8-7-2014 12:00 AM

Enhancement of Biohydrogen Production from Co-Fermentation of Glucose, Starch, and Cellulose

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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering
Science degree in Chemical and Biochemical Engineering
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Enhancement of Biohydrogen Production from Co-Fermentation of Glucose,

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(Thesis Format: Integrated Article)

by

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Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master in Engineering Science

The School of Graduate and Postdoctoral Studies
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Abstract

The aim of this study was to assess the synergistic effects of co-fermentation of glucose, starch, and cellulose using anaerobic digester sludge (ADS) on the biohydrogen (H_2) production and the associated microbial communities. At initial pH of 5.5 and mesophilic temperature of 37 ºC, the H_2 yields were greater by an average of $27 \pm 4\%$ in all the different co-substrate conditions compared to the mono-substrate conditions, which affirmed that co-fermentation of different substrates improved the hydrogen potential. The sensitivity of mesophilic ADS to a temperature shock was also investigated. Unacclimatized mesophilic ADS responded well to a temperature shock of 60ºC which was evident from lower lag phase durations. Interestingly, co-fermentation of starch and cellulose at mesophilic conditions enhanced the hydrogen yield by 26% with respect to mono-substrate, while under thermophilic conditions starch competed with cellulose as the carbon source for the microbial populations and no enhancement in the overall yield was observed.

Co-Authorship Statement

Chapter 3: Co-fermentation of Glucose, Starch, and Cellulose for Mesophilic Biohydrogen Production

Medhavi Gupta, Preethi Velayutham, Elsayed Elbeshbishy, Hisham Hafez, Ehsan Khafipour, Hooman Derakhshani, M. Hesham El Naggar, David B. Levin, George Nakhla

My contributions are as follows:

- Design of research

- Analysis and interpretation of the findings

- Writing the paper

Dr. David Levin and his group at University of Manitoba contributed with their expertise in microbial analysis, including library construction, illumina sequencing, and bioinformatic analysis.

Chapter 4: Sensitivity of Mesophilic Biohydrogen-Producing Cultures to Temperature Shocks

Medhavi Gupta, Noha Nasr, Elsayed Elbeshbishy, Hisham Hafez, M. Hesham El Naggar, George Nakhla

My contributions are as follows:

- Design of research

- Analysis and interpretation of the findings

- Writing the paper

To my parents for their support,

my brother, Nalin, for his unusual words of encouragement,

and my friends for their patience and support

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Nomenclature

MPR   Methane production rate

BESA   Bromoethane sulfonic acid

ADS   Anaerobic digester sludge

PNS   Purple non-sulfur bacteria

C/N   Carbon-to-nitrogen ratio

BM   Buffalo manure

PM   Poultry manure

OFMSW Organic fraction of the municipal solid waste

GW   Greengrocery waste

COD   Chemical oxygen demand

rDNA   Ribosomal deoxyribonucleic acid

TSS   Total suspended solids

VSS   Volatile suspended solids

S/X   Substrate-to-biomass ratio

TCOD   Total chemical oxygen demand

TCD   Thermal conductivity detector

VFA   Volatile fatty acids

FID   Flame ionization detector

SCOD   Soluble chemical oxygen demand

PBS   Phosphate buffered saline

DNA   Deoxyribonucleic acid

PCR   Polymerase chain reaction

P_{max}	Maximum cumulative hydrogen production
R_{max}	Maximum hydrogen production rate
λ	Lag time
Ac/Bu	Acetate-to-butyrate ratio
TVFA	Total volatile fatty acids
OTU	Operational taxonomic unit
PCoA	Principal co-ordinate analysis
MEC	Microbial electrolysis cell
SRB	Sulfate-reducing bacteria
dsDNA	Double-stranded deoxyribonucleic acid
dNTP	Deoxyribonucleotide
CSTR	Continuous stirred tank reactor
DGGE	Denaturing gradient gel electrophoresis

Chapter 1

Introduction

1.1 Background

Most of the world's energy demand today are met with fossil fuels, which are being depleted. Additionally, greenhouse emissions from fossil fuels and other environmental impacts, such as global warming, climate change, ozone layer depletion, etc., are causing an urgent need for renewable energy [Azbar and Levin, 2012]. Hydrogen can address all the above concerns as a viable alternate energy source. It does not contribute to greenhouse effect, producing only heat and water upon combustion and has a high energy yield of 286 kJ/mol, which is at least two times greater than that of any hydrocarbon fuel [Cai et al., 2004].

Among various methods of hydrogen production such as steam reforming of natural gas, water electrolysis, biomass gasification, etc., biological hydrogen production methods are environmentally friendly [Azbar and Levin, 2012; Wang and Wan, 2009]. Among the biological hydrogen production methods, dark fermentation is more attractive than photo-fermentation due to its high utilization efficiency of various organic wastes and feedstocks as substrate and, light-independence [Chen et al., 2006]. Furthermore, in dark fermentation, the hydrogen production rates are much higher compared to photo-fermentation [Azbar and Levin, 2012].

Natural mixed consortia are considered more practical than pure cultures because of simpler operation, ease of bioprocessing in a non-sterile environment and, amenability to broader spectrum of feedstocks due to high microbial diversity, which reduces the process operational costs significantly [Prakasham et al., 2009; Li and Fang, 2007]. A

wide range of hydrolytic and catabolic activities are required while using complex materials and in this regard mixed microbial consortia are useful [Azbar and Levin, 2012].

Renewable carbohydrates-based feedstocks are the preferred organic carbon source for hydrogen-producing fermentations [Hawkes et al., 2002; Azbar and Levin, 2012]. Waste biomass from municipal, agricultural, forestry sectors, industry effluents from pulp/paper and food industries represent an abundant potential source of substrate [Hallenbeck et al., 2009; Azbar and Levin, 2012].

Several researchers have investigated co-digestion of different substrates over the last 15-20 years to evaluate its effects on the performance of anaerobic digestion process by simultaneously treating different organic waste streams. Co-digestion had a distinct positive effect on methane production rate (MPR) (mL/hr) and methane yields [Kim et al., 2003; Esposito et al., 2012].

1.2 Problem Statement

A number of factors limit biohydrogen production including: thermodynamic barriers, product inhibition, branched catabolic pathways, and the nature of substrates [Azbar and Levin, 2012]. Biohydrogen production from simple sugars has been well researched and documented in the literature. Although, it has been documented that carbohydrate-rich "waste" feedstocks are suitable substrates for hydrogen production, relatively few studies have dealt with mixed substrates to explore co-fermentation. Real waste streams have a very complex composition, therefore, studying co-substrate digestion for hydrogen production would provide a better understanding of the microbial physiology, metabolism, and mechanisms of hydrogen production from real wastes

[Hallenbeck et al., 2009]. Hydrogen yields and rates vary considerably even for a specific substrate depending on the inoculum. A more comprehensive understanding of the microbial community structure and its relation to soluble end-products as well as hydrogen yield is required.

Traditionally anaerobic digestion has been performed at mesophiic range, however, when treating complex carbohydrates, hydrolysis is often the rate limiting step at mesophilic temperatures. Treating wastes at their natural temperatures is deemed beneficial due to reduced costs [Donoso-Bravo et al., 2009]. Furthermore since temperature shocks can occur in real life applications, assessing the feasibility of using unacclimatized mesophilic cultures at thermophilic temperatures would reflect real-life situations.

1.3 Research Objectives

The main goal of this study was to investigate co-fermentation of different substrates at both mesophilic and thermophilic conditions. The specific objectives are as follows:

- Assess the synergistic effects of co-fermentation of glucose, starch, and cellulose using ADS on the biohydrogen production

- Characterize changes in the microbial communities of ADS fermentations containing single versus co-substrates

- Assess the response of unacclimatized bio-hydrogen producers to thermophilic conditions, as well as to compare mesophilic and thermophilic co-fermentation of starch and cellulose.

1.4 Thesis Organization

This thesis includes five chapters and conforms to the "integrated article" format as outlined in the Thesis Regulation Guide by the School of Graduate and Postdoctoral Studies (SGPS) of the University of Western Ontario. The thesis consists of the following chapters:

Chapter 1   presents the general introduction and research objectives.

Chapter 2   presents a literature review on anaerobic digestion and bio-hydrogen production.

Chapter 3   presents the impact of co-fermentation of glucose, starch, and cellulose for mesophilic biohydrogen production.

Chapter 4   discusses the sensitivity of mesophilic biohydrogen-producing cultures to temperature shocks.

Chapter 5   summarizes the major conclusions of this research and provides recommendations for further future work based on the results of this study.

1.5 Research Contributions

Various carbohydrate-based feedstocks are potential substrates for biohydrogen production. Such feedstocks are a combinations of different carbohydrates. Although, hydrogen production from single substrates has been studied widely, very few studies have examined co-fermentation of different substrates. The main contributions of this work are:

- Demonstrating the advantages of co-fermentation of glucose, starch, and cellulose, which enhanced biohydrogen production significantly.

- Characterizing the microbial communities and visualizing the evolution of these communities under different substrate conditions.

- Establishing the potential of using mesophilic inoculum at thermophilic conditions for co-fermentation.

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Chapter 2

Literature Review

2.1 Introduction

Energy supply is one of the many challenges faced by humanity in the 21st century. World energy consumption has been projected to increase by 56% between 2010 and 2040 [International Energy Agency, 2013]. The majority of the world's energy demands are met through fossil fuels [Azbar and Levin, 2012]. Greenhouse gas emissions such as carbon dioxide from combustion of fossil fuels and associated global climate change has raised a concern for the environment and human health [Ramachandran et al., 2011; Benemann, 1996]. Development of alternate renewable fuels with lower carbon emissions has become imperative for sustainable development and to meet the increasing demands of an increasing population [Prakasham et al., 2009a; Kyazze et al., 2006]. Hydrogen has been deemed as a promising alternate energy source for the future since during its combustion no carbon dioxide is produced [Masset et al., 2010]. It does not contribute to the greenhouse effect, producing only heat and water upon combustion and has a high energy yield of 286 kJ/mol, which is at least two times greater than that of any hydrocarbon fuel [Cai et al., 2004].

Increase in populations and industrial developments has given rise to large quantities of domestic, industrial, and agricultural wastes generation and proper handling of these wastes is a growing concern doe to threat to air, water and soil [Elbeshbishy, 2011]. Biological hydrogen production from the organic matter present in these wastes is a promising approach to waste management as well as energy generation [Elbeshbishy, 2011; Tenca et al., 2011].

2.2 Biological Hydrogen Production

Biological hydrogen production employs hydrogen producing microorganisms. There are four mechanisms for biohydrogen production: direct biophotolysis, indirect biophotolysis, photo-fermentation, and dark fermentation.

2.2.1 Direct Biophotolysis

Certain bacterial-algal (green algae and cyanobacteria) systems are capable of using solar energy directly to extract electrons and protons from water resulting in evolution of hydrogen (photohydrogen) and oxygen by the following reaction [Levin et al., 2004; Benemann, 1980]:

$$2H_2O + light\ energy \rightarrow 2H_2 + O_2 \hspace{4cm} \text{(Equation 1)}$$

The main disadvantages of this process are that it requires high light intensity, oxygen can be inhibitory and low photochemical efficiency [Das and Veziroglu, 2008].

2.2.2 Indirect Biophotolysis

Cyanobacteria (blue-green algae) can also synthesize hydrogen through photosynthesis by splitting water in a two-step process [Levin et al., 2004]:

$$12H_2O + 6CO_2 + light\ energy \rightarrow C_6H_{12}O_6 + 6O_2 \hspace{2cm} \text{(Equation 2)}$$

$$C_6H_{12}O_6 + 12H_2O + light\ energy \rightarrow 12H_2 + 6CO_2 \hspace{2cm} \text{(Equation 3)}$$
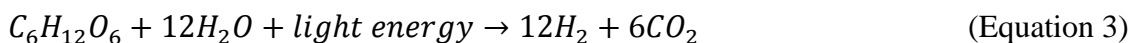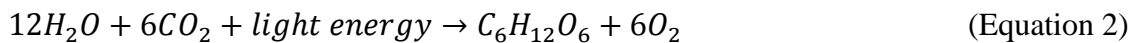
In the first step (aerobic phase), solar energy and water are used to accumulate carbohydrates through the photosynthesis process. In the second step (anaerobic phase), carbohydrates are catabolized for hydrogen production. Due to the multiple steps in indirect biophotolysis, it is less effective than direct biophotolysis [Azbar and Levin, 2012]. The main disadvantage of this process is the need to remove hydrogenase enzymes to avoid degradation of hydrogen [Das and Veziroglu, 2008].

2.2.3 Photo-Fermentation

Purple non-sulfur (PNS) bacteria produce hydrogen under nitrogen deficient conditions due to the presence of nitrogenase, using light energy and reduced compounds (organic acids) [Das and Veziroglu, 2008]:

$$CH_3COOH + 2H_2O + light\ energy \rightarrow 4H_2 + 2CO_2 \qquad\qquad \text{(Equation 4)}$$
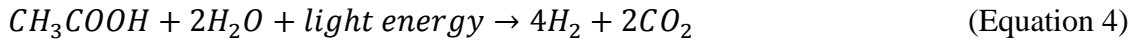
The main disadvantages of this process are the inhibitory effect of oxygen on nitrogenase and the very low (1%-5%) light conversion efficiency [Das and Veziroglu, 2008].

2.2.4 Anaerobic Dark Fermentation

Dark fermentation is a ubiquitous phenomenon under anoxic or anaerobic conditions. Oxidation of organic matter during heterotrophic growth of fermentative bacteria, generates electrons and due to the anoxic environment, oxygen is unavailable, and accordingly other species, e.g., protons, are reduced to molecular hydrogen which acts as an electron acceptor [Das and Veziroglu, 2008]. Anaerobic systems have an advantage over photosynthetic systems in the sense they are simpler, less expensive, and produce hydrogen at faster rates. However, a major drawback is that the hydrogen-producing bacteria are unable to overcome the inherent thermodynamic energy barrier to full substrate utilization [Hallenbeck et al, 2009]. Carbohydrates are the preferred carbon sources for fermentation and the end products vary widely, including acetate, butyrate, propionate, lactic acid, and ethanol [Guo et al, 2010].

Dark fermentation processes produce mixed biogas with primarily hydrogen and carbon dioxide, and may contain methane, carbon monoxide, and hydrogen sulfide [Levin and Azbar, 2012]. Depending on the fermentation pathway and end products, glucose (or its isomer hexoses or its polymers starch and cellulose) yield different

quantities of hydrogen. Majority of hydrogen-producing bacteria are either strict anaerobes (*Clostridia*, mrthylotrophs, rumen bacteria, methanogenic bacteria, archaea), facultative anaerobic bacteria (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and aerobic bacteria (*Alcaligens*, *Bacillus*) [Guo et al., 2010]. A maximum of 4 mol/mol glucose is obtained when acetate is the end-product, and half of this yield/mol glucose is obtained when butyrate is the end product [Hawkes et al, 2002]:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{(Equation 5)}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \text{(Equation 6)}$$
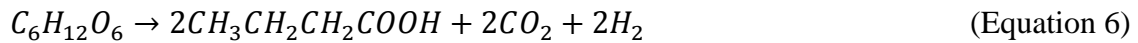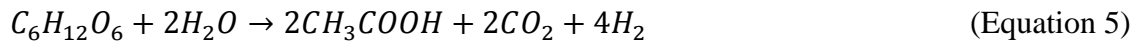
Several microbial populations, known as homoacetogenic bacteria (for example: *Clostridium thermoaceticum* and *Clostridium aceticum*), convert hydrogen and carbon dioxide to acetate, in turn, consuming the hydrogen [Guo et al, 2010]:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$$

Propionate is also a hydrogen-consuming pathway, while ethanol and lactic acid are zero-hydrogen balance pathway [Guo et al, 2010]:

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad \text{(Equation 7)}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad \text{(Equation 8)}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2 \qquad \text{(Equation 9)}$$
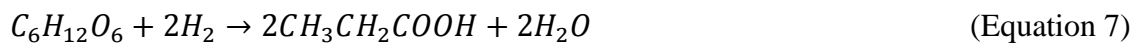
Some microorganisms, known as syntrophic bacteria, can carry out 'impossible' fermentations of some end-products. They are regarded as "impossible" since the Gibbs free energy change is positive under standard conditions, and are only possible at low hydrogen partial pressure conditions [Levin and Azbar, 2012]:

$$Ethanol + H_2O \rightarrow Acetate^- + 2H_2 + H^+ \qquad \text{(Equation 10)}$$

$$Propionate^- + 3H_2O \rightarrow Acetate^- + 3H_2 + H^+ + HCO_3^- \qquad \text{(Equation 11)}$$

$$Butyrate^- + 2H_2O \rightarrow 2Acetate^- + 2H_2 + H^+ \qquad \text{(Equation 12)}$$
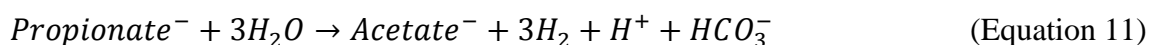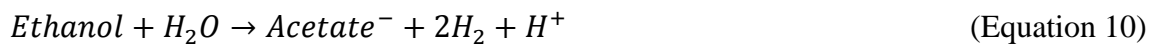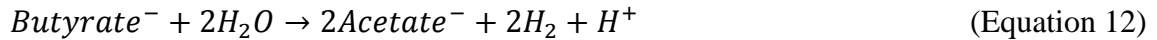
Attaining higher hydrogen yields is the ultimate goal and challenge of fermentative hydrogen research. Process conditions, including inoculum, are an important factor as they controls the formation of end products.

2.3 Factors Affecting Dark Fermentative Hydrogen Production

Several factors influence dark fermentative hydrogen production, including pH, temperature, inoculum, substrate, and hydrogen partial pressure.

2.3.1 pH

pH is an important parameter influencing fermentative hydrogen production [Wang and Wan., 2009]. pH affects not only hydrogen yields, but also impacts metabolic pathways and the structure of microbial communities in mixed cultures.

A pH range of 5-6 has been preferred for food wastes, while a neutral pH for crop-residues and animal manure [Guo et al., 2010]. pH range of 4.7 to 5.7 was reported to be optimal for starch hydrogen fermentation [Lay, 2000]. Yossan et al. [2012] also reported pH 6 to be optimal pH for hydrogen production from palm oil mill effluent with maximum hydrogen yield of 1.06 mmol H_2/ g COD. Masset et al. [2010] reported pH of 5.2 to be optimal for glucose and 5.6 for starch with hydrogen yields of 1.53 and 1.8 mol H_2/mol$_{hexose}$, respectively. At pH lower than 4.1 or higher than 6.1, alcohol production is favored over hydrogen production [Lay, 2000]. pH 5.5 and 6 have been reported to attain better substrate utilization efficiency, cell yield, and hydrogen yields of 1.65 and 1.55 mol H_2/mol$_{hexose}$, respectively [Lee et al., 2008]. Various optimal pHs have been reported in the literature, which could be attributed to difference in the source of inoculum, substrate, and operational temperature. Butyrate and acetate are the favored end products,

but at low pH, butyrate is preferentially produced [Guo et al., 2010]. Acetate-butyrate pathways are favored at pH 4.5-6 while at neutral pH or higher conditions, ethanol and metabolic pathway shift to propionate (hydrogen consuming pathway) are observed [Guo et al., 2010; Fang and Liu, 2002]. Fang and Liu [2002] studied the effect of pH on conversion of glucose by a mixed culture and observed a pH of 5.5 to be optimal with respect to hydrogen yield (2.1 $molH_2/mol_{hexose}$), hydrogen content (64%) in biogas, and specific hydrogen production rate (4.6 L H_2/g-VSS day). At pH higher than 6, reduced hydrogen content in biogas was observed as well as reduction in hydrogen yield and specific production rate. Furthermore, in mixed culture hydrogen production systems, pH higher than 6 leans towards methanogenesis [Fang and Liu, 2002]. Shin and Youn [2005] observed optimal pH to be 5.5 using food waste as substrate and anaerobic digester as seed with hydrogen content, yield and efficiency of decomposition to be 60.5%, 2.2 mol H_2/mol hexose$_{consumed}$ and 90%, respectively. An increase in microbial diversity has also been observed with the increase in pH [Fang and Liu, 2002]. A drastic change in pH can affect the ionization states of the active components of the biomass as well as the substrates, hampering biomass growth [Levin and Azbar, 2012].

2.3.2 Temperature

Temperature is one of the most important parameters affecting both hydrogen potential and microbial metabolisms in mixed cultures [Karlsson et al., 2008; Puhakka et al., 2012]. The optimal temperature for hydrogen production has not been established and contentious results have been reported in the literature. Mesophilic and thermophilic temperatures are commonly used temperatures in the literature [Gadow et al., 2012]. The majority of studies on hydrogen production have been on mesophilic temperatures,

however, thermophilic temperatures have been reported to facilitate higher yields with complex lignocellulosic compounds due to better hydrolysis [Guo et al., 2010]. Thermophilic conditions are also reported to enhance substrate utilization rates and to reduce dissolved hydrogen [Karlsson et al., 2008]. The difference in optimum temperatures could be attributed to the origin of inoculum, the quantity of biodegradable compounds as well as operating conditions [Guo et al., 2010]. Lee et al. [2008] examined mesophilic (37 ºC) and thermophilic (55 ºC) temperatures using starch as substrate and municipal sewage sludge as inoculum, and observed a higher hydrogen yield at mesophilic than at thermophilic. Kargi et al. [2012] used acid hydrolyzed cheese whey starch powder as substrate and mesophilic anaerobic sludge as inoculum, acclimatized at 55 ºC for thermophilic batches, and observed higher hydrogen yields at thermophilic than mesophilic. Yokoyama et al. [2007] examined the effect of different temperatures, 37ºC, 50 ºC, 55 ºC, 60 ºC, 67 ºC, 75 ºC and 85 ºC, using cow waste slurry, and observed optimum hydrogen production at 60 ºC and 75 ºC. The above mentioned authors' also observed differences in the microbial populations at different temperatures. Gadow et al. [2012] evaluated mesophilic, thermophilic, and hyper-thermophilic temperatures for cellulose utilization and observed maximum hydrogen yields at hyper-thermophilic conditions. It has been reported that increasing temperature from 20 ºC -35 ºC, increased the concentration of ethanol, but it decreased with further increasing temperature from 35 ºC to 55 ºC [Wang and Wan, 2009]. Extreme change in temperature affects the activity of essential enzymes therefore, impeding the growth of biomass. Kumar and Das. [2000] studied hydrogen production rates in *Enterobacter clocae* IIT-BT08 and observed increasing hydrogen yield from 15 to 36ºC while afterwards it decreased. Table 2.1 gives

a summary of hydrogen production studies at different temperature conditions. In general, for biohydrogen production, mesophilic temperature range lies between 35 ºC - 37 ºC and thermophilic range between 55 ºC -70 ºC.

2.3.3 Inoculum

The microbial populations are very crucial as they are responsible for degradation of organic compounds to hydrogen and other end-products. Numerous microorganisms have been identified as hydrogen producers, and strictly anaerobic bacteria, mesophilic or thermophilic, are the most common class of bacteria that produce hydrogen. Some facultative anaerobes are also known to give high hydrogen yields [Vertes et al., 2009]. Numerous studies have evaluated hydrogen production potential using mixed communities present in anaerobic digesters [Nasr et al., 2011], compost [Ueno et al., 2001], manure [Akutsu et al., 2008], natural microflora [Puhakka et al., 2012], etc. In addition, pure bacterial isolates have also been studied as mono-cultures or co-cultures. Table 2.1 provides an extensive literature review for hydrogen production using different inoculums.

Utilizing complex materials, requires a wide range of hydrolytic and catabolic activities, which is where mixed microbial populations are useful and more advantageous than pure cultures. Additionally, pure cultures are substrate specific, whereas, mixed cultures have a broader source of feedstock [Wang and Wan, 2009]. Masset et al. [2011] obtained a hydrogen yield of 2 mol/mol hexose using pure isolates of *Clostridium butyricum* and starch as substrate. On the other hand, Akutsu et al. [2008] obtained a higher hydrogen yield of 2.32 mol/mol hexose using mixed waste activated sludge as inoculum and starch as substrate. Datar et al. [2007] achieved hydrogen yield of 3

mol/mol hexose using corn stover as the feedstock and anaerobic digester sludge as inoculum, while Ren [2010] obtained 2.2 mol/mol using *Thermoanaerobacterium thermosaccharolyticum*. Furthermore, during harsh conditions, hydrogen-producing bacteria have a better chance of survival than hydrogen-consuming bacteria. Hydrogen producing bacteria can form protective spores in restrictive environments such as high temperature, extreme acidity and alkalinity, but hydrogen consuming bacteria are not able to withstand such extreme conditions [Zhu and Beland, 2006]. As such, various pretreatment technologies are applied to suppress the activity of hydrogen-consuming bacteria [Sinha and Pandey, 2011]. Acid, base, aeration, freezing and thawing, chloroform, sodium 2-bromoethanesulfonate (BESA), iodopropane, and heat-shock, the most widely used, are some of the pretreatment technologies practiced [Sinha and Pandey, 2011]. When the inoculum was heat pretreated for 30 min at 80°C, Wang et al. [2011] observed an increase in hydrogen yield to 3.37 mol H_2/mol hexose compared to control (2.2 mol H_2/mol hexose) with no pretreatment. In the same study, the authors saw an increase in hydrogen yield to 3.71 and 2.99 mol H_2/mol hexose when the inoculum was alkali pretreated at pH 11 and acid pretreated at pH 4, respectively [Wang et al., 2011]. Zhu and Beland. [2006] tested different pretreatment methods and observed high hydrogen yields of 5.64 and 5.28 mol H_2/mol sucrose$_{added}$ with iodopropane and BESA pretreated sludge, respectively, compared to untreated sludge (5.17 mol H_2/mol sucrose$_{added}$). The above mentioned authors conducted a secondary batch cultivation with alkaline pretreatment (pH 10) and observed higher hydrogen yield of 6.12 mol H_2/mol sucrose$_{added}$ compared to no pretreatment sludge (4.56 mol H_2/mol sucrose$_{added}$). Ren et al.

[2008b] used repeated aeration pretreatment method by maintaining the dissolved oxygen

(<0.5 mg/L) and observed an increase in hydrogen yield by 24%.

Table 2.1. Literature review on hydrogen production from different inoculum

		Inoculum	Substrate	Reactor	Temperature (ºC)	pH	H_2 yield (mol H_2/mol hexose$_{added\ or\ consumed}$)	Ref.
Mixed cultures	Mesophiles	Anaerobic digester sludge	Glucose	Batch with pH control	35	5.5	3.21 mol/ mol hexose consumed	Datar et al., 2007
		Anaerobic digester sludge	Glucose	Batch	37	5.5	1.79	Quemeneur et al., 2011
		Sludge from secondary sedimentation tank	Glucose	CSTR	36	5.5	1.8	Fang et al., 2002
		Cow dung seed	Starch wastewater	Batch	35	7	1.56	Lay et al., 2012
		Anaerobic digester sludge	Corn stover steam explosion under neutral condition	Batch with pH control	35	5.5	2.84	Datar et al., 2007
		Anaerobic digester sludge	Corn stover steam explosion under acidic condition	Batch with pH control	35	5.5	3	Datar et al., 2007
	Thermophiles	Cattle manure	Glucose	Batch	55	5	0.35	Cheong and Hansen, 2007
		Anaerobic mixed cultures	Glucose	Expanded granular sludge bed reactor	70	5.5	0.75	Abreu et al., 2012
		Thermophilic waste activated sludge	Starch (10 g/L)	CSTR HRT 24 hr	55	4.9	2.32	Akutsu et al. 2008
		Thermophilic digested cattle manure				5.4	1.71	

Table 2.1. (Cont.) Literature review on hydrogen production from different inoculum

		Inoculum	Substrate	Reactor	Temperature (ºC)	pH	H_2 yield (mol H_2/mol hexose$_{added\ or\ consumed}$	Ref.
Mixed cultures	Thermophiles	Compost of night solid and organic fractural municipal solid waste	Starch (10 g/L)	CSTR HRT 24 hr	55	5.3	2.13	Akutsu et al. 2008
		Thermophilic acidified potato				4.9	2.02	
		Thermophilic-digested night soil and organic fractural municipal solid waste				5.4	1.38	
Pure cultures	Strict anaerobes	*Clostridium butyricum CWBI1009*	Glucose	Sequenced batch	30	5.2	1.7	Masset et al., 2010
		Clostridium butyricum CWBI1009	Starch	Sequenced batch	30	5.6	2	Masset et al., 2010
		Clostridium termitidis CT1112	Cellulose	Batch	37	7.2	0.62	Ramachandran et al., 2008
		Clostridium beijerinckii	Glucose	Batch	37	6.7	1.45	Masset et al., 2012
		Clostridium saccharaperbutylacetonicum N1-4	Glucose	Batch	37	6	3.1	Alalayah et al., 2008
		Clostridium paraputrificum M-21	Glucose	Batch	45	5.8	1.1	Evvyernie et al., 2001

Table 2.1. (Cont.) Literature review on hydrogen production from different inoculum

		Inoculum	Substrate	Reactor	Temperature (^{o}C)	pH	H$_2$ yield (mol H$_2$/mol hexose$_{added\ or\ consumed}$)	Ref.
Pure cultures	**Strict anaerobes**	*Thermoanaerobacterium thermosaccharolyticum W16*	Glucose	Batch	60	6.5	2.42	Ren et al., 2008a
		Thermoanaerobacterium thermosaccharolyticum W16	Corn stover	Batch	60	7	2.2	Ren et al., 2010
		Caldicellulosiruptor saccharolyticus	Glucose	Batch	70	7	3.4	Mars et al., 2010
		Thermotoga elfi	Glucose	Batch	65	7-7.4	3.33	Van Niel et al., 2002
	Facultative anaerobes	*Klebsiella pneumonia ECU-15*	Glucose	Batch	37	6	2.07	Niu et al., 2010
		Enterobacter aerogenes HO-39	Glucose	Batch	38	6-7.0	1	Yokoi et al., 1995
		Escherichia coli BL-21	Glucose	CSTR	37	6	3.12	Chittibabu et al., 2006
		Enterobacter cloacae IIT-BT08	Glucose	Batch	36	6	2.2	Kumar and Das, 2000
		Rhodopseudomonas palustris P4	Glucose	Batch	37	7	2.76	Oh et al., 2002
	Co-cultures	*Clostridium butyricum* and *Enterobacter aerogenes HO-39*	Sweet potato starch residue	Batch	37	5.25	2.7	Yokoi et al., 2002

Table 2.1. (Cont.) Literature review on hydrogen production from different inoculum

		Inoculum	Substrate	Reactor	Temperature (ºC)	pH	H_2 yield (mol H_2/mol hexose$_{added \, or \, consumed}$)	Ref.
Pure cultures	Co-cultures	*Clostridium beijerinckii* and *Rhodobacter ssphaeroides-RV*	Ground wheat	Annular hybrid bioreactor	32	7-7.5	0.64	Argun et al., 2010
		Clostridium butyricum and Clostridium felsineum	Glucose	Batch	37	5.3	1.71	Masset et al., 2012
		Clostridium pasteurianum and Clostridium felsineum	Glucose	Batch	37	5.3	1.62	Masset et al., 2012
		Clostridium thermocellum and *Thermoanaerobacterium thermosaccharolyticum*	Micro-crystalline cellulose (5 g/L)	Batch	60	6.8	1.8	Liu et al. 2008

2.3.4 Substrates for Fermentative Hydrogen Production

Carbohydrates are the ideal carbon source for fermentative hydrogen production [Hawkes et al., 2002]. A lot of substrates (Table 2.2), majority of which are soluble sugars like glucose and sucrose, have been used for hydrogen producing fermentations due to their ease of degradability, relatively simple structures, presence in several industrial effluents, and presence in polymeric forms [Hallenbeck et al., 2009]. Nevertheless, pure carbohydrate sources are expensive raw materials for large scale hydrogen production, therefore, renewable feedstocks like biomass, agricultural waste by-products, lignocellulosic products, food processing waste, agricultural and livestock effluents, household wastewater, biodiesel industry wastewater, etc., are all more sustainable feedstocks [Hawkes et al., 2002; Elsharnouby et al., 2013; Chong et al., 2009]. Figure 2.1 provides a distribution of usage of pure and real waste substrates reviewed in the literature. Table 2.2 summarizes various substrates examined for fermentative hydrogen production.
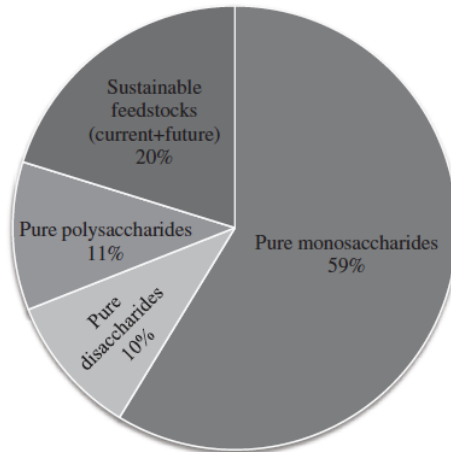
Figure 2.1. Distribution of research in pure vs. real waste substrates [Elsharnouby et al., 2013]

Table 2.2 Summary of various substrates examined for fermentative hydrogen production

	Substrate	Inoculum	Optimal Index (mol/mol)	Reference
Monosaccharide	Glucose	Anaerobic digester sludge	2.69 mol H_2/ mol hexose	Kim and Kim, 2012
	Glucose	Anaerobic digester sludge	2.8 mol H_2/ mol glucose	Hafez et al., 2010
	Xylose	Anaerobic mixed culture	2.25 mol H_2/mol xylose	Lin et al., 2006
	Xylose	*Enterobacter aerogenes* IAM 1183	2.2 mol H_2/mol hexose	Ren et al., 2009
	Arabinose	Mixed culture sludge	1.98 mol H_2/ mol hexose	Danko et al., 2008
	Arabinose	*Escherichia coli strain DJT135*	1.02 mol H_2/ mol hexose	Ghosh et al., 2009
	Galactose	*Enterobacter aerogenes strain HO-38*	0.95 mol H_2/ mol galactose	Yokoi et al., 1995
	Galactose	*Escherichia coli strain DJT135*	0.69 mol H_2/ mol galactose	Ghosh et al., 2009
	Mannose	*Enterobacter aerogenes strain HO-39*	0.98 mol H_2/ mol mannose	Yokoi et al., 1995
	Mannose	*Citrobacter sp. CMC-1*	1.93 mol H_2/ mol mannose	Mangayil et al., 2011
Disaccharide	Sucrose	Anaerobic digester sludge	1.9 mol H_2/mol hexose$_{converted}$	Hussy et al., 2005
	Sucrose	Mixed cultures dominated by *Clostridium pasteurianum*	2.73 mol H_2/ mol sucrose	Zhang et al., 2005
	Maltose	*Enterobacter aerogenes strain HO-38*	2.16 mol H_2/ mol maltose	Yokoi et al., 1995
	Maltose	*Clostridium sp. R1*	3.13 mol H_2/ mol maltose	Ho et al., 2010
	Cellobiose	*Clostridium termitidis*	4.6 mmol H_2/ L culture	Ramachandran et al., 2008
	Cellobiose	*Clostridium sp. R1*	3.5 mol H_2/mol cellobiose	Ho et al., 2010
Polysaccharide	Starch	Soil inoculum	0.59 mol H_2/ mol starch$_{added}$	Logan et al., 2002
	Starch	Paper-mill wastewater sludge	1.1 mol H_2/mol hexose	Lin et al., 2008

	Substrate	Inoculum	Optimal Index	Reference
Polysaccharide	Cellulose	*Clostridium cellulolyticum*	1.7 mol H_2/mol hexose$_{consumed}$	Ren et al., 2007
	Cellulose	*Clostridium termitidis*	0.62 mol H_2/mol hexose	Ramachandran et al., 2008
Real wastes	Potato processing wastewater	Soil inoculum	0.004 mol H_2/ g COD	Van Ginkel et al., 2005
	Molasses	Mixed culture	26.13 mol H_2/ kg COD$_{removed}$	Ren et al., 2006
	Cheese whey	*Clostridium saccharobutylacetonicum ATCC27021*	0.0079 mol H_2/g lactose	Ferchichi et al., 2005
	Sugarbeet juice	Anaerobic digester sludge	1.7 mol H_2/mol hexose$_{converted}$	Hussy et al., 2005
	Food waste and sewage sludge	Anaerobic digester sludge	0.005 mol H_2/ g carbohydrate-COD	Kim et al., 2004
	Wheat starch co-product	Anaerobic digester sludge	1.3 mol H_2/mol hexose$_{consumed}$	Hussy et al., 2003
	Thin stillage	Acclimatized anaerobic digester sludge	0.77 mol H_2/L thin stillage	Nasr et al., 2011
	Sugarcane bagasse	*Clostridium butyricum*	1.73 mol H_2/mol total sugar	Pattra et al., 2008
	Sugar cane bagasse hydrosylate	Elephant dung	0.84 mol H_2/mol total sugar	Fangkum and Reungsang, 2011a

Co-digestion of different substrates has driven several researchers over the last 15-20 years to evaluate its effects on the performance of anaerobic digestion process by simultaneously treating different organic waste streams. Some of the reported advantages of co-digestion are dilution of toxic compounds, improved nutrients balance, improved buffering capacity, and synergistic microbial effects [Esposito et al., 2012b]. Real wastes have been co-digested for methane production. The benefit of

methanogenic co-digestion is primarily due to C/N ratio in the optimal range 20:1 and 30:1, that impact inhibition by ammonia. Another significant benefit of co-digestion is widening the range of bacterial strains taking part in the process [Esposito et al., 2012a]. Kim et al. [2003] investigated the effect of food waste addition on anaerobic digestion of sewage sludge under mesophilic and thermophilic conditions. Co-digestion had a distinct positive effect on methane production rate (MPR) and methane yields. Esposito et al. [2012a] assessed the co-digestion of buffalo manure (BM), poultry manure (PM), organic fraction of the municipal solid waste (OFMSW) and greengrocery waste (GW). Co-digestion of BM and OFMSW resulted in higher methane volumes and decreased the possibility of failure for the biological process. Riano et al. [2011] demonstrated promising results for co-digestion of swine manure with winery wastewater, with a significant increase in the methane yields at different combinations of substrates. Majority of the research on biohydrogen production using dark fermentation has mainly focused on single substrates and very few studies have explored co-digestion of different substrates. Prakasham et al. [2009b] observed a 23% and 9% increase in hydrogen production from glucose-xylose co-fermentation when compared to independent glucose-only and xylose-only experiment, respectively. Xylose co-fermentation with cellulose increased the cellulose conversion efficiency by three times compared to the control without any co-substrate, where nearly no cellulose was utilized [Xia et al., 2012]. Fangkum and Reungsang [2011b] studied the thermophilic co-digestion of xylose and arabinose at 2.5 g/L each concentrations using anaerobic mixed cultures and obtained a maximum hydrogen yield of 2.59 mol H_2/mol-sugar consumed with 95% substrate degradation.

2.3.5 Hydrogen Partial Pressure

It has been reported in many studies that partial pressure of hydrogen is a restrictive factor in hydrogen fermentation process [Guo et al., 2010]. By means of hydrogen production, bacteria re-oxidize reduced ferredoxins and hydrogen carrying coenzymes, and these reactions are unfavorable at high hydrogen concentrations in the liquid phase and cause end-product inhibition [Hawkes et al., 2002]. With the increase in hydrogen concentration, a decrease in hydrogen synthesis and metabolic shifts to the production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine occur [Elbeshbishy et al., 2011]. Lower propionate concentrations were observed at low hydrogen partial pressure [Lee et al., 2012]. Oxidation of long chain fatty acids to volatile fatty acids with hydrogen production is thermodynamically unfavorable with positive Gibbs energy and therefore, very low concentrations of hydrogen are required to overcome this thermodynamic barrier [Guo et al., 2010]. Similarly, additional hydrogen production from acetate is also a thermodynamically unfavorable reaction which is extremely sensitive to hydrogen concentrations.

A number of methods are used to reduce hydrogen partial pressure in the liquid phase. Gas sparging, gas stripping by membrane absorption, ultrasonication, and increased mechanical mixing are some of the techniques used [Elbeshbishy et al., 2011]. Gas sparging has been the most common method to decrease dissolved gas concentrations in hydrogen producing reactors [Elbeshbishy et al., 2011]. Hussy et al. [2003] observed a 48% increase in hydrogen yield from 1.26 to 1.87 mol H_2/mol hexose with nitrogen sparging. Lamed et al. [1988] observed that the hydrogen production in a stirred culture of *Clostridium thermocellum* was 2.8 times greater than

the unstirred one. Liang et al. [2002] investigated the effectiveness of silicone rubber membrane to separate biogas from the liquid medium and observed an improvement in the hydrogen evolution by 10% and the hydrogen yield by 15%. Elbeshbishy et al. [2011] observed an increase in the hydrogen content in the headspace by 31% with the application of ultrasonication technique which removed the dissolved carbon dioxide and hydrogen from the liquid.

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Chapter 3

Co-fermentation of Glucose, Starch, and Cellulose for Mesophilic Biohydrogen Production[1]

3.1 Introduction

Among various biological H_2 production methods, dark fermentation is of great significance to produce H_2 from readily available organic wastes [Wang and Wan, 2009]. Renewable carbohydrate-based feedstocks are the preferred organic carbon source for H_2-producing fermentations [Azbar and Levin, 2012; Hawkes et al., 2002]. Waste biomass from municipal, agricultural, forestry, pulp/paper, and food industries represent an abundant potential source of substrate [Azbar and Levin, 2012; Hallenbeck et al., 2009].

Kleerebezem et al., [2007] outlined the importance and advantages of using mixed culture fermentation. Natural mixed consortia allow bioprocessing in non-sterile environments and have a higher threshold of dealing with mixtures of substrates of variable composition due to high microbial diversity, which reduces the process operational cost significantly [Kleerebezem et al., 2007; Prakasham et al., 2009]. A wide range of hydrolytic and catabolic activities are required while using complex materials, which renders using mixed microbial consortia [Azbar and Levin, 2012]. Several factors influence fermentative H_2 production, irrespective of mixed consortia or pure cultures, including both inoculum and substrate [Wang and Wan, 2009]. The

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[1] This chapter has been submitted to International Journal of Hydrogen Energy in June and is currently under review.

inoculum source and/or type of substrate affect the metabolic pathways of the microbial strain(s) and regulate product formation [Prakasham et al., 2009]. Fermentation of hexose produces H_2 and CO_2 through the acetate and/or butyrate synthesis pathways. However, mixed acid fermentations that synthesize lactate, ethanol, and in some cases formate or propionate produce significantly reduced amounts of H_2 [Hawkes et al., 2002]. Therefore, bacterial metabolism favoring acetate and butyrate production is important [Hawkes et al., 2002].

Numerous studies have examined H_2 production potential of different substrates ranging from simple sugars to more complex substrates such as cellulose. Although biohydrogen production from simple monosaccharide sugars has been well researched, relatively few studies have dealt with co-substrates. To date, the majority of the research on biohydrogen production using dark fermentation has mainly focused on single substrates and very few studies have explored co-fermentation of different substrates. Prakasham et al. [2009] investigated the role of glucose to xylose ratio on fermentative mesophilic biohydrogen production using enriched H_2 producing mixed consortia from buffalo dung compost as inoculum [Prakasham et al., 2009]. They performed batch experiments using overall 5 g/L glucose and xylose independently and at different combinations of glucose and xylose. It was observed that the use of glucose to xylose ratio of 2:3 (on mass basis) was more effective compared to the individual pure sugar fermentation. The glucose-xylose co-fermentation resulted in 23% increase in H_2 production when compared to glucose-only fermentation, and 9% increase in H_2 production when compared to the xylose-only experiment.

Xia et al. [2012] investigated co-substrates, including glucose, xylose, and starch for thermophilic anaerobic conversion of microcrystalline cellulose using anaerobic digestion sludge (ADS) in batch tests [Xia et al., 2012]. A "same substrate-co-substrate" ratio of 10:1 (in terms of COD) was used, with 4 g/L microcrystalline cellulose as substrate and 0.4 g/L of glucose, xylose, or starch dosed individually as co-substrates. Xylose increased the cellulose conversion efficiency by three times compared to the control without any co-substrate, where nearly no cellulose was utilized.

Ren et al. [2008] studied batch fermentation of xylose-glucose mix using *Thermoanaerobacterium thermosaccharolyticum* W16 strain for thermophilic biohydrogen production and observed that the content of glucose in the mixture had an effect on consumption of xylose [Ren et al., 2008]. However, the glucose consumption rate remained essentially constant and was independent of the xylose content. Additionally, the final maximum H_2 yield in the mixture was observed to be 2.37 mol H_2/mol substrate for a glucose:xylose ratio of 4:1, which was not significantly different from the yields obtained using pure monosaccharide substrates (glucose, 2.42 mol H_2/mol substrate; xylose, 2.19 mol H_2/mol substrate). It was also observed that the isolated strains degraded a feedstock consisting of corn-stover hydrosylate as efficiently as the xylose/glucose mix. Lin et al. [2008] conducted a batch study using starch at 20 gCOD/L and seed sludge from paper mill waste-water treatment plant, and achieved a H_2 yield of 2.2 mol H_2/mol hexose [Lin et al.., 2008]. In another study, starch-containing wastewater from a textile factory was used as substrate and cow

dung seed was used as inoculum where maximum H_2 yield of 0.97 mol H_2/mol hexose was obtained at a substrate concentration of 20 gCOD/L and initial pH of 7 [Lay et al., 2011]. Pure culture studies on mesophilic cellulose degradation achieved yields ranging from 0.62-1.7 mol H_2/mol hexose. Ramachandran et al. [2008] achieved 0.62 mol H_2/mol hexose$_{added}$ at 2 g/L initial cellulose concentration [Ramachandran et al., 2008]. Ren et al. [2007] reported the highest mesophilic H_2 production from cellulose with yields of 1.7 mol H_2/mol hexose$_{consumed}$ with initial cellulose concentration of 5 g/L with *Clostridium cellulolyticum.*

It is apparent from the literature review that there are no reports of mixed mesophilic culture on cellulose degradation enhancement by co-fermentation with glucose and starch. The significance of this work stems from the vast majority of cellulosic wastes, which combine starch and cellulose that is known to degrade to glucose. Thus, the premise of this work was based on the synergism of various microbial biohydrogen-producing cultures. We hypothesized that addition of glucose to starch and cellulose would improve their degradation. Thus, the primary objective of this work was to assess the synergistic effects of co-fermentation of glucose, starch, and cellulose using ADS on the biohydrogen production and the associated microbial communities. Detailed microbial characterization using illumina sequencing of the 16S ribosomal (r)DNA V4 hyper-variable region, followed by bioinformatics analyses, was undertaken to characterize changes in the microbial communities of ADS fermentations containing single versus co-substrates.

3. 2 Material and Methods

3.2.1 Seed sludge and substrate

Anaerobically digested sludge was collected from the St. Marys wastewater treatment plant (St. Marys, Ontario, Canada) and used as seed for the experiment. The total suspended solids (TSS) and volatile suspended solids (VSS) of the ADS were 18 and 13 g/L, respectively. The ADS was pretreated at 70 °C for 30 minutes to inhibit methanogens [Nasr et al., 2011]. Glucose, starch, and α-cellulose were added at 2.7 gCOD, individually as mono-substrates, and in combinations in the ratio (1: 1) or (1:1:1), with all possible combinations as co-substrates, with sufficient inorganics and trace minerals [Nasr et al., 2011]. NaHCO$_3$ was used as buffer at 5 g/L.

3.2.2 Experimental design

Batch studies were conducted in serum bottles with a working volume of 200 mL. Experiments were conducted in triplicates for initial substrate-to-biomass (S/X) ratio of 4 gCOD$_{substrate}$/g VSS$_{seed}$. Volume of seed added to each bottle was 50 mL. The TCOD$_{substrate}$ (g/L) to be added to each bottle was calculated based on Equation 1:

$$S/X(\, g\, COD/g\, VSS) = \frac{V_f(L)*\, Substrate\, TCOD(\frac{g}{L})}{V_s(L)*\, Seed\, VSS\, (\frac{g}{L})} \qquad \text{(Equation 1)}$$

Where V_f is the volume of feed and V_s is the volume of seed. 50 mL of seed was added to each bottle and TCOD of substrate to be added was calculated to be 2.7 gCOD. The initial pH value for each bottle was adjusted to 5.5 using HCl. NaHCO$_3$ was added at 5 g/L for pH control. Ten mL samples were collected initially. The headspace was

flushed with nitrogen gas for a period of 2 minutes and capped tightly with rubber stoppers. The bottles were then placed in a swirling-action shaker (Max Q4000, incubated and refrigerated shaker, Thermo Scientific, CA) operating at 180 RPM and maintained temperature of 37 °C. Three control bottles were prepared using ADS without any substrate. Final samples were taken at the end of the batch (187 hours post-inoculation) and the final pH was measured to be 5.1 ± 0.15.

3.2.3 Analytical methods

The biogas production was measured using suitable sized glass syringes in the range of 5-100 mL. The gas in the headspace of the serum bottles was released to equilibrate with the ambient pressure [Nasr et al., 2011]. The biogas composition including hydrogen, methane, and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with thermal conductivity detector (TCD) and a molecular sieve column (Mole sieve 5A, mesh 80/100, 6 ft x 1/8 in). Argon was used as the carrier gas at a flow rate of 30 mL/min and the temperature of the column and the TCD detector were 90 °C and 105 °C, respectively. Volatile fatty acids (VFAs) were analyzed using a gas chromatograph (Varian 8500, Varian Inc., Toronto, Canada) with a flame ionization detector (FID) equipped with a fused silica column (30m x 0.32 mm). Helium was used as carrier gas at a flow rate of 5 mL/min. The temperatures of column were 110 and 250 °C, respectively [Nasr et al., 2011]. Total and soluble chemical oxygen demand (TCOD/ SCOD) were measured using HACH methods and test kits (HACH Odyssey DR/2500 spectrophotometer

manual) [Nasr et al., 2011]. TSS and VSS were analyzed using standard methods [Clesceri et al., 1998].

3.2.4 Microbial analysis

Six replicates (2 mL each) of the ADS from each of the seven treatment conditions were collected into 2 mL vials. Sludge samples were washed using 10X phosphate buffered saline (PBS) buffer. Genomic DNA was extracted from each ADS sample, and the DNAs were subjected to polymerase chain reaction (PCR) amplification of the 16S ribosomal (r) DNA. The resulting amplicons were purified and then subjected to nucleotide sequence analysis using Illumina technology. DNA was extracted from approximately 1 g of sludge sample using E.Z.N.A. DNA isolation kit (OMEGA, biot-tek) according to the manufacturer's instructions and laboratory manuals [Ufnar et al., 2006]. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). DNA samples were normalized to 20 ng/µL, and quality checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. [2009]. Amplicons were verified by agarose gel electrophoresis. The above mentioned techniques are qualitative methods.

3.2.5 Library construction and Illumina sequencing

The following methods are for qualitative analysis for identification. Library construction and Illumina sequencing were performed as described by Derakhshani et

al. [2014]. In brief, the V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers [Caporaso et al., 2012]. The reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. PCR reaction for each sample was performed in duplicate and contained 1.0 μL of pre-normalized DNA, 1.0 μL of each forward and reverse primers (10 μM), 12 μL HPLC grade water (Fisher Scientific, ON, Canada) and 10 μL 5 Prime Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) to remove primers, dNTPs and reaction components. The V4 library was then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (Invitrogen, NY, USA). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, measured by Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers for read1 (5´-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read2 (5´-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3´) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA,

USA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

3.2.6 Bioinformatic analyses

This section and the following section with statistical analysis use techniques for quantitative analysis. Bioinformatic analyses were performed as described by Derakhshani et al. [2014]. In brief, the PANDAseq assembler was used to merge overlapping paired-end Illumina fastq files [Masella et al., 2012]. All the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME (Quantitative Insight Into Microbial Ecology) [Caporaso et al., 2010a]. Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with more than 3 consecutive bases with quality scores below 1e-5 were truncated, and those with a read length shorter than 75 bases were removed from the downstream analysis. Chimeric reads were filtered using UCHIME [Edgar et al., 2011] and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST [Edgar et al., 2010] at 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier [Wang et al., 2007] and aligned with the Greengenes Core reference database [DeSantis et al., 2006] using PyNAST algorithms [Caporaso et al., 2010b]. Phylogenetic tree was built with

FastTree 2.1.3. for further comparisons between microbial communities [Proce et al., 2010].

Within community diversity (α-diversity) was calculated using QIIME. Alpha rarefaction curve was generated using Chao 1 estimator of species richness with ten sampling repetitions at each sampling depth [Chao, 1984]. An even depth of approximately 15,700 sequences per sample was used for calculation of richness and diversity indices. To compare microbial composition between samples, β-diversity was measured by calculating the weighted and unweighted Unifrac distances [Lozupone and Knight, 2005] using QIIME default scripts. Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using PRIMER v6 software [Warwick and Clarke, 2006]. Permutational multivariate analysis of variance (PERMANOVA) was used to calculate P-values and test for significant differences of β-diversity among treatment groups [Anderson, 2005].

3.2.7 Statistical analysis

The UNIVARIATE procedure of SAS (SAS 9.3, 2012) was used to test the normality of residuals for Alfa biodiversity data. Non-normally distributed data were log transformed and then used to assess the effect of sampling date (pre-/post-calving) using MIXED procedure of SAS. Phylum percentage data was also used to evaluate statistical differences among different co-substrates. The MIXED procedure of SAS was utilized, as described above, to test for significant changes in the proportions of different phyla among the groups of interest. All the phyla were divided into two

groups of abundant, above 0.5% of the population, and low-abundance, below 0.5% of the population. The differences between groups were considered significant at $P < 0.05$ while trends were observed at $P < 0.1$.

3.3 Results and Discussion

3.3.1 Biohydrogen production

To understand the effects of different substrates on biohydrogen production using mixed anaerobic consortia, glucose, starch, and cellulose were added individually, as mono-substrates, or in combinations as co-substrates to batch fermentation reactions inoculated with ADS. The overall substrates concentration was maintained at 13.5 gCOD/L in all the bottles, which resulted in initial substrate to biomass ratio of 4 g COD/g VSS. Figure 3.1 shows the cumulative H_2 production for the different substrate conditions. The observed cumulative H_2 production after 187 hours of fermentation was 431, 353, and 53 mL for glucose, starch, and cellulose, respectively, as mono-substrates. A maximum cumulative H_2 production of 499 mL was observed in co-fermentation of glucose and starch, the glucose and cellulose co-fermentation produced 303 mL H_2, the starch and cellulose fermentation produced 269 mL H_2, and co-fermentation of glucose, starch, and cellulose produced 343 mL H_2. As reported above, cellulose-only produced the lowest amount of H_2, and bottles containing cellulose in combination with other substrates yielded lower H_2 production when compared to glucose-only, starch-only, and glucose with starch in combination.

Logan et al. [2002] witnessed lower H_2 gas production with cellulose and potato starch than with glucose and suggested that part of the reason could be due to the degradative abilities of the microbial inoculum relative to the different substrates. In general, it has been reported that glucose is the most preferred substrate for any microbial fermentation [Prakasham et al., 2009], which is in accordance with the data reported in this study. Cellulose degradation at mesophilic temperatures has been deemed unfavorable due to its complex structure and usually requires pre-treatment to hydrolyze cellulose to simple sugars [Hallenbeck et al., 2009]. Most of the cellulose degradation studies have been performed at thermophilic temperatures [Xia et al., 2012]. However, Ramachandran et al. [2008] reported promising cellulose degradation at mesophilic temperatures using pure culture inoculum, *Clostridium termitidis* (10% v/v) at a concentration of 2 g/L of α-cellulose, yielding 0.62 mol H_2/mol hexose [Ramachandran et al., 2008].
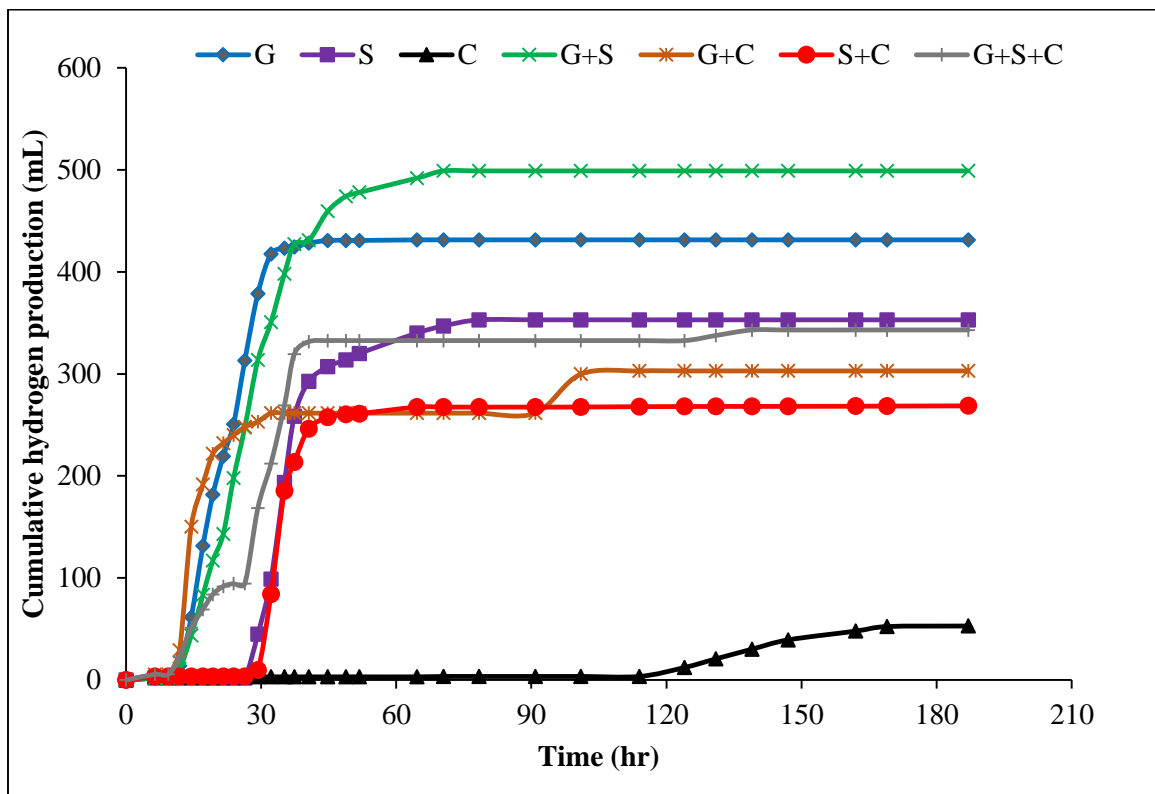
Figure 3.1. Cumulative hydrogen production in cultures grown with different substrates.

As depicted in Figure 3.1, in bottles containing glucose, as a mono-substrate or in combination with other substrates, an initial lag phase in H_2 production of approximately 13 hours was observed. After this phase, a rapid increase in H_2 production was observed followed by a stationary phase. A similar trend was observed in bottles containing starch-only and cellulose-only, but cultures with different substrates displayed lag phases of different durations. Cultures containing starch had a lag phase of approximately 28 hours, while cultures containing cellulose had a lag phase of up to 115 hours. Examining the curves for H_2 production of co-substrate experiments, two or three lag phases and exponential phases were observed, depending

on whether the cultures contained two or three substrates, and the growth phases observed were consistent with the phases observed in mono-substrate cultures. For example, consider the curves for cultures containing the co-substrates glucose, starch, and cellulose: an initial lag phase of ~12 hours was observed followed by an exponential increase in H_2 production. H_2 production plateaued at ~22 hours and then increased rapidly at ~30 hours. A third lag phase was observed at 40 hours and lasted till approximately 124 hours, after which H_2 production increased again for a brief time and then plateaued again at 132 hours.

This data suggest that different substrates, from simple to more complex carbohydrates, were consumed sequentially. Longer lag times for starch and cellulose could be attributed to lower degradability of starch and cellulose when compared to glucose, necessitating an additional hydrolysis step to release fermentable sugars [Masset et al., 2012]. Although, the substrates were consumed sequentially, co-substrate bottles showed enhancement in H_2 production. The observed utilization of these different substrates also suggests that the mixed consortia contained microbial strains which have the potential to degrade glucose, starch, and to some extent, cellulose.

Table 3.1. Synergistic effects of co-substrates. Volumetric hydrogen production (mL H_2/g substrate) calculated from cultures grown with co-substrates based on the hydrogen production from the individual mono-substrates substrates.

Substrate	Expected H_2	Measured H_2	% Difference
	mL/g substrate	mL/g substrate	
Glucose + Starch	157	200	27
Glucose + Cellulose	97	121	25
Starch + Cellulose	81	108	33
Glucose + Starch + Cellulose	112	137	23

Hydrogen yields from individual substrate: 172 mL/g glucose, 141 mL/g starch, 21 mL/g cellulose
[*]Expected H_2 (for glucose + starch) = (172 mL/g glucose) * 0.5 + (141 mL/g starch) * 0.5 = 157 mL/ g substrate

To study the synergistic effects of co-fermenting multiple substrates, specific H_2 production in mL/g substrate was measured from mono-substrate experiments and was then used to estimate the H_2 production in bottles where multiple substrates were used. Interestingly, as depicted in Table 3.1, the measured specific H_2 production when glucose and starch were co-fermented was 200 mL/g substrate which was 27% higher than the estimated H_2 production of 157 mL/g substrate confirming that co-substrate degradation enhanced the H_2 production. This could be attributed to the diversity in the microbial community present in the different substrate conditions which will be discussed in detail in the microbial community analyses section. The kinetics from the

Gompertz equation (Equation 2) for the different substrate conditions was calculated based on (Table 3.2):

$$P = P_{max}\exp\{-\exp\left[\frac{R_{max}e}{P_{max}}(\lambda - t) + 1\right]\} \qquad \text{(Equation 2)}$$

where P is the cumulative H_2 production, P_{max} is the maximum cumulative H_2 production, R_{max} is the maximum H_2 production rate, λ is the lag time, and t is the fermentation time. The coefficient of determination R^2 was 0.99 for all Gompertz data. Mono-substrate glucose, starch, and cellulose had lag phases of 13, 28, and 115 hours, respectively. Bottles containing glucose as a co-substrate had the same lag phase as observed in the glucose-only bottles, that is, 13 ± 2 hours. Bottles containing starch and cellulose as co-substrates had a lag phase similar to that of starch-only bottle, that is, 30 ± 1 hours. According to the Gompertz model, the maximum H_2 production rates for glucose, starch, and cellulose mono-substrate bottles were calculated as 26, 27, and 1 mL/hr, respectively. The H_2 production rate for co-substrates is not considered accurate because of multi-phased gas production.

Table 3.2. Gompertz analysis of hydrogen production from different substrate

Substrate	P	R_m	λ	R^2
	mL	mL/hr	hr	
Glucose	431	26	13	0.99
Starch	353	30	28	0.99
Cellulose	53	1	115	0.98
Glucose + Starch	499	23	15	0.99
Glucose + Cellulose	303	26	10	0.99
Starch + Cellulose	269.0	33.3	30	0.99
Glucose + Starch + Cellulose	343.0	14.3	16	0.99

P: maximum hydrogen production, R_m: maximum hydrogen production rate, λ: lag phase time

3.3.2 Hydrogen Yields

Figure 3.2 shows the hydrogen yields for different substrate conditions. Glucose, starch, and cellulose as mono-substrates resulted in H_2 yields of 1.22, 1.00, and 0.13 mol/mol hexose$_{added}$, respectively. Logan et al. [2002] conducted batch experiment at 26 °C with an initial pH of 6, using soils used for tomato plants as inoculum (32 g/L) and substrate (4 g COD/L), and achieved yields of 0.9, 0.59 and 0.003 mol/mol glucose, starch and cellulose added, respectively. The differences in H_2 yields between this study and the aforementioned Logan's study could be attributed to variation in the mixed culture inoculum and operational temperature. Lay et al. [2001] achieved a H_2 yield of 0.52 mol/mol hexose equivalent$_{added}$ at S/X of 8 g cellulose/g VSS[20]. Significantly higher H_2 yields of 1.7 mol H_2/ mol hexose$_{consumed}$ were reported

in a study by Ren et al. [2007], but this was a pure mesophilic cellulose-degrading bacterium, *Clostridium cellulolyticum*.
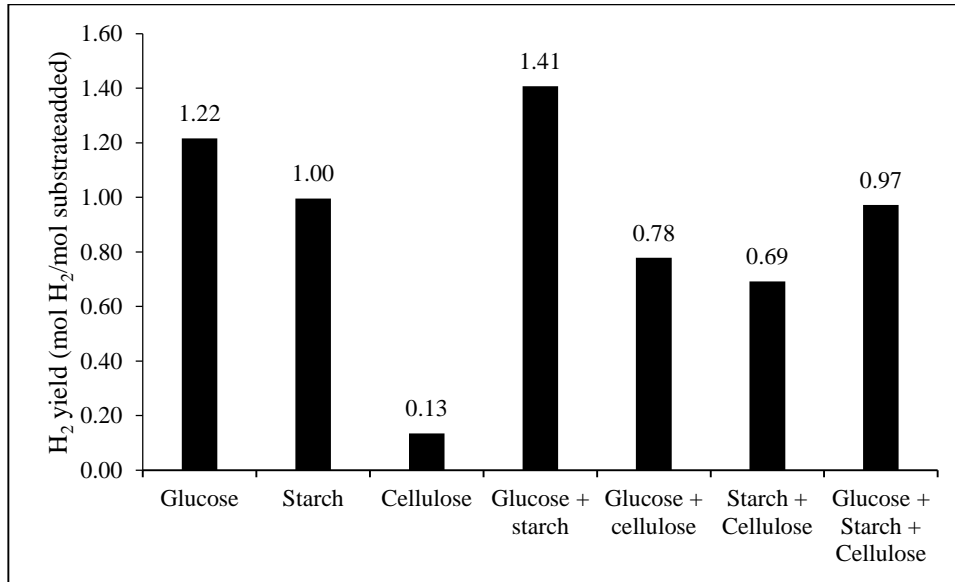
Figure 3.2. Hydrogen yield (mol H_2/mol hexose equiv.) for cultures grown with different substrates. Numbers above the bar graphs indicate the specific calculated yield.

On the other hand, when starch was co-fermented with glucose, a H_2 yield of 1.41 mol/mol was observed which was 27% more than the expected yield (1.11 mol/mol). Furthermore, co-fermentation of glucose-cellulose resulted in a H_2 yield of 0.78 mol/mol, which was 25% higher than the expected yield. Similarly, starch-cellulose co-fermentation resulted in a H_2 yield of 0.69 mol/mol, which was 33% higher than the expected yield. Xia et al. [2012] did a similar co-substrate study at thermophilic conditions with cellulose to co-substrate ratio used of 10:1 and achieved H_2 yields of 0.16 and 0.53 and 0.19 mol H_2/mol hexose$_{added}$ for cellulose-glucose,

cellulose-xylose and cellulose-starch, respectively. Glucose, starch, and cellulose co-substrate resulted in a H_2 yield of 0.97 mol/mol, which was 23% higher than the expected yield. This increase in H_2 yield in all the co-substrate bottles affirms that co-fermentation of different substrates improved the H_2 potential.

Based on the abovementioned results, it is clear that mesophilic cellulose fermentation was associated with low H_2 yields but the addition of glucose to cellulose and/or starch enhanced the fermentation process and thus increased the H_2 yield by at least 23%. Xia et al. [2012] reported maximum cellulose conversion rate and highest H_2 yields when using glucose and xylose as co-substrate, respectively. Interestingly, the H_2 yield was inversely proportional to H_2 production rate for the batches. A similar trend was noticed in another study by Chang et al. [2008] where for the highest H_2 production rate, the lowest H_2 yield was obtained and vice versa. This may be due to mass transfer limitations from the liquid to the biogas, thus increasing dissolved H_2 gas and retarding biohydrogen production processes. However, mass transfer coefficient calculations was beyond the scope of this study.

3.3.3 Volatile fatty acids

Figure 3.3 shows the VFA fractions at the end of the batch experiments for different substrate conditions based on COD. The error bars represent the standard deviation. It is noteworthy that the main VFAs detected in all batches were acetate, butyrate, and propionate. As shown in the Figure 3.3, in glucose–only and starch-only bottles, acetate and butyrate were the predominant fermentation products. In cellulose-

only bottles, propionate was the main product. Quéménur et al., [2011] reported different distribution of metabolic products depending on the substrates with no correlation between H_2 production and butyrate to total VFA (Bu/TVFA), as the butyrate concentrations remained essentially the same in all the different substrate conditions. In this study, for glucose and starch co-substrate bottles, it was observed that acetate was the dominant product, which was consistent with glucose and starch mono-substrate conditions. The theoretical H_2 yield from hexose with acetate formation is 4 mol H_2/mol hexose and 2 mol/ mol hexose for butyrate formation [Hawkes et al., 2002]:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{(Equation 3)}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + CO_2 + 2H_2 \qquad \text{(Equation 4)}$$

Glucose-starch co-substrate bottles had the highest acetate-butyrate ratio (Ac/Bu) while cellulose-only bottle and bottles containing cellulose as co-substrate had relatively lower Ac/Bu ratios. Therefore, the higher acetate to butyrate ratio in the fermentation products would translate to higher H_2 yields. It was also observed that the bottles containing cellulose had higher propionate concentrations when compared to bottles with no cellulose, which suggests that cellulose degradation favors the propionate pathway. Propionate formation pathway has been associated with H_2 consumption, which explains the low H_2 yield and production in cellulose-only bottles [Hawkes et al., 2002]:

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad \text{(Equation 5)}$$
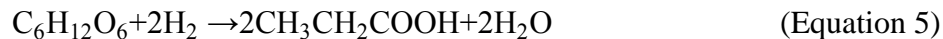
In the bottles containing all three substrates, acetate was the main product and propionate was relatively higher as well which could be due to the presence of cellulose. VFAs contributed on average 60% of the final soluble COD for all the substrate conditions except cellulose-only bottles where only 30% of the SCOD were VFAs. Furthermore, no residual glucose was detected at the end of the batches. This suggests that different intermediates were formed besides the detected VFAs. The microbial community analyses could give an insight on these intermediates formed based on the pathways the microbes take to utilize substrates. Table 3.3 shows the VFA concentrations at the end of the batch experiments for different substrate conditions. Theoretical H_2 production from VFAs produced was calculated based on 0.84 L H_2/ g acetate, 0.58 L H_2/g butyrate and 0.34 L H_2/g propionate (Equations 3, 4, and 5). The theoretical values shown in Table 3.3 were consistent with the H_2 measured during the experiment with a percent difference of 4%. The H_2 yield and the VFAs data support that co-substrate degradation enhanced the H_2 production. Addition of glucose to starch and/or cellulose increased the H_2 yield by favoring the acetate pathway. The CODs mass balances were calculated based on initial and final TCOD as well as the equivalent COD for the H_2 produced (8 g COD/g H_2). The COD mass balance closure of 93±4% verify data reliability.
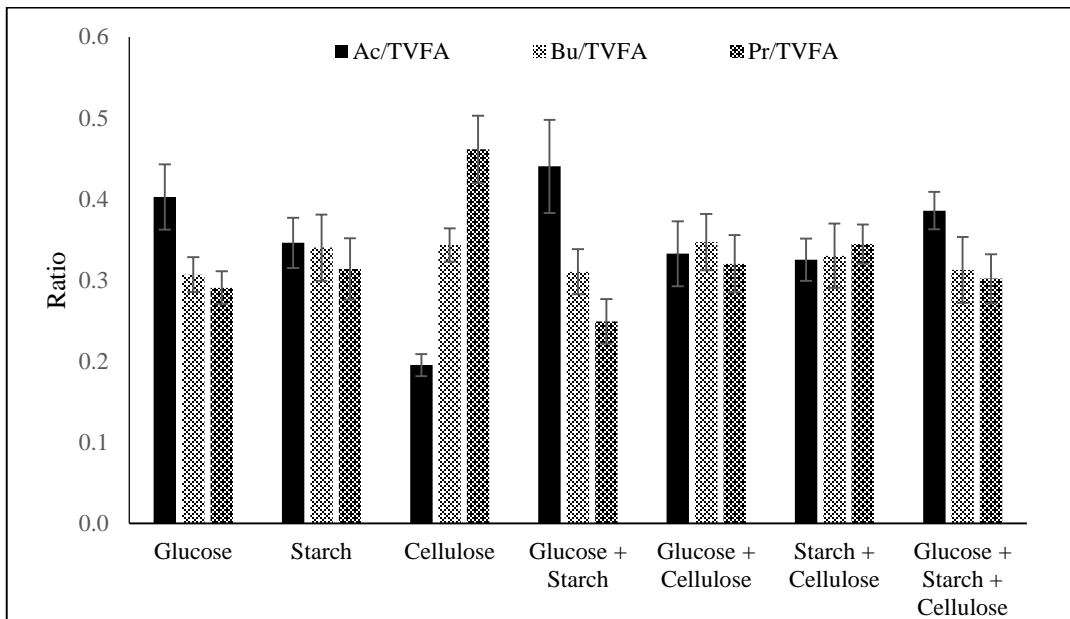
Figure 3.3. VFAs ratios at the fermentation end-point (187 hours post-inoculation) of cultures grown on different substrates.

Table 3.3. Theoretical hydrogen production based on the acetate, butyrate and propionate produced.

Substrate	Acetic acid	Butyric acid	Propionic acid	Theoretical H$_2$				Measured H$_2$	% difference
				From Acetic acid	From Butyric acid	From Propionic acid	Total		
	mg/L	mg/L	mg/L	mL	mL	mL	mL	mL	%
Glucose	2712 ± 271	1215 ± 85	1387 ± 97	412	126	85	452	431	5
Starch	2163 ± 195	1250 ± 150	1391 ± 167	329	129	86	372	353	5
Cellulose	359 ± 25	371 ± 22	601 ± 54	55	38	37	56	53	6
Glucose + Starch	2996 ± 389	1242 ± 112	1202 ± 132	455	129	74	510	499	2
Glucose + Cellulose	1801 ± 216	1105 ± 111	1229 ± 135	274	114	76	312	303	3
Starch + Cellulose	1673 ± 134	998 ± 120	1256 ± 88	254	103	77	280	269	4
Glucose + Starch + Cellulose	2098 ± 126	999 ± 130	1163 ± 116	319	103	72	351	343	2

3.3.4 Microbial community analyses

The microbial communities present in the ADS produced H_2 by digesting complex co-substrates in the serum bottles. Figure 3.4 shows amplification of the 16S rDNA V4 region using the 515 F and 806 R primers, as demonstrated by the presence of the PCR products of the expected size (300-350 bp). A total of 1,579,849 16S rDNA sequences were generated from the overall 48 samples. The sequences, which share at least 97% sequence similarity to current nucleotide database of the National Centre for Biotechnology Information using the BLAST algorithm [Drancourt et al., 2004], resulted in a large number of operational taxonomic units (OTUs) per sample, and thus revealed microbial communities with a wide range of species richness.

OTUs within 11 genera and 4 families were identified in samples of ADS cultured with mono-substrates. OTUs within 14 genera, 1 order, and 1 phylum were identified in samples of ADS cultured with di-substrates, and four of these OTUs (1 phylum, 1 order, and 2 genera) were unique to the di-substrate samples. OTUs within 12 genera, 5 families, 1 order, and 1 phylum were identified in samples of ADS cultured with tri-substrates. The taxonomic diversity in the microbial communities was identified using the QIIME software that creates rarefaction curves between the average numbers of sequence per treatment vs. rarefaction measures [Caporaso et al., 2010]. The greatest taxonomic diversity was observed in mono-substrate glucose and the seed control. In contrast, the lowest taxonomic diversity was detected in the microbial community grown on cellulose-only. Glucose-starch co-substrate showed

greater diversity than starch-alone. The OTUs of co-substrates glucose-cellulose; and

starch-cellulose were not significantly different from each other. However, the OTU
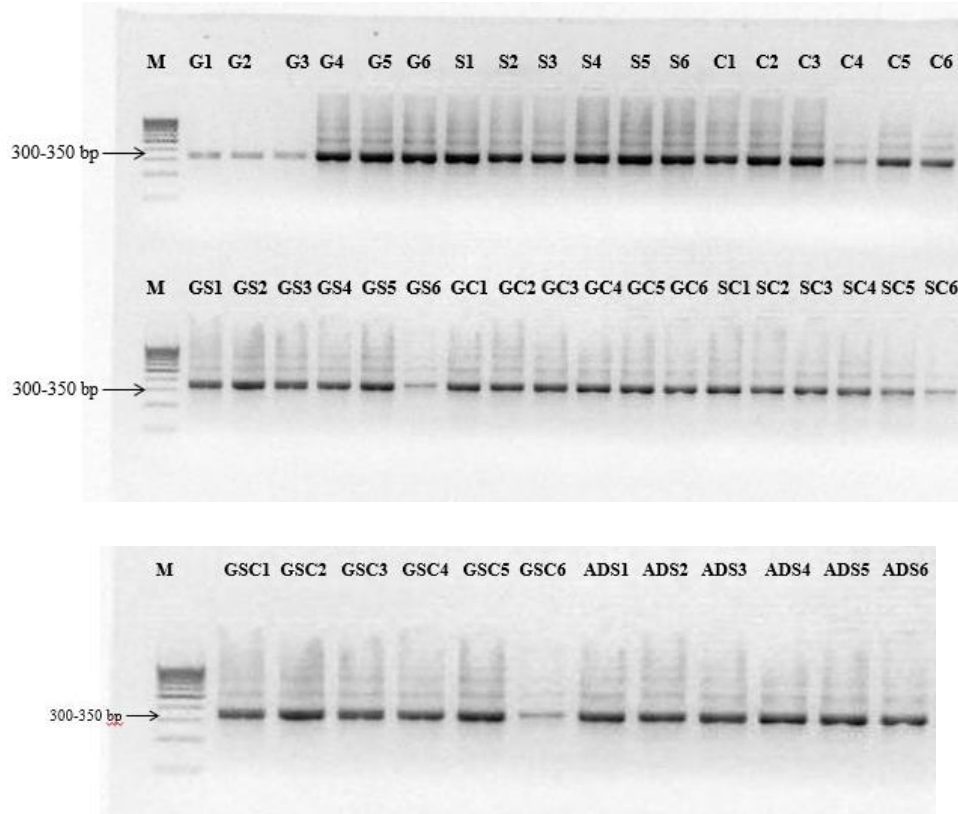
Figure 3.4. PCR products generated by PCR amplification of16S rRNA genes from

DNA extracted from cultures grown with different substrates. G: Glucose; S: Starch;

C: Cellulose; GS:Glucose-Starch; GC:Glucose-Cellulose; SC:Starch-Cellulose;

GSC:Glucose-Starch-Cellulose; ADS:ADS control. Numbers 1 to 6 indicate 6

replicates.

composition of co-substrate containing glucose-starch-cellulose had greater values

than those of cellulose-only, glucose-cellulose; and starch-cellulose. These rarefaction

curves revealed that glucose-alone supported the growth of more diverse microbial

consortia than co-substrates. Xia et al. [2012] observed the identical trend with highest diversity in seed control and bottle supplemented with glucose co-substrate with cellulose.

Figure 3.5 illustrates the unweighted UniFrac and Principal Co-ordinate analysis (PCoA) technique which identified relationships between the overall microbial compositions in bottle with different substrate (mono- or co-substrate). The PCoA helped to clearly define the species similarity and diversity among different bottles. Axis 1 of the PCoA plot explained 15.1% of the variation, while axis 2 explained 7.1% of the variation between the different bathes. The visual representation implies that the different bottles with common substrate composition shared OTU diversity, and clustered together. Glucose-only and the seed control had a large number of common OTUs. Glucose-starch co-substrate and starch-only manifested close correlation with each other due to presence of the common substrate, starch, in both. Similarly, cellulose-only and co-substrate starch and cellulose contained significant species-similarity. Co-substrate glucose-starch-cellulose and co-substrate glucose-cellulose were similar and clustered closely. The PCoA analysis indicated that the separation and similarity of bacterial communities is associated with the combination of co-substrates in the serum bottle reactors. Although the greatest taxonomic diversity was observed in glucose batches, it was also evident that at higher taxonomic levels co-substrates support similar species diversity as the related mono-substrates.

Figure 3.6a shows that the observed species number followed a similar trend as H₂ yield with respect to different substrate conditions. A linear relationship was observed between the number of observed species and H₂ yield, that is, the increase in H₂ yield is associated with increased number of observed species (Figure 3.6b). It must
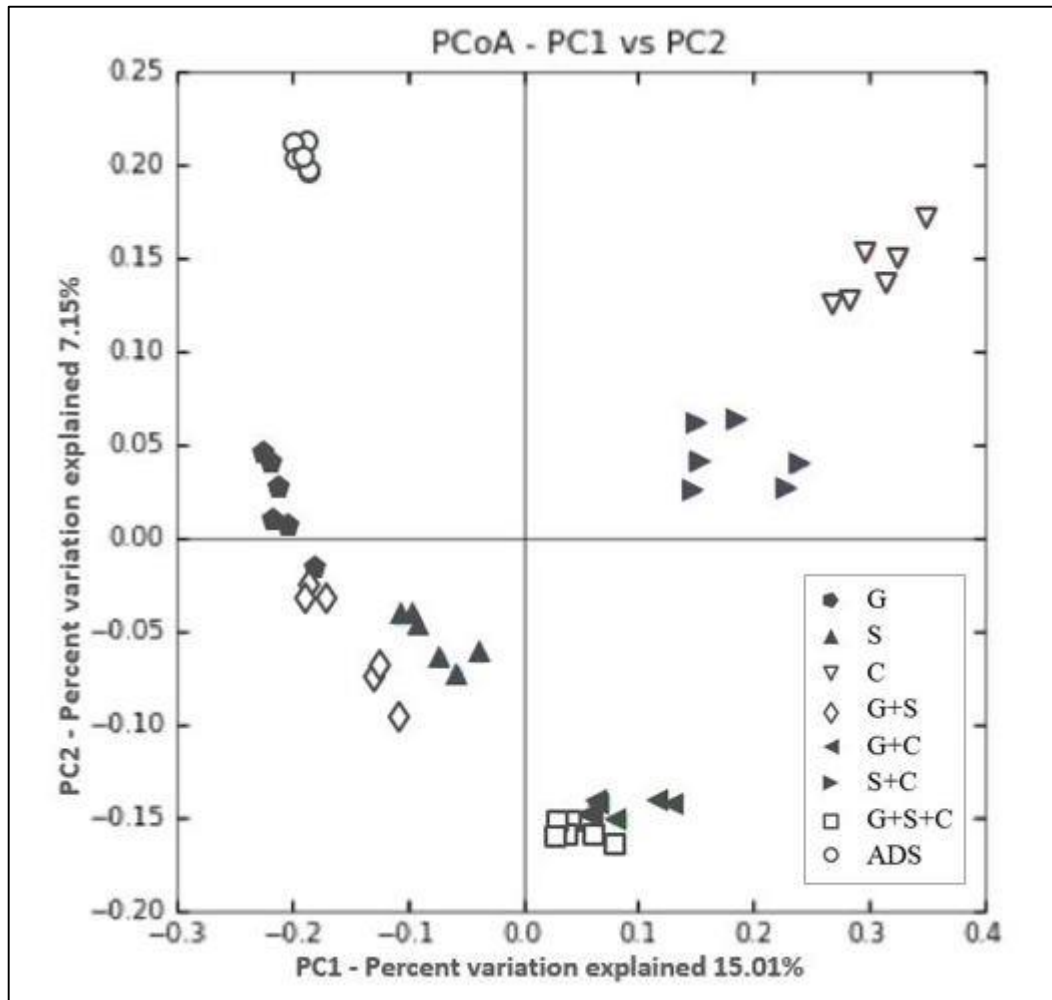
Figure 3.5. Principle co-ordinate analysis of unweighted UniFrac distances

be asserted that to the best of the authors knowledge, never before has microbial diversity been correlated statistically with a bioreactor performance measure.
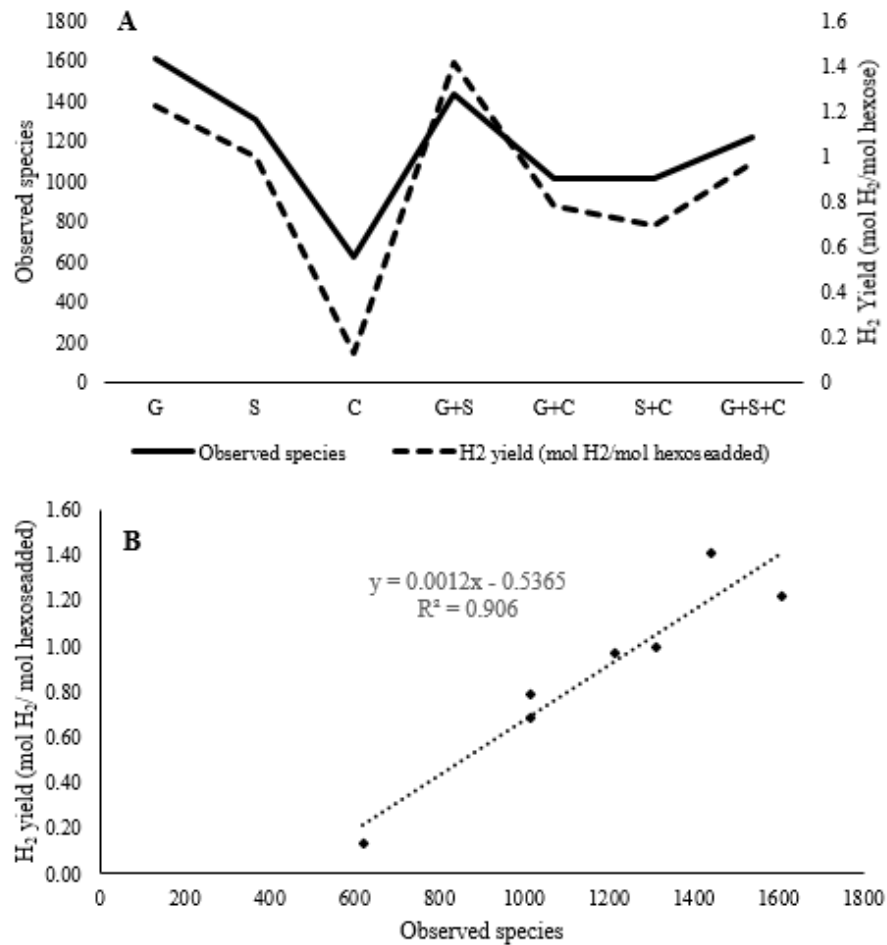
Figure 3.6. A) Trend of observed species and H_2 yield; **B)** Relationship between observed species and H_2 yield

OTUs in the Phyla *Bacteroides, Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes, Synergistes* and *Thermotogae* were common in mono- and co-substrate bottles, in agreement with the study by Xia et al. [2012] which analyzed thermophilic H_2 production using anaerobic digester sludge. However, OTUs in the Phyla *Acidobacteria, Actinobacteria,* and *Bacteroidetesee* were unique to only the co-substrate conditions, and were absent in mono-substrate conditions. Table 3.4 gives a

breakdown of the taxa that were enriched relative to the seed control to give a distribution of the microbial communities' in different substrate conditions.

Table 3.4. OTU enrichment in cultures grown with different substrates relative to seed control

OTU	G	S	C	G+S	G+C	S+C	G+S+C
	Enrichment/Seed control						
Clostridia *(c)*			100			23	10
Clostridiaceae *(f)*	13	6				10	7
Clostridium (g)	53	20		51	10		9
Ruminococcaceae *(f)*	18			8	10	10	16
Ruminococcus (g)		46			37	24	42
Ethanoligenens (g)	1830			638			
Streptococcus (g)		6032					
Lachnospiraceae *(f)*			2175		281	827	227
Bacteroides (g)		314	595		728	671	555
Parabacteroides (g)		228	342		490	546	665
Oscillospira (g)				51	128		118
Bifidobacterium (g)				4666			
Desulfovibrio (g)					170	111	

OTUs in the genus *Clostridium* (Family Clostridiaceae) showed increases of 53- and 20-fold, in mono-substrate glucose and starch bottles, compared to the ADS seed control. Glucose-starch cultures displayed a 51-fold increase in *Clostridium* species (sp.)*,* while glucose-cellulose and glucose-starch-cellulose had 10 and 9-fold increases in *Clostridium* sp., respectively. OTUs in the Family *Clostridiaceae* were also enriched in glucose, starch, glucose-starch, glucose-cellulose, and glucose-starch-cellulose cultures. *Clostridium* sp. have been well established as a H_2 producers, and these bacteria are known to produce the highest H_2 yields [Hawkes et al., 2002]. Fang et al. [2002] reported that, the majority of the species identified in a mesophilic, H_2-

producing sludge were *Clostridium* sp., and these bacteria have been studied for H_2 production with a variety of substrates and feedstocks.

OTUs in the genus *Ethanoligenens* were observed to increase by 1830 in mono-substrate cultures containing glucose and by 638-fold co-substrate cultures containing glucose and starch. However, OTUs in the genus *Ethanoligenens* were not observed in other cultures. *Ethanoligenes* sp. are a dominant H_2 producing bacteria with strong viability and competitive abilities in microbial communities under non-sterile conditions [Xing et al., 2008]. Xing et al. [2008] observed high H_2 production rates and greater pH tolerance by *Ethanoligenens* sp. using glucose as substrate at mesophilic conditions. *Ethanoligenens* sp. are also known to produce acetate and ethanol as end-products [Azbar and Levin, 2012]. It is well established that acetate pathway is associated with increased molar yield of H_2 [Azbar and Levin, 2012]. The presence of this strain explains maximum H_2 yields and production of acetate as an end-product in cultures containing glucose-only or in cultures containing glucose-starch co-substrates. Ethanol could be one of the intermediates formed contributing to the remaining 40% of the final SCOD.

OTUs in the Family *Ruminococcaceae* were also commonly observed in glucose-only cultures and all cultures containing glucose-co-substrates, and showed considerable fold-enrichment suggesting that OTUs in the *Ruminococcaceae* have the capacity to thrive under different substrate conditions. OTUs in the genus *Ruminococcus*, however, were observed in high numbers in starch-only cultures, as well as in cultures containing di-substrates (glucose-cellulose and starch-cellulose) and

tri-substrates (glucose-starch-cellulose). *Ruminococcus* sp. are well known as obligate H_2 producing bacteria found in the rumen of cattle [Ho et al., 2011]. They are known to produce extracellular hydrolytic enzymes that can break down cellulose and hemicelluloses, and can ferment both hexose and pentose sugars [Ntaikou et al. 2008]. In a study by Ntaikou et al. [2008], *Ruminococcus albus* was enriched successfully on glucose, cellobiose, xylose, and arabinose, which are the main products of cellulose and hemicellulose hydrolysis, with H_2, acetic acid, formic acid, and ethanol as the main fermentation end-products. Additionally, it was reported that formate produced from glucose consumption was further converted to H_2 and CO_2 (Equation 6) by the enzyme H_2 formate lyase:

$$HCOOH \rightarrow CO_2 + H_2 \hspace{3cm} \text{(Equation 6)}$$

The presence of this species in glucose, starch and, co-substrate cultures strongly suggests that it enhanced the hydrolysis of the complex starch and cellulose and later utilized the soluble end-products for H_2 production.

Enrichment of OTUs in the Phylum *Lachnospiraceae* was common in cultures containing cellulose, either as a mono- or co-substrate. There was a 2175 fold enrichment of *Lachnospiraceae* sp. in cellulose-only cultures, and 281, 827, and 227 fold-increases in glucose-cellulose, starch-cellulose, and glucose-starch-cellulose cultures, respectively. Significant enrichment in OTUs from the Class *Clostridia* were also detected in cellulose-only, starch-cellulose, and glucose-starch-cellulose cultures. Both *Clostridia* and *Lachnospiraceae* belong to the Phylum *Firmicutes*, which are

known to be H_2 producing microbes [Azbar and Levin, 2012]. Nissilä et al. [2011] identified *Clostridia* and *Lachnospiraceae* as thermophilic, cellulolytic, H_2-producing microorganisms enriched from rumen fluid. The presence of these bacterial strains in both studies could be attributed to the presence of cellulose as a substrate.

OTUs in the genus *Bifidobacterium* belongs to the Phylum *Actinobacteria*. *Bifidobacterium* sp. displayed a 4666-fold enrichment in glucose-starch cultures. Cheng et al. [2008] identified *Bifidobacterium* sp. in a starch-fed, dark fermentation reactor and suggested that *Bifidobacterium* sp. could hydrolyze starch into di-saccharides (maltose) or monosaccharides (glucose), which were then consumed by *Clostridium* species for H_2 production. This signifies the synergistic effect of this culture with other microbial cultures present. Chouari et al. [2005] investigated bacterial contribution in the total microbial community in anaerobic digester sludge and found that 27.7 % of the OTU distribution belonged to *Actinobacteria* and *Firmicutes* which represented the most abundant Phyla. OTU in the genus *Streptococcus* displayed a 6032- fold enrichment in starch-only cultures. *Streptococcus* sp. are facultative anaerobes, H_2 producers, and have been characterized by their diverse metabolic activity [Badiei et al., 2012]. *Streptococcus* sp. have been observed in a number of H_2 production studies [Badiei et al., 2012; Cheng et al., 2008; Song et al., 2012]. Song et al. [2012] proposed that the mutualism and symbiosis relations of *Streptococcus* and other mixed bacteria were of vital importance for fermentative H_2 production.

OTUs in the genera *Bacteroides* and *Parabacteroides* showed significant fold-enrichments in starch-only and cellulose-only cultures, as well as in glucose-cellulose, starch-cellulose, and glucose-starch-cellulose cultures. *Bacteroides* and *Parabacteroides* are important H_2-producers and both belong to the Phylum *Bacteroidetes*, which is one of the most abundant Phyla found in ADS [Chouari et al., 2005]. *Bacteroides* sp. have been identified in microbial electrolysis cells (MEC) as efficient Fe(III)-reducing fermentative bacteria, as well as biohydrogen producers in cultures containing cellulosic feedstocks [Ho et al., 2012; Wang et al., 2012]. Increases in their populations suggest they are capable of utilizing complex carbohydrates in varying substrate conditions.

OTUs in the genus *Desufovibrio* were enriched in glucose-cellulose and starch-cellulose cultures. *Desufovibrio* sp. are sulfate-reducing bacteria (SRB) that are metabolically versatile in nature and can exist in low sulfate concentration environments where they grow fermentatively and produce H_2, CO_2, and acetate in syntropy with other organisms [Plugge et al., 2011]. In a recent study by Martins et al. [2013], it was reported that *Desulfovibrio* sp. have extremely high hydrogenase activity, and *Desulfovibrio vulgaris* was shown to produce H_2 from lactate, ethanol, and/or formate [Martins et al., 2013]. Increased H_2 yields in the co-substrate cultures could be attributed to the presence of these species, as they have the potential to synthesize H_2 from fermentation end-products such as formate, ethanol and lactate.

From the extensive microbial characterization conducted in this study, it is evident that microbial diversity correlates well with H_2 yield. Furthermore, synergies

between various microbial communities appear to enhance biohydrogen yield, despite the reduction in maximum biohydrogen production rate.

3.4 Conclusions

It can be concluded from this study that there were synergistic effects of co-fermentation of glucose, starch, and cellulose using ADS. The following conclusions can be drawn:

- Glucose addition to starch and/or cellulose favored the acetate pathway. Cellulose degradation was associated with the propionate synthesis pathway.

- Co-fermentation improved the H_2 potential and the yields were greater by an average of $27 \pm 4\%$ than expected.

- OTUs in the Phyla *Bacteroides, Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes, Synergistes* and *Thermotogae* were common in mono- and co-substrate bottles, and OTUs in the Phyla *Acidobacteria, Actinobacteria,* and *Bacteroidetesee* were unique to only the co-substrate conditions.

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Chapter 4

Sensitivity of Mesophilic Biohydrogen-Producing Cultures to Temperature

Shocks[2]

4.1 Introduction

Dark fermentative hydrogen production is light independent and can utilize complex carbohydrate-rich substrates [Puhakka et al., 2012; Hawkes et al., 2002]. Several environmental parameters control the hydrogen potential including pH, substrate, nutrients, inoculum, and temperature [Puhakka et al., 2012]. Of all the aforementioned parameters, temperature is the most important factor as it influences the activity of the hydrogen producers and the mechanism of hydrogen production [Wang et al., 2009].

The optimal temperature for hydrogen production has not been established and contentious results have been reported in the literature. Table 4.1 summarizes literature reports that studied mesophilic and thermophilic temperature conditions. Lee et al. [2008] examined mesophilic (37 ℃) and thermophilic (55 ℃) temperatures using starch as substrate (16 gCOD/L), pH of 8.5, municipal sewage sludge as inoculum, and observed a higher yield at 37℃ than at 55℃ (0.96 vs. 0.26 mol H_2/mol hexose). Zhang et al. [2003] examined starch as substrate at pH 7 using hydrogen-producing sludge from a completely stirred fermenter treating sucrose wastewater (operated at 37℃ and pH 5.5) and obtained a yield of 0.55 mol H_2/mol hexose at thermophilic temperature

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[2] This chapter is under review for publication in International Journal of Hydrogen Energy.

(55ºC) and 0.33 mol H_2/ mol hexose at 37ºC. Puhakka et al. [2012] studied a comparison between mesophilic (37 ºC) and thermophilic (55 ºC) temperatures using intermediate temperature (45 ºC) hot spring sample as inoculum and glucose as substrate, and obtained yield of 1.25 and 1.0 mol H_2/mol hexose for mesophilic and thermophilic conditions, respectively. Kim and Kim [2012] used mesophilic anaerobic digester sludge acclimatized with glucose at 60ºC, starch as substrate, and observed a H_2 yield of 1.78 mol H_2/mol hexose at 60ºC. Kargi et al. [2012] used acid hydrolyzed cheese whey starch powder as substrate at pH 7, and mesophilic anaerobic sludge as inoculum, acclimatized at 55 ºC for thermophilic batches, and observed H_2 yields of 0.47 and 0.81 mol H_2/mol hexose at mesophilic (35 ºC) and thermophilic (55 ºC) temperatures, respectively [Kargi et al., 2012].

The majority of research in bio-hydrogen production has been focused on single substrates with the exception of few co-fermentation studies. Role of glucose-xylose combination was studied by Prakasham et al. [2009] and an increase of 23% and 9% in the H_2 production was observed when compared to glucose-only and xylose-only, respectively. Xia et al. [2012] studied co-fermentation of microcrystalline cellulose with glucose, starch, and xylose for biohydrogen production at pH 6.6 using anaerobic digester sludge acclimatized to 55ºC. Cellulose-only yielded 0.03 mol/mol hexose, whereas, yields of 0.16, 0.19, and 0.53 mol H_2/mol hexose was observed for cellulose-glucose, cellulose-starch and, cellulose-xylose, respectively. Ren et al. [2008] studied thermophilic hydrogen production from xylose-glucose mixture using *Thermoanaerobacterium thermosaccharolyticum* W16 strain and observed hydrogen

yields of the mixture (2.37 mol H_2/mol substrate) to be not significantly different from the mono-substrate conditions (glucose, 2.42 mol H_2/mol substrate; xylose, 2.19 mol H_2/mol substrate).

Starch and cellulose are the major components in many agricultural and food-industry wastes and wastewaters [O-Thong et al., 2011]. The initial hydrolysis is known to be the rate-limiting step in anaerobic fermentation of complex carbohydrates. Thermophilic fermentation processes have demonstrated to enhance degradation kinetics, and production rates as well as destruction of pathogens [Shin et al., 2004; Cheong and Hansen, 2007; O-Thong et al., 2011]. Most of the literature studies have used thermophilic sludge for thermophilic hydrogen production or mesophilic sludge acclimatized to thermophilic temperatures. Mesophilic temperature range lies between 35 ℃ -37 ℃ and thermophilic range between 55 ℃ -70 ℃. In real-life applications, temperature shocks, which deleteriously impact microbial cultures, can occur in spite of temperature controlled systems. Mesophilic digester are more widely used and can undergo temperature shocks due to varying feedstock, feedstock strength, auto-thermal reactions, etc. Thus the aforementioned studies using thermophilic and/or acclimatized mesophilic cultures do not reflect real-life conditions. Thus, in light of the limited comparative co-fermentation studies, the main objectives of this study are to assess the response of unacclimatized bio-hydrogen producers to thermophilic conditions, as well as to compare mesophilic and thermophilic co-fermentation of starch and cellulose. In this study, starch and cellulose were used as mono-substrate and in combination as co-substrates (1:1 ratio) to make a

comparative assessment between mesophilic (37ºC) and thermophilic (60ºC) biohydrogen production using anaerobic digester sludge acquired from a mesophilic digester.

Table 4.1. Mesophilic and thermophilic studies in the literature

Inoculum	Substrate	pH	Reactor	Temp. (ºC)	H$_2$ yield	Reference
Municipal sewage sludge	Starch (16 gCOD/L)	8.5	Batch	37	0.96	Lee et al. 2008
				55	0.28	
Mesophilic sucrose fed wastewater	Starch (4.6 g/L)	7	Batch	37	0.33	Zhang et al. 2003
				55	0.55	
Sediment from hot (45ºC) spring	Glucose (9 g/L)	6.5	Batch	37	1.25	Puhakka et al. 2012
		7.5		55	1.00	
Mesophilic anaerobic digester sludge acclimatized with glucose (10 gCOD/L), pH 5.5 at 60ºC	Starch (3 gCOD/L)	6.8	Batch	60	1.78	Kim and Kim 2012
	Glucose (3 gCOD/L)				2.69	
Mesophilic anaerobic digester sludge				37	1.60	
Mesophilic anaerobic digester sludge acclimatized with glucose at 55ºC	Ground wheat starch acid-hydrolyzed (18 g/L)	7	Batch	55	2.40	Cakir et al. 2010
Thermophilic waste activated sludge		4.9			2.32	
Thermophilic digested cattle manure		5.4			1.71	
Compost of night solid and organic fractural municipal solid waste	Starch (10 g/L)	5.3	CSTR HRT 24 hr	55	2.13	Akutsu et al. 2008
Thermophilic acidified potato		4.9			2.02	
Thermophilic-digested night soil and organic fractural municipal solid waste		5.4			1.38	

Inoculum	Substrate	pH	Reactor	Temp. (°C)	H_2 yield	Reference
Sludge compost acclimatized	Cellulose powder (5 g/L)	6.6 / 6.4	Batch / Chemostat HRT 3 day	60	2.00 / 2.00	Ueno et al. 2001
Co-culture *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum*	Micro-crystalline cellulose (5 g/L)	6.8	Batch	60	1.80	Liu et al. 2008
Rumen fluid acclimatized	Cellulose (5 g/L)	7	Batch	60	0.32	Nissila et l. 2011
Anaerobic digester sludge acclimatized using microcrystalline cellulose and glucose (10:1) for 12 d at 55ºC	Micro-crystalline cellulose (4 g/L)	6.6	Sequential batch	55	0.03	Xia et al. 2012
	Microcrystalline cellulose (4 g/L) + Glucose (0.4 g/L)				0.16	
	Microcrystalline cellulose (4 g/l) + Starch (0.4 g/L)				0.19	
	Microcrystalline cellulose (4 g/l) + Xylose (0.4 g/L)				0.53	
Mesophilic anaerobic sludge	Cheese whey starch powder acid hydrolyzed (10.77 g/L)	7	Batch	35	0.47	Kargi et al. 2012
Anaerobic sludge acclimatized at 55ºC				55	0.81	

Inoculum	Substrate	pH	Reactor	Temp. (ºC)	H_2 yield	Reference
Sediment sample from geothermal spring 60ºC	Cassava starch (5 g/L)	5.5	Batch	60	0.90	O-Thong et al. 2011
	Cassava starch (5 g/L)		Repeated batch		1.68	
	Cassava starch processing wastewater (9.2 g/L)		CSTR-Fed-batch-5d HRT		2.04	

4.2 Materials and Methods

4.2.1 Seed sludge and substrate

Mesophilic anaerobically digested sludge (ADS) was collected from the St. Marys wastewater treatment plant (St. Marys, Ontario, Canada) and used as seed for the experiment. The total suspended solids (TSS) and volatile suspended solids (VSS) of the ADS were 18 and 13 g/L, respectively for the mesophilic experiment and 20 and 14 g/L for thermophilic experiment. The ADS was pretreated at 70°C for 30 minutes to inhibit methanogens [Nasr et al., 2011]. Starch and cellulose were added at 2.7 gCOD, individually as mono-substrates, and in combinations as co-substrates for the mesophilic experiment. For the thermophilic experiment starch and α-cellulose were added at 2.8 gCOD. Sufficient inorganics and trace minerals were added to the media [Hafez et al., 2010]. NaHCO$_3$ was used as buffer at 5 g/L.

4.2.2 Experimental design

Batch studies were conducted in serum bottles with a working volume of 200 mL. Experiments were conducted in triplicates for an initial substrate-to-biomass (S/X) ratio of 4 gCOD$_{substrate}$/g VSS$_{seed}$. The volume of seed added to each bottle was 50 mL. The TCOD$_{substrate}$ (g/L) to be added to each bottle was calculated based on Equation 1:

$$S/X(\text{ g COD/g VSS}) = \frac{V_f(L) * \text{Substrate TCOD}(\frac{g}{L})}{V_s(L) * \text{Seed VSS }(\frac{g}{L})} \qquad \text{(Equation 1)}$$

Where V_f is the volume of feed and V_s is the volume of seed. 50 mL of seed was added to each bottle and TCOD of substrate to be added was calculated to be 2.7 and 2.8

gCOD for mesophilic and thermophilic experiments, respectively. The initial pH for each bottle was adjusted to 5.5 using HCl. NaHCO$_3$ was added at 5 g/L for pH control. Ten-mL samples from each bottle were collected initially. The headspace was flushed with nitrogen gas for a period of 2 minutes and capped tightly with rubber stoppers. The bottles were then placed in a swirling-action shaker (Max Q4000, incubated and refrigerated shaker, Thermo Scientific, CA) operating at 180 RPM and maintained temperature of 37 and 60°C for mesophilic and thermophilic experiments, respectively. Three control bottles were prepared using ADS without any substrate. Final samples were taken at the end of the batch.

4.2.3 Analytical methods

The biogas production was measured using glass syringes in the range of 5-100 mL. The gas in the headspace of the serum bottles was released to equilibrate with the ambient pressure [Nasr et al., 2011]. The biogas composition including hydrogen, methane, and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with thermal conductivity detector (TCD) and a molecular sieve column (Mole sieve 5A, mesh 80/100, 6 ft x 1/8 in). Argon was used as the carrier gas at a flow rate of 30 mL/min and the temperature of the column and the TCD detector were 90°C and 105°C, respectively. Volatile fatty acids (VFAs) were analyzed using a gas chromatograph (Varian 8500, Varian Inc., Toronto, Canada) with a flame ionization detector (FID) equipped with a fused silica column (30m x 0.32 mm). Helium was used as carrier gas at a flow rate of 5 mL/min. The temperatures of column were 110 and 250°C, respectively [Hafez et al., 2010]. Total and soluble

chemical oxygen demand (TCOD/ SCOD) were measured using HACH methods and test kits (HACH Odyssey DR/2500 spectrophotometer manual) [Nasr et al., 2011]. TSS and VSS were analyzed using standard methods [Clesceri et al., 1998].

4.3 Results and Discussion

4.3.1 Biohydrogen production

Preheated mesophilic anaerobic digester sludge was tested for biohydrogen production under mesophilic and thermophilic temperatures without acclimatization, where starch and cellulose were added individually and in combination in equal ratios. The initial substrate to biomass ratio was 4 gCOD/ gVSS. The overall substrate concentration in all bottles was maintained at 13.5 and 14 g/L for mesophilic and thermophilic experiments, respectively. Figure 4.1 shows the cumulative H_2 production for the different substrates and temperature conditions. The highest cumulative H_2 production after 337 hours was observed for thermophilic starch-only (415 mL), followed by mesophilic starch-only (353 mL). Co-substrate starch-cellulose gave 224 mL thermophilically and 269 mL mesophilically. Lastly, thermophilic cellulose-only gave 167 mL and mesophilic gave a minimal of 53 mL. It is evident that mesophilic ADS responded well to the temperature increase as reflected by the good thermophilic H_2 production from complex starch and cellulose. The error bars are shown to present the reproducibility of the experimental results and are based on the standard deviation.
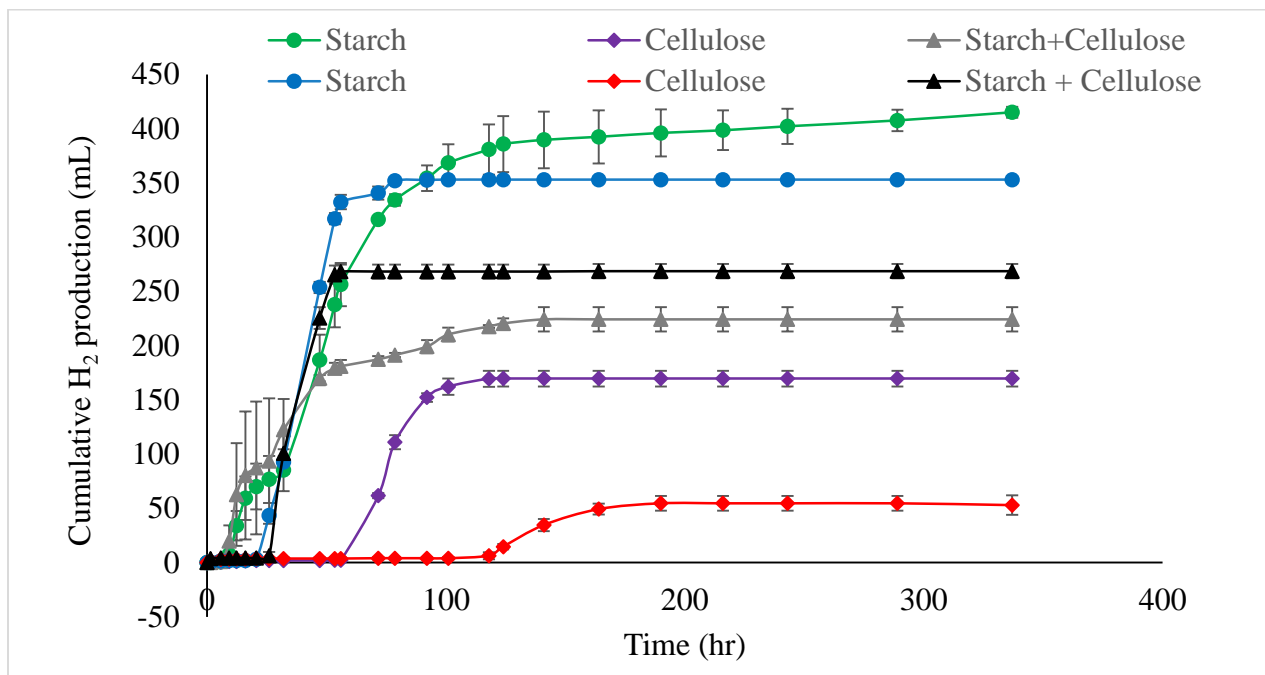
Figure 4.1. Cumulative hydrogen production. Solid symbols are thermophilic and

hollow symbols are mesophilic

It can also be observed that the lag phase for thermophilic starch and starch-cellulose biodegradation was less than 10 hours as compared with 26 hours at mesophilic conditions. Cellulose batches under both thermophilic and mesophilic conditions exhibited longer lag phases of 72 and 120 hour, respectively. It can be inferred from the above observations that thermophilic temperature shortened the lag phase for both starch and cellulose, although cellulose required more acclimatization time than starch. This observation of decrease in lag time for thermophilic conditions was in contrast to what has been reported in the literature [Shin et al., 2004; Cakir et al., 2010; Zhang et al., 2003; Lee et al., 2008]. It is also interesting to note that most of the studies in literature (Table 4.1) performed the experiments at around neutral pH as

opposed to this study. Relatively higher yields observed in this study using unacclimatized seed sludge suggests that pH is an important parameter to consider while designing experiments. It has been reported that the initial pH is an important factor in H_2 production which affects the duration of lag phase [Puhakka et al., 2012]. For mono-substrate starch and cellulose, higher H_2 production was obtained under thermophilic conditions, but in the case of co-substrate starch-cellulose, mesophilic performed better than thermophilic.

4.3.2 Hydrogen Yields

Table 4.2 shows the H_2 yields for the different substrates and temperature conditions.

Table 4.2. H_2 yields

	Substrate	Cumulative H_2 (mL)	Hydrogen Yield mol H_2/mol hexose$_{added}$
Mesophilic	Starch	353	1.00 ± 0.01
	Cellulose	53	0.13 ± 0.02
	Starch + Cellulose	269	0.69 ± 0.02
Thermophilic	Starch	415	1.13 ± 0.01
	Cellulose	170	0.42 ± 0.02
	Starch + Cellulose	224	0.58 ± 0.03

The maximum H_2 yield of 1.13 mol H_2/mol hexose$_{added}$ was observed for starch only at 60ºC whereas, the mesophilic yield was 1 mol H_2/mol hexose$_{added}$. The

thermophilic cellulose-only yield of 0.42 mol H_2/mol hexose$_{added}$ was almost 3 times the mesophilic yield (0.13 mol H_2/mol hexose$_{added}$). Starch-cellulose combination gave yields of 0.58 and 0.69 mol H_2/mol hexose$_{added}$ for thermophilic and mesophilic, respectively. Co-fermentation of starch-cellulose at thermophilic temperature did not show any enhancement in yield, however mesophilic co-fermentation increased the yield by 26% with respect to the estimated mono-substrate yields. Xia et al. [2012] conducted a study using thermophilic anaerobic digester sludge with microcrystalline cellulose as substrate and in combination with starch with a ratio of 10:1, and achieved H_2 yields of 0.19 mol H_2/mol hexose$_{added}$. The above mentioned study considered starch to compete with cellulose as the substrate for the microbial community and observed the lowest cellulose conversion with no improvement in the overall yield of co-fermentation. The authors' hypothesized that all the H_2 production occurred only due to starch consumption and no cellulose was utilized, and starch is not a suitable co-substrate for cellulose digestion at thermophilic conditions. This could explain the relatively lower yield in the thermophilic co-fermentation of starch-cellulose as compared with mesophilic conditions observed in our study. Lee et al. [2008] conducted an experiment using seed sludge at mesophilic (37 ºC) and thermophilic (55ºC) temperature, and observed H_2 yields of 0.96 and 0.28 mol H_2/mol starch. Kim and Kim [2012] did a similar study and used mesophilic seed sludge at thermophilic temperatures and assessed H_2 production potential from starch. The aforementioned authors achieved a yield of 1.78 mol H_2/mol hexose, however the initial pH was 6.8 and the mesophilic seed sludge was acclimatized first. Ueno et al. [2001] achieved 2 mol H_2/mol hexose using cellulose powder as substrate (5 g/L) and anaerobic

microflora from sludge compost acclimatized to thermophilic (60ºC) temperature at a pH of 6.6. These higher yields are due to using thermophilic sludge which has enriched thermophiles sustainable at higher temperatures as opposed to the temperature shocked mesophilic biomass used in this study. Nissila et al. [2011] observed yield of 0.32 mol H_2/mol hexose using cellulose (5 g/L) as substrate at pH 7 and using cow rumen fluid as inoculum at 60ºC.

4.3.3 Volatile fatty acids

Figure 4.2 shows the VFA fractions at the end of the batch for thermophilic and mesophilic experiments for different substrate conditions based on COD. The error bars represent the standard deviation.
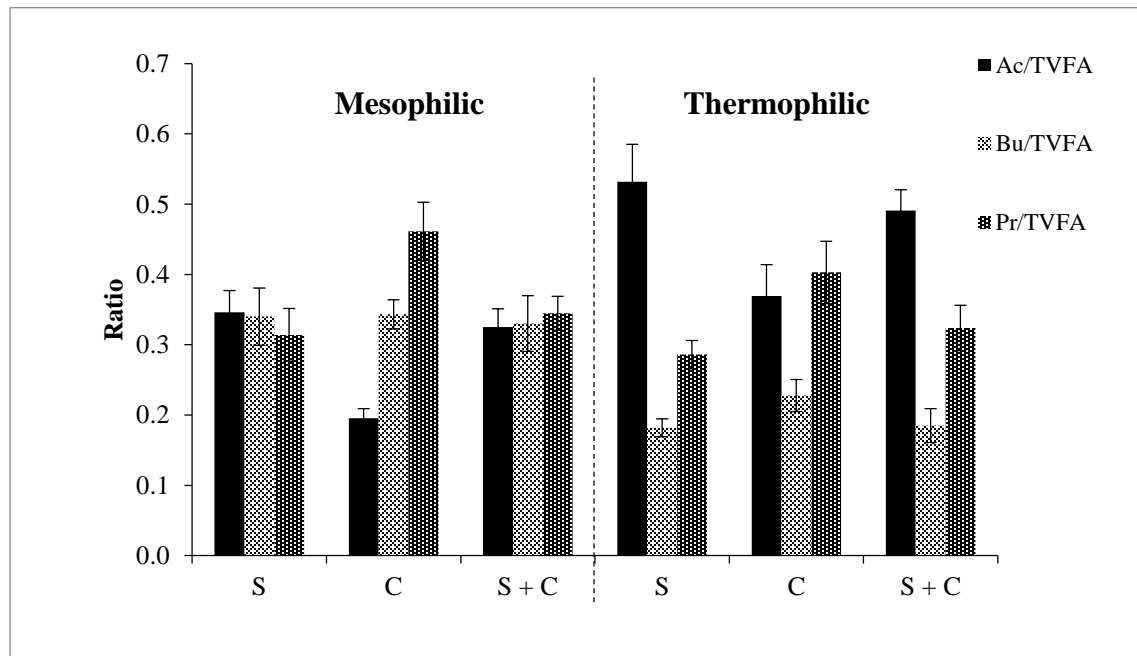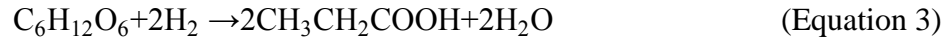

Figure 4.2. VFAs ratios at the fermentation end-point

The main VFAs detected in all the experiments were acetate, butyrate and, propionate. Acetate pathway (Equation 1) of H_2 production from hexose gives the maximum yield of 4 mol/mol hexose, and 2 mol/ mol hexose is obtained with butyrate (Equation 2) as the fermentation product [Hawkes et al., 2002]. Propionate production from hexose is associated with H_2 consumption (Equation 3)

$$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3COOH+2CO_2+4H_2 \qquad \text{(Equation 1)}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH+CO_2+2H_2 \qquad \text{(Equation 2)}$$

$$C_6H_{12}O_6+2H_2 \rightarrow 2CH_3CH_2COOH+2H_2O \qquad \text{(Equation 3)}$$

As shown in Figure 4.2, acetate was the main fermentation product in both thermophilic and mesophilic starch-only and starch-cellulose batches which rationalizes the higher H_2 production potential relative to cellulose-only. The discrepancies between mesophilic and thermophilic results come from butyrate ratios. In the thermophilic batches, butyrate was not the favorable product, while on the other hand in the mesophilic experiments there was significant butyrate production. Thermophilic experiments had higher acetate/butyrate (HAc/HBu) ratios of 7:1, 4:1, and 7:1 compared to mesophilic conditions where ratios of 3:1, 1:1, and 2:1 for starch-only, cellulose-only, and starch-cellulose batches, respectively, were observed. These results are consistent with the literature as thermophiles are associated with higher acetate production while decreasing butyrate, ethanol, and lactic acid during fermentation processes [O-Thong et al., 2011]. This shift to acetate production is favorable since acetate formation gives twice the H_2 yield compared to butyrate

formation [O-Thong et al., 2009]. Propionate concentrations were predominant in both mesophilic and thermophilic cellulose-only bottles. However, in the mesophilic cellulose-only batch, propionate concentration was the highest while acetate concentration was lowest. It can be inferred that cellulose degradation favors the propionate pathway with low H_2 production. Shin et al. [2004] evaluated H_2 production using mesophilic and thermophilic acclimatized acidogenic cultures at pH 5.5 from food waste and observed negligible propionate concentrations at thermophilic temperature (55ºC) compared to mesophilic temperature (35 ºC) which explains lower hydrogen production and yields from mesophilic cellulose-only batch compared to the thermophilic batch.

VFAs contributed on an average 60% of the final soluble COD for thermophilic conditions, while at mesophilic conditions, cellulose-only contributed 30% and starch and starch-cellulose contributed on an average 64% of the final soluble SCOD. This suggests that besides the detected VFAs, different intermediates or solvents were produced. Puhakka et al. [2012] conducted a similar study at mesophilic (37ºC) and thermophilic (55ºC) temperatures using glucose as substrate (9 g/L) and sediment sample from a geothermal hot spring (45ºC) as the inoculum, and observed different distribution of soluble metabolites at the two different temperature conditions, where butyrate was produced in low concentrations at 37ºC and not detected at 55ºC. Additionally, the aforementioned authors observed less acetate at 37ºC as compared to 55ºC. In addition to the aforementioned+ metabolites, formate,

lactate and ethanol were the other prominent metabolites observed, which could have accounted for the soluble COD in this study.

Based on 0.84 L H_2/g acetate, 0.58 L H_2/ g butyrate and 0.34 L H_2 consumed/ g propionate (Equation 1, 2, and 3), theoretical H_2 production from VFAs was calculated. The theoretical values shown in Table 4.3 were consistent with the H_2 measured during the experiment with an average percent difference of 4% and 11% for mesophilic and thermophilic, respectively. Interestingly, the measured H2 production was lower than theoretical for mesophilic conditions while for thermophilic conditions, the theoretical H_2 production was lower than the measured. This may be attributed to further conversion of VFAs to other alcohols such as ethanol, acetone and butanol. Based on initial and final TCOD as well as equivalent COD for the H_2 produced (8 gCOD/g H_2), the COD mass balances were calculated. The COD mass balance closures of 90±4% and 91±5% for thermophilic and mesophilic, respectively, verify the data reliability.

Table 4.3. Theoretical hydrogen production based on the acetate, butyrate, and propionate produced

	Substrate	Acetic acid	Butyric acid	Propionic acid	Theoretical H_2	Measured H_2	% difference
		mg/L	mg/L	mg/L	mL	mL	%
Mesophilic	Starch	2163 ± 195	1250 ± 150	1391 ± 167	372	353	5
	Cellulose	359 ± 25	371 ± 22	601 ± 54	56	53	6
	Starch + Cellulose	1673 ± 134	998 ± 120	1256 ± 88	280	269	4
Thermophilic	Starch	2389 ± 161	481 ± 59	911 ± 64	357	415	14
	Cellulose	969 ± 36	351 ± 12	748 ± 35	137	170	19
	Starch + Cellulose	1537 ± 24	341 ± 32	719 ± 84	225	224	0

4.4 Conclusions

In real-life applications, temperature shocks can occur in mesophilic digesters due to change in feedstock, strength, auto-thermal reactions, etc., and therefore this study provides a preliminary understanding of the response of mesophilic sludge to a thermophilic temperature shocks. Based on the findings in this study, the following conclusions can be drawn:

- Additional step of acclimatization of mesophilic seed sludge is not required as the microbial communities present can withstand temperature shocks.

- pH around 5.5 was observed to be ideal for thermophilic conditions as lower lag phases were observed.

- Maximum H_2 yield of 1.13 mol H_2/mol hexose$_{added}$ was observed for starch only at 60ºC whereas, at 37 ºC the yield was 1 mol H_2/mol hexose$_{added}$. The thermophilic cellulose-only yield of 0.42 mol H_2/mol hexose$_{added}$ was almost 3 times the mesophilic yield (0.13 mol H_2/mol hexose$_{added}$).

- Mesophilic co-fermentation of starch-cellulose increased the yield by 26% with respect to the estimated mono-substrate yields. On the other hand, thermophilic co-fermentation did not show any enhancement and this observation was attributed to starch being a more preferable substrate compared to cellulose as the carbon source for the microbial communities present at thermophilic conditions.

- Higher HAc/HBu ratios were observed at thermophilic conditions compared to mesophilic conditions.

- Cellulose degradation favored the propionate pathway. However, at thermophilic conditions lower levels of propionate were detected as compared to mesophilic conditions.

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Chapter 5

Conclusions and Recommendations

5.1 Conclusions

The following conclusions can be drawn, based on the findings of this study:

5.1.1 Effect of co-fermentation of glucose, starch, and cellulose for mesophilic biohydrogen production

- The substrates were utilized sequentially from simple to more complex carbohydrates.

- Glucose addition to starch and/or cellulose favored the acetate pathway. Cellulose degradation was associated with the propionate synthesis pathway.

- Co-fermentation improved the H_2 potential and the yields were greater by an average of $27 \pm 4\%$ than expected.

- OTUs in the Phyla *Bacteroides, Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes, Synergistes* and *Thermotogae* were common in mono- and co-substrate bottles, and OTUs in the Phyla *Acidobacteria, Actinobacteria,* and *Bacteroidetesee* were unique to only the co-substrate conditions.

- A linear relationship was observed between the number of observed species and H_2 yield, that is, the increase in H_2 yield is associated with increased number of observed species.

5.1.2 Sensitivity of mesophilic biohydrogen-producing cultures to temperature shocks

- Additional step of acclimatization of mesophilic seed sludge is not required as the microbial communities present can withstand temperature shocks.

- pH around 5.5 was observed to be ideal for thermophilic conditions as lower lag phases were observed.

- Maximum H_2 yield of 1.13 mol H_2/mol hexose$_{added}$ was observed for starch only at 60ºC whereas, at 37 ºC the yield was 1 mol H_2/mol hexose$_{added}$. The thermophilic cellulose-only yield of 0.42 mol H_2/mol hexose$_{added}$ was almost 3 times the mesophilic yield (0.13 mol H_2/mol hexose$_{added}$).

- Mesophilic co-fermentation of starch-cellulose increased the yield by 26% with respect to the mono-substrate yields. On the other hand, thermophilic co-fermentation did not show any enhancement and this observation was attributed to starch being a more preferable substrate compared to cellulose as the carbon source for the microbial communities present at thermophilic conditions.

- Higher HAc/HBu ratios were observed at thermophilic conditions compared to mesophilic conditions. Cellulose degradation favored the propionate pathway. However, at thermophilic conditions lower levels of propionate were detected as compared to mesophilic conditions.

5.2 Recommendations

Based on the findings of this study, further research should include:

- Assessment of different substrates such as xylose, arabinose, and cellobiose in different mixing combinations in conjunction with glucose, starch, and cellulose..

- Comprehensive kinetic analysis to elucidate the effect of co-substrates using mixed cultures.

- Microbial characterization of temperature shocked cultures to understand the microbiology.

- Scale-up to fed-batch and/or continuous system reactors for better control of operational conditions and continuous hydrogen production.

Curriculum Vitae

Name	Medhavi Gupta
Education	Masters in Engineering Science
	Chemical and Biochemical Engineering
	University of Western Ontario, London, Ontario
	Expected date of graduation: August 2014
	Bachelor of Science [Honours], Biochemistry and Biotechnology
	University of Windsor, Windsor, Ontario
Awards and Scholarships	Western Graduate Research Scholarship
	2012-2014
	Fully funded Masters' tuition recipient
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	Windsor International Student Employment (W.I.S.E.) Award
	2008-2012
	Entrance Scholarship for International Students
	2008-2009
Research Experience	**Graduate Research** (2012-Present)
	Department of Chemical and Biochemical Engineering,
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- Thesis: Studying the effect of co-fermentation of glucose, starch and, cellulose wastes for biohydrogen production
- Experience in running Continuous Stirred Tank Reactor (CSTR), Fed-Batch, and Batch experiments
- Trained in Water Quality Analysis, Culturing anaerobes, Denaturing Gradient Gel Electrophoresis (DGGE) Analysis
- Bio-reactor design and manufacturing supervision

Undergraduate Research (2011-2012)
Department of Chemistry and Biochemistry,
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- Thesis: Studying Effect of Neutral Sphingomyelinase2 (NSMase2) on Low Density Lipoprotein (LDL) Receptors
- Skills developed: Fluorescence Microscopy, Cell Culture, Flow Cytometry, Cloning, Protein Purification, Protein Electrophoresis and Immunoblotting

Work Experience	Industrial Projects (2013-2014) • Treatability study of the effect of alkaline hydrolysis pre-treatment conditions on methane production • Experimental design, and execution of biomethanation potential (BMP) tests including data analysis and reporting • Conduct Vector Attraction Reduction (VAR) test to monitor volatile/fixed solids reduction • Participated in evaluation of biohydrogen production from cellulosic wastes
Teaching Experience	Lab Coordinator, Dr. Nakhla Lab (2013-Present) University of Western Ontario • In charge of lab safety, equipment's, devices, updating lab procedures • Supervised and trained new recruits on general water quality analysis Graduate Teaching Assistant (2013-2014) Department of Chemical and Biochemical Engineering, University of Western University • Bioprocess Engineering • Bioreaction Engineering

Publications and Conferences

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