

Western  Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

8-7-2014 12:00 AM

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

Medhavi Gupta
The University of Western Ontario

Supervisor
Dr. George Nakhla
The University of Western Ontario

Graduate Program in Chemical and Biochemical Engineering
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Engineering Science
© Medhavi Gupta 2014

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>

 Part of the [Biochemical and Biomolecular Engineering Commons](#), and the [Environmental Engineering Commons](#)

Recommended Citation

Gupta, Medhavi, "Enhancement of Biohydrogen Production from Co-Fermentation of Glucose, Starch, and Cellulose" (2014). *Electronic Thesis and Dissertation Repository*. 2230.
<https://ir.lib.uwo.ca/etd/2230>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

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

(Thesis Format: Integrated Article)

by

Medhavi Gupta

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
The University of Western Ontario
London, Ontario, Canada

©Medhavi Gupta 2014

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 (H₂) production and the associated microbial communities. At initial pH of 5.5 and mesophilic temperature of 37 °C, the H₂ 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 60°C 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.

Keywords

Biohydrogen, anaerobic digestion, co-fermentation, batch, substrate-to-biomass ratio, mixed culture, microbial community analysis

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

Acknowledgements

I would like to express my special appreciation and thanks to my advisors' Dr. George Nakhla, Dr. M. Hesham El Nagggar, and Dr. Hisham Hafez for their constant guidance and support. Dr. Nakhla, you have been a tremendous mentor for me and I would like to thank you for always encouraging, in your own ways, and allowing me to grow as a research engineer. I have learnt a lot from you and I have a lot more to learn.

I would like to extend my gratitude to Dr. Elsayed Elbeshbishy for his continued patience, encouragement, and advice during the course of this work. I would also like to thank my colleague and a good friend, Noha Nasr, for her friendship and continuous support throughout the course of this work. I have learnt a lot from you and it has been a pleasure working alongside you, so much so that all those countless sleepless nights were somewhat fun. I'd also like to thank the team members, old and new – Dr. Ahmed Eldyasti, Bipro Dhar, Maritza Gomez, Nan Yang, Basem Horoun, August Wang, Kai Li and Joseph Donohue for all their help and being a wonderful team. I would also like to thank the engineering staff, in particular Nada, for always cheering and encouraging me.

The financial support from Western Engineering through the Western Engineering Scholarship is gratefully acknowledged. I also want to thank Dr. David Levin from University of Manitoba and his team, Preethi Velayutham and Thinesh Peranantham for their contribution and help in this work.

Finally, I would like to thank my parents and brother, Nalin, for their love and support. I would like to thank my friends, Rishabh, Frank, and Navleen, for their never ending support and uncanny levels of patience during my journey.

Table of Contents

Abstract.....	ii
Co-Authorship Statement.....	iii
Acknowledgements.....	v
Table of Contents.....	vi
List of Tables.....	ix
List of Figures.....	x
Nomenclature.....	xi
Chapter 1: Introduction.....	1
1.1 Background.....	1
1.2 Problem Statement.....	2
1.3 Research Objectives.....	3
1.4 Thesis Organization.....	4
1.5 Research Contributions.....	4
1.6 References.....	5
Chapter 2: Literature Review.....	7
2.1 Introduction.....	7
2.2 Biological Hydrogen Production.....	8
2.2.1 Direct Biophotolysis.....	8
2.2.2 Indirect Biophotolysis.....	8
2.2.3 Photo-Fermentation.....	9
2.2.4 Anaerobic Dark Fermentation.....	9
2.3 Factors Affecting Dark Fermentative Hydrogen Production.....	11

2.3.1	pH.....	11
2.3.2	Temperature.....	12
2.3.3	Inoculum.....	14
2.3.4	Substrates for Fermentative Hydrogen Production.....	21
2.3.5	Hydrogen Partial Pressure.....	25
2.4	References.....	26
Chapter 3: Co-fermentation of Glucose, Starch, and Cellulose for Mesophilic		
Biohydrogen Production.....		
3.1	Introduction.....	39
3.2	Materials and Methods.....	43
3.2.1	Seed Sludge and Substrate.....	43
3.2.2	Experimental Design.....	43
3.2.3	Analytical Methods.....	44
3.2.4	Microbial Analysis.....	45
3.2.5	Library Construction and Illumina Sequencing.....	45
3.2.6	Bioinformatic Analyses.....	47
3.2.7	Statistical Analysis.....	48
3.3	Results and Discussion.....	49
3.3.1	Biohydrogen Production.....	49
3.3.2	Hydrogen Yields.....	55
3.3.3	Volatile Fatty Acids.....	57
3.3.4	Microbial Community Analyses.....	62
3.4	Conclusions.....	72

3.5	References.....	72
Chapter 4: Sensitivity of Mesophilic Biohydrogen-Producing Cultures to		
	Temperature Shocks.....	80
4.1	Introduction.....	80
4.2	Materials and Methods.....	87
	4.2.1 Seed Sludge and Substrate.....	87
	4.2.2 Experimental Design.....	87
	4.2.3 Analytical Methods.....	88
4.3	Results and Discussion.....	89
	4.3.1 Biohydrogen Production.....	89
	4.3.2 Hydrogen Yields.....	91
	4.3.3 Volatile Fatty Acids.....	93
4.4	Conclusions.....	98
4.5	References.....	99
Chapter 5: Conclusions and Recommendations.....		
5.1	Conclusions.....	103
	5.1.1 Effect of co-fermentation of glucose, starch, and cellulose for mesophilic biohydrogen production.....	103
	5.1.2 Sensitivity of mesophilic biohydrogen-producing cultures to temperature shocks.....	104
5.2	Recommendations	105
	Curriculum Vitae.....	106

List of Tables

Table 2.1 Literature review on hydrogen production from different inoculum.....	17
Table 2.2 Summary of various substrates examined for fermentative hydrogen production.....	22
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.....	53
Table 3.2. Gompertz analysis of hydrogen production from different substrate.....	55
Table 3.3. Theoretical hydrogen production based on the acetate, butyrate and propionate produced.....	61
Table 3.4. OTU enrichment in cultures grown with different substrates relative to seed control.....	67
Table 4.1 Mesophilic and thermophilic studies in the literature.....	84
Table 4.2 H ₂ yields.....	91
Table 4.3. Theoretical hydrogen production based on the acetate, butyrate, and propionate produced	97

List of Figures

Figure 2.1 Distribution of research in pure vs. real waste substrates.....	21
Figure 3.1 Cumulative hydrogen production in cultures grown with different substrates.....	51
Figure 3.2. Hydrogen yield (mol H ₂ /mol hexose equiv.) for cultures grown with different substrates.....	56
Figure 3.3 VFAs ratios at the fermentation end-point (187 hours post-inoculation) of cultures grown on different substrates.....	60
Figure 3.4. PCR products generated by PCR amplification of 16S rRNA genes from DNA extracted from cultures grown with different substrates.....	63
Figure 3.5 Principle co-ordinate analysis of unweighted UniFrac distances.....	65
Figure 3.6 A) Trend of observed species and H ₂ yield; B) Relationship between observed species and H ₂ yield.....	66
Figure 4.1 Cumulative hydrogen production. Solid symbols are thermophilic and hollow symbols are mesophilic.....	90
Figure 4.2 VFAs ratios at the fermentation end-point.....	93

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 mesophilic 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.

1

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.

1.6 References

1. Azbar, N., Levin, D. State of the art and progress in production of biohydrogen. Bentham Science Publishers; 2012.
2. Cai, M., Liu, J., Wei, Y., 2004. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. *Environ. Sci. Technol.* 38: 3195-3202.
3. Chen, X., Sun, Y., Xiu, Z., Li, X., Zhang D., 2006. Stoichiometric analysis of biological hydrogen production by fermentative bacteria. *Int J Hydrogen Energy.* 31: 539-549.
4. Donoso-Bravo, A., Retamal, C., Carballa, M., Ruiz-Filippi, G., Chamy, R., 2009. Influence of temperature on the hydrolysis, acidogenesis and methanogenesis in mesophilic anaerobic digestion: parameter identification and modelling application. *Water Sci Technol.* 60: 9-17.
5. Esposito, G., Frunzo, L., Panico, A., Pirozzi, F., 2012. Enhanced bio-methane production from co-digestion of different organic substrates. *Environ Technol.* 33: 2733-2740.
6. Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I., 2002. Sustainable fermentative hydrogen production: challenges for process optimization. *Int J Hydrogen Energy.* 27:1339-1347.

7. Hallenbeck, P.C., Ghosh, D., Skonieczny, M.T., Yargeau, V., 2009. Microbiological and engineering aspects of biohydrogen production. *Indian J Microbiol*; 49: 48-59.
8. Kim, H.W., Han, S.K., Shin, H.S., 2003. The optimization of food waste addition as a co-substrate in anaerobic digestion of sewage sludge. *Waste Management and Research*. 21: 515-526.
9. Li, C., Fang, H.H.P., 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Crit Rev Environ Sci Technol*. 37:1-39.
10. Prakasham, R.S., Brahmaiah, P., Sathish, T., Rao, K.R.S.S., 2009. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *Int J Hydrogen Energy*. 34:9354-9361.
11. Wang, J., Wan, W., 2009. Kinetic models for fermentative hydrogen production: A review. *Int J Hydrogen Energy*. 34: 3313-3323.

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 due 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

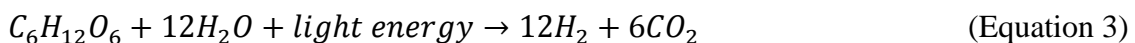
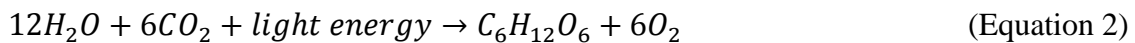
Certain bacterial-algal (green algae and cyanobacteria) systems are capable of using solar energy directly to extract electrons and protons from water resulting in evolution of hydrogen (photohydrogen) and oxygen by the following reaction [Levin et al., 2004; Benemann, 1980]:



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

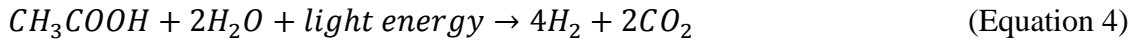
Cyanobacteria (blue-green algae) can also synthesize hydrogen through photosynthesis by splitting water in a two-step process [Levin et al., 2004]:



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]:



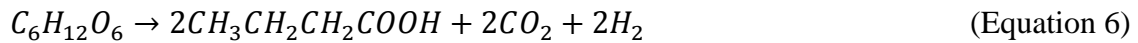
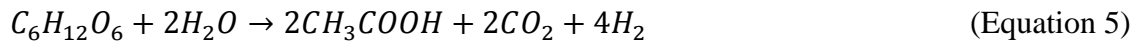
The main disadvantages of this process are the inhibitory effect of oxygen on nitrogenase and the very low (1%-5%) light conversion efficiency [Das and Veziroglu, 2008].

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

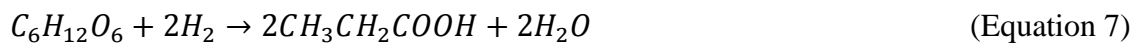
quantities of hydrogen. Majority of hydrogen-producing bacteria are either strict anaerobes (*Clostridia*, mrthylotrophs, rumen bacteria, methanogenic bacteria, archaea), facultative anaerobic bacteria (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and aerobic bacteria (*Alcaligenes*, *Bacillus*) [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]:



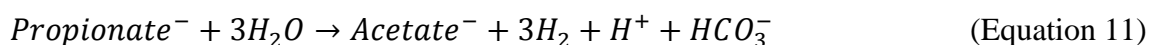
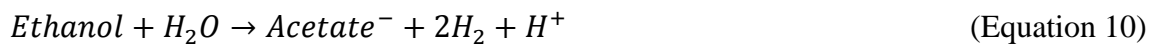
Several microbial populations, known as homoacetogenic bacteria (for example: *Clostridium thermoaceticum* and *Clostridium aceticum*), convert hydrogen and carbon dioxide to acetate, in turn, consuming the hydrogen [Guo et al, 2010]:

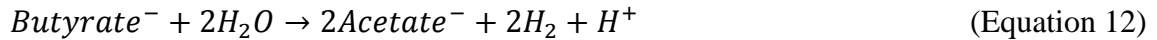


Propionate is also a hydrogen-consuming pathway, while ethanol and lactic acid are zero-hydrogen balance pathway [Guo et al, 2010]:



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]:





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 H₂/ 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 H₂/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 H₂/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 ($2.1 \text{ molH}_2/\text{mol}_{\text{hexose}}$), hydrogen content (64%) in biogas, and specific hydrogen production rate ($4.6 \text{ L H}_2/\text{g-VSS day}$). 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%, $2.2 \text{ mol H}_2/\text{mol hexose}_{\text{consumed}}$ 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 °C) and thermophilic (55 °C) 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 °C for thermophilic batches, and observed higher hydrogen yields at thermophilic than mesophilic. Yokoyama et al. [2007] examined the effect of different temperatures, 37°C, 50 °C, 55 °C, 60 °C, 67 °C, 75 °C and 85 °C, using cow waste slurry, and observed optimum hydrogen production at 60 °C and 75 °C. 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 °C -35 °C, increased the concentration of ethanol, but it decreased with further increasing temperature from 35 °C to 55 °C [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 cloacae* IIT-BT08 and observed increasing hydrogen yield from 15 to 36°C 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 °C - 37 °C and thermophilic range between 55 °C -70 °C.

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 H₂/mol hexose compared to control (2.2 mol H₂/mol hexose) with no pretreatment. In the same study, the authors saw an increase in hydrogen yield to 3.71 and 2.99 mol H₂/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 H₂/mol sucrose_{added} with iodopropane and BESA pretreated sludge, respectively, compared to untreated sludge (5.17 mol H₂/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 H₂/mol sucrose_{added} compared to no pretreatment sludge (4.56 mol H₂/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 (°C)	pH	H₂ yield (mol H₂/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 (°C)	pH	H₂ yield (mol H₂/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	<i>Clostridium butyricum</i> CWBI1009	Glucose	Sequenced batch	30	5.2	1.7	Masset et al., 2010
		<i>Clostridium butyricum</i> CWBI1009	Starch	Sequenced batch	30	5.6	2	Masset et al., 2010
		<i>Clostridium termitidis</i> CT1112	Cellulose	Batch	37	7.2	0.62	Ramachandran et al., 2008
		<i>Clostridium beijerinckii</i>	Glucose	Batch	37	6.7	1.45	Masset et al., 2012
		<i>Clostridium saccharoperbutylacetonicum</i> N1-4	Glucose	Batch	37	6	3.1	Alalayah et al., 2008
		<i>Clostridium paraputrificum</i> 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 (°C)	pH	H₂ yield (mol H₂/mol hexose^{added} or consumed)	Ref.
Pure cultures	Strict anaerobes	<i>Thermoanaerobacterium thermosaccharolyticum</i> W16	Glucose	Batch	60	6.5	2.42	Ren et al., 2008a
		<i>Thermoanaerobacterium thermosaccharolyticum</i> W16	Corn stover	Batch	60	7	2.2	Ren et al., 2010
		<i>Caldicellulosiruptor saccharolyticus</i>	Glucose	Batch	70	7	3.4	Mars et al., 2010
		<i>Thermotoga elfi</i>	Glucose	Batch	65	7-7.4	3.33	Van Niel et al., 2002
	Facultative anaerobes	<i>Klebsiella pneumonia</i> ECU-15	Glucose	Batch	37	6	2.07	Niu et al., 2010
		<i>Enterobacter aerogenes</i> HO-39	Glucose	Batch	38	6-7.0	1	Yokoi et al., 1995
		<i>Escherichia coli</i> BL-21	Glucose	CSTR	37	6	3.12	Chittibabu et al., 2006
		<i>Enterobacter cloacae</i> IIT-BT08	Glucose	Batch	36	6	2.2	Kumar and Das, 2000
		<i>Rhodospseudomonas palustris</i> P4	Glucose	Batch	37	7	2.76	Oh et al., 2002
	Co-cultures	<i>Clostridium butyricum</i> and <i>Enterobacter aerogenes</i> 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 (°C)	pH	H₂ yield (mol H₂/mol hexose^{added or consumed})	Ref.
Pure cultures	Co-cultures	<i>Clostridium beijerinckii</i> and <i>Rhodobacter sphaeroides-RV</i>	Ground wheat	Annular hybrid bioreactor	32	7-7.5	0.64	Argun et al., 2010
		<i>Clostridium butyricum</i> and <i>Clostridium felsineum</i>	Glucose	Batch	37	5.3	1.71	Masset et al., 2012
		<i>Clostridium pasteurianum</i> and <i>Clostridium felsineum</i>	Glucose	Batch	37	5.3	1.62	Masset et al., 2012
		<i>Clostridium thermocellum</i> and <i>Thermoanaerobacterium thermosaccharolyticum</i>	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.

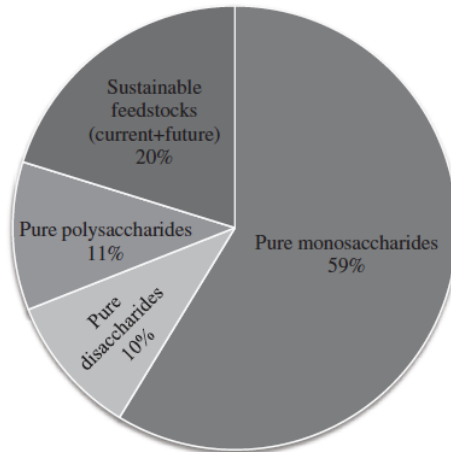


Figure 2.1. Distribution of research in pure vs. real waste substrates [Elsharnouby et al., 2013]

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	<i>Enterobacter aerogenes</i> 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	<i>Escherichia coli</i> strain DJT135	1.02 mol H ₂ / mol hexose	Ghosh et al., 2009
	Galactose	<i>Enterobacter aerogenes</i> strain HO-38	0.95 mol H ₂ / mol galactose	Yokoi et al., 1995
	Galactose	<i>Escherichia coli</i> strain DJT135	0.69 mol H ₂ / mol galactose	Ghosh et al., 2009
	Mannose	<i>Enterobacter aerogenes</i> strain HO-39	0.98 mol H ₂ / mol mannose	Yokoi et al., 1995
	Mannose	<i>Citrobacter sp. CMC-1</i>	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 <i>Clostridium pasteurianum</i>	2.73 mol H ₂ / mol sucrose	Zhang et al., 2005
	Maltose	<i>Enterobacter aerogenes</i> strain HO-38	2.16 mol H ₂ / mol maltose	Yokoi et al., 1995
	Maltose	<i>Clostridium sp. R1</i>	3.13 mol H ₂ / mol maltose	Ho et al., 2010
	Cellobiose	<i>Clostridium termitidis</i>	4.6 mmol H ₂ / L culture	Ramachandran et al., 2008
	Cellobiose	<i>Clostridium sp. R1</i>	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	<i>Clostridium cellulolyticum</i>	1.7 mol H ₂ /mol hexose _{consumed}	Ren et al., 2007
	Cellulose	<i>Clostridium termitidis</i>	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	<i>Clostridium saccharobutylaceticum</i> 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	<i>Clostridium butyricum</i>	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 H₂/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 H₂/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.

2.4 References

1. Abreau, A.A., Karakashev, D., Souza, D.Z., Alves, M.M., 2012. Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. *Biotechnol Biofuels*. 5:1-12.
2. Akutsu, Y., Li, Y.Y., Tandukar, M., Kubota, K., Harada, H., 2008. Effects of seed sludge on fermentative characteristics and microbial community structures in thermophilic hydrogen fermentation of starch. *Int J Hydrogen Energy*. 33: 6541-6548.
3. Alalayah, W.M., Kalil, M.S., Kadhum, A.A.H., Jahim, J.M., Alauj, N.M., 2008. Hydrogen production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). *Int J Hydrogen Energy*. 33: 7392-7396.
4. Argun, H. and Kargi, F., 2010. Bio-hydrogen production from ground wheat starch by continuous combined fermentation using annular-hybrid bioreactor. *Int J Hydrogen Energy*. 35: 6170-6178.
5. Azbar, N., Levin, D. State of the art and progress in production of biohydrogen. Bentham Science Publishers; 2012.

6. Benemann, J.R., 1980. Bioengineering aspects of biophotolysis. *Enzyme Microbiol Technol.* 2: 103-111.
7. Benemann, J., 1996. Hydrogen biotechnology: Progress and prospects. *Nat Biotechnol.* 14: 1101-1103.
8. Cai, M., Liu, J., Wei, Y., 2004. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. *Environ. Sci. Technol.* 38: 3195-3202.
9. Cheong, D.Y., Hansen, C.L., 2007. Feasibility of hydrogen production in thermophilic fermentation by natural anaerobes. *Bioresour Technol.* 98: 2229-2239.
10. Chittibabu, G., Nath, K., Dad, D., 2006. Feasibility studies on the fermentative hydrogen production by *recombinant Escherichia coli* BL-21. *Process Biochem.* 41: 682-688.
11. Chong, M.L., Sabaratnam, V., Shirai, Y., Hassan, M.A., 2009. Biohydrogen production from biomass and industrial wastes by dark fermentation. *Int J Hydrogen Energy.* 34: 3277-3287.
12. Danko, A.S., Abreu, A.A., Alves, M.M., 2008. Effect of arabinose concentration on dark fermentation hydrogen production using different mixed cultures. *Int J Hydrogen Energy.* 33: 4527-4532.
13. Das, D., Veziroglu, T.N., 2008. Advances in biological hydrogen production processes. *Int J Hydrogen Energy.* 33: 6046-6057.
14. Datar, R., Huang, J., Maness, P.C., Mohagheghi, A., Czernik, S., Chornet, E., 2007. Hydrogen production from the fermentation of corn stover biomass

- pretreated with a steam-explosion process. *Int J Hydrogen Energy*. 32: 932-939.
15. Elbeshbishy, E., 2011. Novel application of ultrasonication for biohydrogen and biomethane production. Ph.D. Thesis, University of Western Ontario, London, Ontario, Canada.
 16. Elbeshbishy, E., Hafez, H., Nakhla, G., 2011. Hydrogen production using sono-biohydrogenator. *Int J Hydrogen Energy*. 36: 1456-1465.
 17. Elsharnouby, O., Hafez, H., Nakhla, G., El Naggar, M.H., 2013. A critical literature review on biohydrogen production by pure cultures. *Int J Hydrogen Energy*. 38, 4945-4966.
 18. Esposito, G., Frunzo, L., Panico, A., Pirozzi, F., 2012a. Enhanced bio-methane production from co-digestion of different organic substrates. *Environ Technol*. 33 (24): 2733-2740.
 19. Esposito, G., Frunzo, L., Giordano, A., Liotta, F., Panico, A., Pirozzi, F., 2012b. Anaerobic co-digestion of organic wastes. *Rev Environ Sci Biotechnol*. 11: 325-341.
 20. Evvyernie, D., Morimoto, K., Karita, S., Kimura, T., Sakka, K., Ohmiya, K., 2001. Conversion of Chitinous waste to hydrogen gas by *Clostridium paraputrificum* M-21. *J Biosci Bioengineering*. 91: 339-343.
 21. Fang, H.H.P., Zhang, T., Liu, H., 2002. Microbial diversity of a mesophilic hydrogen-producing sludge. *Appl Microbiol Biotechnol*. 58: 112-118.

22. Fang, H.H.P., Liu, H., 2002. Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresour Technol.* 82: 87-93.
23. Fangkum, A., Reungsang, A., 2011a. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effect of initial pH and substrate concentration. *Int J Hydrogen Energy.* 36: 8687-8696.
24. Fangkum, A., Reungsang, A., 2011b. Biohydrogen production from mixed xylose/arabinose at thermophilic temperature by anaerobic mixed cultures in elephant dung. *Int J Hydrogen Energy.* 36: 13928-13938.
25. Ferchichi, M., Crabbe, E., Gil, G.H., Hintz, W., Almadidy, A., 2005. Influence of initial pH on hydrogen production from cheese whey. *J Biotechnol.* 120: 402-409.
26. Gadow, S.I., Li, Y.Y., Liu, Y., 2012. Effect of temperature on continuous hydrogen production of cellulose. *Int J Hydrogen Energy.* 37: 15465-15472.
27. Ghosh, D., Hallenbeck, P.C., 2009. Fermentative hydrogen yields from different sugars by batch cultures of metabolically engineered *Escherichia coli* DJT135. *Int J Hydrogen Energy.* 34: 7979-7982.
28. Guo, X.M., Trably, E., Latrille, E., Carrere, H., Steyer, J., 2010. Hydrogen production from agricultural waste by dark fermentation: A review. *Int J Hydrogen Energy.* 35: 10660-10673.
29. Hafez, H., Nakhla, G., El Naggar, M.H., Elbeshbishy, E., Baghchehsaraee, B., 2010. Effect of organic loading rate on a novel hydrogen bioreactor. *Int J Hydrogen Energy.* 35: 81-92.

30. Hallenbeck, P.C., Ghosh, D., Skonieczny, M.T., Yargeau, V., 2009. Microbiological and engineering aspects of biohydrogen production. *Int J Microbiol.* 49: 48-59.
31. Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I., 2002. Sustainable fermentative hydrogen production: challenges for process optimization. *Int J Hydrogen Energy.* 27:1339-1347.
32. Ho, K.L., Chen, Y.Y., Lee, D.J., 2010. Biohydrogen production from cellobiose in phenol and cresol-containing medium using *Clostridium* sp. R1. *Int J Hydrogen Energy* 35: 10239-10244.
33. Hussy, I., Hawkes, F.R., Dinsdale, R., Hawkes, D.L., 2003. Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. *Biotechnol Bioeng.* 84: 619-626.
34. Hussy, I., Hawkes, F.R., Dinsdale, R., Hawkes, D.L., 2005. Continuous fermentative hydrogen production from sucrose and sugarbeet. *Int J Hydrogen Energy.* 30: 471-483.
35. International Energy Outlook, July 2013. Independent Statistics and Analysis. U.S. Energy Information Administration.
36. Kargi, F., Eren, N.S., Ozmihci, S., 2012. Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations. *Int J Hydrogen Energy.* 37: 8338-8342.
37. Karlsson, A., Vallin, L., Ejertsson, J., 2008. Effects of temperature, hydraulic retention time and hydrogen extraction rate on hydrogen production from the

- fermentation of food industry residues and manure. *Int J Hydrogen Energy*. 33: 953-962.
38. Kim, H.W., Han, S.K., Shin, H.S., 2003. The optimization of food waste addition as a co-substrate in anaerobic digestion of sewage sludge. *Waste Management and Research*. 21: 515-526.
39. Kim, S.H., Han, S.K., Shim, H.S., 2004. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *Int J Hydrogen Energy*. 29: 1607-1616.
40. Kim, D.H., Kim, M.S., 2012. Thermophilic fermentative hydrogen production from various carbon sources by anaerobic mixed cultures. *Int J Hydrogen Energy*. 37:2021-27.
41. Kyazze, G., Martinez-Perez, N., Dinsdale, R., Premier, G.C., Hawkes, F.R., Guwy, A.J., Hawkes, D.L., 2006. Influence of substrate concentration on the stability and yield of continuous biohydrogen production. *Biotechnol Bioeng*. 93: 971-979.
42. Kumar, N., Das, D., 2000. Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BL 08. *Process Biochem*. 35: 589-593.
43. Lamed, R.J., Lobos, J.H., Su, T.M., 1988. Effects of stirring and hydrogen on fermentation products of *Clostridium thermocellum*. *Appl Environ Microbiol*. 1216-1221.
44. Lay, J.J., 2000. Modelling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol Bioeng*. 68: 269-278.

45. Lay, C.H., Kuo, S.Y., Sen, B., Chen, C.C., Chang, J.S., Lin, C.Y., 2012. Fermentative biohydrogen production from starch-containing textile wastewater. *Int J Hydrogen Energy*. 37: 2050-2057.
46. Lee, K.S., Hsu, Y.F., Lo, Y.C., Lin, P.J., Lin, C.Y., Chang, J.S., 2008. Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *Int. J. Hydrogen Energy*. 33, 1565-1572.
47. Lee, K.S., Tseng, T.S., Liu, Y.W., Hsiao, Y.D., 2012. Enhancing the performance of dark fermentative hydrogen production using a reduced pressure fermentation strategy. *Int J Hydrogen Energy*. 37: 15556-15562.
48. Levin, D.B., Pitt, L., Love, M., 2004. Biohydrogen production: prospects and limitations to practical application. *Int J Hydrogen Energy*. 29: 173-185.
49. Liang, T., Cheng, S., Wu, K., 2002. Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. *Int J Hydrogen Energy*. 27: 1157-1165.
50. Lin, C., Cheng, C., Fermentative hydrogen production from xylose using anaerobic mixed microflora. *Int J Hydrogen Energy*. 31: 832-840.
51. Lin, C.Y., Chang, C.C., Hung, C.H., 2008. Fermentative hydrogen production from starch using natural mixed cultures. *Int. J. Hydrogen Energy*. 33 (10), 2445-2453.
52. Liu, Y., Yu, P., Song, X., Qu, Y., 2008. Hydrogen production form cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. *Int J Hydrogen Energy*. 33: 2927-2933.

53. Logan, B.E., Oh, S.E., Kim, I.S., Ginkel, S.V., 2002. Biological hydrogen production measured in batch anaerobic respirometers. *Environ. Sci. Technol.* 36 (11), 2530-2535.
54. Mangayil, R., Santala, V., Karp, M., 2011. Fermentative hydrogen production from different sugars by *Citrobacter* sp. CMC-1 in batch culture. *Int J Hydrogen Energy.* 36: 15187-15194.
55. Mars, A.E., Veuskens, T., Budde, M.A.W., van Doeveren, F.N.M., Lips, S.J., Bakker, R.R., de Vrije, T., Claassen, A.M., 2010. Biohydrogen production from untreated and hydrolyzed steam peels by extreme thermophiles *Caldicellulosiruptor saccharoluticus* and *Thermotoga neopolitana*. *Int J Hydrogen Energy.* 35: 7730-7737.
56. Masset, J., Hiligsmann, S., Hamilton, C., Beckers, L., Franck, F., Thonart, P., 2010. Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently isolated strain of hydrogen-producing *Clostridium butyricum* CWBI1009. *Int J Hydrogen Energy.* 35: 3371-3378.
57. Masset, J., Calusinska, M., Hamilton, C., Hiligsmann, S., Joris, B., Wilmotte, A., Thonart, P., 2012. Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium* spp. *Biotechnol Biofuels.* 5:1-15.
58. Nasr, N., Elbeshbishy, E., Hafez, H., Nakhla, G., El-Naggar, M.H., 2011. Biohydrogen production from thin stillage using conventional and acclimatized anaerobic digester sludge. *Int J Hydrogen Energy.* 36: 12761-12769.

59. Niu, K., Zhang, X., Tan, W.S., Zhu, M.L., 2010. Characteristics of fermentative hydrogen production with *Klebsiella pneumonia* ECU-15 from anaerobic sewage sludge. *Int J Hydrogen Energy*. 35: 71-80.
60. Oh, Y.K., Seol, E.H., Lee, E.Y., Park, S., 2002. Fermentative hydrogen production by a new chemoheterotrophic bacterium *Rhodopseudomonas palustris* P4. *Int J Hydrogen Energy*. 27: 1373-1379.
61. Patra, S., Sangyoka, S., Boonmee, M., Teungsang, A., 2008. Bio-hydrogen production from the fermentation of sugarcane bagasse hydrosylate by *Clostridium butyricum*. *Int J Hydrogen Energy*. 33: 5256-5265.
- Wang, W., Xie, L., Luo, G., Zhou, Q., 2013. Enhanced fermentative hydrogen production from cassava stillage by co-digestion: The effects of different co-substrates. *Int J Hydrogen Energy*. 38: 6980-6988.
62. Prakasham, R.S., Sathish, T., Brahmaiah, P., Rao, S., Rao, R.S., Hobbs, P.J., 2009a. Biohydrogen production from renewable agri-waste blend: Optimization using mixed design. *Int J Hydrogen Energy*. 34: 6143-6148.
63. Prakasham, R.S., Brahmaiah, P., Sathish, T., Rao, K.R.S.S., 2009b. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *Int J Hydrogen Energy*. 34:9354-9361.
64. Puhakka, J.A., Karadag, D., Nissila, M.E., 2012. Comparison of mesophilic and thermophilic anaerobic hydrogen production by hot spring enrichment culture. *Int J Hydrogen Energy*. 37: 16453-16459.

65. Quemeneur, M., Hamelin, J., Benomar, S., Guidici-Orticoni, M.T., Latrille, E., Steyer, J.P., Trably, E., 2011. Changes in hydrogenase genetic diversity and proteomic patterns in mixed-culture dark fermentation of mono-, di- and tri-saccharides. *Int J Hydrogen Energy*. 36: 11654-11665.
66. Ramachandran, U., Wrana, N., Cicek, N., Sparling, R., Levin, D.B., 2008. Hydrogen production and end-product synthesis patterns by *Clostridium termitidis* strain CT1112 in batch fermentation cultures with cellobiose or α -cellulose. *Int J Hydrogen Energy*. 33: 7006-7012.
67. Ramachandran, U., Wrana, N., Cicek, N., Sparling, R., Levin, D.B., 2011. Isolation and characterization of hydrogen- and ethanol-producing *Clostridium* sp. strain URNW. *Can J Microbiol*. 57: 236-243.
68. Ren, N., Li, J., Li, B., Wang, Y., Liu, S., 2006. Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *Int J Hydrogen Energy*. 31: 2147-2157.
69. Ren, Z., Ward, T.E., Logan, B.E., Regan, J.M., 2007. Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species. *J. Appl. Microbiol*. 6 (103), 2258-2266.
70. Ren, N., Cao, G., Wang, A., Lee, D.J., Guo, W., Zhu, Y., 2008a. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energy*. 33: 6124-6132.

71. Ren, N., Guo, W., Wang, X., Xiang, W., Liu, B., Wang, X., Chen, Z., 2008b. Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. *Int J Hydrogen Energy*. 33: 4318-4324.
72. Ren, Y., Wang, J., Liu, Z., Ren, Y., Li, G., 2009. Hydrogen production from the monomeric sugars hydrolyzed from hemicellulose by *Enterobacter aerogenes*. *Renewable Energy*. 34: 2774-2779.
73. Ren, N., Cao, G., Guo, W., Wang, A., Zhu, Y., Liu, B., Xu, J., 2010. Biological hydrogen production from corn stover by moderately thermophile *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energy*. 35: 2708-2712.
74. Riano, B., Molinuevo, B., Garcia-Gonzalez, M.C., 2011. Potential for methane production from anaerobic co-digestion of swine manure with winery wastewater. *Bioresour Technol*. 102: 4131-4136.
75. Shin, H.S., Youn, J.H., 2005. Conversion of food waste into hydrogen by thermophilic acidogenesis. *Biodegradation*. 16: 33-44.
76. Sinha, P., Pandey, A., 2011. An evaluation report and challenges for fermentative biohydrogen production. *Int J Hydrogen Energy*. 36: 7460-7478.
77. Tenca, A., Schievano, A., Perazzolo, F., Adani, F., Oberti, R., 2011. Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: Maximizing stable production without pH control. *Bioresour Technol*. 102: 8582-8588.

78. Ueno, Y., Haruta, S., Ishii, M., Igarashi, Y., 2001. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. *Appl Microbiol Biotechnol.* 57: 555-562.
79. van Niel, E.W.J., Budde, M.A.W., de Haas, G.G., van der Val, F.J., Claassen, P.A.M., Stams, A.J.M., 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharaolyticus* and *Thermotoga elfii*. *Int J Hydrogen Energy.* 27: 1391-1398.
80. Van Ginkel, S.W., Oh, S.E., Logan, B.E., 2005. Biohydrogen gas production from food processing and domestic wastewaters. *Int J Hydrogen Energy.* 30: 1535-1542.
81. Vertes, A., Qureshi, N., Blaschek, H., Yukawa, H. Biomass to biofuels: Strategies for global industries. John Wiley and Sons, Ltd; 2009.
82. Wang, Y., Ai, P., Hu, C., Zhang, Y., 2011. Effects of pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose. *Int J Hydrogen Energy.* 36: 390-396.
83. Wang, J., Wan, W., 2009. Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy.* 34: 799-811.
84. Xia, Y., Cai, L., Zhang, T., Fang, H.H.P., 2012. Effects of substrate loading and co-substrates on thermophilic anaerobic conversion of microcrystalline cellulose and microbial communities using high-throughput sequencing. *Int. J. Hydrogen Energy.* 37: 13652-13659.

85. Yokoyama, H., Waki, M., Moriya, N., Yasuda T., Tanaka, Y., Haga, K., 2007. Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. *Appl Microbiol Biotechnol.* 74: 474-483.
86. Yokoi, H., Maki, R., Hirose, J., Hayashi, S., 2002. Microbial production of hydrogen from starch-manufacturing wastes. *Biomass Bioenergy.* 22: 389-395.
87. Yokoi, H., Ohkawara, T., Hirose, J., Hayashi, S., Takasaki Y., 1995. Characteristics of hydrogen production by aciduric *Enterobacter aerogenes* Strain HO-39. *J Fermentation Bioengineering.* 80: 571-574.
88. Yossan, S., O-Thong, S., Prasertsan, P., 2012. Effect of initial pH, nutrients and temperature on hydrogen production from palm oil mill effluent using thermotolerant consortia and corresponding microbial communities. *Int J Hydrogen Energy.* 37: 13806-13814.
89. Zhang, Y., Liu, G., Shen, J., 2005. Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. *Int J Hydrogen Energy.* 30: 855-860.
90. Zhu, H., Beland, M., 2006. Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *Int J Hydrogen Energy.* 31:1980-1988.

Chapter 3

Co-fermentation of Glucose, Starch, and Cellulose for Mesophilic Biohydrogen Production¹

3.1 Introduction

Among various biological H₂ production methods, dark fermentation is of great significance to produce H₂ from readily available organic wastes [Wang and Wan, 2009]. Renewable carbohydrate-based feedstocks are the preferred organic carbon source for H₂-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 H₂ production, irrespective of mixed consortia or pure cultures, including both inoculum and substrate [Wang and Wan, 2009]. The

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 H₂ and CO₂ 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 H₂ [Hawkes et al., 2002]. Therefore, bacterial metabolism favoring acetate and butyrate production is important [Hawkes et al., 2002].

Numerous studies have examined H₂ 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 H₂ 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 H₂ production when compared to glucose-only fermentation, and 9% increase in H₂ 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 H₂ yield in the mixture was observed to be 2.37 mol H₂/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 H₂/mol substrate; xylose, 2.19 mol H₂/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 H₂ yield of 2.2 mol H₂/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 H₂ yield of 0.97 mol H₂/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 H₂/mol hexose. Ramachandran et al. [2008] achieved 0.62 mol H₂/mol hexose_{added} at 2 g/L initial cellulose concentration [Ramachandran et al., 2008]. Ren et al. [2007] reported the highest mesophilic H₂ production from cellulose with yields of 1.7 mol H₂/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]. NaHCO₃ 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:

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

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. NaHCO₃ 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/ μ L, 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 μ L of pre-normalized DNA, 1.0 μ L of each forward and reverse primers (10 μ M), 12 μ L HPLC grade water (Fisher Scientific, ON, Canada) and 10 μ L 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 H₂ production for the different substrate conditions. The observed cumulative H₂ production after 187 hours of fermentation was 431, 353, and 53 mL for glucose, starch, and cellulose, respectively, as mono-substrates. A maximum cumulative H₂ production of 499 mL was observed in co-fermentation of glucose and starch, the glucose and cellulose co-fermentation produced 303 mL H₂, the starch and cellulose fermentation produced 269 mL H₂, and co-fermentation of glucose, starch, and cellulose produced 343 mL H₂. As reported above, cellulose-only produced the lowest amount of H₂, and bottles containing cellulose in combination with other substrates yielded lower H₂ production when compared to glucose-only, starch-only, and glucose with starch in combination.

Logan et al. [2002] witnessed lower H₂ 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 H₂/mol hexose [Ramachandran et al., 2008].

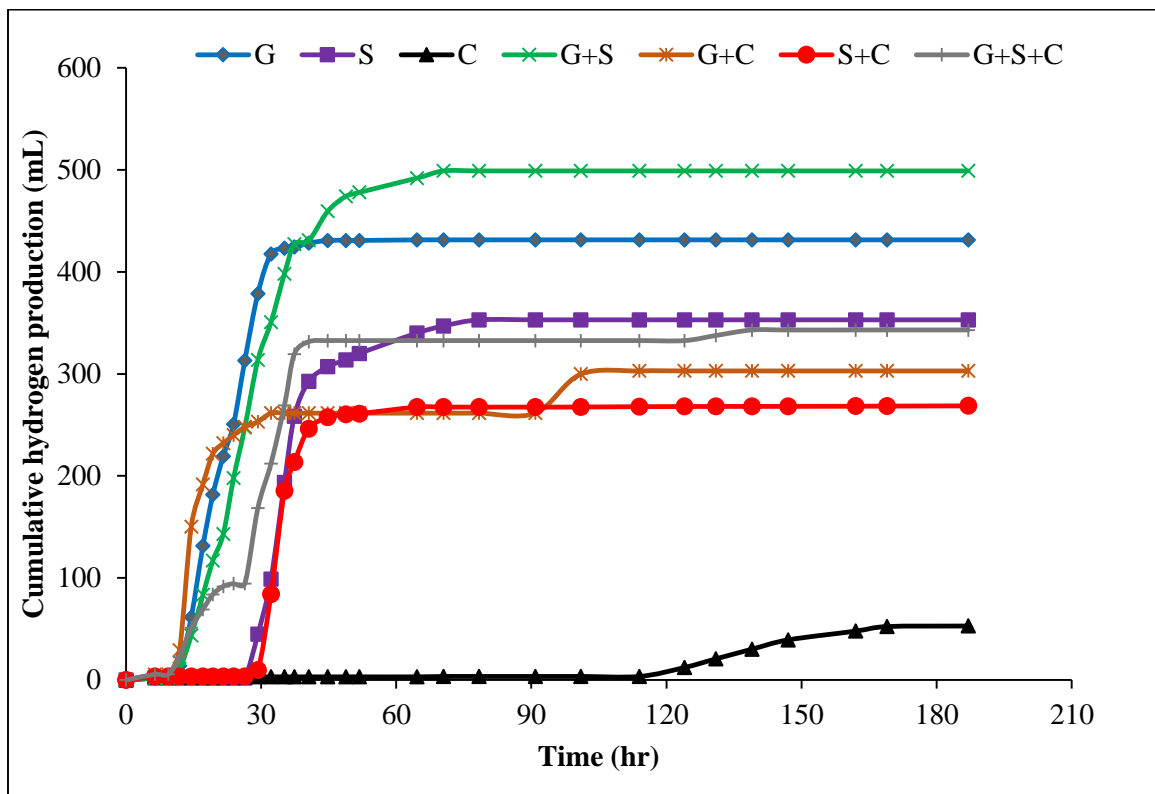


Figure 3.1. Cumulative hydrogen production in cultures grown with different substrates.

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 H₂ production. H₂ 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 H₂ 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 H₂ 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 H₂/g substrate) calculated from cultures grown with co-substrates based on the hydrogen production from the individual mono-substrates substrates.

Substrate	Expected H₂	Measured H₂	% 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 H₂ (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 H₂ production in mL/g substrate was measured from mono-substrate experiments and was then used to estimate the H₂ production in bottles where multiple substrates were used. Interestingly, as depicted in Table 3.1, the measured specific H₂ production when glucose and starch were co-fermented was 200 mL/g substrate which was 27% higher than the estimated H₂ production of 157 mL/g substrate confirming that co-substrate degradation enhanced the H₂ 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):

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

where P is the cumulative H₂ production, P_{max} is the maximum cumulative H₂ production, R_{max} is the maximum H₂ production rate, λ is the lag time, and t is the fermentation time. The coefficient of determination R² 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 H₂ production rates for glucose, starch, and cellulose mono-substrate bottles were calculated as 26, 27, and 1 mL/hr, respectively. The H₂ 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 H₂ 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 H₂ 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 H₂ yield of 0.52 mol/mol hexose equivalent_{added} at S/X of 8 g cellulose/g VSS²⁰. Significantly higher H₂ yields of 1.7 mol H₂/ mol hexose_{consumed} were reported

in a study by Ren et al. [2007], but this was a pure mesophilic cellulose-degrading bacterium, *Clostridium cellulolyticum*.

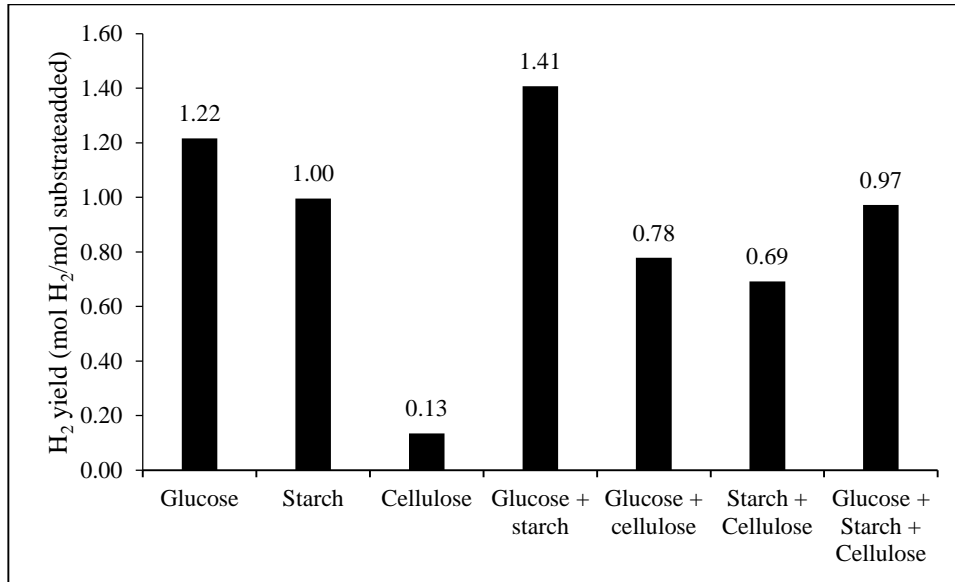


Figure 3.2. Hydrogen yield (mol H₂/mol hexose equiv.) for cultures grown with different substrates. Numbers above the bar graphs indicate the specific calculated yield.

On the other hand, when starch was co-fermented with glucose, a H₂ 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 H₂ yield of 0.78 mol/mol, which was 25% higher than the expected yield. Similarly, starch-cellulose co-fermentation resulted in a H₂ 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 H₂ yields of 0.16 and 0.53 and 0.19 mol H₂/mol hexose_{added} for cellulose-glucose,

cellulose-xylose and cellulose-starch, respectively. Glucose, starch, and cellulose co-substrate resulted in a H₂ yield of 0.97 mol/mol, which was 23% higher than the expected yield. This increase in H₂ yield in all the co-substrate bottles affirms that co-fermentation of different substrates improved the H₂ potential.

Based on the abovementioned results, it is clear that mesophilic cellulose fermentation was associated with low H₂ yields but the addition of glucose to cellulose and/or starch enhanced the fermentation process and thus increased the H₂ yield by at least 23%. Xia et al. [2012] reported maximum cellulose conversion rate and highest H₂ yields when using glucose and xylose as co-substrate, respectively. Interestingly, the H₂ yield was inversely proportional to H₂ production rate for the batches. A similar trend was noticed in another study by Chang et al. [2008] where for the highest H₂ production rate, the lowest H₂ yield was obtained and vice versa. This may be due to mass transfer limitations from the liquid to the biogas, thus increasing dissolved H₂ gas and retarding biohydrogen production processes. However, mass transfer coefficient calculations was beyond the scope of this study.

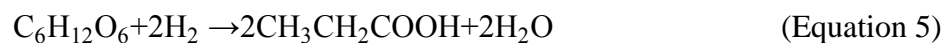
3.3.3 Volatile fatty acids

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 H₂ 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 H₂ yield from hexose with acetate formation is 4 mol H₂/mol hexose and 2 mol/ mol hexose for butyrate formation [Hawkes et al., 2002]:



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 H₂ 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 H₂ consumption, which explains the low H₂ yield and production in cellulose-only bottles [Hawkes et al., 2002]:



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 $93\pm 4\%$ verify data reliability.

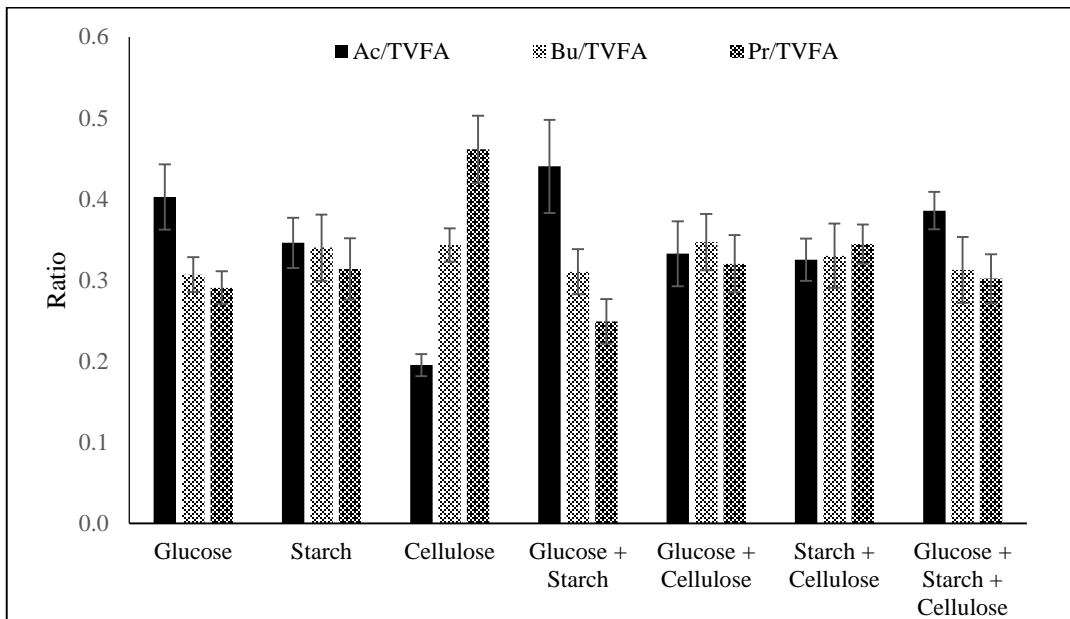


Figure 3.3. VFAs ratios at the fermentation end-point (187 hours post-inoculation) of cultures grown on different substrates.

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

Substrate	Acetic acid	Butyric acid	Propionic acid	Theoretical H ₂			Total	Measured H ₂	% difference
				From Acetic acid	From Butyric acid	From Propionic acid			
				mg/L	mg/L	mg/L			
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 H₂ 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

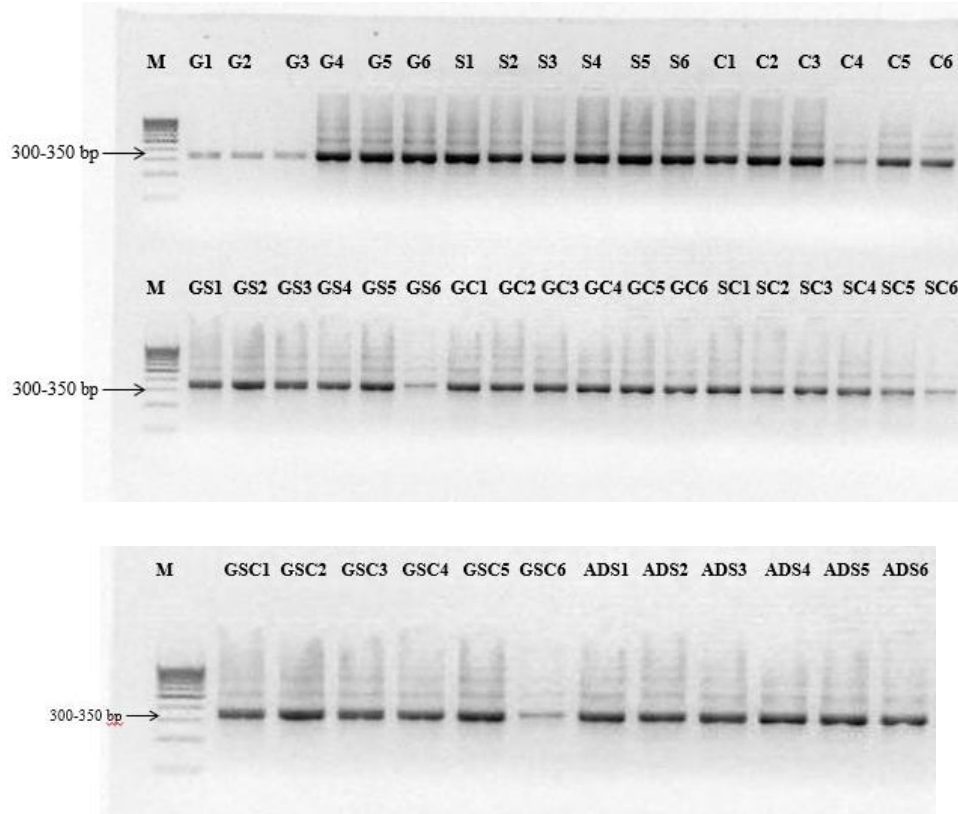


Figure 3.4. PCR products generated by PCR amplification of 16S rRNA genes from DNA extracted from cultures grown with different substrates. G: Glucose; S: Starch; C: Cellulose; GS: Glucose-Starch; GC: Glucose-Cellulose; SC: Starch-Cellulose; GSC: Glucose-Starch-Cellulose; ADS: ADS control. Numbers 1 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

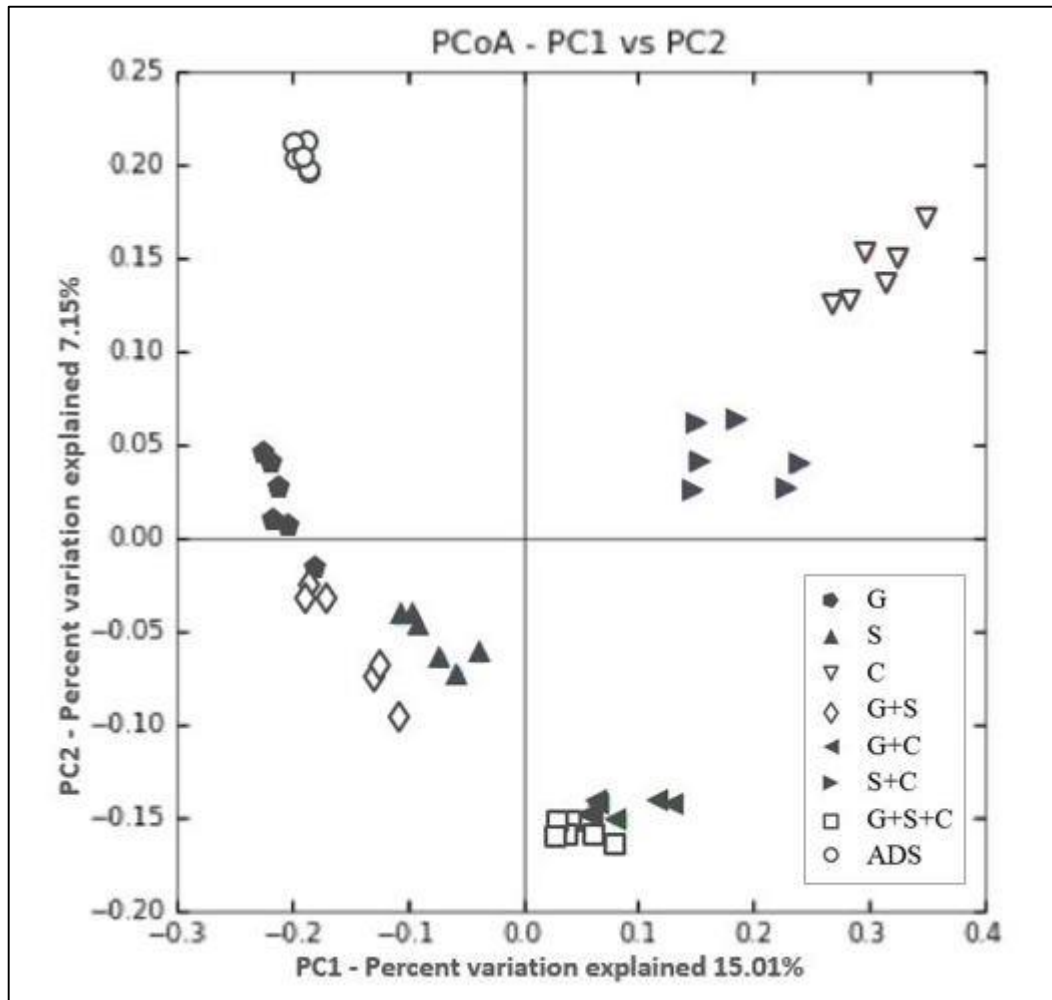


Figure 3.5. Principle co-ordinate analysis of unweighted UniFrac distances

be asserted that to the best of the authors knowledge, never before has microbial diversity been correlated statistically with a bioreactor performance measure.

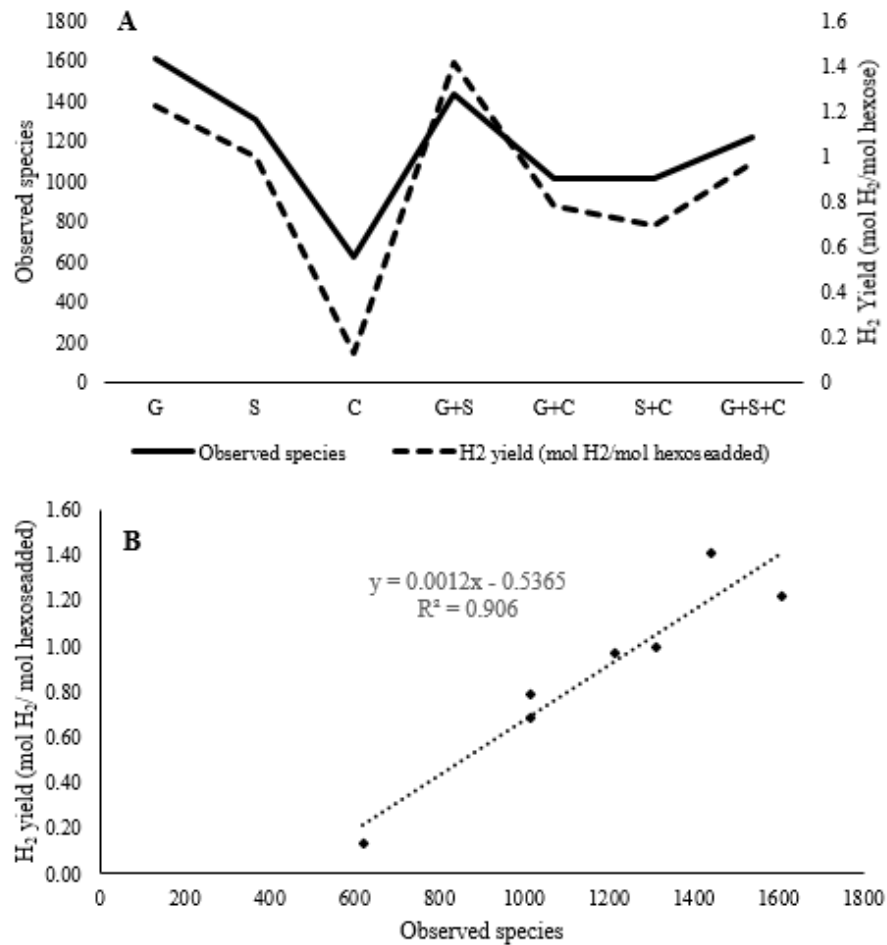


Figure 3.6. **A)** Trend of observed species and H₂ yield; **B)** Relationship between observed species and H₂ yield

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 H₂ production using anaerobic digester sludge. However, OTUs in the Phyla *Acidobacteria*, *Actinobacteria*, and *Bacteroidetes* 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						
<i>Clostridia</i> (c)			100			23	10
<i>Clostridiaceae</i> (f)	13	6				10	7
<i>Clostridium</i> (g)	53	20		51	10		9
<i>Ruminococcaceae</i> (f)	18			8	10	10	16
<i>Ruminococcus</i> (g)		46			37	24	42
<i>Ethanoligenens</i> (g)	1830			638			
<i>Streptococcus</i> (g)		6032					
<i>Lachnospiraceae</i> (f)			2175		281	827	227
<i>Bacteroides</i> (g)		314	595		728	671	555
<i>Parabacteroides</i> (g)		228	342		490	546	665
<i>Oscillospira</i> (g)				51	128		118
<i>Bifidobacterium</i> (g)				4666			
<i>Desulfovibrio</i> (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 H₂ producers, and these bacteria are known to produce the highest H₂ yields [Hawkes et al., 2002]. Fang et al. [2002] reported that, the majority of the species identified in a mesophilic, H₂-

producing sludge were *Clostridium* sp., and these bacteria have been studied for H₂ 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 H₂ 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 H₂ 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 H₂ [Azbar and Levin, 2012]. The presence of this strain explains maximum H₂ 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 H₂ 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 H₂, 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 H₂ and CO₂ (Equation 6) by the enzyme H₂ formate lyase:



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 H₂ 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 H₂ producing microbes [Azbar and Levin, 2012]. Nissilä et al. [2011] identified *Clostridia* and *Lachnospiraceae* as thermophilic, cellulolytic, H₂-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 disaccharides (maltose) or monosaccharides (glucose), which were then consumed by *Clostridium* species for H₂ 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, H₂ producers, and have been characterized by their diverse metabolic activity [Badiei et al., 2012]. *Streptococcus* sp. have been observed in a number of H₂ 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 H₂ 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 H₂-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 *Desulfovibrio* were enriched in glucose-cellulose and starch-cellulose cultures. *Desulfovibrio* 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 H₂, CO₂, 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 H₂ from lactate, ethanol, and/or formate [Martins et al., 2013]. Increased H₂ yields in the co-substrate cultures could be attributed to the presence of these species, as they have the potential to synthesize H₂ 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 H₂ 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 H₂ 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 *Bacteroidetes* were unique to only the co-substrate conditions.

3.5 References

1. Anderson, M.J., 2005. PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance. Department of Statistics, University of Auckland, New Zealand.
2. Azbar, N., Levin, D., 2012. State of the art and progress in production of biohydrogen. Bentham Science Publishers.

3. Badiei, M., Jahim, J.M., Anuar, N., Abdullah, S.R.S., Su, L.S., Kamaruzzaman, M.A., 2012. Microbial community analysis of mixed anaerobic microflora in suspended sludge of ASBR producing hydrogen from palm oil mill effluent. *Int. J. Hydrogen Energy*. 37 (4), 3169-3176.
4. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Pena, A.G.; Goodrich, J.K.; Gordon, J.I., 2010a. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7 (5), 335-336; DOI 10.1038/nmeth.f.303.
5. Caporaso, J.G.; Bittinger, K.; Bushman, F.D.; DeSantis, T.Z.; Andersen, G.L.; Knight, R., 2010b. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*. 26 (2), 266-267.
6. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M., 2012. Ultra-high-throughput microbial community analysis on the illumina HiSeq and MiSeq platforms. *The ISME Journal*., 6 (8), 1621-1624.
7. Chao, A., 1984. Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*. 11 (4), 265-270.
8. Chang, J.J., Wu, J.H., Wen, F.S., Hung, K.Y., Chen, Y.T., Hsiao, C.L., Lin, C.Y., Huang, C.C., 2008. Molecular monitoring of microbes in a continuous hydrogen-producing system with different hydraulic retention time. *Int. J. Hydrogen Energy*. 33 (5), 1579-1585.
9. Cheng, C.H., Hung, C.H., Lee, K.S., Liao, P.Y., Liang, C.M., Yang, L.H., Lin, P.J., Lin, C.Y., 2008. Microbial diversity structure of a starch-feeding

- fermentative hydrogen production reactor operated under different incubation conditions. *Int. J. Hydrogen Energy*. 33 (19), 5242-5249.
10. Chouari, R., Paslier, D.L., Daegelen, P., Ginestet, P., Weissenbach, J., Sghir, A., 2005. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. *Environ. Microbiol.* 7 (8), 1104-1115.
 11. Clesceri, L.S.; Greenberg, A.E.; Eaton, A.D., 1998. APHA, AWWA, WEF. Standard methods for the examination of water and wastewater. 20th ed.; American Public Health Association: Washington.
 12. Derakhshani, H., Khazanehei, H.R., Tun, H.M., Alqarni, S., Cardoso, F.C., Plaizier, J.C., Khafipour, E., Looor, J.J., 2014. The microbiome composition of the rumen is altered during the peripartal period in dairy cattle. *PloS One*. In press.
 13. DeSantis, T.Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E.L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Anderson, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72 (7), 5069-5072.
 14. Drancourt, M., Berger, P., Raoult, D., 2004. Systematic 16S rDNA sequencing of atypical clinical isolates identified 27 new bacterial species associated with humans. *J Clin Microbiol.* 42 (5), 2197-2202.
 15. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 27 (16), 2194-2200.

16. Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.*, 26 (19), 2460-2461.
17. Fang, H.H.P., Zhang, T., Liu, H., 2002. Microbial diversity of a mesophilic hydrogen-producing sludge. *Appl. Microbiol. Biotechnol.* 58 (1), 112-118.
18. Hallenbeck, P.C., Ghosh, D., Skonieczny, M.T., Yargeau, V., 2009. Microbiological and engineering aspects of biohydrogen production. *Indian J. Microbiol.* 49 (1), 48-59.
19. Hawkes, F.R., Dinsdale, R., Kawkes, D.L., Hussy, I., 2002. Sustainable fermentative hydrogen production: challenges for process optimization. *Int. J. Hydrogen Energy.* 27 (11-12), 1339-1347.
20. Ho, C.H., Chang, J.J., Lin, J.J., Chin, T.Y., Mathew, G.M., Huang, C.C., 2011. Establishment of functional rumen bacterial consortia (FRBC) for simultaneous biohydrogen and bioethanol production from lignocellulose. *Int. J. Hydrogen Energy.* 36 (19), 12168-12176.
21. Ho, K.L., Lee, D.J., Su, A., Chang, J.S., 2012. Biohydrogen from cellulosic feedstock: Dilution-to-stimulation approach. *Int. J. Hydrogen Energy.* 37 (20), 15582-15587.
22. Khafipour, E., Li, S., Plaizier, J.C., Krause, D.O., 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl. Environ. Microbiol.* 75 (22), 7115-7124.
23. Kleerebezem, R., Loosdrecht, M.C.M.V., 2007. Mixed culture biotechnology for bioenergy production. *Curr. Opin. Biotechnol.* 18 (3), 207-212.

24. Lay, J.J., 2001. Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. *Biotechnol. Bioeng.* 74 (4), 280-287.
25. Lay, C.H., Kuo, S.Y., Sen, B., Chen, C.C., Chang, J.S., Lin, C.Y., 2011. Fermentative biohydrogen production from starch-containing textile wastewater. *Int. J. Hydrogen Energy.* 37 (2), 2050-2057.
26. Lin, C.Y., Chang, C.C., Hung, C.H., 2008. Fermentative hydrogen production from starch using natural mixed cultures. *Int. J. Hydrogen Energy.* 33 (10), 2445-2453.
27. Logan, B.E., Oh, S.E., Kim, I.S., Ginkel, S.V., 2002. Biological hydrogen production measured in batch anaerobic respirometers. *Environ. Sci. Technol.* 36 (11), 2530-2535.
28. Lozupone, C.; Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71 (12), 8228-8235.
29. Martins, M., Pereira, I.A.C., 2013. Sulfate-reducing bacteria as new microorganisms for biological hydrogen production. *Int. J. Hydrogen Energy.* 38 (19), 12294-12301.
30. Masella, A.P.; Bartram, A.K.; Truszkowski, J.M.; Brown, D.G.; Neufeld, J.D., 2012. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics.*, 13 (1), 1-7.
31. Masset, J., Calusinska, M., Hamilton, C., Hiligsmann, S., Joris, B., Wilmotte, A., Thonart, P., 2012. Fermentative hydrogen production from glucose and

- starch using pure strains and artificial co-cultures of *Clostridium spp.* *Biotechnology for Biofuels*. 5 (35), 1-15.
32. Nasr, N., Elbeshbishy, E., Hafez, H., Nakhla, G., El-Naggar, M.H., 2011. Biohydrogen production from thin stillage using conventional and acclimatized anaerobic digester sludge. *Int. J. Hydrogen Energy*. 36 (20), 12761-12769.
33. Nissilä, M.E., Tähti, H.P., Rintala, J.A., Puhakka, J.A., 2011. Thermophilic hydrogen production from cellulose with rumen fluid enrichment culture: Effects of different heat treatments. *Int. J. Hydrogen Energy*. 36 (2), 1482-1490.
34. Ntaikou, I., Gavala, H.N., Kornaros, M., Lyberatos, G., 2008. Hydrogen production from sugars and sweet sorghum biomass using *Ruminococcus albus*. *Int. J. Hydrogen Energy*. 33 (4), 1153-1163.
35. Plugge, C.M., Zhang, W., Scholten, J.C.M., Stams, A.J.M., 2011. Metabolic flexibility of sulfate-reducing bacteria. *Front. Microbiol.* 2 (81), 1-8.
36. Price, M.N.; Dehal, P.S.; Arkin, A.P., 2010. FastTree 2—approximately maximum-likelihood trees for large alignments. *PloS one*. 5 (3), e9490.
37. Quéménur, M., Hamelin, J., Benomar, S., Guidici-Orticoni, M.T., Latrille, E., Steyer, J.P., Trably, E., 2011. Changes in hydrogenase genetic diversity and proteomic pattern in mixed-culture dark fermentation of mono-, di- and tri-saccharides. *Int. J. Hydrogen Energy*. 36 (18), 11654-11665.
38. Prakasham, R.S., Brahmaiah, P., Sathish, T., Rao, K.R.S.S., 2009. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *Int. J. Hydrogen Energy*. 34 (23), 9354-9361.

39. Ramachandran, U., Wrana, N., Cicek, N., Sparling, R., Levin, D.B., 2008. Hydrogen production and end-product synthesis pattern by *Clostridium termitidis* strain CT1112 in batch fermentation cultures with cellobiose or α -cellulose. *Int. J. Hydrogen Energy*. 33 (23), 7006-7012.
40. Ren, Z., Ward, T.E., Logan, B.E., Regan, J.M., 2007. Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species. *J. Appl. Microbiol.* 6 (103), 2258-2266.
41. Ren, N., Cao, G., Wang, A., Lee, D.J., Guo, W., Zhu, Y., 2008. Dark fermentation of xyloses and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int. J. Hydrogen Energy*. 33 (21), 6124-6132.
42. Song, Z.X., Dai, Y., Fan, Q.L., Li, X.H., Fan, Y.T., Hou, H.W., 2012. Effects of pretreatment method of natural bacteria source on microbial community and bio-hydrogen production by dark fermentation. *Int. J. Hydrogen Energy*. 37 (7), 5631-5636.
43. Ufnar, J.A., Wang, S.Y., Christiansen, J.M., Yampara-Iquise, H., Carson, C.A., Ellender, R.D., 2006. Detection of the *nifH* gene of *Methanobrevibacter smithii*: a potential tool to identify sewage pollution in recreational waters. *Journal of Applied Microbiology*. 101 (1), 44-52.
44. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73 (16), 5261-5267.

45. Wang, J., Wan, W., 2009 Factors influencing hydrogen production: a review. *Int. J. Hydrogen Energy*. 34 (2), 799-811.
46. Wang, A., Liu, L., Sun, D., Ren, N., Lee, D.J., 2010. Isolation of Fe(III)-reducing fermentative bacterium *Bacteroides* sp. W7 in the anode suspension of a microbial electrolysis cell (MEC). *Int. J. Hydrogen Energy*. 35 (7), 3178-3182.
47. Xia, Y.; Cai, L., Zhang, T., Fang, H.H.P., 2012. Effects of substrate loading and co-substrates on thermophilic anaerobic conversion of microcrystalline cellulose and microbial communities using high-throughput sequencing. *Int. J. Hydrogen Energy*. 37 (18), 13652-13659.
48. Xing, D., Ren, N., Wang, A., Li, Q., Feng, Y., Ma, F., 2008. Continuous hydrogen production of auto-aggregative *Ethanoligenens harbinense* YUAN-3 under nonsterile conditions. *Int. J. Hydrogen Energy*. 33 (5), 1489-1495.
49. Warwick, R.; Clarke, K. PRIMER 6. PRIMER-E Ltd, Plymouth. 2006.

Chapter 4

Sensitivity of Mesophilic Biohydrogen-Producing Cultures to Temperature

Shocks²

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 °C) and thermophilic (55 °C) temperatures using starch as substrate (16 gCOD/L), pH of 8.5, municipal sewage sludge as inoculum, and observed a higher yield at 37°C than at 55°C (0.96 vs. 0.26 mol H₂/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°C and pH 5.5) and obtained a yield of 0.55 mol H₂/mol hexose at thermophilic temperature

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

(55°C) and 0.33 mol H₂/ mol hexose at 37°C. Puhakka et al. [2012] studied a comparison between mesophilic (37 °C) and thermophilic (55 °C) temperatures using intermediate temperature (45 °C) hot spring sample as inoculum and glucose as substrate, and obtained yield of 1.25 and 1.0 mol H₂/mol hexose for mesophilic and thermophilic conditions, respectively. Kim and Kim [2012] used mesophilic anaerobic digester sludge acclimatized with glucose at 60°C, starch as substrate, and observed a H₂ yield of 1.78 mol H₂/mol hexose at 60°C. 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 °C for thermophilic batches, and observed H₂ yields of 0.47 and 0.81 mol H₂/mol hexose at mesophilic (35 °C) and thermophilic (55 °C) 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 H₂ 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 55°C. Cellulose-only yielded 0.03 mol/mol hexose, whereas, yields of 0.16, 0.19, and 0.53 mol H₂/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 H₂/mol substrate) to be not significantly different from the mono-substrate conditions (glucose, 2.42 mol H₂/mol substrate; xylose, 2.19 mol H₂/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 °C -37 °C and thermophilic range between 55 °C -70 °C. 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 (37°C) and thermophilic (60°C) 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. (°C)	H ₂ yield	Reference
Municipal sewage sludge	Starch (16 gCOD/L)	8.5	Batch	37 55	0.96 0.28	Lee et al. 2008
Mesophilic sucrose fed wastewater	Starch (4.6 g/L)	7	Batch	37 55	0.33 0.55	Zhang et al. 2003
Sediment from hot (45°C) spring	Glucose (9 g/L)	6.5 7.5	Batch	37 55	1.25 1.00	Puhakka et al. 2012
Mesophilic anaerobic digester sludge acclimatized with glucose (10 gCOD/L), pH 5.5 at 60°C	Starch (3 gCOD/L) Glucose (3 gCOD/L)	6.8	Batch	60	1.78 2.69	Kim and Kim 2012
Mesophilic anaerobic digester sludge				37	1.60	
Mesophilic anaerobic digester sludge acclimatized with glucose at 55°C	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	Batch	60	2.00	Ueno et al. 2001
		6.4	Chemostat HRT 3 day		2.00	
Co-culture <i>Clostridium thermocellum</i> and <i>Thermoanaerobacterium thermosaccharolyticum</i>	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 55°C	Micro-crystalline cellulose (4 g/L)				0.03	
	Microcrystalline cellulose (4 g/L) + Glucose (0.4 g/L)	6.6	Sequential batch	55	0.16	Xia et al. 2012
	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 55°C				55	0.81	

Inoculum	Substrate	pH	Reactor	Temp. (°C)	H₂ yield	Reference
	Cassava starch (5 g/L)		Batch		0.90	
Sediment sample from geothermal spring 60°C	Cassava starch (5 g/L)	5.5	Repeated batch	60	1.68	O-Thong et al. 2011
	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]. NaHCO₃ 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:

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

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. NaHCO₃ 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 H₂ production for the different substrates and temperature conditions. The highest cumulative H₂ 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 H₂ 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.

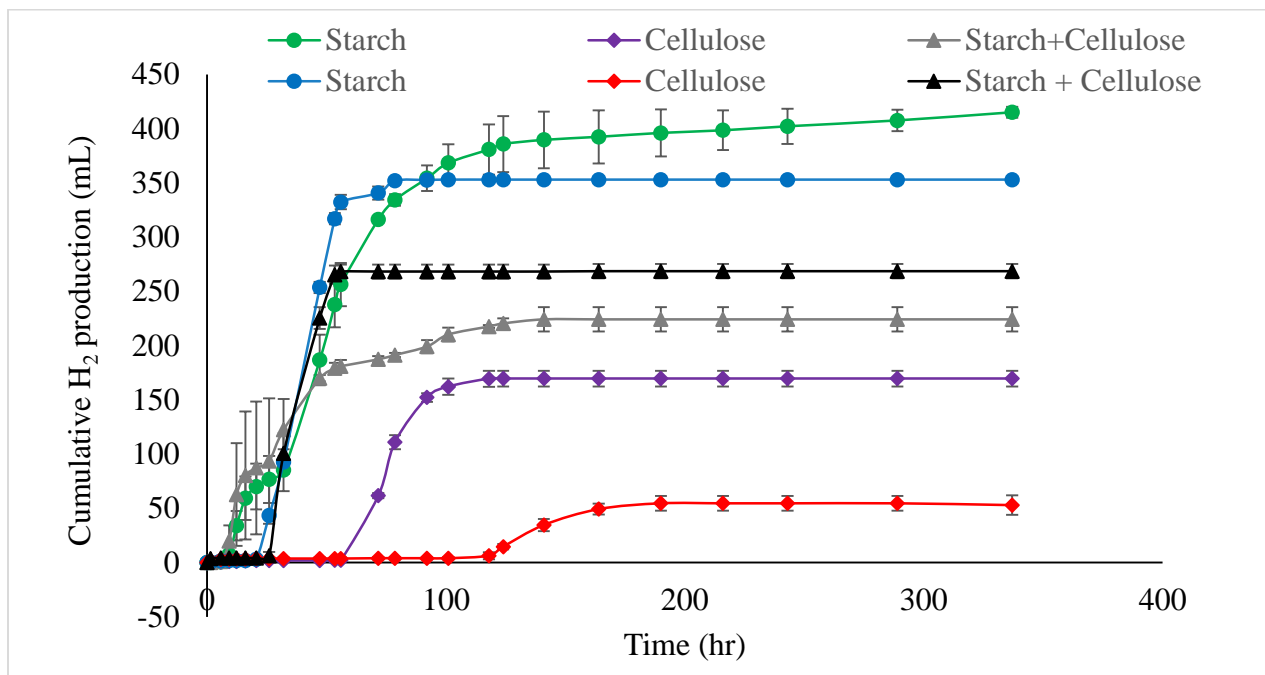


Figure 4.1. Cumulative hydrogen production. Solid symbols are thermophilic and hollow symbols are mesophilic

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 H₂ production which affects the duration of lag phase [Puhakka et al., 2012]. For mono-substrate starch and cellulose, higher H₂ 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 H₂ yields for the different substrates and temperature conditions.

Table 4.2. H₂ 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 H₂ yield of 1.13 mol H₂/mol hexose_{added} was observed for starch only at 60°C whereas, the mesophilic yield was 1 mol H₂/mol hexose_{added}. The

thermophilic cellulose-only yield of 0.42 mol H₂/mol hexose_{added} was almost 3 times the mesophilic yield (0.13 mol H₂/mol hexose_{added}). Starch-cellulose combination gave yields of 0.58 and 0.69 mol H₂/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 H₂ yields of 0.19 mol H₂/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 H₂ 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 °C) and thermophilic (55°C) temperature, and observed H₂ yields of 0.96 and 0.28 mol H₂/mol starch. Kim and Kim [2012] did a similar study and used mesophilic seed sludge at thermophilic temperatures and assessed H₂ production potential from starch. The aforementioned authors achieved a yield of 1.78 mol H₂/mol hexose, however the initial pH was 6.8 and the mesophilic seed sludge was acclimatized first. Ueno et al. [2001] achieved 2 mol H₂/mol hexose using cellulose powder as substrate (5 g/L) and anaerobic

microflora from sludge compost acclimatized to thermophilic (60°C) 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 H₂/mol hexose using cellulose (5 g/L) as substrate at pH 7 and using cow rumen fluid as inoculum at 60°C.

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.

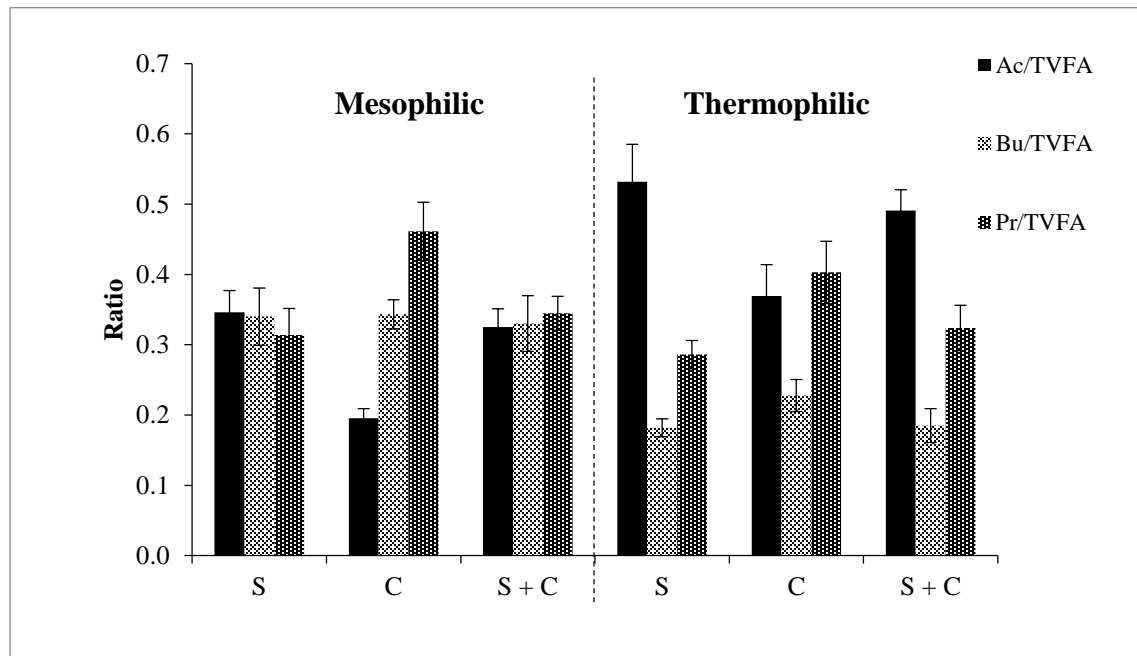
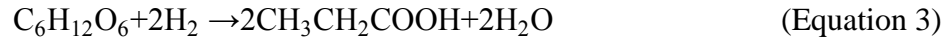


Figure 4.2. VFAs ratios at the fermentation end-point

The main VFAs detected in all the experiments were acetate, butyrate and, propionate. Acetate pathway (Equation 1) of H₂ 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 H₂ consumption (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 H₂ 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 H₂ 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 H₂ production. Shin et al. [2004] evaluated H₂ production using mesophilic and thermophilic acclimatized acidogenic cultures at pH 5.5 from food waste and observed negligible propionate concentrations at thermophilic temperature (55°C) compared to mesophilic temperature (35 °C) 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 (37°C) and thermophilic (55°C) temperatures using glucose as substrate (9 g/L) and sediment sample from a geothermal hot spring (45°C) as the inoculum, and observed different distribution of soluble metabolites at the two different temperature conditions, where butyrate was produced in low concentrations at 37°C and not detected at 55°C. Additionally, the aforementioned authors observed less acetate at 37°C as compared to 55°C. 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 H₂/g acetate, 0.58 L H₂/ g butyrate and 0.34 L H₂ consumed/ g propionate (Equation 1, 2, and 3), theoretical H₂ production from VFAs was calculated. The theoretical values shown in Table 4.3 were consistent with the H₂ 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 H₂ 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 H₂ produced (8 gCOD/g H₂), 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 H₂ yield of 1.13 mol H₂/mol hexose_{added} was observed for starch only at 60°C whereas, at 37 °C the yield was 1 mol H₂/mol hexose_{added}. The thermophilic cellulose-only yield of 0.42 mol H₂/mol hexose_{added} was almost 3 times the mesophilic yield (0.13 mol H₂/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.

4.5 References

1. Puhakka, J.A., Karadag, D., Nissilä., 2012. Comparison of mesophilic and thermophilic anaerobic hydrogen production by hot spring enrichment culture. *Int J Hydrogen Energy*. 37: 16453-16459.
2. Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I., 2002. Sustainable fermentative hydrogen production: challenges for process optimization. *Int J Hydrogen Energy*. 27:1339-1347.
3. Wang, J., Wan, W., 2009. Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy*. 34: 799-811
4. Lee, K.S., Hsu, Y.F., Lo, Y.C., Lin, P.J., Lin, C.Y., Chang, J.S., 2008. Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *Int J Hydrogen Energy*. 33: 1565-1572.
5. Zhang, T., Liu, H., Fang, H.H.P., 2003. Biohydrogen production from starch in wastewater under thermophilic condition. *J Environ Management*. 69: 149-156.
6. Kim, D.H., Kim, M.S., 2012. Thermophilic fermentative hydrogen production from various carbon sources by anaerobic mixed cultures. *Int J Hydrogen Energy*. 37:2021-2027.
7. Kargi, F., Eren, N.S., Ozmihci, S., 2012. Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations. *Int J Hydrogen Energy*. 37:8338-8342.

8. Prakasham, R.S., Brahmaiah, P., Sathish, Rao K.R.S.S., 2009. Fermentative biohydrogen production by mixed anaerobic consortia: Impact of glucose to xylose ratio. *Int J Hydrogen Energy*. 34:9354-9361.
9. Xia, Y., Cai, L., Zhang, T., Fang, H.H.P., 2012. Effects of substrate loading and co-substrates on thermophilic anaerobic conversion of microcrystalline cellulose and microbial communities revealed using high-throughput sequencing. *Int J Hydrogen Energy*. 37: 13652-13659.
10. Ren, N., Cao, G., Wang, A., Lee, D.J., Guo, W., Zhu, Y., 2008. Dark fermentation of xylose and glucose mix using *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energy*. 33:6124-6132.
11. O-Thong, S., Hniman, A., Prasertsan, P., Imai, T., 2011. Biohydrogen production from cassava wastewater by thermophilic mixed cultures. *Int J Hydrogen Energy*. 34:3409-3416.
12. Shin, H.S., Youn, J.H., Kim, S.H., 2004. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *Int J Hydrogen Energy*. 29:1355-1363.
13. Cheong, D.Y., Hansen, C.L., 2007. Feasibility of hydrogen production in thermophilic mixed fermentation by natural anaerobes. *Bioresour. Technol*. 98:2229-2239.
14. Cakir, A., Ozmihci, S., Kargi, F., 2010. Comparison of bio-hydrogen production from hydrolyzed wheat starch by mesophilic and thermophilic dark fermentation. *Int J Hydrogen Energy*. 35:13214-13218.

15. Akutsu, Y., Li, Y.Y., Tandukar, M., Kubota, K., Harada, H., 2008. Effects of seed sludge on fermentative characteristics and microbial community structures in thermophilic hydrogen fermentations of starch. *Int J Hydrogen Energy*. 33:6541-6548.
16. Ueno, Y., Haruta, S., Ishii, M., Igarashi, Y., 2001. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. *Appl. Microbiol. Biotechnol.* 57:555-562.
17. Liu, Y., Yu, P., Song, X., Qu, Y., 2008. Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. *Int J Hydrogen Energy*. 33:2927-2933.
18. Nissila, M.E., Tahti, H.P., Rintala, J.A., Puhakka, J.A., 2011. Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: Effects of different heat treatments. *Int J Hydrogen Energy*. 36:1482-1490.
19. Nasr, N., Elbeshbishy, E., Hafez, H., Nakhla, G., El Naggar, M.H., 2011. Biohydrogen production from thin stillage using conventional and acclimatized anaerobic digester sludge. *Int J Hydrogen Energy*. 36:12761-12769.
20. Hafez, H., Nakhla, G., El Naggar, M.H., Elbeshbishy, E., Baghchehsaraee, B., 2010. Effect of organic loading on a novel hydrogen bioreactor. *Int J Hydrogen Energy*. 35:81-92.
21. Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998. APHA, AWWA, WEF. *Standard methods for the examination of water and wastewater*. 20th ed.; American Public Health Association: Washington.

22. O-Thong, S., Prasertsan, P., Birkeland, N.K., 2009. Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis. *Bioresour. Technol.* 100:909-918.

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 H₂ 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 *Bacteroidetes* were unique to only the co-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.

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 H₂ yield of 1.13 mol H₂/mol hexose_{added} was observed for starch only at 60°C whereas, at 37 °C the yield was 1 mol H₂/mol hexose_{added}. The thermophilic cellulose-only yield of 0.42 mol H₂/mol hexose_{added} was almost 3 times the mesophilic yield (0.13 mol H₂/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 <i>Expected date of graduation: August 2014</i> 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 2012-2014 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, University of Western Ontario <ul style="list-style-type: none">• 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, University of Windsor <ul style="list-style-type: none">• 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

Gupta, M., Velayutham, P., Elbeshbishy, E., Hafez, H., EKhafipour, E., Derakhshani, H., El Nagggar, M.H., Levin, D.B., George, Nakhla. (2014) Co-fermentation of glucose, starch, and cellulose for mesophilic biohydrogen production. *Submitted to International Journal of Hydrogen Energy*.

Gupta, M., Nasr, N., Elbeshbishy, E., Hafez, H., El Nagggar, M.H., Nakhla, G. (2014) Sensitivity of mesophilic biohydrogen-producing cultures to temperature shocks. *Submitted to International Journal of Hydrogen Energy*.

Junior, A.D.N.F., Zaiat, M., **Gupta, M.**, Elbeshbishy, E., Hafez, H., Nakhla, G. (2014) Impact of organic loading rate on biohydrogen production in an up-flow anaerobic packed bed reactor (UAnPBR). *Bioresource Technology*. 164: 371-379.

Nasr, N., **Gupta, M.**, Elbeshbishy, E., Hafez, H., El Nagggar, M.H., Nakhla, G. (2014) Biohydrogen production from pretreated corn cobs. *Submitted to International Journal of Hydrogen Energy*.

Gupta, M., Elbeshbishy, E., Hafez, H., George, Nakhla., El Nagggar, M.H. (2014) Effect of co-substrate digestion on mesophilic biohydrogen production using anaerobic digester sludge. Poster in proceedings of 2nd Waterloo International Conference, March 17, Waterloo, Ontario, Canada. 2nd best poster presentation award.

Gupta, M., Velayutham, P., Elbeshbishy, E., Hafez, H., Khafipour, E., Derakhshani, H., El Naggari, M.H., Levin, D.B., George, Nakhla. (2014) Mesophilic biohydrogen production and microbial community analyses from co-fermentation of glucose, starch, and cellulose. Poster in Advanced Biofuels Symposium, May 27-29, 2014, Ottawa, Ontario, Canada.