed at a pH value of 5.5. Hydrogen consumption was detected in glycerol samples at initial pH adjustments of 5.5 and 6.5. The CH_4 production instead of the H_2 production was dominant in the control and glycerol fed cultures with increasing the initial pH levels. Under all the pH conditions under investigated, Ac^-, But^-, EtOH, and i-Prop were the main metabolites detected in the glucose control. The amount of the glucose degradation byproducts varied with initial pH adjustment. In comparison, the major metabolites from glycerol degradation were Ac^-, EtOH, and 1,3 PDO. The glucose degradation rate was a function of the initial pH level and only small changes in the glycerol degradation were observed for initial pH values of 5.5, 6.5, and 7.5. An analysis of the glucose controls demonstrated the H_2-producing ability of the mixed cultures. The highest H_2 yield was 0.33 ± 0.03 mol H_2 mol^{-1} glycerol at an initial pH of 5.5.

4.6 References

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Chapter 5: EFFECTS OF LONG-CHAIN FATTY ACIDS (LCFAs) ON H_2 PRODUCTION FROM GLYCEROL IN MIXED ANAEROBIC CULTURES

5.1 Introduction

Using fossil fuels such as coal, petroleum, and natural gas are contributing factors to many global environmental issues such as rising sea levels, air pollution, greenhouse gas emissions, and global warming. These environmental problems which are associated with a negative impact on all life are responsible for restricting the economic growth of both developing and developed countries. In addition, decreasing fossil fuels inventories is coupled with increasing global demand. This increasing demand and global pollution caused by fossil fuels has driven the need for alternatives of renewable energy sources such as solar, wind, and bio-based energy. Hydrogen (H_2) production from renewable feedstocks is a viable carbon free energy source which can minimize the greenhouse effect caused by the consumption of fossil fuels. Hydrogen has a higher heating value of 141.9 MJ kg^{-1} when compared to the fossil fuels, such as gasoline, 47.5 MJ kg^{-1}, and diesel, 44.8 MJ kg^{-1} (Dincer, 2012).

According to Balat (2008) and Onozaki et al. (2006), H_2 is produced almost exclusively by employing energy intensive processes which include the steam reforming of CH_4 or the partial oxidation of hydrocarbon fuels. Kothari et al. (2008) reported that fossil fuels feedstocks such as natural gas, heavy oils, and coal are employed to produce H_2 production with percent contributions of 48%, 30%, and 18%, respectively. Unlike these energy intensive processes, H_2 production using biological methods will minimize the use of polluting producing fossil fuels. Moreover, renewable biomass feedstocks can be utilized to produce biohydrogen (bio-H_2). Furthermore, the utilization of waste biomass is a means of minimizing the cost of bio-H_2 production.

During bio-H_2 production by dark fermentation, fermentative bacteria utilize carbohydrates from a variety of sources, such as agriculture and municipal wastes. When compared with other bio-H_2 production processes, the growth rate for dark fermentation microorganisms is larger (Holladay et al., 2009; Nath and Das, 2004). Glucose has been used as a model substrate for bio-H_2 production (Silva-Illanes et al., 2017; Ray et al.,

2008), and the theoretical H_2 yield is 4 mol H_2 mol^{-1} glucose when acetic acid is the only byproduct via dark fermentation. However, glucose is a valuable chemical in the food industry, and it is not economically practical for industrial bio-H_2 production.

Glycerol ($C_3H_8O_3$), a by-product from the manufacture of biodiesel, has been considered as an alternative substrate for bio-H_2 production by the dark fermentative process (Silva-Illanes et al., 2017; Tapia-Venegas et al., 2015; Temudo et al., 2008). Along with the rapid growth of the biodiesel industry, the cost for crude glycerol decreased to 0.17 US$·$kg^{-1}$ in 2019 (da Silva Ruy et al., 2020); whereas for model substrates such as glucose, the cost is approximately 0.45 US$·$kg^{-1}$ in 2019. Glycerol is a pollutant at threhold levels and cannot be discharged into the environment. Through the dark fermentative process, crude glycerol can be utilized by microorganisms with some pretreatments depending upon the heavy metal content (Viana et al., 2012; Chatzifragkou and Papanikolaou, 2012). Theoretically, 3 mol of H_2 can be produced from glycerol when acetate acid is the only by-product (Equation 5.1). Therefore, converting the waste by-product (glycerol) into H_2 is more economically viable when compared to glucose.

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \quad (5.1)$$

Using mixed anaerobic cultures to produce H_2 has been reported using organic wastes as feedstocks. According to Ray et al. (2008), the low H_2 yield obtained in mixed cultures is related to H_2-consuming populations such as hydrogenotrophic methanogens, sulfate-reducing bacteria, and homoacetogens (Equation 5.2-5.4). Various methods have been employed to inhibit anaerobic H_2 consuming microorganisms and hence, improve the H_2 yield. For example, the pH level is an important parameter which is related to the dominant microbial populations. An optimal H_2 yield was observed at a pH of 5.5 (Silva-Illanes et al., 2017). Thermal shock is another factor which was used to control undesirable populations and improve the H_2 yield from 0.11 to 0.41 mol H_2 mol^{-1} glycerol (Temudo et al., 2008). Adding chemicals such as long chain fatty acids (LCFAs) or 2-bromo-ethane sulfonate (BES) has been utilized to inhibit H_2 consuming methanogens and increase the H_2 yield in glucose fed anaerobic mixed cultures (Ray et al., 2008; Chowdhury et al., 2007; Kim, Han, and Shin, 2004). The growth of

methanogenic bacteria, such as *Methanosaeta* and *Methanosarcina*, can be controlled at a pH of 3.0 (Park et al., 2005) while acetogenic bacteria (H_2 producers) are able to survive at a pH less than 6.5 (Huang et al., 2003). Thermal shock of microorganisms is a nonreversible process but this process is unsuitable in the large-scale systems because of energy costs. Although BES is a methanogenic inhibitor, major disadvantages of using this chemical include toxic effects if discharged into the environmnet and it is produced from fossil fuels. In comparison, LCFAs are renewable, biodegradable microbial organic inhibitors (Lalman and Bagley, 2001; Barclay et al., 1994). According to Ma et al. (2015) and Palatsi et al. (2012), LCFAs are able to adsorb on microbial cell membranes before transportation across the membranes and into the cell for the further degradation. Depending on the degree of inhibition caused by the LCFA, a delay or lag-phase is observed during the degradation of a substrate. For example, LCFAs are very slowly biodegradable by H_2 producing acetogens but these acetogens can experience a lag-phase in growth and eventually they adapt and begin to degrade LCFAs (Rinzema et al., 1994). Combining LCFAs with low pH conditions is a means of enhancing the inhibition of H_2-consuming populations and to subsequently improve the H_2 yield.

$$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O \quad (5.2)$$

$$SO_4^{-2} + H^+ + 4H_2 \rightarrow HS^- + 4H_2O \quad (5.3)$$

$$2HCO_3^- + 2H^+ + 4H_2 \rightarrow CH_4COOH + 4H_2O \quad (5.4)$$

Linoleic (C18:2) acid (LA) and oleic (C18:1) acid (OA) are two unsaturated LCFAs which have been reported to inhibit aceticlastic methanogenic organisms (Lalman and Bagley, 2000, 2001) and to improve H_2 yields when mixed anaerobic cultures were fed with glucose (Ray et al., 2008; Ray et al., 2010). Lalman and Bagley (2000, 2001) also demonstrated that stearic (C18:0) acid (SA), OA, and LA slightly inhibited hydrogenotrophic methanogenesis. LCFAs shorter than 18 carbon atoms such as palmitic (C16:0) acid (PA), myristic (C14:0) acid (MA), and lauric (C12:0) acid (LUA) are produced from the anaerobic biodegradation of C18:1 and C18:2 LCFAs. According to Ababouch et al. (1994), LUA exerts the strongest inhibitory effect when compared with other saturated fatty acids. At a concentration of 860 mg L^{-1}, LUA was able to decrease

the activity of aceticlastic methanogens by 50% and a mixture of saturated LCFAs which include MA and LUA at threshold levels was able to suppress methanogenesis (Koster and Cramer, 1987).

One objective of the work in this chapter was to determine and compare the effects of both saturated and unsaturated C18 LCFAs on H_2 production from glycerol in mixed anaerobic cultures at 37 ºC and with an initial pH of 5.5. Another objective was to determine and compare the effects of LCFAs shorter than those containing 18 carbons (PA, MA, and LUA) on H_2 production from glycerol at 37 ºC and with an initial pH of 5.5.

5.2 Materials and methods

5.2.1 Chemicals

Glycerol (≥ 99.0%, Sigma-Aldrich, Co., Oakville, Ontario, Canada) was the feedstock used in this study. The purity of LUA, MA, PA, SA, OA, and LA is provided in Section 3.1. Other chemicals which were used to prepare the bottle reactors are listed in Section 3.1.

5.2.2 Inoculum source

The anaerobic inocula used in study were obtained from anaerobic bioreactors at an EtOH producing facility and a municipal wastewater treatment facility located in Chatham, Ontario. The cultures were mixed (1:1 ratio) and maintained in a 5-L (4 L liquid and 1 L gas space) anaerobic mother reactor. The mother reactor was maintained according to the description provided in Section 3.2. Cultures from the mother reactor were diluted with basal medium to achieve a concentration of 2,000 mg L^{-1} VSS in 160 mL serum bottle reactors. The chemical composition of the basal media solution is provided in Section 3.3.

5.2.3 Experimental design

Experiments were designed to examine the effects of LUA, MA, PA, SA, OA, and LA on H_2 production from glycerol at 37±1ºC and the initial pH of 5.5. Each LCFA

concentration employed in this study was 2,000 mg L^{-1}. Control cultures were prepared with the LCFAs and with only glycerol. The experimental methods used in this study were adapted from Lalman and Bagley (2000). Batch experiments were conducted in 160 mL serum bottle reactors. The preparation of serum bottle reactors was described in Section 3.3. The cultures (2,000 mg L^{-1} VSS) were fed with 5,110 mg L^{-1} glycerol on day 0 and fed again with the same amount of glycerol after 4 days. The first glycerol injection was designated as day 0. After the first substrate injection, the total liquid volume of each serum bottle was 50 mL. After 4 days, all the serum bottles were opened, purged with nitrogen gas (99.99%) for 3 minutes and the pH was adjusted to the initial value of 5.5. Next, all the serum bottles were resealed and fed with glycerol in the anaerobic glovebox. The serum bottle headspace was injected with 20 mL of glovebox atmosphere. The duration of this study was set as 8 days. Headspace gas and liquid samples were withdrawn every 24 hours.

The LCFA stock solution (100,000 mg L^{-1}) was prepared using the saponification method reported by Rinzema et al. (1994). Preparation of LCFA stock solution is described in Section 3.5.5. The LCFAs were added 24 hours before adding glycerol as a means to aid in the LCFA adsorption onto the cell surface (Saddy, 2011; Veeravalli, 2014).

5.2.4 Analytical methods

Headspace gas samples (25 μL) were analyzed using a Varian 3800 gas chromatograph (GC) (Varian Inc., Palo Alto, USA) as described in Section 3.5.3. The liquid samples (1 mL) filtration processes were described in Section 3.5. VFAs were analyzed using an UltiMate 3000 high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, USA) as described in Section 3.5.1. Glycerol and alcohols were analyzed using the HPLC equipped with a refractive index detector (RI-101, Shodex, Tokyo, Japan) as described in Section 3.5.2.

The method outlined by Tukey (1949) was used for multiple comparisons of the H_2 yields. The Tukey method is used to compare pairs of the sample means which are significantly different from each other. This method was conducted by using a one-way

analysis of variance (ANOVA) test in Minitab 15 (Minitab Inc., State College, PA) to generate a table of pairwise comparison.

5.3 Results

5.3.1 Hydrogen production

In control cultures injected with only LUA, MA, PA, SA, OA, or LA, H_2 production was not detected. The inhibitory effect of the LCFA chemical structure on the H_2 yield was examined in cultures fed glycerol. This includes the effect of LCFA carbon chain length for saturated structures as well as the degree of carbon-carbon bond unsaturation for structures containing 18 carbons.

Hydrogen was produced and accumulated in all the samples fed with glycerol plus the different LCFAs. After the first glycerol injection, the control, MA, and PA treated cultures rapidly produced H_2 and reached maximum yields of approximately 0.33 ± 0.03, 0.31 ± 0.01, and 0.39 ± 0.03 mol H_2 mol^{-1} glycerol on day 3, respectively (Figure 5.1). In contrast, in the LUA treated cultures the maximum H_2 yield was approximately 0.24 ± 0.01 mol H_2 mol^{-1} glycerol on day 2 (Figure 5.1). After reaching a maximum yield, H_2 production decreased in the control cultures; however, in the LUA, MA, and PA treated cultures, only a slight reduction was observed on day 4. After glycerol was injected again, a peak H_2 yield was attained on day 7 followed by a decrease on day 8 in the control cultures. Unlike the H_2 production trend observed for the first injection, H_2 accumulated from day 5 to day 8, when the cultures were fed with glycerol treated with LUA, MA, or PA. Although H_2 accumulation was observed in the LUA, MA, and the PA treated cultures, the daily H_2 yield for the second glycerol injection was less than the yield for the first injection. Moreover, when the cultures were fed glycerol plus PA, the H_2 yield improved when compared to the control cultures for the first and second glycerol injections. Also, the H_2 yields were significantly different between the controls and PA treated cultures (Table 5.1). When compared to controls, the H_2 yield of LUA treated cultures was low after both glycerol injections (Table 5. 1).

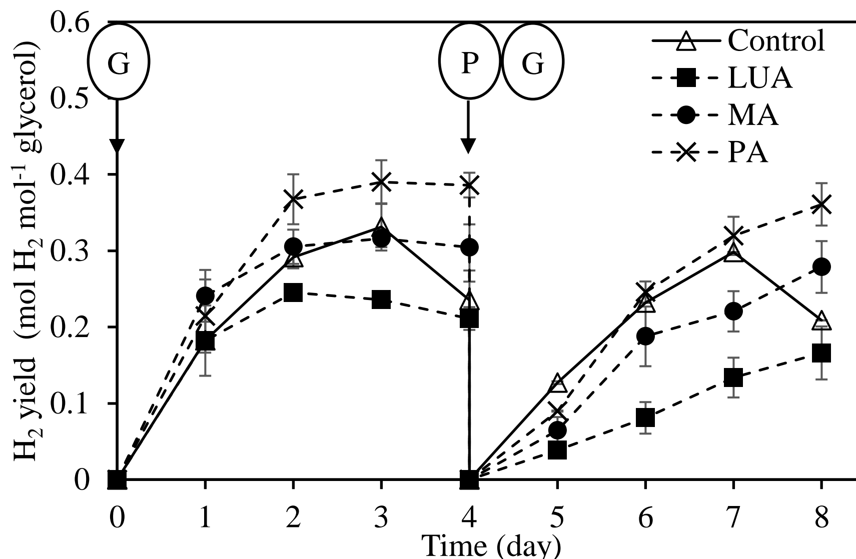


Figure 5.1: Hydrogen production profiles for mixed cultures receiving LUA, MA, PA, or no LCFA (controls) plus glycerol at a pH of 5.5 (G = Glycerol feeding; P = Nitrogen purging).

Note: The average and SD for triplicate samples are shown in the figure.

The H_2 production profiles for cultures receiving C18-LCFAs (SA, OA, or LA) plus glycerol are shown in Figure 5.2. After the first feeding period, the maximum H_2 yield for the LA treated cultures was approximately 0.45 ± 0.01 mol H_2 mol^{-1} glycerol on day 3. In comparison, the SA and OA treated cultures reached peak values of approximately 0.29 ± 0.00 and 0.42 ± 0.01 mol H_2 mol^{-1} glycerol on day 2, respectively. Unlike the control cultures, the H_2 yields for the SA, OA, and LA treated cultures decreased after reaching a maximum level. After the second glycerol injection, the H_2 yield peaked at approximately 0.46 ± 0.03 mol H_2 mol^{-1} glycerol on day 7 when cultures were fed with glycerol plus LA. For the OA treated cultures, the H_2 yield increased to 0.40 ± 0.04 mol H_2 mol^{-1} glycerol on day 8. However, the H_2 production for SA treated cultures was less when compared to the OA and LA treated cultures. In comparison to the control cultures fed with only glycerol, the H_2 yield increased in the presence of OA or LA. The maximum H_2 yield of LA treated cultures observed after the second glycerol injection were higher than the yield after the first glycerol injection (Figure 5.3). The maximum H_2 yields for the OA and LA treated cultures were statistical different when

compared to the yield for control cultures for the first and second glycerol feeding (Table 5.1). An improvement in H_2 production after both glycerol injections was observed for the OA and LA treated cultures. In comparison, a substantial decrease in the H_2 yield was observed for the SA treated cultures.

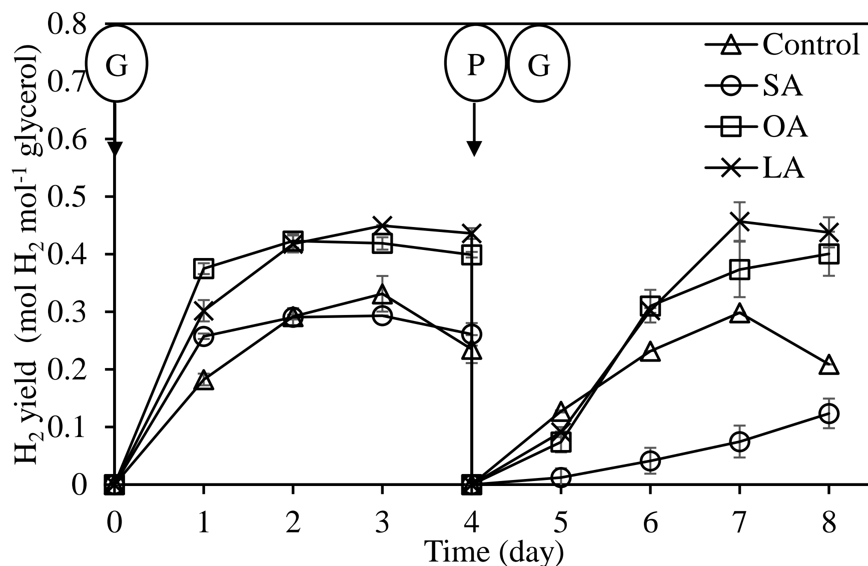


Figure 5.2: Hydrogen production profiles for mixed cultures receiving SA, OA, LA, or no LCFA (controls) plus glycerol at a pH of 5.5 (G = Glycerol feeding; P = Nitrogen purging).

Note: The average and SD for triplicate samples are shown in the figure.

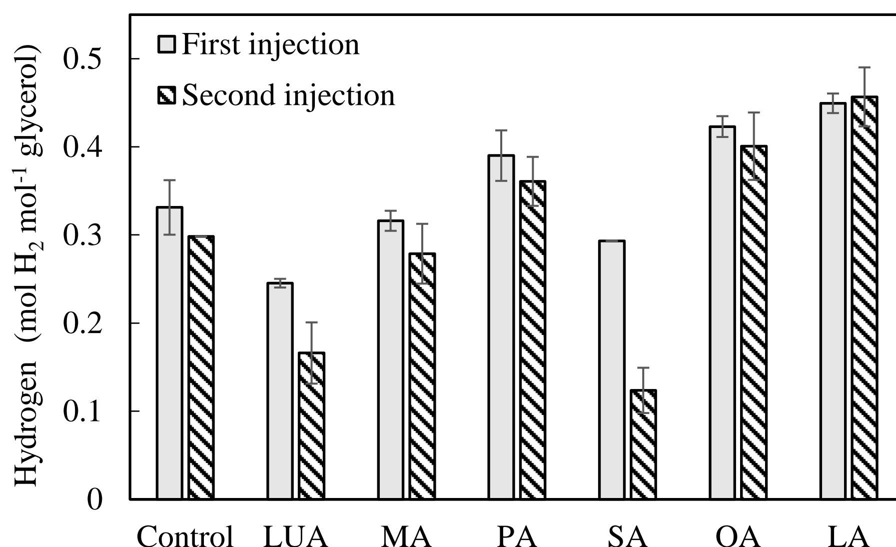


Figure 5.3: Comparing maximum hydrogen yields after the first and second glycerol injections for mixed cultures receiving LUA, MA, PA, SA, OA, LA, or no LCFA (control) plus glycerol at a pH of 5.5.

Note: The average and SD for triplicate samples are shown in the figure.

Table 5.1: Tukey pairwise comparisons of maximum hydrogen yields after the first and second glycerol injections for mixed cultures receiving LUA, MA, PA, SA, OA, LA, or no LCFA (control) plus glycerol

	Mean H_2 yield in the first glycerol injection (mol H_2 mol^{-1} glycerol)	Grouping	Mean H_2 yield in the second glycerol injection (mol H_2 mol^{-1} glycerol)	Grouping
LA	0.45±0.01	A	0.46±0.03	A
OA	0.42±0.01	A B	0.40±0.04	A B
PA	0.39±0.02	B	0.36±0.03	B
Control	0.33±0.04	C	0.30±0.00	C
MA	0.32±0.01	C	0.28±0.03	C
SA	0.29±0.00	C D	0.17±0.02	D
LUA	0.24±0.01	D	0.12±0.03	D

Notes: 1. Statistical comparison using a 95% confidence interval and as reported by Tukey (1949)

2. Data with different letters indicate significant difference and A>B>C>D

5.3.2 VFAs production

The major VFAs produced were La^-, Ac^-, Pr^-, and But^-. La^- levels of less than 200 mg L^{-1} was produced in both glycerol injections when the mixed cultures were inhibited with MA or PA. The La^- concentration reached a peak level of approximately 465 mg L^{-1} on day 6 and with a lower concentration in LUA treated cultures (Figure 5.4 (a)). In the LUA treated cultures, Ac^- accumulated to approximately 750 mg L^{-1} on day 8. In comparison, Ac^- accumulated after the first glycerol injection and was reduced to less than 110 mg L^{-1} on day 8 in the controls, MA, and PA treated cultures (Figure 5.4 (b)). Propionate production was observed 3 days after the first glycerol injection, and the level increased to approximately 365 mg L^{-1} on day 8 in cultures injected with PA. In other cultures, the Pr^- level was less than 150 mg L^{-1} (Figure 5.4 (c)). After the second glycerol injection, the But^- concentration increased from low levels to approximately 900, 720, and 965 mg L^{-1} in the controls, the MA, and the PA treated cultures, respectively (Figure 5.4 (d)). In cultures fed with glycerol and inhibited by various LCFAs, Ac^- production was the major short chain VFA produced within 4 days after the first glycerol injection. Butyrate production was the major VFA produced after the second glycerol feeding in the controls and cultures treated with MA or PA (Figure 5.4).

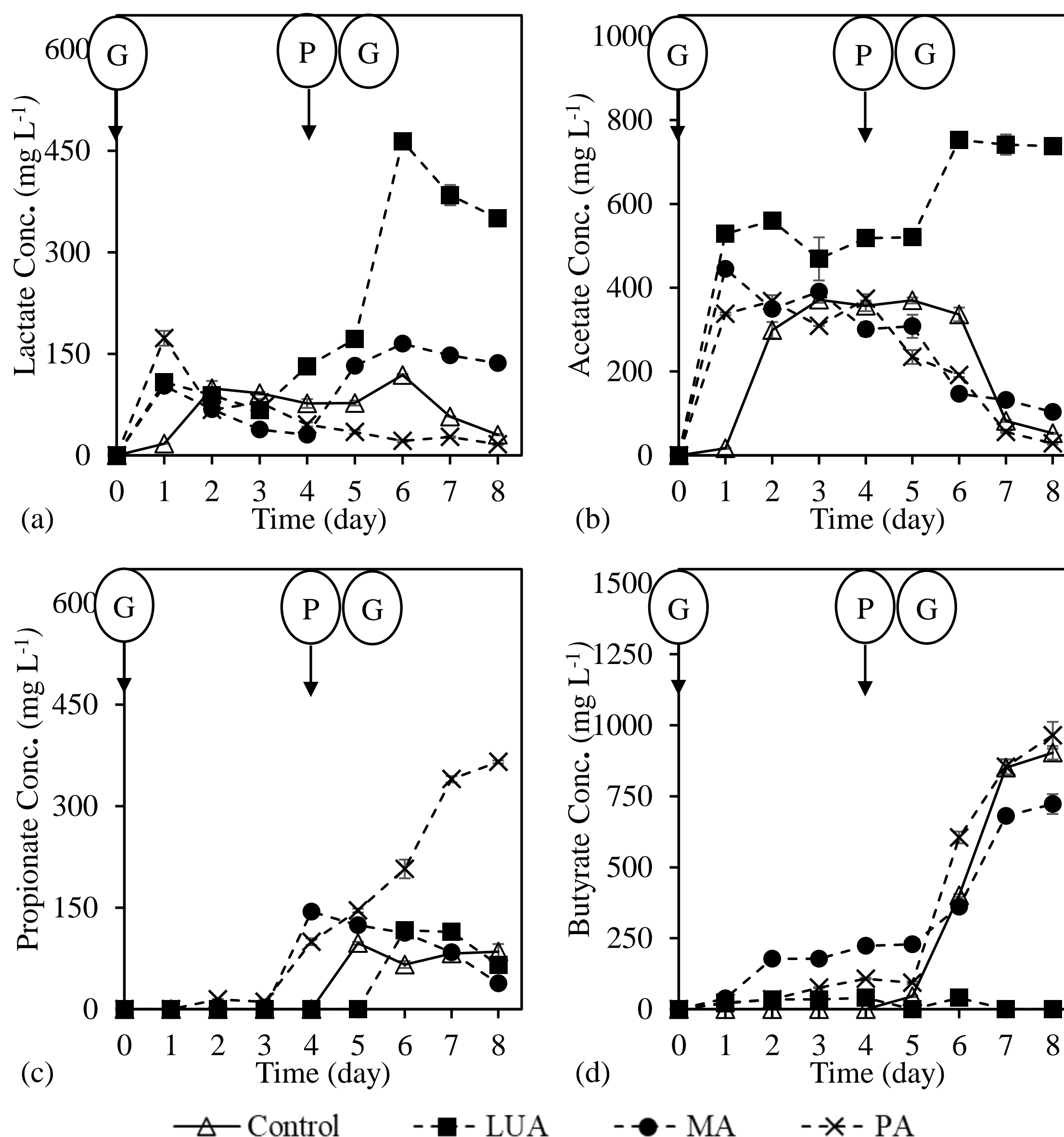
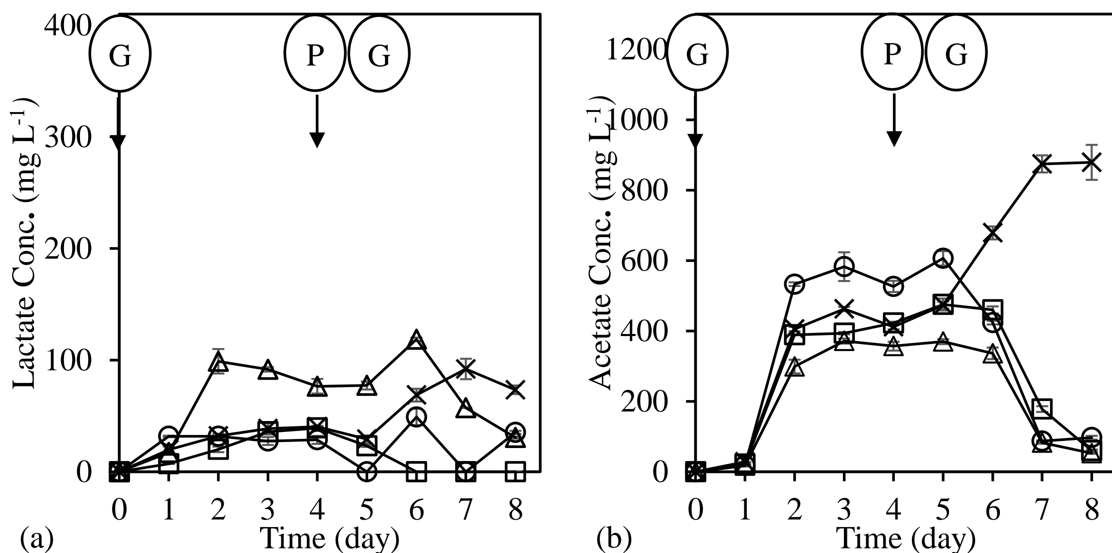


Figure 5.4: VFAs production for mixed cultures receiving LUA, MA, PA, or no LCFA (controls) plus glycerol at a pH of 5.5 (Lactate production is shown in a; Acetate production is shown in b; Propionate production is shown in c; Butyrate production is shown in d; G = Glycerol feeding; P = Nitrogen purging).

Note: The average and SD for triplicate samples are shown in the figure.

La^- production (less than 120 mg L^{-1}) was detected under all the different feeding conditions (Figure 5.5(a)). In the LA treated cultures, the Ac^- concentration increased and then maintained a level of approximately 450 mg L^{-1} after the first glycerol injection.

After the second glycerol feeding, the Ac^- concentration increased to approximately 875 mg L^{-1} within three days (Figure 5.5 (b)). When the cultures were fed with glycerol and plus SA, the Ac^- concentration (approximately 400 mg L^{-1}) was less than that for the OA treated cultures (approximately 540 mg L^{-1}) after the first glycerol injection. After the second glycerol injection, the Ac^- concentration decreased to less than 100 mg L^{-1} for the SA and OA treated cultures (Figure 5.5 (b)). Propionate (approximately 100 mg L^{-1}) was only detected in the OA treated cultures on day 2 after the first glycerol feeding; however, in the control and LA treated cultures, the Pr^- levels were approximately 90 and 110 mg L^{-1}, respectively, on day 8 after the second glycerol feeding. Propionate production was not observed in the SA treated samples (Figure 5.5 (c)). In the LA treated cultures, But^- production was not detected. After the second glycerol injection, the But^- concentration increased in the controls and in the SA and OA treated cultures. The But^- concentration in the control and OA treated cultures increased to approximately 850 and 815 mg L^{-1}, respectively, on day 7. Acetate was a major metabolite in cultures fed glycerol and inhibited by LA. In cultures fed with glycerol and inhibited by SA or PA, the Ac^- concentration was dominant after the first glycerol injection while But^- production was dominant after the second injection (Figure 5.5).



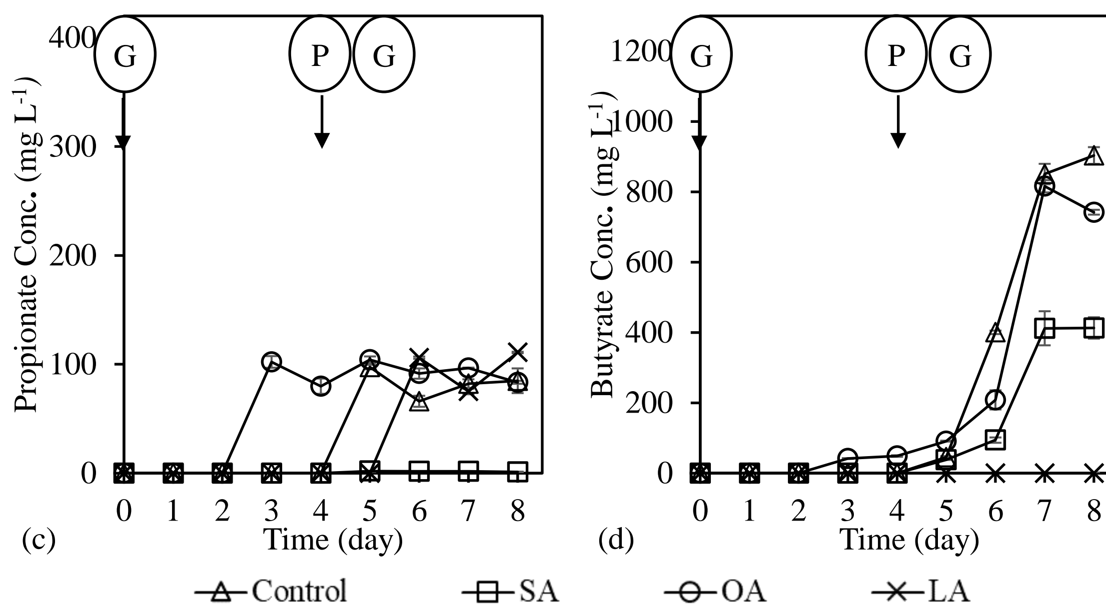


Figure 5.5: VFAs production for mixed cultures receiving SA, OA, LA, or no LCFA (controls) plus glycerol at a pH of 5.5 (Lactate production is shown in a; Acetate production is shown in b; Propionate production is shown in c; Butyrate production is shown in d; G = Glycerol feeding; P = Nitrogen purging).

Note: The average and SD for triplicate samples are shown in the figure.

5.3.3 Alcohol production

Two main alcohols detected included EtOH and 1,3-PDO. After the first glycerol injection, elevated EtOH concentrations were observed with levels reaching approximately 600 and 650 mg L^{-1} in cultures treated with MA or PA, respectively. After the second glycerol injection, the EtOH concentrations increased and reached approximately 1,100 and 890 mg L^{-1} in MA and PA treated cultures, respectively. In comparison, a lower EtOH level was detected in the LUA treated cultures (Figure 5.6 (a)). In cultures receiving glycerol plus LA, the EtOH concentration increased and reached approximately 850 mg L^{-1} on day 4 after the first substrate feeding. After adding glycerol again, the EtOH concentration increased to more than 80% and reached approximately 1,600 mg L^{-1} on day 8 (Figure 5.7 (a)). In cultures inhibited by SA or OA, the EtOH concentrations reached approximately 650 mg L^{-1} on day 2 and maintained a similar level until day 4. On day 8, in the SA or OA treated cultures, the accumulated EtOH

concentration reached 910 and 1050 mg L^{-1}, respectively (Figure 5.7 (a)). Moreover, the EtOH production accumulated to approximately 1,300 mg L^{-1} in the control cultures after the second glycerol injection (Figure 5.6 (a)). In cultures fed glycerol plus LA, larger quantities of EtOH were produced when compared to the SA or OA treated cultures after the second glycerol injection.

The quantity of 1,3-PDO detected was significant in cultures fed with glycerol plus the different LCFAs. In the LUA treated cultures, the concentration of 1,3-PDO reached approximately 1,420 mg L^{-1} and remained at this level after the first glycerol feeding. After the second glycerol feeding, the 1,3-PDO concentration increased to approximately 2,450 mg L^{-1} on day 8 (Figure 5.6 (b)). Also, elevated 1,3-PDO concentrations were observed in the MA and PA treated cultures with levels between 1,100 and 1,300 mg L^{-1} after the first glycerol injection. After adding glycerol again, the 1,3-PDO concentrations accumulated to approximately 2,025 and 1,960 mg L^{-1} in cultures treated with MA or PA, respectively (Figure 5.6 (b)). When cultures were treated with SA or OA, 1,3-PDO production increased after the second injection of glycerol, and the 1,3-PDO concentrations accumulated to approximately 2,050 and 1,990 mg L^{-1} on day 8, respectively. In comparison, when mixed cultures were inhibited by LA, the quantity of 1,3-PDO production was less than other LCFA treated cultures with the concentration reaching approximately 1,530 mg L^{-1} on day 8 (Figure 5.7 (b)).

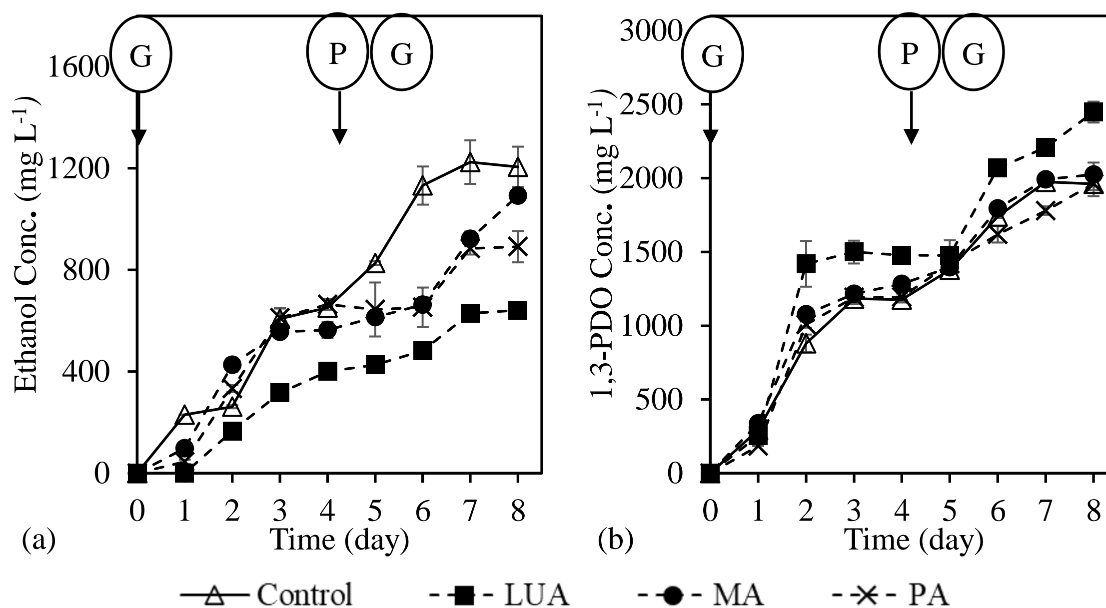


Figure 5.6: Alcohols production for mixed cultures receiving LUA, MA, PA, or no LCFA (controls) plus glycerol at a pH of 5.5 (Ethanol production is shown in a; 1,3-PDO production is shown in b; G = Glycerol feeding; P = Nitrogen purging).

Note: The average and SD for triplicate samples are shown in the figure.

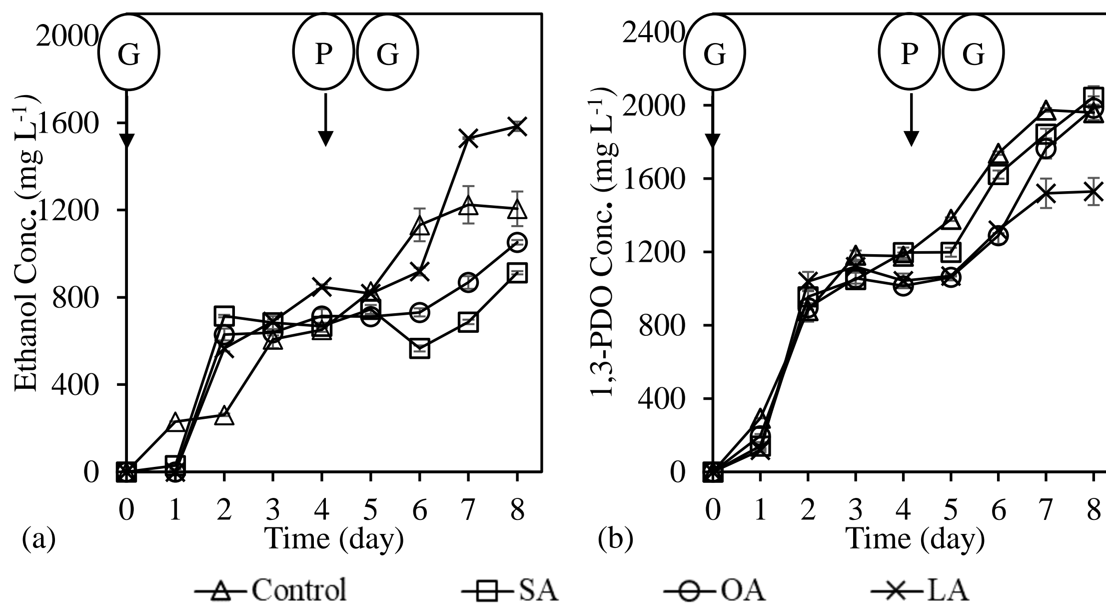


Figure 5.7: Alcohols production for mixed cultures receiving SA, OA, LA, or no LCFA (controls) plus glycerol at a pH of 5.5 (Ethanol production is shown in a; 1,3-PDO production is shown in b; G = Glycerol feeding; P = Nitrogen purging).

Note: The average and SD for triplicate samples are shown in the figure.

5.3.4 Electron balance

When the maximum H_2 yield was observed after the first and second glycerol injections, the major fermentative metabolites were reported as a percent of electron equivalents in Figure 5.8 and 5.9. An accounting of the metabolite electron balance shows that the sum of percent electron equivalents was in the range of 85% and 95%. Three days after the first glycerol injection, 99% of glycerol was depleted and the electron equivalents from glycerol degradation were directed and distributed into gas and liquid metabolites. In the control cultures and cultures treated with MA or PA, approximately 20% and 55% the electron equivalents were directed to EtOH and 1,3-PDO, respectively (Figure 5.8 (a)). In comparison, approximately 10% and 67% electron equivalents were directed to EtOH and 1,3-PDO in cultures treated with LUA. Additionally, the percent electron equivalents directed toward the formation of alcohols were between 72% and 77% but less than 12% of the electron equivalents were directed to VFAs in control samples and samples treated with LUA, MA, or PA (Figure 5.8 (a)). In the MA and PA treated cultures, higher H_2 yields (equivalence of approximately 4.5% and 5.5% electron equivalents) were observed with higher EtOH and lower 1,3-PDO levels. In contrast, the lowest H_2 yield was associated with the highest 1,3-PDO level in cultures contained LUA (Figure 5.8 (a)). In the LUA, MA, and PA treated cultures, glycerol was not completely degraded 4 days after the second glycerol injection. Approximately 20% residual glycerol was observed in cultures treated with LUA (Figure 5.8 (b)). In the PA treated cultures, approximately 25% of the electron equivalents were diverted into But^- after the second glycerol injection. A higher H_2 yield (approximately 5.0% electron equivalents) and a lower alcohol level (approximately 42% electron equivalents) were observed in the PA treated cultures. In comparison, in the MA treated cultures, a lower quantity of electron equivalents was directed to But^- (approximately 15% electrons) after the second glycerol injection. A higher alcohol level (approximately 50% electron equivalents) and a lower H_2 yield (approximately 4.0% electron equivalents) were observed in the MA treated cultures (Figure 5.8 (b)). In comparison with the control samples, most of glycerol was depleted after the second glycerol feeding and large

quantities of electron equivalents were distributed to But^- (approximately 25% electrons) and alcohols (approximately 55% electrons).

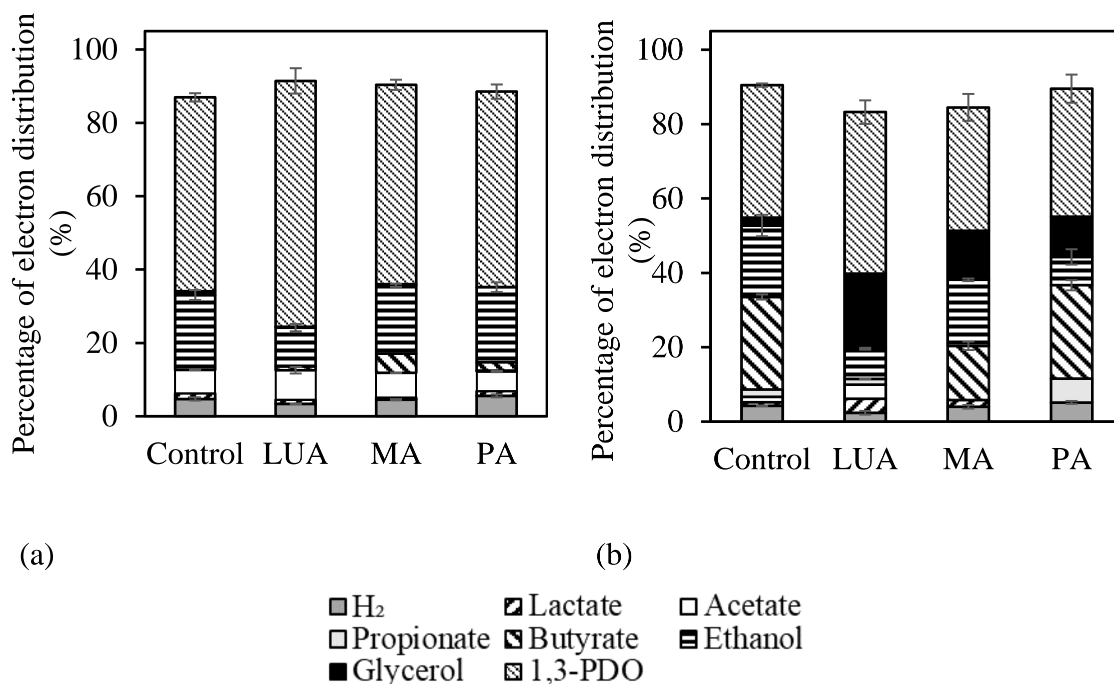


Figure 5.8: Percent electron distribution profiles based on the maximum H_2 yield after the first and second glycerol injections in mixed cultures receiving LUA, MA, PA, or no LCFA (controls) plus glycerol at a pH of 5.5 (First glycerol injection is shown in a; Second glycerol injection is shown in b).

Note: The average and SD for triplicate samples are shown in the figure.

When SA or LA were added to the cultures, maximum H_2 yields were observed on day 3 after the first glycerol injection while the maximum H_2 yield was observed on day 2 in the OA treated cultures. More than 95% of glycerol was consumed 3 days after the first glycerol injection (Figure 5.9(a)). EtOH and 1,3-PDO were a major electron sink in the controls and cultures treated with SA, MA, or LA. Also, the percent electron equivalents converted into Ac^- ranged from approximately 6.0% to 9.0% in the controls and cultures treated with SA, MA, or LA. In the LA treated cultures, a larger fraction of electron equivalents was directed to Ac^- (7.9%) and H_2 (6.5%) when compared to quantity directed to Ac^- (6.5%) and H_2 (4.7%) in the control samples (Figure 5.9 (a)). After injecting glycerol again, the maximum H_2 yield was observed on day 7 for LA

treated cultures and day 8 for SA and OA treated cultures. When the maximum H_2 yield was detected, large quantities of undegraded glycerol were observed in these cultures. Approximately 38% of residual glycerol was observed in the SA treated cultures (Figure 5.9 (b)). In the LA treated cultures, the largest portion of electron equivalents were directed to H_2 (6.6%) and approximately 7.9% and 41% directed to Ac^- and alcohols, respectively. In comparison, larger quantities of electron equivalents were directed into But^- instead of Ac^- in the controls and cultures inhibited by SA or OA (Figure 5.9 (b)).

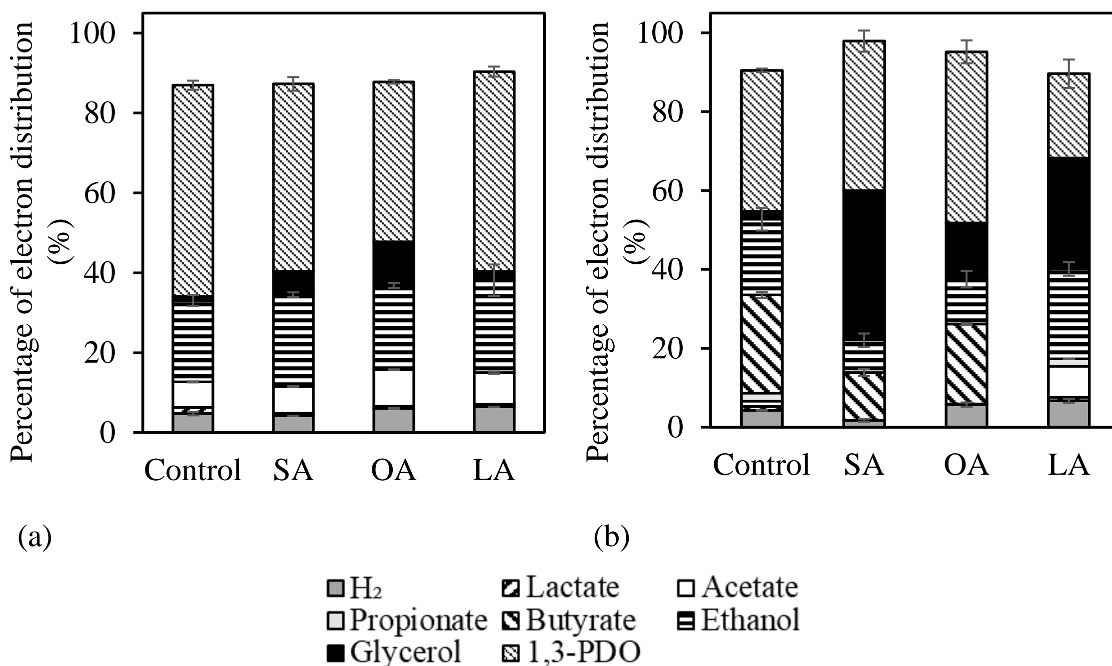


Figure 5.9: Percent electron distribution profiles based on the maximum H_2 yield after the first and second glycerol injections in mixed cultures receiving SA, OA, LA, no LCFA (controls) plus glycerol at a pH of 5.5 (First glycerol injection is shown in a; Second glycerol injection is shown in b).

Note: The average and SD for triplicate samples are shown in the figure.

5.4 Discussion

LCFAs at threshold concentrations are inhibitory to anaerobic microorganisms (Sousa et al., 2009). Many studies have investigated the impact of unsaturated C18 LCFAs (OA and LA) on H_2 production using cultures fed glucose (Ray et al., 2008;

Chowdhury et al., 2007; Lalman and Bagley, 2000) and saturated LCFAs such as SA on hydrogenotrophic methanogenesis (Lalman and Bagley, 2000). LCFAs shorter than 18 carbons such as LUA, MA, and PA are able to suppress methanogenesis (Saady, 2011). In this work, 6 LCFAs (LUA, MA, PA, SA, OA, and LA) were used to examine their effects on H_2 and metabolites production in mixed anaerobic cultures fed glycerol at 37 ºC and an initial pH of 5.5. Glycerol was fed on two occasions as a means of establishing the continued inhibitory effect of LCFAs on suppressing H_2 consumers. When compared to controls, the H_2 consumption observed on day 4 was not significantly different in cultures inhibited by each LCFA. When the cultures were inhibited by a saturated LCFA, the H_2 yield increased with increasing the carbon chain length except for the SA treated cultures. In comparison, the H_2 yield did not improve in cultures treated with MA, SA, or LUA. When the cultures were treated with a saturated LCFA, the H_2 yield was less than the yield in unsaturated LCFA treated cultures. After the first glycerol injection, the data demonstrated that the effect of PA, OA, or LA improved the H_2 yield from 0.33 ± 0.03 to 0.39 ± 0.03, 0.42 ± 0.01, and 0.45 ± 0.01 mol H_2 mol $^{-1}$ glycerol, respectively. The H_2 yields for the LA, OA, or PA treated cultures were significantly different when compared to the control cultures (Table 5.1). In similar studies, other researchers have reported that H_2 yields increased in the presence of PA, OA, or LA when cultures were fed glucose and maintained at 37 ºC at an initial pH value of 5.0 (Saady, 2011; Ray et al., 2008; Grukar, 2005). The highest H_2 yield of 0.46 ± 0.03 mol H_2 mol $^{-1}$ glycerol was observed in the LA treated cultures after the second glycerol injection; however, this yield was not significantly different from the yield in the OA treated cultures (Table 5.1). The maximum H_2 yield (0.46 ± 0.03 mol H_2 mol $^{-1}$ glycerol) in cultures received LA was greater than the yields shown in Table 5.2. Although enriched pure cultures have been used to produce H_2 from glycerol (Silva-Illanes et al., 2017; Ito, et al., 2005), employing mixed anaerobic cultures is advantageous because feedstock sterilization is not required.

After adding glycerol, the VFAs levels were less than the amount of alcohols produced and the main alcohols were EtOH and 1,3-PDO. Similar results were reported by Akutsu et al. (2009), Seifert et al. (2009), and Temudo et al. (2008). In each LCFA treated culture, different quantities of residual glycerol were detected after the second

glycerol injection, while most of glycerol was consumed in the control cultures three days after the glycerol injections. The slow glycerol degradation may have been caused by the accumulation of alcohols and acids which were produced after the first and second

Table 5.2: H_2 yields from glycerol degradation reported by different studies

Inoculum	Operating conditions	Glycerol concentration (mg L^{-1})	H_2 yield (mol H_2 mol $^{-1}$ glycerol)	References
Anaerobic cultures	pH 6.5 35 ºC	4,000	0.11	Akutsu et al. (2009)
Anaerobic mixed cultures	pH 6.0 37 ºC	10,000	0.41	Seifert et al. (2009)
Anaerobic mixed cultures	pH 5.5 37 ºC	5,000	0.40	Tapia-Venegas et al. (2015)
Anaerobic mixed cultures	pH 5.5 37 ºC	5,110	0.46	This study

Note: The main operating conditions and glycerol concentrations are also listed

glycerol injection. Additionally, the inhibitory effect of SA on glycerol degradation was significantly reduced after the second glycerol injection. Acetate (approximately 880 mg L^{-1}) was the main VFA which accumulated in the LA treated cultures while But^- (approximately 965 and 815 mg L^{-1}) was abundant in the PA or OA treated cultures after the second glycerol injection. In comparison to the controls, more electron equivalents (based on electron balance) were directed into producing EtOH (23%) and H_2 (6.4%) with lesser amount directed to 1,3-PDO (50%) in the LA treated cultures after the first glycerol injection. Similarly, Tapia-Venegas et al. (2015) reported elevated Ac^- levels (29.3 - 40.8% COD) and EtOH concentrations (23.5-35.6% COD) were associated with increasing H_2 yields (3.2-6.1 mmol H_2 $gCOD^{-1}$). In comparison, in the LUA treated cultures, the largest quantity of electron equivalents was directed to 1,3-PDO (67%); however, approximately 3.4% electron equivalents were directed to producing H_2 after the first glycerol feeding. Moscoviz et al. (2016) reported that the H_2 production was less than 1% of the total COD when the 1,3-PDO production was dominant (60-74%$_{\text{total COD}}$) at an analyzed range of pH levels between 5 and 9. Adding LA diverted electron equivalents to Ac^- production instead of producing 1,3-PDO and improve the H_2 yield.

5.5 Conclusion

In this study, the impact of selected LCFAs (LUA, MA, PA, SA, OA, and LA) on H_2 and metabolites production from glycerol degradation were analyzed in mixed cultures via dark fermentation at 37 ℃ with an initial pH value of 5.5. All the LCFAs demonstrated some degree of inhibition based on byproducts distribution trend in comparison with the control cultures. In cultures receiving PA, OA, or LA, the H_2 yields increased in comparison to the controls. OA and LA were most effective in enhancing the H_2 production with the highest yield of 0.42 ± 0.01 and 0.46 ± 0.03 mol H_2 mol $^{-1}$ glycerol, respectively. For both glycerol injections, Ac^-, EtOH, and 1,3-PDO production were dominant metabolites in the LA treated cultures. In contrast, Ac^- was the most abundant VFA after the first glycerol injection while But^- production was dominant after the second glycerol injection in cultures treated with PA or OA. The impact of adding LUA increased the production of 1,3-PDO after both glycerol injections. When the maximum H_2 yields were observed after the second glycerol feeding, glycerol was not fully degraded in the LCFA treated cultures.

5.6 References

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Chapter 6: USING A STATISTICAL APPROACH TO OPTIMIZE H_2 PRODUCTION FROM GLYCEROL BY LINOLEIC ACID INHIBITED MIXED ANAEROBIC CULTURES

6.1 Introduction

Rapidly depleting fossil fuel supplies coupled with the negative environmental impacts resulting from using these energy supplies are major issues facing many nations as they strive for increasing economic growth. Negative effects such as increasing air pollution, greenhouse gases production, and global warming, have motivated researchers to develop alternative energy sources.

Hydrogen (H_2), an environment friendly energy source when produced from renewable energy, is carbon neutral and only water is produced during combustion. In terms of combustion efficiency, H_2 is approximately 3-fold (120-142 MJ kg^{-1}) greater than that for fossil fuels, such as gasoline (47.5 MJ kg^{-1}) and diesel (44.8 MJ kg^{-1}) (Patel et al., 2018; Dincer, 2012). Many commercial processes utilized for producing H_2, for example steam reforming and hydrocarbon pyrolysis, are energy-intensive and depend on fossil fuels (Midilli et al., 2005). Producing H_2 from renewable sources is an alternative energy solution which can alleviate social and environmental issues linked to using fossil fuels. Among the different H_2 production processes, biological processes have been widely studied because of the many advantages associated with using these technologies. Biological processes can utilize waste products, generated from various sources such as the food industry and the agricultural residues (Roy and Das, 2016).

Microbial processes which can be employed to produce H_2 include fermentation (photo or dark), biophotolysis (direct or indirect), and microbial electrolysis. When compared with the other methods, H_2 production by dark fermentation can employ different substrates and use a wide range of microorganisms. Dark fermentation requires no energy input and the low operating and maintenance costs are advantageous when compared to the light-dependent process. In addition, the higher microbial growth rate and larger H_2 synthesis rate are additional advantages when utilizing the dark fermentation process (Toledo-Alarcón et al., 2018; Holladay et al., 2009).

Many researchers have reported using glycerol ($C_3H_8O_3$), a low-value by-product from the biodiesel industry, as a substrate for H_2 production by dark fermentation (Silva-Illanes et al., 2017; Zahedi et al., 2016; Tapia-Venegas et al., 2015). Along with the rapid growth of the biodiesel industry, the cost for crude glycerol was 0.17 US$·kg^{-1} in 2019 (da Silva Ruy et al., 2020) whereas for model substrates such as glucose, the cost is approximately 0.45 US$·kg^{-1} in 2019. The theoretical H_2 yield is 3.0 mol H_2 per mol glycerol when Ac^- is the only reduced byproduct during degradation (Equation 6.1). When Ac^-, EtOH, and 1,3-PDO are by-products from the glycerol degradation, the theoretical H_2 yield is 1.8 mol per mol glycerol (Equation 6.2). However, the theoretical H_2 yield is difficult to achieve because of the effect by factors such as pH, feeding substrate, temperature, and the composition of inoculum (Toledo-Alarcón et al., 2018; Wang and Wan, 2009a). Wu et al. (2011) reported that various concentrations of glycerol were examined to assess the impact on H_2 production. The work by Wu et al. (2011) demonstrated that the H_2 yield increased with increasing glycerol concentration less than 50 g L^{-1}. However, when glycerol concentration was larger than 50 g L^{-1}, the H_2 formation was significantly decreased. Similarly, Mangayil et al. (2012) analyzed a range of glycerol concentrations from 0.5 g L^{-1} to 5 g L^{-1} and reported an optimum glycerol concentration of 1 g L^{-1} with a H_2 yield of 1.1 mol H_2 mol^{-1} glycerol at pH of 6.5. Paranhos and Silva (2020) concluded that a balance between the glycerol concentration and the retention time was necessary towards optimizing H_2 production. Based on these studies, the glycerol concentration is considered as an important factor for optimizing H_2 production.

$$C_3H_8O_3 + H_2O \rightarrow CH_3COOH + 3H_2 + CO_2 \quad (6.1)$$

$$5C_3H_8O_3 + 2H_2O \rightarrow CH_3COOH + CH_3CH_2OH + 2CH_2(CH_2OH)_2 + 9H_2 + 5CO_2 \quad (6.2)$$

Employing mixed anaerobic cultures are advantageous because they can utilize many substrates and can adapt to environmental stresses, such as pH and temperature changes (Wang and Wan, 2009a; Temudo et al., 2007). Mixed anaerobic cultures contain H_2-producing and H_2-consuming bacteria. Hydrogenotrophic methanogens, sulfate-reducing bacteria, and homoacetogens are major H_2 consumers (Li and Fang, 2007;

Stams et al., 2005; Conrad and Wetter, 1990). Various pretreatments, such as thermal shock, acidic treatment, and chemical inhibitors can suppress H_2 consumers while preserving the activity of H_2 producers in mixed anaerobic cultures.

According to Li and Fang (2007), thermal shock (100 ºC) pretreatment was effective in suppressing H_2-consumers and enriching H_2-producers such as *Clostridium sp*. Temperatures from 50 to 100 ºC coupled with a thermal shock duration of 20 to 30 min was reported to supress H_2-consumers (Wong et al., 2014; Yin et al., 2014; Baghchehsaraee et al.,2008; Duangmanee et al., 2007). However, thermal shock requires a significant energy input which is uneconomical and therefore, unsustainable for large-scale H_2 production systems. The pH level is another critical parameter that affects H_2-consuming bacteria in mixed cultures. Fang et al. (2004) reported that the optimal pH range is between 5.2 and 6.8 for H_2 production in the mixed cultures. Also, methanogenic growth is restricted when the pH level is less than 6 (Liu et al., 2008; Park et al., 2005). Moreover, the range of pH values from 5.8 to 7.0 is favorable for homoacetogens growth (Huang et al., 2003). Culture treatment such as adding an inhibitor can enhance H_2 production and reduce the activity of H_2 consumers.

Long chain fatty acids (LCFAs) such as oleic acid (OA) and linoleic acid (LA), are inhibitors which have been reported to inhibit methanogens (Ray et al., 2010; Lalman and Bagley, 2000). LCFAs are abundant, biodegradable, and non-toxic organic inhibitors (Lalman and Bagley, 2001; Hwu and Lettinga, 1997). According to Ma et al. (2015) and Palatsi et al. (2012), LCFAs are able to adsorb onto cell membranes before transporting into cell membranes. The transporting process of LCFAs is slow and the degradation of LCFAs by β-oxidation takes place inside the cells (Rinzema et al., 1994). The degradation rates of feedstocks such as carbohydrates are impaired by selected LCFAs because of their inhibitory action on microorganisms (Rinzema et al., 1994). According to Ray et al. (2010) and Chowdhury et al. (2007), methanogenic inhibition by adding LA caused an increase in H_2 and volatile fatty acids (VFAs) production in mixed anaerobic cultures. Ray et al. (2010) reported a H_2 yield of 3.38 mol H_2 mol^{-1} glucose when the LA concentration was increased to 2.0 g L^{-1} at an initial pH of 5.0 and a temperature of 37 ºC. Combining a low pH together with adding LA can aid in increasing the inhibitory effects

on H_2 consumers.

The impact of temperature on fermentative H_2 production is considered an important factor which can affect the function of mixed anaerobic cultures. A review by Li and Fang (2007) has indicated that H_2 production was favourable under mesophilic (30 to 40 ºC) and thermophilic (50 to 64 ºC) temperatures. Evidence from several studies have shown that when operation temperature was set at approximately 37 ºC, higher H_2 yields were obtained in mixed cultures fed with glycerol (Tapia-Venegas et al., 2015; Akutsu et al., 2009; Seifert et al., 2009; Selembo et al., 2009). When the temperature was increased, the hydrolysis rate and the cellular activity were also increased in response to improving H_2 production (Chong et al., 2009; Lee et al., 2008). Based on the current research, the variables selected for optimizing H_2 yield in this study were the initial pH level, the substrate (glycerol) concentration, and the temperature. These varables can be modelled using a variety of statistical methods for optimizing H_2 production (Veeravalli, 2014; Varrone et al., 2012; Ray et al., 2010).

Response surface methodology (RSM) is a valuable tool for modelling and analysis. RSM explores independent factors to generate their individual and interactive effects as well as the optimal conditions to achieve a maximum response (Varrone et al., 2012). Box-Behnken design (BBD) is an RSM method which is commonly used for optimization analysis. The advantages of BBD are better predictability and fewer experiments are required when compared to other methods with the same number of factors (Bae et al., 2005). The objective of this study was to employ the BBD to optimize the H_2 yield from glycerol degradation using LA inhibited mixed anaerobic cultures. The three factors considered include the initial pH level, the glycerol concentration, and the temperature. Based on the data in chapter 5, 2,000 mgL^{-1} LA plus glycerol fed to anaerobic cultures maintained at 37 ºC and at an initial pH value of 5.5 was most effective in increasing the H_2 yield to 0.46 ± 0.03 mol H^2 mol^{-1} when compared to the glycerol fed control cultures. These conditions were employed to proceed with the BBD study.

6.2 Materials and experimental methods

6.2.1 Chemicals

Glycerol (≥ 99.0%, Sigma-Aldrich, Co., Oakville, Ontario, Canada) was the feedstock used in this study. LA (≥ 95.0%, TCI Chemical Industry Co., Portland, USA) was an inhibitor used in this study. Other chemicals which were used to prepare serum bottle reactors were listed in Section 3.1.

6.2.2 Inoculum source

The anaerobic inocula used in the experiments were provided by anaerobic bioreactors located at an ethanol producing facility and a municipal wastewater treatment facility located in Chatham, Ontario. Cultures were maintained in a 5-L (4 L liquid and 1 L gas space) mother reactor. The operation and maintenance conditions for the mother reactor are described in Section 3.2. Mixed anaerobic cultures were removed from the mother reactor and diluted with basal medium to achieve a concentration of 2,000 mg L^{-1} VSS in the 160 mL serum bottle reactors. The chemical composition of the basal media solution is provided in Section 3.3.

6.2.3 Hydrogen production study

The experimental methods used in this study were adapted from Lalman and Bagley (2000). All the BBD experimental conditions were conducted in serum bottle reactors (160 mL) under anaerobic conditions. Preparation of the serum bottle reactors as well as gas and liquid samples removal are described in Section 3.3-3.4. All the BBD design conditions were examined in triplicate. The LA inhibitor stock solution (100,000 mg L^{-1}) was prepared using the method reported by Rinzema et al., (1994). Preparation of the LA stock solution is described in Section 3.5.5. A calculated amount of the LA stock solution was added into each serum bottle reactor to achieve a concentration of 2,000 mg L^{-1}. LA was added 24 hours before adding glycerol as a means to aid in the LCFA adsorption process (Veeravalli, 2014; Saddy, 2011). The range for the initial pH levels, the temperatures and the glycerol concentrations were in accordance with the

experimental design. The duration of this study was 4 days. Headspace gas samples were withdrawn every 24 hours to determine the quantity of H_2 produced.

6.3 Experimental methods

6.3.1 Experimental design

A 3-factor and 3-level Box-Behnken experimental design was used to optimize the H_2 yield (Box and Behnken, 1960). The three design factors and three levels are shown in Table 6.1. The factors with related experimental levels were selected based on literature values and results from screening studies. The effects of the initial pH levels were reported in previous studies which were considered in selecting the pH range for the study (Silva-Illanes et all., 2017; Mangayil et al., 2012; Ray et al., 2010; Ray et al., 2008; Park et al., 2005; Fang et al., 2004). The three levels of glycerol concentrations selected were based on the results from screening experiments. Glycerol concentrations from 350 mg L^{-1} to 5,110 mg L^{-1} were analyzed in a previous study and a mid-point concentration of 2,600 mg L^{-1} was selected based on the highest H_2 yield. Data for the screening study for different glycerol concentrations is provided in Appendix D. Three temperature levels were selected to optimize the H_2 yield. A mesophilic temperature of 37 ºC is commonly used in fermentative H_2 production (Tapia-Venegas et al., 2015; Akutsu et al., 2009; Seifert et al., 2009; Selembo et al., 2009). The temperature of 22 ºC was the selected ambient environmental temperature. An elevated temperature of 52 ºC was employed by Sittijunda and Reungsang (2012) and Liu et al. (2003).

Table 6.1: Levels and factors selected for the experimental design

Levels	Factors		
	A	B	C
	Initial pH	Glycerol (mg l^{-1})	Temperature (ºC)
-1	5.5	1300	22
0	6.5	2600	37
+1	7.5	5110	52

Note: Glycerol represents the glycerol concentration used in experiments

The 15 experimental conditions (#1 to #15) are shown in Table 6.2. Experiments conducted under the same conditions were designated as the central points to assess the error or statistical noise of the magnitude in the BBD analysis. The H_2 yields (Y) were obtained from experimental results and considered as the response variable. The selected experimental factors and the response variable were modelled using Minitab 15 (Minitab Inc., State College, PA). The BBD data was used to generate a quadratic polynomial equation (Equation 6.3).

$$Y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j=2}^{3} a_{ij} X_i X_j \qquad (6.3)$$

Where X_i's represent input variables that influence the response variable Y (H_2 yield), a_0 is an offset term, a_i is the ith linear coefficient, a_{ii} is the quadratic coefficient, and a_{ij} is the ij^{th} interaction coefficient. The input values of X_1, X_2, and X_3 are the selected factors of the initial pH, glycerol concentration, and temperature, respectively (Table 6.2).

Table 6.2: Design matrix for selected factors and response at different factor levels

	Factors			Response	
	Initial pH	Glycerol (mg L^{-1})	Temperature (ºC)	H_2 yield (mol H_2 mol^{-1} glycerol)	
Expt.#	X_1	X_2	X_3	Y (Average)	SD
1	5.5	2600	22	0.16	0.01
2	5.5	2600	52	0.86	0.02
3	6.5	1300	22	0.11	0.00
4	6.5	2600	37	0.55	0.01
5	6.5	1300	52	0.62	0.03
6	7.5	5110	37	0.28	0.01
7	5.5	1300	37	0.44	0.02
8	7.5	1300	37	0.16	0.01
9	6.5	5110	22	0.15	0.01
10	5.5	5110	37	0.45	0.04
11	6.5	2600	37	0.54	0.01
12	7.5	2600	52	0.23	0.01
13	7.5	2600	22	0.05	0.02
14	6.5	5110	52	0.53	0.04
15	6.5	2600	37	0.54	0.01

6.3.2 Analytical methods

The serum bottles headspace gas samples (25 µL) were analyzed using a Varian 3800 gas chromatograph (GC) (Varian Inc., Palo Alto, USA). The GC was equipped with a thermal conductivity detector (TCD) and a 2 m long × 3.175 mm diameter ShinCarbon ST (RESTEK, USA) packed column. The operating temperatures of the TCD, injector, and column oven were 200 ºC, 150 ºC, and 200 ºC, respectively. Nitrogen gas (99.99%, Praxair, ON) was used as the carrier gas with a flow rate of 15 mL min^{-1}.

An analysis of variance (ANOVA) was used to determine the significant effect of the variables in the response surface model of the BBD. The significance (p values < 0.05) was included in the modified response surface model (Wang and Wan, 2009b). The Anderson-Darling (AD) statistic is a tool employed to assess the reliability of fitting the model to the experimental results whether the residuals are normal distributed (Myers et al., 2016; Ray et al., 2010). The residual is the difference between the experimental results and the predicted values at each factor level (Myers et al., 2016). The AD test was used to assess the accuracy of the BBD model. The D-optimality analysis is a numerical method used to optimize the response variable based on the model (Del Castillo et al., 1996). The D-optimality analysis employs a numerical algorithm to calculate the D-optimality values for all combinations of various factor levels in the model (Titterington, 1975). The D-optimality value can vary between zero (minimum desirability) and one (maximum desirability) for the three factors under consideration. The largest D-optimality value indicates the optimal conditions of three selected factors for the maximum response.

6.4 Results and discussion

Based on the BBD design matrix, the experimental results at each design point, and the response variable (H_2 yield) are shown in Table 6.2. The experiments were conducted in triplicates and hence, the total number of experiments was 45, including the central points. The response variable (the H_2 yield) was used to compute the response surface model (Equation 6.3) and to determine the coefficients for the multiple regression expression.

6.4.1 Impacts of selected factors on the response variable

The impact of the three factors on the response variable (H_2 yield) is shown in Figure 6.1. The main effect plots indicated that increasing the H_2 yields was correlated with increasing the temperature. The H_2 yields were significantly improved as the temperature was increased from 22 to 37 ºC. A similar study reported that the H_2 yield increased from 0.3 to 1.0 mol H_2 mol^{-1} glycerol with a temperature increase from 25 to 40 ºC (Mangayil et al., 2012). In this study, the highest H_2 yield of 1.1 ± 0.1 mol H_2 mol^{-1} glycerol was observed when temperature was increased from 37 ºC to 52 ºC. In comparison, increasing H_2 yields have been reported with decreasing the pH. Many studies have reported maximum H_2 yields were observed in a pH range of 5.0 and 6.5 from glycerol degradation (Silva-Illanes et al., 2017; Akutsu et al., 2009; Seifert et al., 2009; Selembo et al., 2009). According to Fang and Liu (2002), adjusting the pH condition affected the H_2 production rate, metabolic pathways, and microbial community. In the work reported by Fang and Liu (2002), decreasing the pH level to 5.5 resulted in a H_2 yield of 2.1 ± 0.1 mol H_2 mol^{-1} glucose. In another study, Veeravalli (2014) examined a pH range from 5.0 to 7.0 and reported a maximum H_2 yield of 2.6 ± 0.15 mol H_2 mol^{-1} hexose at a pH of 5.0 in mixed cultures inhibited by 2,000 mg L^{-1} LA. At the glycerol mid-point concentration of 2,600 mg L^{-1}, the highest H_2 yield was less than the highest yields affected by other two factors (Figure 6.1). Mangayil et al. (2012) reported that the H_2 yield increased to 1.1 ± 0.1 mol H_2 mol^{-1} when the glycerol concentration decreased from 5,000 to 1,000 mg L^{-1}, and a concentration less than 1,000 mg L^{-1} resulted in a reduction of the H_2 yield. According to Ray et al. (2010), the H_2 yield was influenced by multiple glucose additions. These researchers concluded that increasing H_2 yields were observed adding a second glucose feed to anaerobic mixed cultures. In similar studies, researchers have reported the glycerol concentration is a significant parameter affecting H_2 production (de Oliveira Paranhos and Silva, 2020; Seifert et al., 2009).

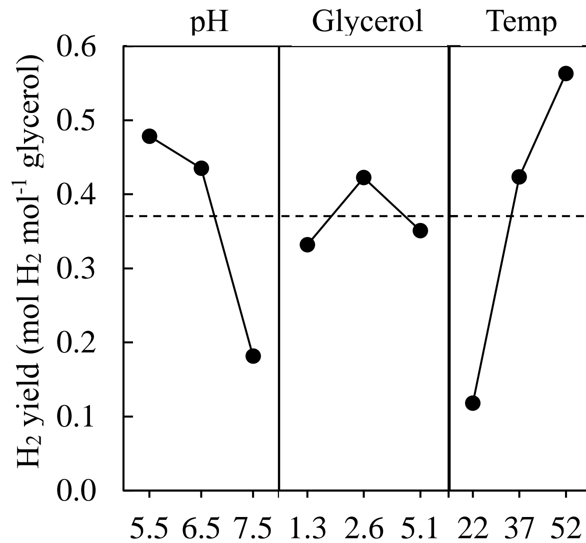

Figure 6.1: Main effect plots of three factors for H_2 yield in a three-factor and three-level Box-Behnken design (BBD).
Notes: pH = the initial pH, Glycerol = glycerol concentration (g L^{-1}), and Temp = temperature (ºC).

Interaction effects between the different factors on the H_2 yield is shown in Figure 6.2. When 2,600 mg L^{-1} glycerol was fed to the cultures, the H_2 yields peaked at pH of 5.5. The H_2 yields under lower pH conditions were greater than the yields at a pH of 7.5 (Figure 6.2 (a)). Combination effects of factors between the glycerol concentration and pH have been reported for H_2 production from glycerol degradation (Mangayil et al., 2012; Varrone et al., 2012). Increasing the H_2 yield was observed by increasing the temperature and reducing the pH (Figure 6.2 (b)). According to Ngo et al. (2011), increasing the H_2 yield was observed with the temperature increasing to 75 ºC and reducing the initial pH to 6.8 in pure cultures. Increasing the H_2 yield was observed at 37 ºC at each glycerol concentration. A small increase in the H_2 yield was observed for cultures maintained at 22 ºC and fed 5,110 mg L^{-1} glycerol in comparison to the other concentrations. A similar small increase was observed for cultures fed 1,300 mg L^{-1} glycerol and maintained at 52 ºC (Figure 6.2 (c)).

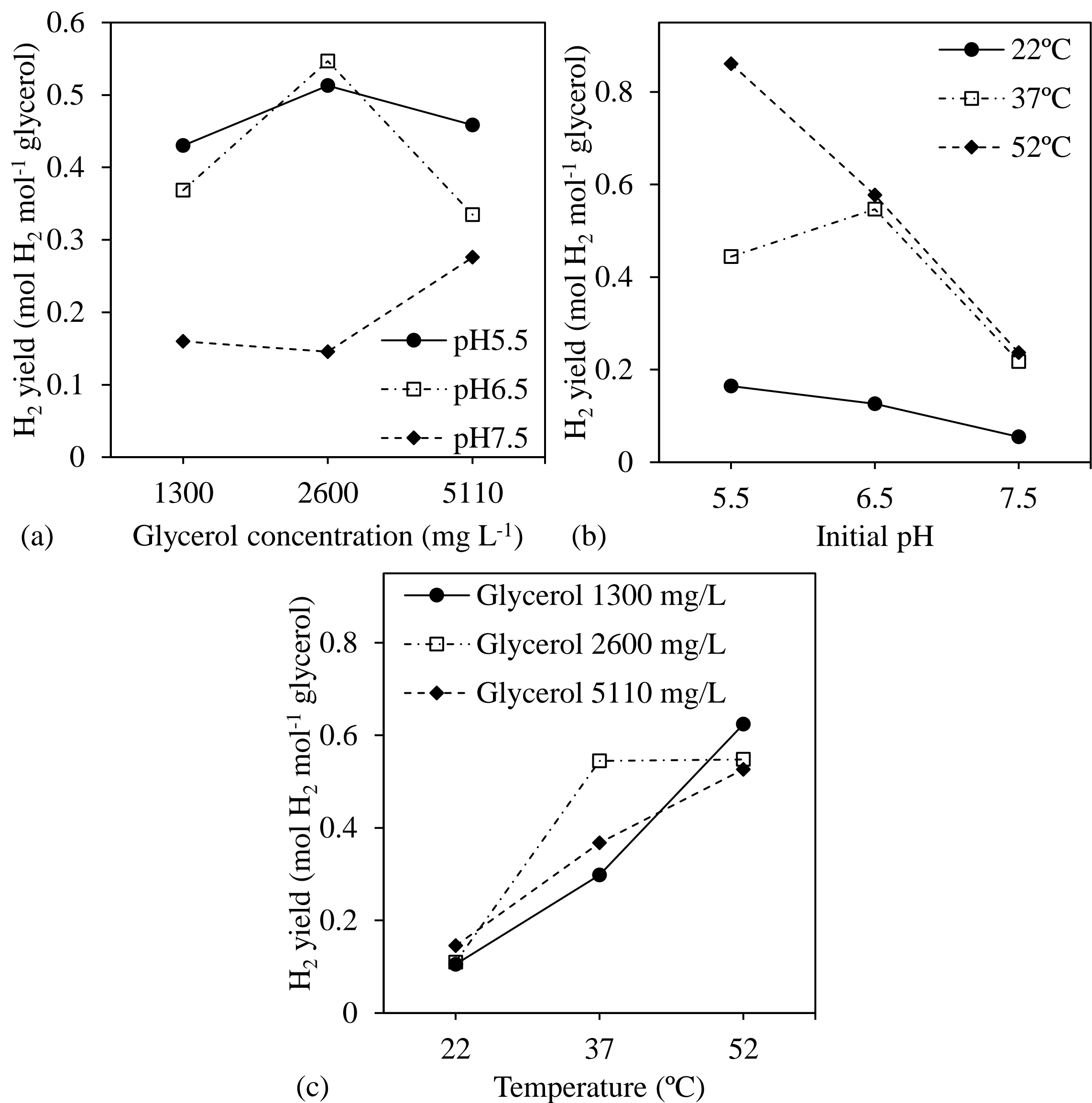

Figure 6.2: Interaction effects of two factors for H_2 yield in a three-factor and three-level Box-Behnken design (BBD).

The three-dimensional (3D) surface plots (Figure 6.3) show the relation between the initial pH, the temperature, the glycerol concentration, and the response variable (H_2 yield). Each plot shows the influence of changing two factors on the H_2 yield. Interaction between the initial pH and glycerol concentration demonstrated that increasing the H_2 yield was due to the combined effect of two variables. The combined effect of the initial pH and glycerol concentration revealed that the low pH and the mid-level glycerol concentration were associated with high H_2 yields (Figure 6.3 (a)). Similarly, Mangayil et

al. (2012) analyzed a pH range from 5.5 to 8.0 and a range of glycerol concentration from 500 to 5,000 mg L^{-1}. These researchers reported a H_2 yield of 1.1 ± 0.1 mol H_2 mol^{-1} from crude glycerol optimal conditions of 1 g L^{-1} glycerol and at a pH 6.5. Ray et al. (2010) reported that improved H_2 yields were observed when the initial pH values were reduced from 7.6 to 5.0 with increasing the number of glucose additions in mixed cultures inhibited by a LCFA range from 0 to 2,000 mg L^{-1}. Other studies also reported that decreasing the pH condition is related to increasing the H_2 yields (Fernandes et al., 2010; Leitão et al., 2006; Fang and Liu, 2002). Interaction between the glycerol concentration and the temperature demonstrated that increasing the temperature and the optimal glycerol concentration were closely associated with improving the H_2 yield (Figure 6.3 (b)). In comparison, work by other researchers has shown that increasing the temperatures from 24 to 40 ºC combined with decreasing the glycerol concentrations from 5,000 to 1,000 mg L^{-1}, resulted in increasing the H_2 yield (Mangayil et al., 2012). In comparison, Varrone et al. (2012) reported that increased H_2 yields were observed at a lower temperature level in a range of 37 to 39 ºC and glycerol concentration from 12,000 to 18,000 mg L^{-1}.

When the initial pH was set at a lower level of 5.5 with a high temperature at 52 ºC, the combined effect showed a significantly positive response on the H_2 yield (Figure 6.3 (c)). Similarly, Sittijunda and Reungsang (2012) reported that a maximum H_2 yield of 0.3 mol H_2 mol^{-1} glycerol was detected at 55 ºC and a pH of 5.5. In comparison, an optimal pH of 7.9 and a glycerol concentration of 15,000 mg L^{-1} was reported for H_2 production from crude glycerol by mixed anaerobic cultures (Varrone et al., 2012). In this study, the glycerol concentration had less of an effect on the H_2 yield when compared to the other two factors. The highest yield of 0.86 ± 0.02 mol H_2 mol^{-1} glycerol was observed when mixed LA inhibited cultures were fed 2,600 mg L^{-1} glycerol and maintained at 52 ºC and a pH of 5.5. This yield is approximately 29% of the theoretical yield shown in Equation 6.1.

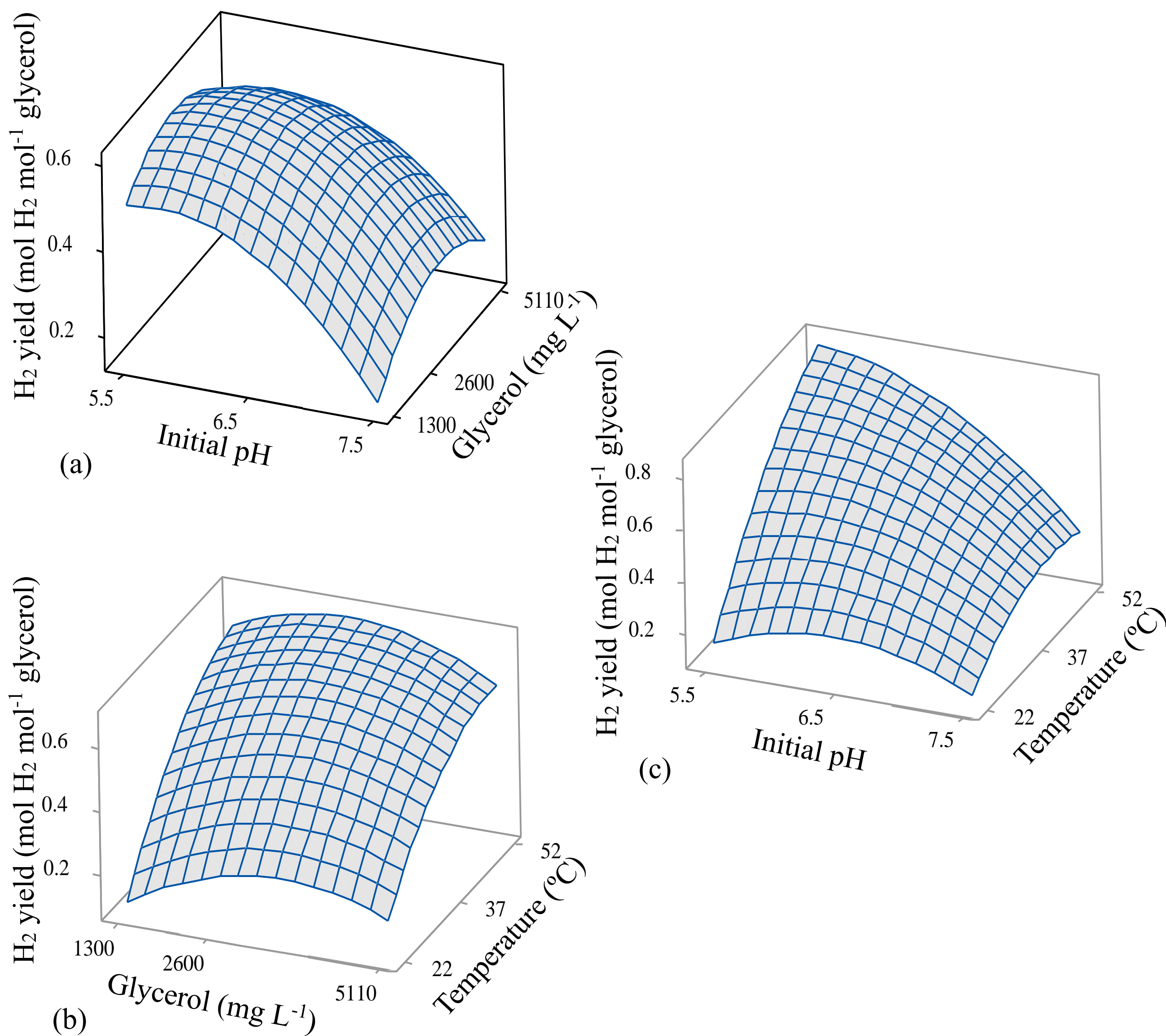

Figure 6.3: 3D Response surface plots for (a) Interaction effects between the initial pH and glycerol concentration. (b) Interaction effects between glycerol concentration and temperature. (c) Interaction effects between initial pH and temperature.

6.4.2 Modeling and optimizing on the response variable

The optimization study was conducted based on the results obtained from Table 6.2 to develop a full quadratic model (Equation 6.3) using the ANOVA analysis. The ANOVA output is shown in Table 6.3. The p-value (the probability) of model which is

less than 0.05 indicates the model is statistically significant. The p-values for the initial pH (X_1) and temperature (X_3) which were less than 0.05 indicate these two factors were statistically significant. However, the p-value for the glycerol concentration (X_2) was statistically insignificant ($p > 0.05$). The BBD model includes square factors represented by X_i^2 and 2-way interaction factors are represented by X_iX_i . Square and 2-way interaction factors were statistically significant with p-values less than 0.05.

$$Y = -6.794 + 1.644X_1 + 0.000132X_2 + 0.10728X_3 - 0.1166X_1^2 - 0.000X_2^2 - 0.000440X_3^2 + 0.000013X_1X_2 - 0.008623X_1X_3 - 0.00001X_2X_3 \quad (6.3)$$

Where Y represents the H_2 yield (mol H_2 mol^{-1} glycerol), X_1, X_2, and X_3 are the selected factors of the initial pH, glycerol concentration (mg L^{-1}), and temperature (ºC), respectively.

Table 6.3: ANOVA results for the experimental response at each factor level

Source	DF[a]	SS[b]	MS[c]	F-value	P-value[d]
Model	9	2.24955	0.24995	153.46	0.000
X_1	1	0.52952	0.52952	325.10	0.000
X_2	1	0.00240	0.00240	1.47	0.233
X_3	1	1.18233	1.18233	725.88	0.000
X_1^2	1	0.15070	0.15070	92.52	0.000
X_2^2	1	0.10067	0.10067	61.81	0.000
X_3^2	1	0.10866	0.10866	66.71	0.000
X_1X_2	1	0.00770	0.00770	4.73	0.037
X_1X_3	1	0.20078	0.20078	123.27	0.000
X_2X_3	1	0.01418	0.01418	8.71	0.006
Error	35	0.05701	0.00163		
Total	44	2.30656			

Note: a. DF = degrees of freedom, b. SS = sum of squares, c. MS = Mean squares, d. P-value <0.05 indicates that a factor is significant. X_1 = initial pH; X_2 = glycerol concentration; X_3 = temperature

In Figure 6.4 (a), the experimental results were compared with the model predicted values in a scatter plot. This comparison indicated a reasonable correlation at each level of the H_2 yield. The residuals were calculated based on the difference between the experimental results and predicted values. The residuals were used to assess the suitability of fitting the model to the experimental results using the AD statistic. The normal probability plot of the residuals (Figure 6.4 (b)) is approximately linear which is

supportive that the residuals are normally distributed. In Figure 6.4 (b), an AD value of 0.181 was less than the value of 0.735 for the sample size of 45 at a 5% level of significance (Stephens, 1974) and a p-value of 0.909 was higher than 0.05. The AD value and p-value confirmed the residuals are normal distribution. This indicates the predicted values are correlated with the experimental results.

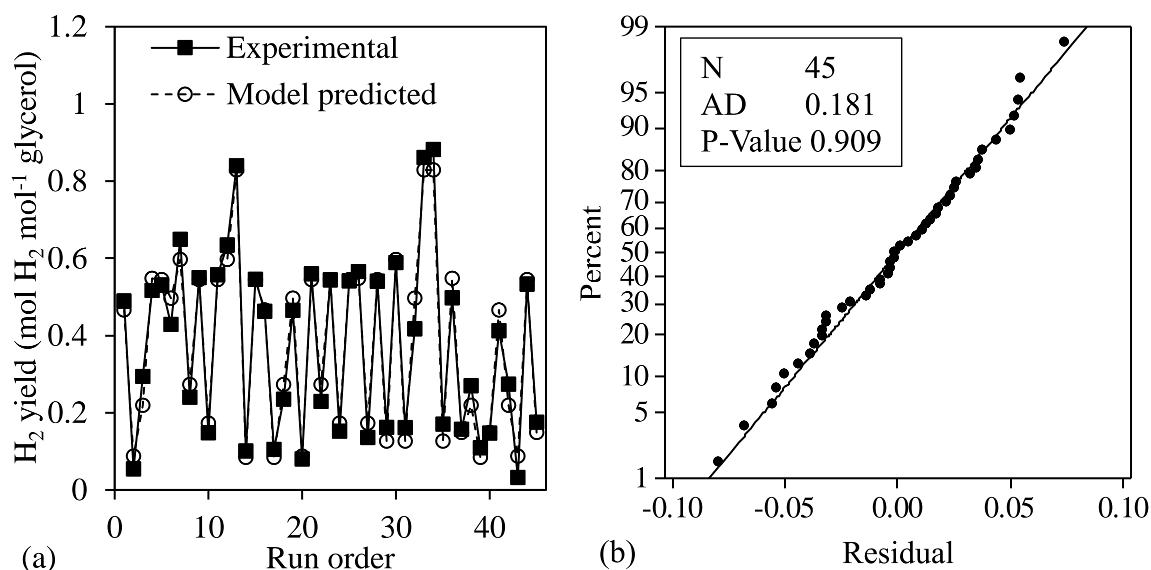

Figure 6.4: Accuracy evaluation of the BBD model. (a) Scatter plot of the H_2 yield at each experimental order. (b) Anderson-Darling normality plot residuals.

Note: N = the number of experiments; AD = Anderson-Darling statistic; P-Value = level of confidence.

The D-optimality index was used to determine the maximum H_2 yield (Y) within the selected ranges of three factors under consideration (Figure 6.5). The high and low levels of three factors are limitation for the D-optimality. The maximum yield of 0.84 mol H_2 mol^{-1} glycerol was predicted at 52 ºC and a pH of 5.5 when the glycerol concentration was 2,710 mg L^{-1}. The predicted value is approximately 2% less than the highest value obtained from experimental results. Several studies reported H_2 yields ranging from 0.11 to 0.41 mol H_2 mol^{-1} glycerol for mixed anaerobic cultures with an initial pH of 6.5 and a temperature of approximately 37 ºC (Akutsu et al., 2009; Seifert et al., 2009; Selembo et al., 2009). In comparison, a higher yield of approximately 0.84 mol H_2 mol^{-1} glycerol was observed in this study. In other studies, Seifert et al. (2009)

concluded that the H_2 yield of 0.41 mol H_2 mol^{-1} glycerol reached a maximum with increasing the glycerol concentration from 5 to 10 g L^{-1}. Varrone et al. (2012) reported that the maximum H_2 yield of 0.96 mol H_2 mol^{-1} glycerol was observed at 37 ºC, an initial pH of 7.9, and a glycerol concentration of 15.0 g L^{-1}. Moreover, Mangayil et al. (2012) demonstrated that the H_2 production from glycerol significantly decreased when the temperature increased above 46 ºC. In contrast, a significantly high yield of 2.73 mol H_2 mol^{-1} glycerol was reported at 75 ºC by using a pure culture (*Thermotoga neapolitana*) in a fermentative batch reactor (Ngo et al., 2011).

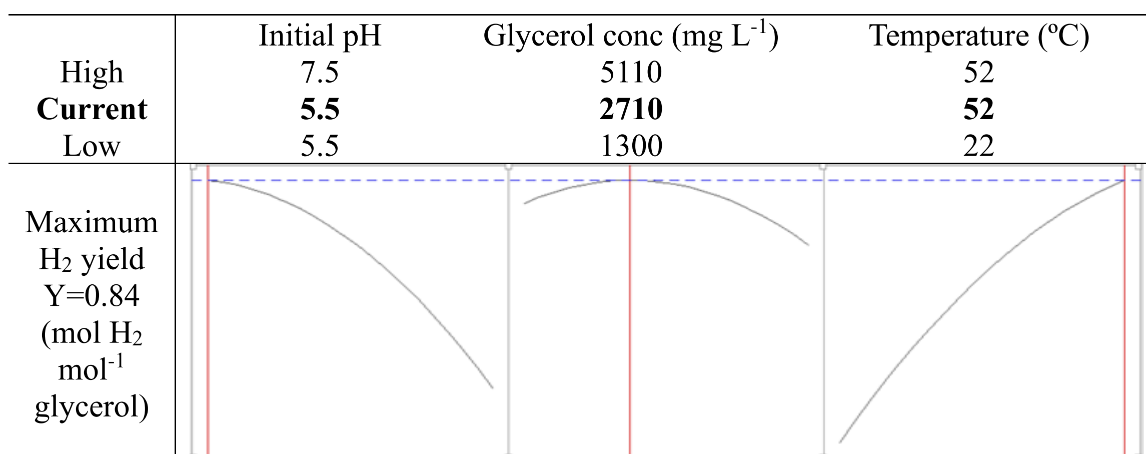

Figure 6.5: Optimization plot to address the optimum levels of selected factors for the maximum H_2 yield (mol H_2 mol^{-1} glycerol).

Notes: The values in bold are the optimum levels for achieving the maximum H_2 yield.

6.5 Conclusion

A three-factor and three-level BBD model based on the RSM was utilized to maximize the H_2 yield from glycerol degradation in LA inhibited mixed anaerobic cultures. The initial pH level, glycerol concentration, and temperature were the three factors selected to develop the model. The LA concentration selected in this study was based on the conclusions from chapter 5.

Based on the ANOVA analysis, the designed model was statistically significant with a p-value less than 0.05. Fitting the model to the experimental results was conducted using the AD statistic. The three-dimensional (3D) surface plots demonstrated the effects of the three factors and the response variable (H_2 yield). The maximum experimental H_2

yield of 0.86 mol H_2 mol^{-1} glycerol was approximately 2% higher than the value predicted using D-optimality analysis. At the highest H_2 yield obtained, the optimum levels of selected factors significantly impacted H_2 production during glycerol degradation.

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Chapter 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Glycerol, a low-value by-product from the biodiesel industry, is contaminated with metals and LCFAs and cannot be directly discharged to the environment. The biodiesel industry is rapid growing together with production of glycerol. Glycerol is considered as an economical and competitive substrate for H_2 production through dark fermentation. Lab-scale experiments were conducted to demonstrate H_2 production from glycerol. The impact of the factors various was determined using different analytical and statistical methods.

The main objective of chapter 4 was to determine the effects of the initial pH on H_2 production from glycerol through dark fermentation in mixed anaerobic cultures. In this chapter, the degradation of two substrates (glucose and glycerol) was examined at selected pH levels of 5.5, 6.5, and 7.5. Decreasing the initial pH affected the H_2 production as well as the other metabolites production in glucose and glycerol fed cultures. Hydrogen production was optimum at pH = 5.5 in both glucose and glycerol samples. When the initial pH level increased, CH_4 production was dominant instead of H_2 production. At the initial pH of 5.5, the highest H_2 yield of 0.33 ± 0.03 mol H_2 mol^{-1} glycerol was observed with major metabolites such as Ac^-, EtOH, and 1,3-PDO. The glycerol degradation rate was not a function of the initial pH level and was less than the glucose degradation rate at each analyzed pH condition. Hydrogen consumption was observed in all the glycerol samples. The pH level of 5.5 was determined the optimum pH condition for H_2 production in mixed anaerobic cultures fed glycerol.

Chapter 5 focused on further increasing the H_2 yield using inhibitors together with glycerol at an initial pH of 5.5. LCFAs are microbial organic inhibitors. Six LCFAs (LUA, MA, PA, SA, OA, and LA) were selected based on the number of carbon atoms as well as the carbon chain length and the carbon-carbon bond saturation and unsaturation. Control samples receiving each inhibitor did not produce H_2. Hydrogen production was detected in samples fed only glycerol as well as glycerol cultures inhibited with different LCFAs. In mixed cultures receiving PA, OA, or LA, the H_2 yields increased when

compared to cultures fed only glycerol. LA enhanced H_2 production with a yield of 0.46 ± 0.03 mol H_2 mol^{-1} glycerol. When compared to the H_2 yield for the work in chapter 4, the yield increased approximately 29%. Acetate, EtOH, and 1,3-PDO were major metabolites in cultures fed glycerol plus LA. When compared to the glycerol only cultures, lower H_2 yields were observed in samples inhibited by LUA or SA. 1,3-PDO production was observed to increase in the LUA and SA fed cultures. After the second glycerol injection, the production of major VFAs varied in glycerol cultures and cultures fed glycerol plus PA or OA. An electron balance was used to account for the distribution of electron equivalents from the substrate. After the first glycerol injection in the LA treated cultures, the electron equivalents directed to H_2 was approximately 6.5% and approximately 7.9% to Ac^-. EtOH (approximately 23.1%) and 1,3-PDO (approximately 50.0%) were the major electron sinks. In samples inhibited by other LCFAs, the electron distribution pattern showed that most of the electrons were directed to produce VFAs and alcohols. After the second glycerol injection, negligible quantity of residual glycerol was observed in cultures inhibited by each LCFA. The H_2 yields were not increased after the second glycerol feeding when compared to the yields after the first feeding in each LCFA treated cultures. In comparison of the other LCFAs, LA was an effective inhibitor to improve the H_2 yield at the initial pH of 5.5.

Chapter 6 is focused on maximizing H_2 yield using a three-factor and three-level BBD model in cultures fed glycerol and LA. The initial pH levels (5.5 to 7.5), glycerol concentrations (1,300 to 5,110 mg L^{-1}), and operational temperatures (22 to 52 ºC) were the three factors selected in this study. The impact of the main and interactive effects of the different factors was evaluated using an ANOVA. The relationship between the different factors on the H_2 yield is shown in the 3-D surface plots. The highest H_2 yield observed was 0.86 ± 0.02 mol H_2 mol^{-1} glycerol at 55 ºC, a pH of 5.5, and a glycerol concentration of 2,600 mg L^{-1}. Based on Equations 6.1 and 6.2, a yield of 0.86 mol H_2 mol^{-1} glycerol was approximately 29% and 48%, respectively, of the theoretical H_2 yield. The D-optimality analysis was used to predict the optimal conditions for maximizing the H_2 yield based on the BBD model. The predicted result was 0.84 mol H_2 mol^{-1} glycerol at 55 ºC, a pH of 5.5, and a glycerol concentration of 2,710 mg L^{-1}. At the highest H_2 yield

obtained, the optimum levels of selected factors had a significant impact on H_2 production.

7.2 Recommendations

This thesis demonstrated that pure glycerol can be used to produce H_2 using mixed anaerobic cultures. The work validated that H_2 production, and the maximization of the H_2 yield was feasible using lab-scale batch experiments utilizing glycerol and LA. Although many factors and conditions were considered and analyzed in these studies, there are many remaining which must be addressed in future studies. For future studies, the recommendations are summarized as follows:

1) Use refined and unrefined crude glycerol feedstock as a substrate for H_2 production.
2) Identify H_2-producing microorganisms in mixed cultures fed with glucose or glycerol.
3) Enrich H_2-producing bacterial community in the environment with glycerol.
4) Use LCFA acclimated cultures to establish if the parent LCFA and LCFA degradation byproducts are effective inhibitors.
5) Conduct continuous flow studies to determine the impact of operational parameters such as the solids retention time and the organic loading rate,

Treating or not treating the crude glycerol to remove or not removing impurities such as heavy metals may impact the cost of heavy metals micro-nutrients in the fermentation media. Utilizing a heavy metal containing glycerol feed will require analysis of the heavy metal content as a means to establish the presence of toxic heavy metals. Another issue to address is using the crude glycerol and attempting to meet the heavy metal requirement for preparing the basal media. This could be troublesome especially if the crude glycerol has to be diluted to meet the required glycerol concentration and heavy metal concentration.

Microbial community analysis is key to identifying H_2-producing bacteria. Screening for H_2-producing bacteria would allow for the determination of

microorganisms which are responsible for H_2 production. Enriching these microorganisms in the environment with crude glycerol will indicate their survivability for producing H_2. These steps will lead to increase the feasibility of using a continuous reactor for the scaling up of a H_2 production process.

Using LCFA acclimated H_2-producing bacteria for a continuous reactor could overcome limitations, such as the reactor operation such as constant pH, reducing the H_2 partial pressure, and by-products accumulation. Based on the present studies, the optimal conditions, which were obtained at the highest H_2 yield, can be employed as a guide towards the design of a laboratory scale continuous flow reactor system. In continuous flow reactors, varying the HRT can be employed to improve the H_2 yield.

APPENDICES

Appendix A: Additional data

Checking the performance of mixed cultures after two times of glycerol feeding.

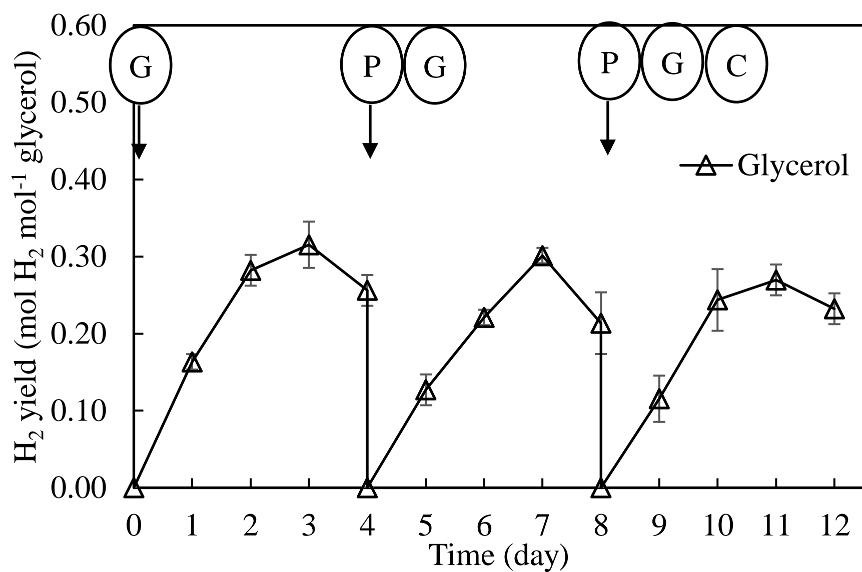

Figure A.1: Hydrogen production profiles for mixed cultures receiving glycerol at a pH of 5.5 *(G = Glycerol feeding; P = Nitrogen purging; C = Centrifuge)*

Appendix B: Calibration curves

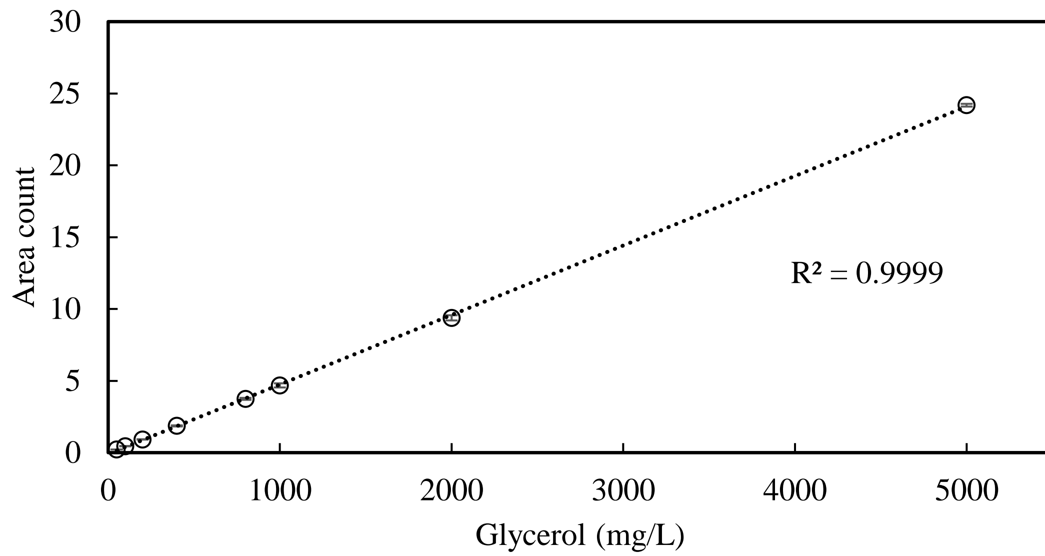

Figure B.1: Glycerol calibration curve

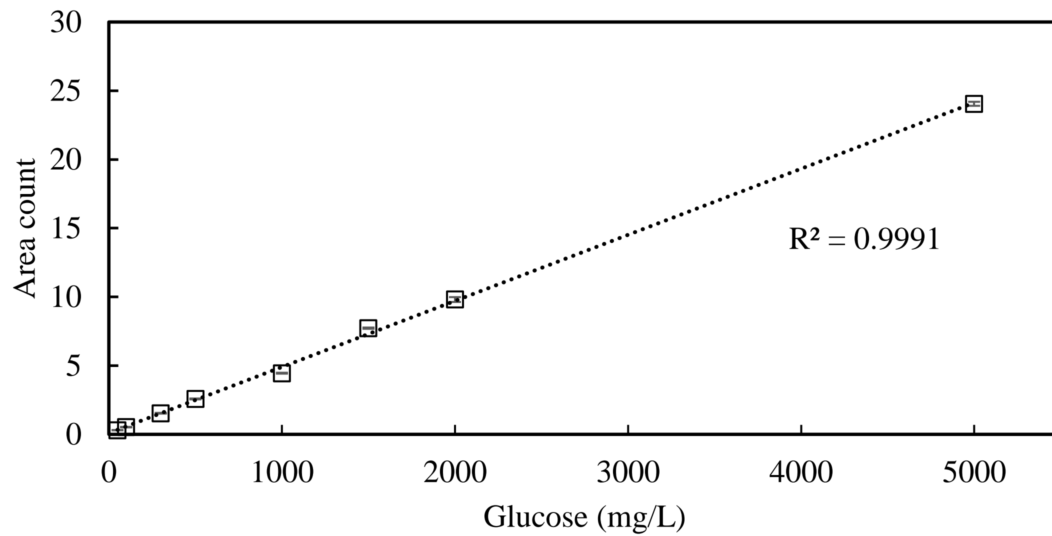

Figure B.2: Glucose calibration curve

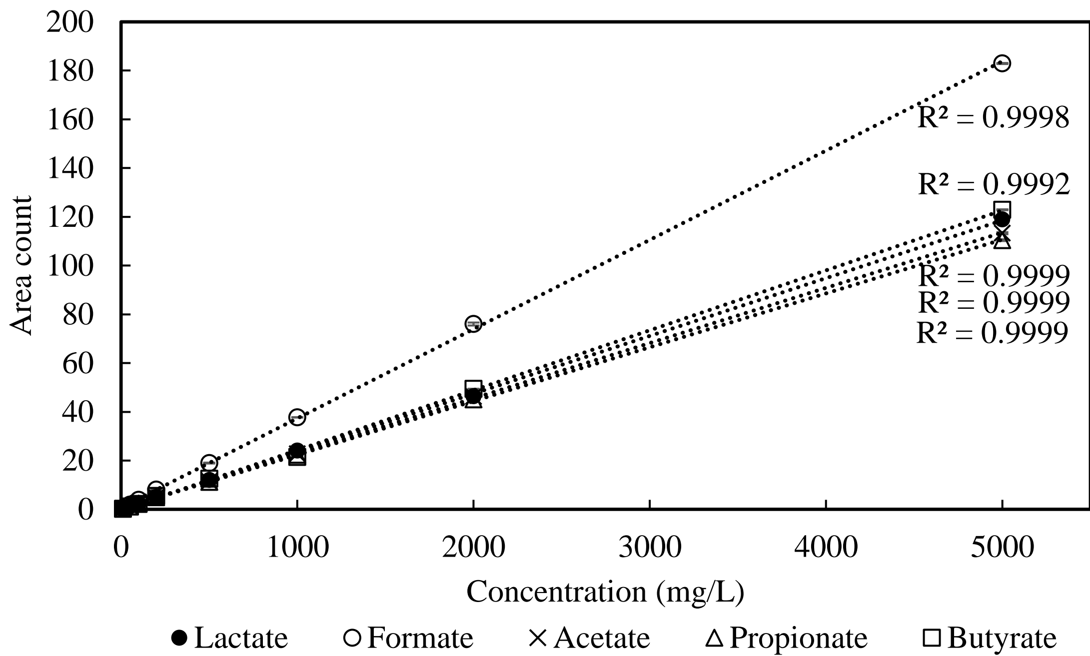

Figure B.3: Volatile fatty acids (VFAs) calibration curve

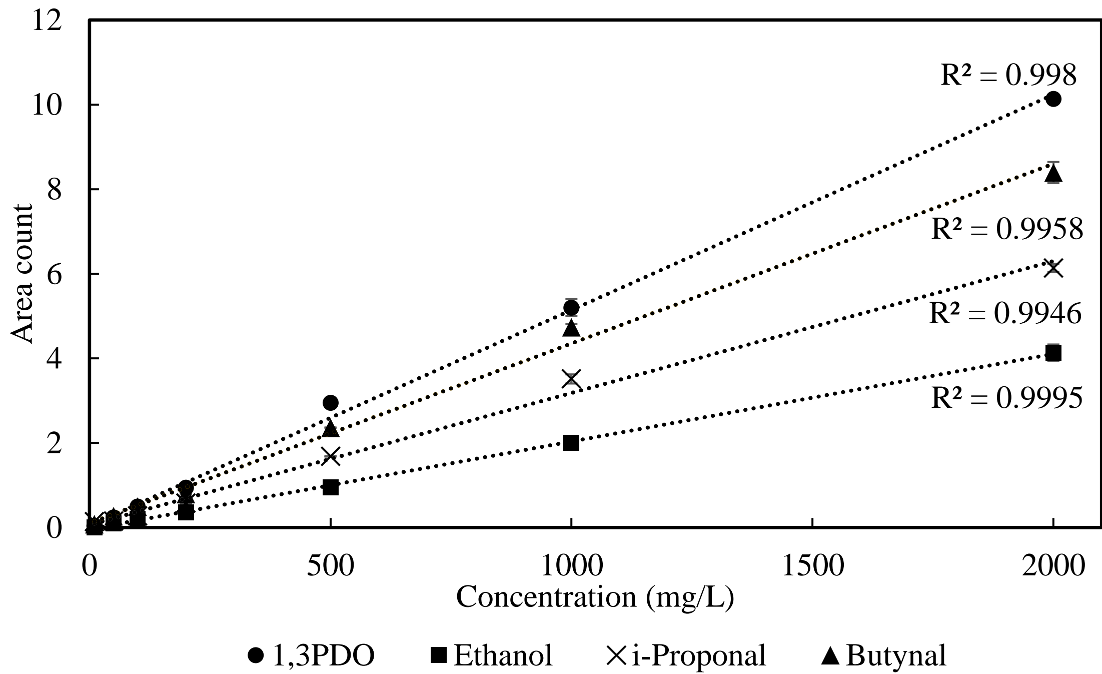

Figure B.4: Alcohols calibration curve

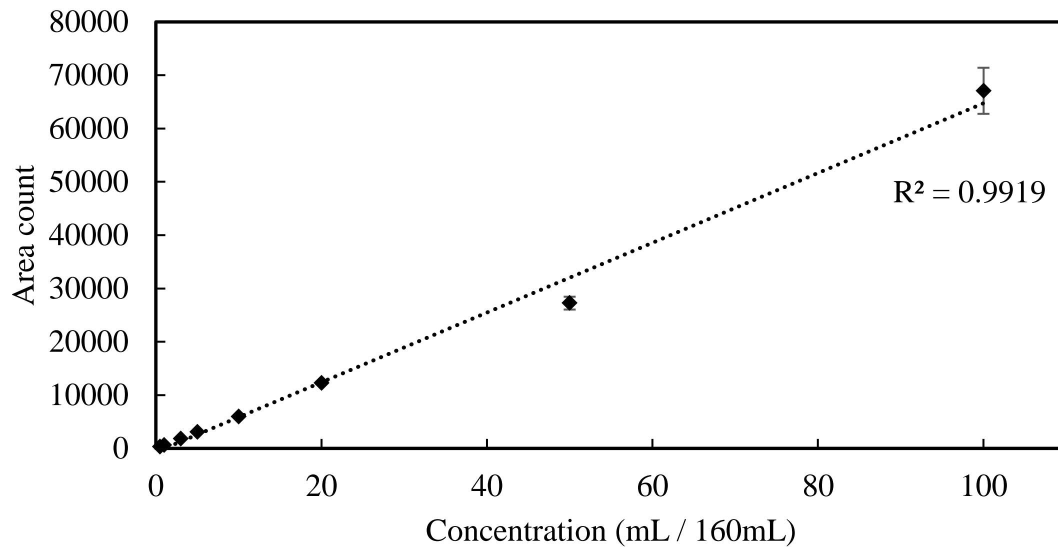

Figure B.5: H_2 calibration curve

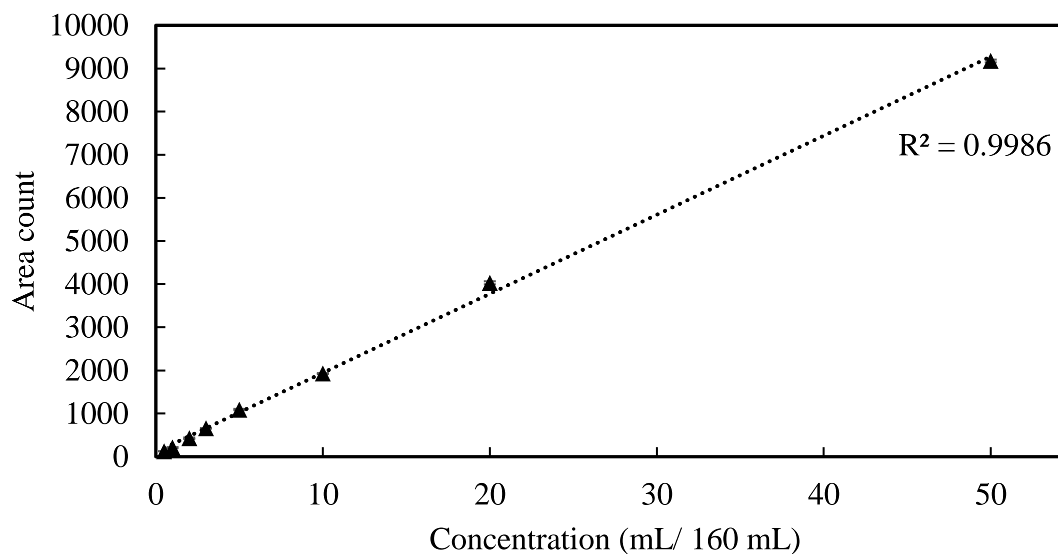

Figure B.6: CH_4 calibration curve

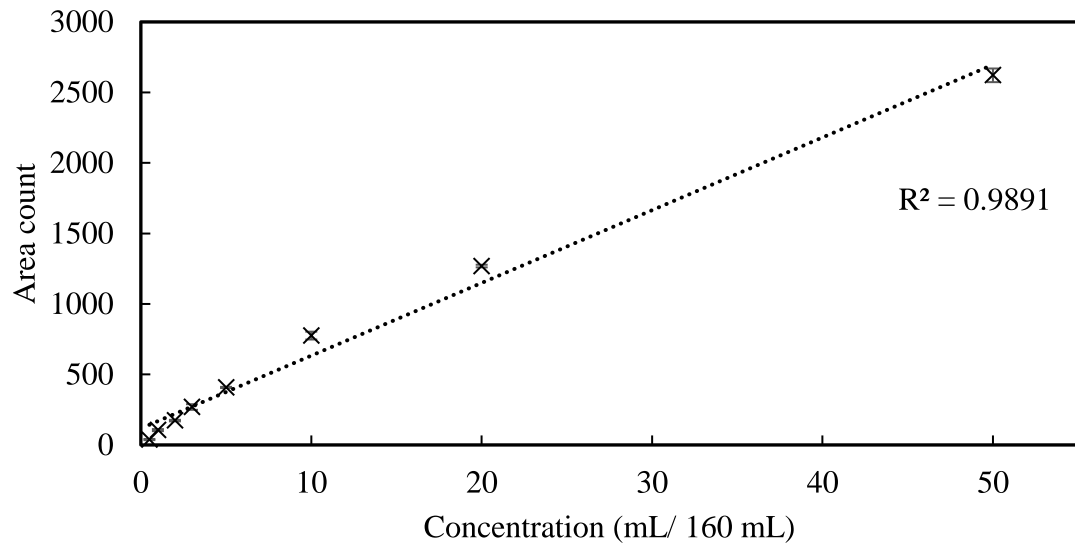

Figure B.7: CO_2 calibration curve

Appendix C: Example of the electron balance

The electron balance was done on the assumption bellow:

$$\sum \text{Substrate}_0 = \sum \text{Substrate}_t + \sum \text{Byproducts}_t$$

Example for LA treated glycerol samples:

On day 0,

$$\sum \text{Substrate}_0 = \frac{\text{Glycerol concentration (mg/L)} \times \text{reactor volume (L)}}{\text{Glycerol molecular weight (g/mol)} \times 1000} \times 14 \frac{\text{e}^-}{\text{mol}}$$

$$= 3.89 \times 10^{-2}\ \text{e}^-$$

On day 4,

Byproducts	Molecular weight (g/mol)	Concentration (mg/L)	Concentration (mol)	Electron equivalent per mol	Electron equivalent (e^-)
HLa	90	0.0081	2.25×10^{-5}	12	2.70×10^{-4}
HFr	46	0.0049	1.36×10^{-5}	2	2.72×10^{-5}
HAc	60	0.1235	3.43×10^{-4}	8	2.74×10^{-3}
EtoH	46	0.3321	9.23×10^{-4}	12	1.10×10^{-2}
1,3-PDO	76	0.4078	1.13×10^{-3}	16	1.81×10^{-2}
H_2	2	0.4359	1.21×10^{-3}	2	2.42×10^{-3}

Note: Each concentration is obtained from experimental results.

Therefore,

$$\sum \text{Byproducts}_t = 3.46 \times 10^{-2}\ \text{e}^-$$

$$\sum \text{Substrate}_t = 3.89 \times 10^{-2} \text{e}^-$$

$$\%Electron\ equivalent\ recovery = \frac{3.46 \times 10^{-2}\ \text{e}^-}{3.89 \times 10^{-2}\ \text{e}^-} \times 100 = 89\%$$

Appendix D: Screening of the glycerol concentration

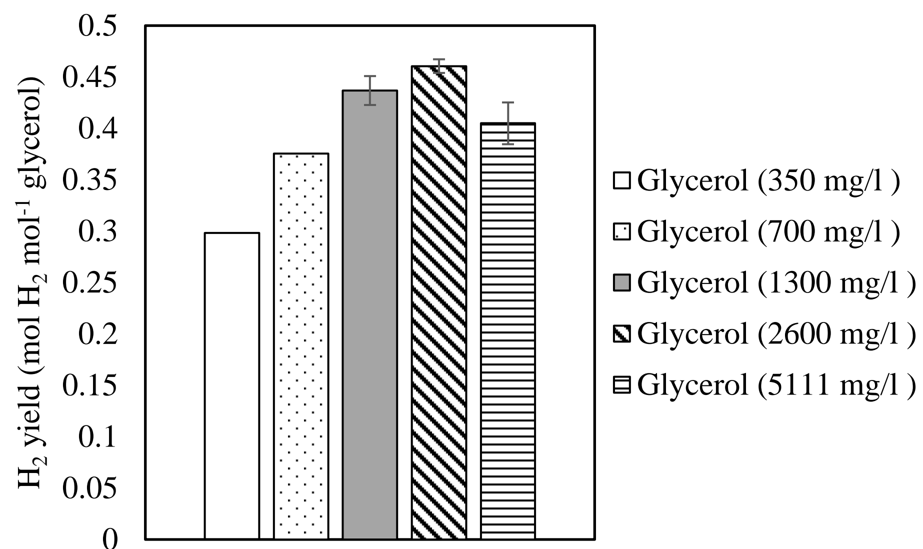

Figure D.1: The effect of glycerol concentrations

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