August 2016

Biohydrogen and biomethane production from lignocellulosic biomass

Chinaza Okeoghene Akobi
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

Abstract

The main purpose of this study was to investigate the impact of furfural on mixed cultures during fermentative hydrogen production from lignocellulosic biomass. Small batch studies using synthetic lignocellulosic hydrolysate grown on mesophilic mixed cultures, revealed a threshold furfural concentration of greater than 1 g/L with enhancement to the yields (from the control) observed at 0.5 g/L furfural (at initial substrate-to–biomass (S°/X°) ratios of 0.5 and 1 gCOD/gVSS) and at both 0.5 g/L and 1 g/L furfural (at S°/X° of 2 and 4 gCOD/gVSS). This study was scaled-up from 200 mL to 11 L working volume batches, using half the substrate concentration of the small batch studies, at an S°/X° of 4 gCOD/gVSS in order to determine the Monod microbial kinetics of mixed cultures in the presence of furfural at both mesophilic and thermophilic temperatures. A 45 % enhancement at 1 g/L furfural was observed in the mesophilic experiment but a 50 % reduction at the same furfural concentration was observed at thermophilic conditions both relative to the yields from their respective controls. Enhanced kinetics observed in the control without furfural at both temperatures emphasized that although furfural is indeed an inhibitor, it can be broken down at low concentrations by mesophilic hydrogen-producers to increase hydrogen yields. Liquid and solid real waste hydrolysates obtained from poplar wood biomass treated using twin-screw extrusion technology were evaluated for their biohydrogen potential and the feasibility of a two-stage anaerobic digestion process. This study proved that acidification of the first-stage biohydrogen production process brought about a 50 % increase (on average) in $TVFA/SCOD_{initial}$ which enhanced methane yields in the second-stage. In the two-stage anaerobic digestion process, energy yields were 33 % and 18 % higher, while feedstock COD removal efficiencies were 16 % and 14 % higher than the single-stage BMP tests for the liquid and solid samples respectively.

Keywords

Furfural, biohydrogen, biomethane, mesophilic, thermophilic, lignocellulosic hydrolysates, batch, kinetics, twin screw extrusion, anaerobic digestion

Co-Authorship Statement

Chapter 3: Biological hydrogen production from synthetic lignocellulosic hydrolysate using mesophilic anaerobic digester sludge: Impact of furfural

Chinaza Okeoghene Akobi, Hisham Hafez, George Nakhla,

This chapter has been submitted to the *Bioresource Technology* journal

Chinaza Okeoghene Akobi: experimental design, laboratory work, data analysis and interpretation, and paper writing.

Dr. Hisham Hafez: paper review

Dr. George Nakhla: supervision, critical data interpretation, paper review, and corrections.

Chapter 4: Impact of furfural on biological hydrogen production kinetics from synthetic lignocellulosic hydrolysate using mesophilic and thermophilic mixed cultures

Chinaza Okeoghene Akobi, Hisham Hafez, George Nakhla

A version of this chapter has been submitted to the *Renewable Energy* journal

Chinaza Okeoghene Akobi: experimental design, laboratory work, data analysis and interpretation, modeling, and paper writing.

Dr. George Nakhla: supervision, critical data interpretation, paper review, and corrections.

Dr. Hisham Hafez: paper review.

Chapter 5: Single-stage and two-stage anaerobic digestion of extruded lignocellulosic biomass

Chinaza Okeoghene Akobi, Hisham Hafez, George Nakhla

This chapter has been submitted to the *Applied Energy* journal.

Chinaza Okeoghene Akobi: experimental design, laboratory work, data analysis and interpretation, and paper writing.

Dr. George Nakhla: supervision, critical data interpretation, paper review, and corrections.

Dr. Hisham Hafez: paper review.

To my husband, Tochukwu for being a great support system for me these past few months,

To my son, Naeto for his adorable smiles, patience and understanding,

To my parents & parents-in-law for their endless love and prayers.

Acknowledgments

First and foremost, I thank God Almighty for His sufficient grace, favour and mercy towards me and for seeing me through a successful completion of this program.

I would like to express my heartfelt gratitude to my supervisor, Dr. George Nakhla, for the research opportunity, support, guidance, and mentorship throughout my studies. I also like to thank Dr. Hisham Hafez, for his recommendations and advice especially during the GreenField project. I am grateful to all members of Dr. Nakhla's research group, especially to Maritza Gomez-flores for her tremendous help with the kinetic modeling, Noha Nasr and Medhavi Gupta for being ever so helpful and to Hyeongu Yeo and Basem Haroun whom I worked with and learned a lot from, during the latter part of my program. My thanks also go to Mingu Kim and Mohammad Chowdhury for their support during my experimental work and to Eric Liu for his assistance with some data analysis. Special thanks to my long time friend, Nosakhare Imuwahen, for taking out time to carefully edit my work.

I am grateful to Ashley Jokhu, Cole Handsaeme, Brian Dennis, Paul Sheller, Stephen Mallinson and Nada Brkljac for their cheerfulness and unmatched willingness to help and support me in the little way they could. The financial support from Western Engineering, Mitacs and GreenField Specialty Alcohols is gratefully acknowledged.

I also want to appreciate the tremendous love, support and encouragement from my husband, Tochukwu Akobi and son, Naeto Akobi as well as the prayers, advice and kind words of my parents, Engr. and Mrs G. O. Anyachebelu as well as my parents-in –law, Rev and Rev. (Mrs) I. Akobi. Shout out to my siblings, friends and family for their constant support and words of encouragement.

I appreciate you all.

Funding Acknowledgments

The author would like to acknowledge the financial support provided by GreenField Specialty Alcohols Inc., Mitacs Inc., and Natural Sciences and Engineering Research Council of Canada (NSERC) throughout the course of this work.

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Nomenclature

$C_{H2,\ i}$	Fraction of gas in headspace of reactor in present interval
$C_{H2,\ i-1}$	Fraction of gas in headspace of reactor in preceding interval
IC50	Inhibitor concentration that elicited 50 % reduction in maximum production rate
K_s	Saturation constant or half-velocity constant (g/L)
P	H_2 production potential (mL)
R_{max}	Maximum H_2 production rate (L/d or ml/h)
$V_{G,i}$	Total gas volume accumulated (mL)
$V_{H2,i}$	Present cumulative H_2 gas volume (mL)
$V_{H2,i-1}$	Preceding cumulative H_2 gas volume (mL)
$V_{h,i}$	Total volume of the headspace of the reactor (mL)
$Y_{X/S}$	Biomass yield (g VSS g COD)
μ_{max}	Maximum specific growth rate (h^{-1} or d^{-1})
λ	Lag phase (h or d)

Abbreviations

ANOVA	Analysis of Variance
APE	Average Percent Error
BHP	Biohydrogen Potential
BMP	Biomethane Potential
COD	Chemical Oxygen Demand
HBA	Hydroxylbenzoic acid
HMF	5-Hydroxylmethyl furfural
HSD	Honestly Significant Difference
RID	Refractive Index Detector
RMSE	Root Mean Square Error
RPM	Revolutions Per Minute
SCOD	Soluble Chemical Oxygen Demand
SHPR	Specific Hydrogen Production Rate
SMPR	Specific Methane Production Rate
SPSS	Statistical Package for the Social Sciences
SSE	Sum of Square Errors
S°/X°	Substrate to Biomass
TCD	Thermal Conductivity Detector
TSE	Twin Screw Extruder
TSS	Total Suspended Solids
TVFA	Total Volatile Fatty Acids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

CHAPTER 1

1 Introduction

1.1 Background

Biohydrogen has been described as the key energy carrier for the future. It is produced from carbohydrate-rich substrates through biological means using microorganisms such as bacteria or algae. Lignocellulosic materials (such as corn stover, sugar cane bagasse, and poplar wood) are generally found in abundance as agricultural or industrial by-products with little or no commercial value. In agriculture, most of these wastes are left unused on the fields after harvest creating environmental problems and a waste of potential renewable resource.

Lignocellulosic biomass consists of complex carbohydrate (sugar) compounds and not just simple sugars (Monlau et al., 2013). These molecules need to be broken down using pretreatment methods into simpler forms for easy conversion to hydrogen gas. Dilute acid pretreatment has been widely used with respect to biohydrogen production as it is considered the easiest, most efficient, and cost-effective method that produces high sugar yields and favorably changes the structure of the substrate to facilitate fermentation (Mosier et al., 2005; Willfor et al., 2005; Wyman et al., 2005). However, this process generates fermentation inhibitors such as furan derivatives (aldehydes such as furfural and hydroxylmethylfurfural), ketones, phenols and organic acids. Furfural is considered to be a limiting factor in the biological conversion of lignocellulosic materials due to its adverse inhibitory effects on the microorganism's membrane integrity and biohydrogen production rates and yields (Siqueira and Reginatto, 2015).

This research was divided into three parts. Chapter 3 employs the use of mesophilic cultures to assess the impact of 0.5 g/L, 1 g/L, 2 g/L and 4 g/L furfural on biohydrogen production rates and yields, from synthetic lignocellulosic hydrolysate with sugars concentration of 65.4 g/L, at initial

substrate-to-biomass (S°/X°) ratios of 0.5, 1, 2 and 4 gCOD/gVSS. This study was scaled up from 250 mL bottles to 15 L anaerobic continuously-stirred tank reactors operated as batch system using half the concentration of the substrate utilized in Chapter 3, with furfural concentrations of 0 g/L, 1 g/L and 4 g/L which were run in parallel with strict pH control. Samples were taken with time in order to investigate sugars degradation and metabolites formation with time as well as model microbial growth and product formation in the presence of furfural using Monod kinetics ran on MATLAB® software which form Chapter 4 of this thesis. Having tested synthetic lignocellulosic waste in two studies, the author opted to try a real waste hydrolysate. Biohydrogen and biomethane tests were carried out on hydrolysate samples obtained from the Twin Screw Extruder (TSE) treating poplar wood biomass, operated at GreenField Specialty Alcohols Inc. A single-stage biomethane protential (BMP) test and a two-stage anaerobic digestion process (first-stage biohydrogen potential (BHP) test followed by a second-stage BMP test) were evaluated.

1.2 Problem statement

Over the past few decades, a lot of research has been carried out on biological hydrogen production. There has also been increased focus on the use of lignocellulosic wastes for biogas production as they are available, renewable, cheap, and rich in sugars. The pretreatment of this biomass prior to fermentation have been shown to liberate potential refractory compounds such as furfural which adversely affect biogas production rates and yields.

It is of importance to thoroughly understand furfural's threshold inhibition levels and how this compound affects microbial growth as well as biogas production rates and yields using mixed cultures at mesophilic and thermophilic temperatures. The knowledge of these parameters would enable the optimization of biogas production in order to improve the efficiency of the fermentation

process as well as provide a basis for better reactor design, more efficient control and effective scale-up of process systems.

1.3 Research objectives

The main goals of this research were:

- To assess the impact of furfural on biohydrogen production from lignocellulosic hydrolysate using mixed cultures
- To ascertain the threshold furfural concentrations that resulted in the maximum hydrogen production, rates and yields
- To obtain the kinetic parameters of mixed mesophilic and thermophilic cultures as well as metabolites formation in the presence of furfural
- To prove that acidification in the first-stage biohydrogen production process increases COD removal efficiency, anaerobic biodegradability and energy yields in the second-stage BMP process compared with a single-stage BMP process

1.4 Thesis organization

This thesis comprises six 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 six chapters are as follows:

Chapter 1 presents the general introduction including research objectives and research contributions.

Chapter 2 presents the literature review on biohydrogen and biomethane production from lignocellulosic wastes

Chapter 3 presents my first research article that has been submitted to the *Bioresource Technology* journal entitled "Biological hydrogen production from synthetic lignocellulosic hydrolysate using mesophilic anaerobic digester sludge: Impact of furfural"

Chapter 4 presents my second research article that has been modififed and submitted to the *Renewable Energy* journal entitled "Impact of furfural on biological hydrogen production kinetics from synthetic lignocellulosic hydrolysate using mesophilic and thermophilic mixed cultures"

Chapter 5 presents my third research article that has been submitted to the *Applied Energy* journal entitled "Single-stage and two-stage anaerobic digestion of extruded lignocellulosic biomass"

Chapter 6 summarizes the main conclusions and recommendations for future work based on the results from all aspects of this research.

1.5 Research contributions

Furfural, as a degradation by-product of lignocellulosic biomass pretreatment, has not been thoroughly studied in terms of inhibition threshold concentration and its effects on the kinetic parameters of mixed cultures. Therefore, the main contributions of this research include:

- Emphasizing the impact of S°/X° and furfural inhibition on fermentative hydrogen production using mixed cultures
- Providing the microbial and product formation kinetics of mesophilic and thermophilic cultures grown on lignocellulosic hydrolysate

- Demonstrating the impact of acidification by comparing a single-stage biomethane production process with a second-stage biomethane production process preceded by first-stage biohydrogen production step using poplar wood biomass

1.6 References

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CHAPTER 2

2 Literature Review

2.1 Introduction

With increasing worldwide concern over the use of fossil fuels to generate electricity and power machinery, there has emerged the need to consider alternative sources of energy. Global technologies for energy production and supply, at present, rely heavily on fossil fuels causing rapid depletion of these resources and increasing carbon dioxide (CO_2) emissions as energy consumption continues to rise (IEA, 2010; Kapdan and Kargi, 2006). It is believed that the biological production of hydrogen (biohydrogen), which is a renewable form of energy, can mitigate this trend and alleviate concerns involved with fossil fuel use. This process makes use of readily available, low-value substrates (non-food crops or wastes) as feedstocks thereby eliminating the competition for land used to produce food or feed (Monlau et al., 2013a).

2.2 Why Biohydrogen?

Bioethanol, biobutanol, biodiesel and biohydrogen are biofuels which are viable alternative fuels compared to carbon-based fuels (Carere et al., 2008). Biohydrogen has been singled out from this bunch, as it is considered to be a clean energy carrier since it produces only water as waste product when it burns thus implying zero CO_2 emissions (Sung et al. 2003). Biological hydrogen production methods are thus more environmentally-friendly, and less energy intensive compared to current methods of production such as steam reforming of hydrocarbons and electrolysis of water (Kapdan and Kargi, 2006). Biohydrogen has therefore attracted global attention as it has the prospects of becoming an inexhaustible and low cost renewable energy carrier. Even though research in this area is still in its infancy, there is increased world interest as the prospects seem promising (Show et al., 2012).

Forsberg (2007) considers hydrogen to be the ultimate transport fuel due to its non-polluting nature. It also possesses high energy (about three times more energy per unit mass than petrol or diesel (Pattra et al., 2008)) which can be stored in fuel cells to produce electricity that can be used to power cars, appliances and machinery. One kg of hydrogen has a high heating value of 142 MJ and can replace almost 3.55 L of conventional diesel in terms of energy value (Koroneos et al., 2004; Sarma et al., 2013). Hydrogen can either be used in internal combustion engines or in fuel cells for energy generation (Brar and Sarma, 2013). Currently, most of the hydrogen produced is generated using steam reforming of oil and gas (Armor, 1999) or coal gasification (Stiegel and Ramezan, 2006) which are fossil-fuel reliant, unsustainable, incur high production costs, and cause environmental pollution. Hydrogen can also be produced from renewable sources (especially wastes). While utilizing these wastes to produce valuable products (such as energy recovery), issues associated with waste treatment and land pollution caused by disposal to landfills, can be simultaneously resolved (Duff and Murray, 1996; Panagiotopoulous et al., 2009; Saratale et al., 2008). Thus, biological hydrogen (biohydrogen) production from waste is fast-gaining significant global attention. For these reasons, biohydrogen has been described as the key energy carrrier for the future (Kapdan and Kargi, 2006).

2.3 Biological hydrogen production

Biohydrogen is hydrogen produced from biological means using microorganisms such as bacteria or algae. Hydrogen can be produced biologically through the following processes:

2.3.1 Photolysis*:*

This is a light-dependent process that occurs when cyanobacteria (or algae) split water during photosynthesis into hydrogen and oxygen in the presence of sunlight. The advantage of this process is that water is the main feed material which is inexpensive and readily available. Also, 98 % pure hydrogen gas has been produced using the direct photolysis method

(Hankamer et al., 2007). The downside to this process is that it requires a large bioreactor, micro-organisms have to do more metabolic work, and the hydrogen yields are low.

2.3.2 Photo fermentation: This is another light-dependent method in which 90 % of product gas is hydrogen and the process releases no hydrogen sulphide or carbon monoxide. Here, photo-heterotrophs (eg. purple bacteria) convert organic acids in the presence of sunlight into H_2, CO_2 and carbon compounds. The main enzymes utilized by these bacteria are the nitrogenases that require nitrogen-scarce conditions for hydrogen production. Disadvantages of this process include the use of costly bio-reactors, dependence on ATP-consuming nitrogenases and lack of efficiency of light-harvesting antennae (Mathews and Wang, 2009).

2.3.3 Microbial Electrolysis Cell (MEC) is another promising technique employed for biohydrogen production. In this method, microorganisms oxidize substrates such as acetate to give protons, electrons, and by-products such as bicarbonate. This reaction occurs at the anode chamber of the cell, where the protons are then reduced to hydrogen at the cathode, either by adding voltage to the circuit through power supply or by setting the anode potential using a potentiostat (Nam et al., 2011; Xiao et al., 2012). The major challenges of the MEC technology include low hydrogen production rates and high energy inputs (Kadier et al., 2016).

2.3.4 Dark fermentation is the main light-independent process for biohydrogen production. In this method, anaerobic bacteria consume sugars to produce H_2, CO_2, and organic acids. It is considered the most favorable process since hydrogen is produced at a higher rate and at low cost (Show et al., 2012). The process can be carried out in simple reactors, requires no light energy and can be used on a wide range of substrates at non-aseptic conditions (Hallenbeck et al., 2012; Wang and Wan, 2008; Valdez-Vazquez *et al.*, 2005).

A yield of 12 mol H_2 per mol hexose is the maximum stoichiometric yield that can be obtained using glucose as the model substrate (Macaskie and Redwood, 2008):

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12\ H_2 + 6CO_2 \qquad 2.\ 1$$

In biohydrogen production, no organism has been known to be capable of performing this conversion with this much efficiency. Thermodynamically, the maximum yield for dark fermentation is 4 mol H_2 per mol hexose (the Thauer limit, Thauer et al., 1977), where carbohydrates are converted into hydrogen gas and organic pollutants such as volatile fatty acids (VFAs) and alcohols (Macaskie and Redwood, 2008). This value is the maximum theoretical hydrogen yield that can be obtained through the acetic pathway (See equation 2.2). Other reactions commonly encountered from glucose as substrate during dark fermentative hydrogen production are presented in equations 2.3 - 2.6. According to equation 2.3, the butyrate pathway involves hydrogen production with a theoretical maximum yield of 2 mol H_2/mol glucose (Guo et al., 2010).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad 2.2$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad 2.3$$

The propionate pathway is, however, a hydrogen consuming pathway as presented in equation 2.4

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad 2.4$$

Ethanol and lactate are also products that are observed from glucose degradation but their pathways produce no hydrogen as presented in equations 2.5 and 2.6 respectively (Guo et al., 2010).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad 2.5$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2 \qquad 2.6$$

Xylose is the most abundant sugar present in the hemicellulosic fraction of lignocellulosic biomasses (Barakat et al., 2012). Using xylose, a five carbon sugar, as model substrate, maximum hydrogen yields that can be obtained from the acetic and butyric acid pathways become 3.33 mol H_2/mol xylose and 1.67 mol H_2/mol xylose, respectively, as presented in equations 2.7 and 2.8 (Kongjan et al., 2010).

$$C_5H_{10}O_5 + 1.67H_2O \rightarrow 1.67CH_3COOH + 1.67CO_2 + 3.33H_2 \quad 2.7$$

$$C_5H_{10}O_5 \rightarrow 0.83CH_3CH_2CH_2COOH + 1.67CO_2 + 1.67H_2 \quad 2.8$$

The propionate pathways from xylose degradation consumes 1.67 mol H_2/mol xylose as shown in equation 2.9.

$$C_5H_{10}O_5 + 1.67H_2 \rightarrow 1.67CH_3CH_2COOH + 1.67H_2O \quad 2.9$$

As shown in the above equations, higher yields are associated with the production of acetate and lower yields are linked with the production of propionate and other end products like lactic acid and alcohols. In practice, even the dark fermentative process does not produce up to the maximum thermodynamic theoretical yield of 4 mol H_2 per mol hexose (Zhang, 2011). It can only occur in an ideal case where all the carbon substrates are fully utilized along the right pathways without any diversion to the formation of other fermentation products (e.g organic acids). A second stage process is usually required to recover the energy residues remaining in the effluent. Some of these second stage processes could include photo-fermentation, anaerobic digestion, and/or microbial fuel cells (Barakat et al., 2012; Claassen et al., 2000; Zhang, 2011).

Previous laboratory studies have highlighted the gains in dark fermentation including high hydrogen production rates, reduced demand for energy and ease of operation and sustainability

(Claassen et al., 1999; Hallenbeck and Benemann, 2002; Nandi and Sengupta, 1998; Nath and Das, 2004). Interest is geared towards dark fermentation as a sustainable method for hydrogen production (Ntaikou et al., 2009) and is the method employed in this study.

2.4 Feedstock

Low-cost renewable substrates are necessary in establishing a cost-effective technology for hydrogen production (Zhang, 2011). Biomasses that are rich in carbohydrates are the most-suitable feedstock for biohydrogen production. Annually, worldwide production of lignocellulosic materials has increased from about 10 - 50 billion metric tonnes (Claassen et al., 1999) to over 220 billion metric tonnes (Chandra et al., 2012; Cui et al., 2012). Production (Ntaikou et al, 2010). Lignocellulosic materials are generally low cost, carbohydrate-rich and are found in abundance as agricultural or industrial by-products with little or no commercial value). In agriculture, most of these wastes are left unused on the fields after harvest creating environmental problems and a waste of potential renewable resource (Pan et al., 2010). It has been identified as a promising substrate for biological hydrogen production, as it is rich in carbohydrates (sugars) which are known to produce large amounts of hydrogen.

Lignocellulose or lignocellulosic biomass refers to dry matter found in plants. It consists mainly of carbohydrate polymers (cellulose, hemicelluloses) and an aromatic polymer (lignin) which vary in quantity and quality depending on the plant or feed material (Aman, 1993). The carbohydrate polymers consist of sugar monomers (six carbon and five carbon sugars). Typically, lignocellulose comprises 30 % – 70 % cellulose, 15 % – 30 % hemicellulose and 10 % - 25 % lignin, indicating that cellulose is the most abundant fraction. Table 2.1 presents the biochemical composition of some lignocellulosic biomasses.

Cellulose consists of glucose units linked in linear chains and is the main component of plant cell walls. Cellulose is insoluble in most solvents, including water, due to strong hydrogen bonds and is also very resistant to hydrolysis (Galbe and Zacchi, 2012). Hemicelluloses comprise short, branched chains of several pentoses, mainly xylose and arabinose and hexoses, e.g., mannose, galactose and glucose. Hemicelluloses bind cellulose fibrils to form microfibrils, which improve the cell wall's stability. They are cross-linked with lignin, creating a complex bond which is resistant to microbial degradation (Ladisch et al., 1983; Lynch, 1992). Hemicellulose is more hydrophilic and as such easier to hydrolyze than cellulose (Horn et al., 2012; Wyman et al., 2004). Lignin is the major non-carbohydrate component of lignocellulosic materials. It is closely attached to cellulose and hemicellulose through a variety of chemical bonds and is responsible for the remarkable strength of plants. It is a cross-linked hydrophobic polymer, insoluble and resistant to anaerobic breakdown and its presence affects the degradability of the lignocellulosic biomass (Monlau et al., 2013a).

Lignocellulosic materials also consist of valuable components such as extractives and fatty acids (Galbe and Zacchi, 2012). Removing the rind from biomass before processing lowers the lignin content (Pattra et al., 2008). Lignin is the most recalcitrant of all the plant cell components, and as such the higher the proportion of lignin the lower the bioavailability of the substrate. Lignin molecules reduce the surface area available to enzymatic penetration and activity (Haug, 1993).

Lignocellulosic biomass can be broadly classified into three categories:

a) Virgin biomass – e.g trees, bushes, grasses (all naturally occurring terrestrial plants)

b) Waste biomass – These include low-value by-products of industrial sectors:

    I. Agricultural: corn stalks, sugarcane bagasse, rice straw (Monlau et al., 2013a)

II. Forestry: saw and paper mill discards (Appels et al., 2011)

c) Energy crops – crops with high biomass yields which are produced to serve as raw material for second generation biofuel (i.e. as feedstock for combustion or conversion to biofuels) e.g switch grass, mischantus, elephant grass and Jerusalem artichoke (Cheng et al., 2011).

Table 2:1: Biochemical composition of some lignocellulosic biomasses

Lignocellulosic compounds	Celluloses (%)	Hemicelluloses (%)	Lignin (%)	References
Wheat straw	39.6	26.6	21	Monlau et al, 2013a
Wheat bran	42.5	21.2	3.4	
Rye straw	38	36.9	17.6	
Rice straw	32	18	11.2	
Poplar wood	44.5	22.5	19.5	
Barley straw	37.5	25.3	16	
Maize bran	39.8	29.7	2.6	
Maize stover	41.7	18.9	26.1	
Corn stover	36.8	30.6	23.1	
Corn cob	38.9	42.2	10.9	Pan et al, 2010
Corn stalk	34.1	30.6	7.8	Chen et al, 2005
Sugarcane bagasse	33.6	23.9	4.3	Pattra et al, 2008
Corn cobs	45	35	15	Sun et al., 2002
Grasses	25-40	35-50	10-30	
Leaves	15-20	80-85	0	
Cotton seed hairs	80-95	5-20	0	
Paper	85-99	0	0-15	
Switch grass	45	31.4	12	

2.5 Hydrogen fermentation process parameters

2.5.1 Temperature

Fermentative hydrogen production has been studied at mesophilic (20 – 40 ˚C), thermophilic (50 – 65 ˚C) and hyperthermophilic temperatures (>70 ˚C). It has been reported that higher hydrogen yields and shorter lag times are associated with higher temperatures. Also, it was stated that better hydrogen yields and hydrogen production rates were achieved at thermophilic than at mesophilic

temperatures (Elsharnouby et al., 2013). Chairattanamanokorn et al (2009) reported a maximum hydrogen yield of 430 mL/L of pretreated sugarcane bagasse at a temperature of 55 ˚C and short lag time of 7.9 h and a low hydrogen production rate of 184 ml/l at 25 ˚C after a longer lag time of 37.6 h. Kargi et al. (2012) also reported that thermophilic hydrogen fermentation yielded higher cumulative hydrogen production (171 mL), yield (111 mL H_2/g total sugar) and rate (3.46 L H_2/L/h) compared to the mesophilic fermentation due to the inactivity of hydrogen consumers at high temperature.

2.5.2 pH

pH is an important parameter in fermentative hydrogen production. Various studies have reported an optimum pH for hydrogen production from carbohydrates of around 5.2 to 7 and from hydrolysates from 5.5 to 8 (Nissila et al., 2014). Lay (2000) reported that hydrogen was produced from starch in a chemostat reactor within a pH of 4.7 and 5.7 and alcohol production rate was observed to be greater than hydrogen production rate when pH was lower than 4.3 or greater than 6.1. A pH of 5.5 has previously been reported to be optimal for biohydrogen production from sugarcane bagasse hydrolysate by *Clostridium butyricum* (Pattra et al., 2008), from sucrose using mixed cultures with hydrogen conversion efficiency of 27.5 % (Sung et al., 2003) and from food waste using mixed cultures with hydrogen yield and decomposition efficiency of 2.2 mol H_2/mol hexose $_{consumed}$ and 90 % respectively (Shin and Youn, 2005). A pH of 5.5 was therefore selected for use in this study.

2.5.3 Partial pressure

The conversion of acetate to hydrogen is thermodynamically unfavorable at moderate temperatures and is strongly determined by the hydrogen partial pressure. Pathways that produce hydrogen are sensitive to hydrogen concentration which could cause end-product inhibition (Nath and Das,

2004). As the concentration of hydrogen increases, hydrogen synthesis decreases and metabolic pathways shift towards the production of more reduced substrates such as lactate and ethanol (Levin et al., 2004). Gas sparging, ultrasonication, increased mechanical mixing and membrane - absorption technologies are some of the techniques used to reduce the hydrogen partial pressure in a liquid (Elbeshbishy et al, 2011; Van Groenestijn et al. 2002). Specific hydrogen production rate increased from 1.4 mL H_2/min/g biomass to 3.1 mL H_2/min/g biomass after sparging with nitrogen (Mizuno et al., 2000). Elbeshbishy et al. (2011) observed a 31 % increase in the hydrogen content of the reactor headspace after eliminating the dissolved carbon dioxide and hydrogen from the liquid using ultrasonication technique. Higher hydrogen production, rates and yields were generally achieved when fermentation was performed under lower hydrogen partial pressures (Hallenbeck and Bennmann, 2002; Nath and Das., 2004).

2.5.4 Reactor design and metabolic engineering

Reactor design and metabolic engineering are other factors to consider in order to maximize hydrogen production and yields as well as optimize the entire fermentation process (Maeda et al., 2008; Veit at al., 2008). Several reactor designs for biogas production have been studied. Antonopoulou et al. (2008) investigated sequential hydrogen and methane production from cheese whey in a two-stage anaerobic digestion process using a continuous stirred tank reactor (CSTR) for the first stage hydrogen production and an anaerobic baffled reactor for the second stage methane production and observed a hydrogen and methane production rate of 7.5 L H_2/d at an HRT of 24 h and 75.6 L CH_4/d at an HRT of 4.4 d respectively. Han et al. (2005) also performed a two-stage anaerobic digestion process of food waste using a leaching-bed reactor (LBR) in the first-stage and an up-flow anaerobic sludge blanket (UASB) for the second stage and observed a maximum efficiency of 71 % by adjusting dilution rate from 4.5 to 2.5 d^{-1} in the acidogenic

hydrogenesis stage and a 95 % COD removal efficiency at loading rates as high as 13 gCOD/Ld in the methanogenesis stage. Park et al. (2010) studied the two-stage anaerobic process from 28 g/L COD of dilute molasses using packed bed reactors (PBR) in both stages and observed the highest hydrogen and methane production rates of 2.8 L H_2/L-reactor/d at an optimum HRT of 6 h and 1.48 LCH_4/L-reactor/d at an optimum HRT of 6 d respectively. Metabolic engineering involves optimizing cell processes in order to increase the production of a desired compound. Maeda et al. (2008) metabolically engineered *Eschericia coli* in order to enhance hydrogen production and observed a 141-fold increase in hydrogen production from formate, a 50 % increase in hydrogen yield and a three-fold increase in hydrogen production from glucose. Veit et al. (2008) engineered a synthetic ferredoxin-dependent NAD(P)H:H_2 pathway model system in *Escherichia coli* BL21 (DE3) and experimentally evaluated the thermodynamic limitations of nucleotide pyridine-dependent H_2 synthesis under closed batch conditions and observed NADPH-dependent H_2 accumulation at a maximum partial H_2 pressure equal to a biochemically effective intracellular NADPH/NADP+ of 13:1.

2.5.5 Inhibitory compounds

Compounds that result from the degradation of lignocellulosic biomass such as furfural, HMF, acetic acid and phenolic compounds, may inhibit dark fermentative hydrogen production. They are known to decrease enzyme activities, inhibit protein and RNA synthesis, breakdown DNA, decrease intracellular pH and damage microbial membranes (Nissila et al., 2014) which in turn decrease hydrogen yields. Monlau et al. (2013b) studied hydrogen using different volume fractions (4 % to 35 %) of dilute acid hydrolysate obtained from sun-flower stalks containing 1.2 g/L furfural, 0.1 g/L HMF and 0.02 g/L phenolic compounds in the presence of 5 gVS/L glucose.

Volumes higher than 15 % hydrolysate showed 0 mol H_2/mol hexose equivalent indicating the inhibitory effect of the compounds on hydrogen production at higher concentrations.

Hydrolysates can however be detoxified using charcoal, cation exchange resin (Sainio et al., 2011), overliming ($Ca(OH)_2$) (Larsson et al, 1999) or with yeasts (Chang et al., 2011). Sainio et al. (2011) investigated the use of cation exchange resin, neutral polymer adsorbent and granular activated carbon (GAC) to remove furfural, HMF and acetic acid from a synthetic hydrolysate containing 20 % sulphuric acid with GAC showing the highest adsorption capacity for all the inhibitors. Larsson et al. (1999) studied various detoxification methods with the aim of improving cell growth and ethanol production by *Saccharomyces cerevisiae* using a dilute-acid hydrolysate of spruce as substrate. Ion exchange and treatment with $Ca(OH)_2$ were observed to be among the most efficient methods while treatment with 0.1 % sulphite was among the least efficient methods. Futhermore, enzyme detoxification with phenoloxidase laccase was reported to be the only detoxification method that removed the phenolic compounds while anion exchange at pH 10 was the most effective at removing all of the aliphatic acids, furan derivatives and phenolic compounds. The latter method however resulted in the loss of fermentable sugars.

2.5.6 Hydrolysate concentration

Hydrogen yields and production rates increase with increasing hydrolysate concentrations up to a certain extent after which VFAs accumulate and either inhibit hydrogen producers or decrease pH below acceptable range for hydrogen producers. High substrate concentrations could cause substrate inhibition, increase lag phase and hydrogen partial pressure (Nissila et al., 2014). Kongjan et al. (2010) investigated hydrogen production by an extreme thermophilic mixed culture from wheat straw hydrolysate in batches. The aforementioned authors observed longer lag phases with no hydrogen produced at hydrolysate concentrations as high as 30 % (v/v) stating that high

hydrolysate concentrations inhibited fermentative hydrogen production. Fan et al. (2006) reported an increase in hydrogen partial pressure in the reactor when substrate concentration increased from 0 to 50 g/L during fermentative hydrogen production from beer lees biomass by cow dung.

2.6 Biomass pretreatment

It is relatively difficult to access the cellulose and hemicellulose polymers of lignocellulosic materials to yield sugars. The main difficulties lie in the following (Galbe and Zacchi, 2012):

a) complex structure of materials making hydrolysis challenging
b) mixture of pentose and hexose sugars, which can cause fermentation problems (pentoses are not readily fermented)
c) formation of several compounds that may adversely affect fermentation.
d) by-products that originate from the biomass itself such as aromatic compounds or aliphatic acids causing inhibition of the fermentation process.

For these reasons, it is very difficult for hydrogen-producing bacteria to produce hydrogen from untreated or raw biomass (Dalia and Yuval, 2003). However, these complex compounds need to be broken down into simpler forms for easy conversion to hydrogen gas. In order to enhance the yield and rate of biohydrogen production, lignocellulosic biomass must therefore undergo pretreatment.

Pretreatment is considered the most important step in biomass conversion to energy as it has a large impact on subsequent steps in the process. Several pretreatment methods exist:

- Mechanical/Physical: chipping, grinding, milling, extrusion, ultrasonic/radiation (Monlau et al., 2013a)

- Thermal: steam explosion, hydrothermal (liquid hot water) (Harmsen et al., 2010; Kumar et al., 2009)
- Chemical: acid, alkaline, ionic liquids, AFEX (Ammonia Fiber Explosion), Organosolv (Dadi et al., 2007; Datta, 1981; Harmsen et al., 2010; Mcmillan, 1994; Mosier et al., 2005; Teymouri et al., 2005; Wyman et al., 2005)
- Biological: enzymes/microbial (Panagiotopoulos et al., 2012; Sun and Cheng, 2002)

Tables 2.2a and 2.2b present various pretreatment methods that have been investigated on a range of lignocellulosic biomasses for biohydrogen and biomethane production respectively. These studies show significant energy gains after pretreatment without taking into account the energy imput for the pretreatment process. Acids, particularly**,** dilute acid pretreatments have been widely employed with respect to biohydrogen production as it produces high sugar yields and favorably changes the structure of the substrate components, thus facilitating easy fermentation (Chang et al, 2011; Cui et al., 2009; Nissila et al., 2014; Panagiotopoulos et al., 2012; Pattra et al, 2008). Dilute acid (0.4 % - 2 % H_2SO_4) at temperatures between 160 – 220 ˚C are typically employed (Wilfor et al, 2005). With concentrated acids, there is the need to recover the acids used and to neutralize the hydrolysates before fermentation can occur (Nissila et al, 2012). Dilute acid pretreatment can be used to achieve high sugar and energy yields from recalcitrant materials such as hardwood and softwood (Galbe and Zacchi, 2012). Of all the pretreatment methods, acid pretreatment is considered the easiest, most efficient and a potentially cost-effective method, even to replicate on an industrial scale (Harmsen et al., 2010; Mosier et al., 2005).

Figure 2.1 shows the impact of pretreatment on the structure of a lignocellulosic material.

Table 2.2 a: Effect of biomass pretreatment on biohydrogen production

Lignocellulosic biomass	Pretreatment method	Pretreatment conditions	BioH_2 yield (L H_2/$kg_{VSadded}$)	Energy from pretreated biomass (MJ/kgVSadded)	Energy from raw biomass (MJ/kgVSadded)	Energy gain (%)	References
Wheat bran	Steam explosion + acid	0.27 MPa, 60 min, 0.01M HCl	86	0.93	0.55	69	Pan et al., 2008
	Acid	0.01M HCl, boiled 30 min	81	0.87	0.55	58	
	Acid + irradiation	0.01M HCl + 9 min microwave (880 W)	93	1	0.55	81	
Corn straw	Steam explosion + enzymatic	1.5 MPa, 10min + cellulase (25 FPU/g)	*68	*0.73	-	-	Li & Chen, 2007
Corn cob	Dilute Acid	1 % HCl/100 ˚C/30min	108	1.16	0.14	728	Pan et al., 2009
Corn stalks	Steam explosion	1.6 Mpa, 5 min	*63.7	*5.25	-	-	Lu et al., 2009
	Alkaline	0.5 % NaOH	57	0.62	0.03	1966	Zhang et al., 2006
	Acid	0.2 % HCl, boiled 30min	150	1.62	0.03	5300	
Sweet sorghum stalk	Alkaline	0.4 % NaOH, 20 °C, 24 h	127	1.37	0.56	144	Shi et al., 2010
Beer lees	Acid	2 % (w/v) HCl	*53	*0.57	*0.03	1800	Cui et al., 2009
Poplar leaves	Acid	4 %(w/v) HCl	*33.5	*0.36	*0.16	125	
	Enzymatic	2 % (v/v) viscozyme L	*45	*0.49	*0.16	206	Cui et al., 2010
Maize leaves	Microbial	Aerobic bacterium *Bacillus amyloliquefaciens*	73.13	0.78	0.18	333	Ivanova et al., 2009
Bagasse	Alkaline + enzymatic	4 % NaOH (w/v), 100˚C, 2h + Cellulase, 20 FPU/g	300	3.23	-	-	Chairattana-manokom et al., 2009
	Physical + Microbial	2 mm/100 ˚C, 2h + cellulase (20PFU/g)	31.36	0.34	-	-	

*Values based on Dry Matter (DM) (not kgVS)

Table 2.2 b: Effect of biomass pretreatment on biomethane production

Lignocellulosic biomass	Pretreatment method	Pretreatment conditions	BioCH$_4$ yield (L H$_2$/kg$_{VSadded}$)	Energy from pretreated biomass (MJ/kgVSadded)	Energy from raw biomass (MJ/kgVSadded)	Energy gain (%)	References
Newsprint	Acid	30 % acetic acid + 2 % HNO_3	271	10.78	3.86	179	Xiao & Clarkson, 1997
Rice straw	Microbial	*Polyporus ostreiformis* (Brown-rot fungus)	295	11.74	8.91	32	Ghosh & Bhattacharyya,1999
		Phanerochaete chrysosporium (White-rot fungus)	328	13.05	8.91	46	
	Alkaline + physical	2 % NH_3, 90 °C, 10 mm	245	9.75	7.56	29	Zhang & Zhang 1999
Wheat straw	Physical	0.4 mm (Grinding)	248	9.87	6.45	53	Sharma et al., 1988
	Steam explosion	170 °C, 10 min	361	14.36	10.98	31	Bauer et al., 2009
Corn stover	Alkaline	2 % NaOH (w/w), 20 °C, 3 days	215	8.55	4.554	89	Zheng et al., 2009
Bermuda grass	Physical	0.4 mm (Grinding)	228	9.07	5.45	66	Sharma et al., 1988

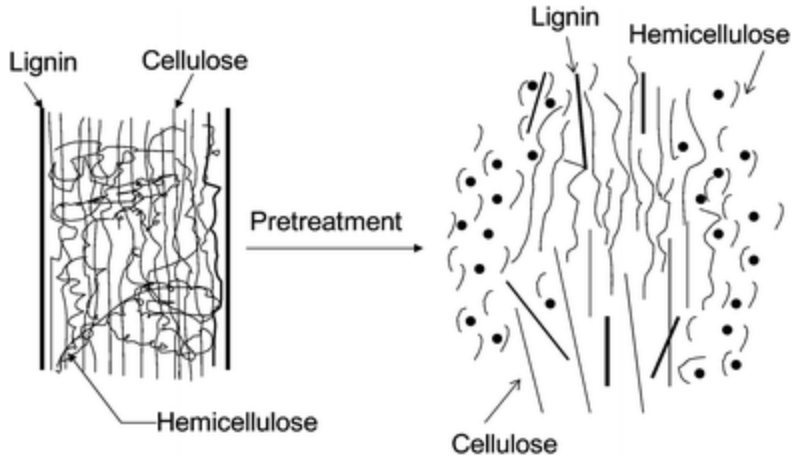
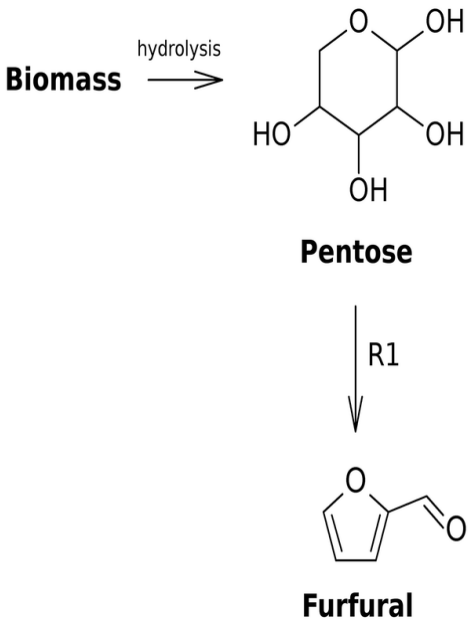


Figure 2.1: Schematic diagram showing the impact of pretreatment on lignocellulosic biomass
(Source: Google images)

2.7 Formation and inhibition of Furfural

During pretreatment of lignocellulosic materials using chemical treatments such as dilute acids, several decomposition by-products are formed in addition to the fermentable sugars which may be harmful to microorganisms and interfere with fermentation (Barakat et al., 2012; Siqueira and Reginatto, 2015). These compounds include organic acids such as acetic acid; furan derivatives such as furfural and hydroxylmethylfurfural (HMF) (degradation products formed from pentoses and hexoses respectively), and phenolic compounds such as vanillin, syringaldehyde and 4-hydroxylbenzoic acid (HBA) (formed from lignin degradation) (Barakat et al., 2012; Galbe and Zacchi, 2012; Siqueira and Reginatto, 2015). Of all the compounds mentioned, furfural and HMF (furan derivatives) are thought to strongly inhibit hydrogen production compared to the others with furfural being even more toxic than HMF (Haroun et al., 2016). Furfural is the main degradation product of pentoses and is formed by the Maillard reaction as a by-product of the hydrolysis of cellulosic matter at high temperatures and pressures (see Figure 2.2) (Navarro, 1994).



R1 is a dehydration step where 1 molecule of water is lost at high temperatures and pressures.

Figure 2.2: Schematic diagram showing furfural formation from biomass
(Source: Google images)

Furfural is an inhibitor of interest due to the adverse effects it has on microbial cells and membranes. Mechanisms have been proposed to describe by-product toxicity which include cellular membrane damage, chemical reactivity with cellular content, accumulation of reactive oxygen species, inhibition of metabolism, reduction in cell growth rate and cell membrane permeability (Allen et al., 2010; Almeida et al., 2007; Zaldivar et al., 1999). Toxicity is thought to be related to their chemical structures and hydrophobicity (Barakat et al., 2012). The hydrophobicity of furan compounds enables their passage into microorganism's cytoplasm. Furfural inhibits NADH-dependent enzymes (such as pyruvate dehydrogenase), which are vital to the main metabolic pathways (Modig et al., 2002; Palmquist and Hahn-Hagerdal, 2000). The aforementioned authors further stated that furfural can be reduced to furfuryl alcohol consuming NADH, which in turn reduces hydrogen and metabolites production that also require NADH.

The knowledge of the inhibitory level and impact of furfural and other pretreatment by-products is necessary for hydrogen fermentation in order to reduce their concentrations in the hydrolysates so

as to maximize biogas production (Barakat et al., 2012; Kumar et al., 2014; Siqueira and Reginatto, 2015).

2.8 Microorganisms for hydrogen production

The use of microorganisms is gaining widespread attention as a cost-efficient way to produce hydrogen (Kotay and Das, 2008). Both pure and mixed cultures have been studied for biohydrogen production. *Clostridium butyricum, C. acetobutyricum, C. Saccharoperbutylacetonicum and C. pasteurianum* have been investigated as pure cultures with high efficiency for hydrogen production (Hawkes et al., 2002; Pattra et al., 2008). Mixed cultures from natural environments such as soil, animal waste, and anaerobic sludge have also been used to produce energy. They are easier to use, simpler to operate and can act on a wide range of substrates in contrast to using pure cultures (Li and Chen, 2007). They also do not require aseptic conditions (Ntaikou et al., 2010). Very few studies have been performed to ascertain the inhibitory effect of furfural on fermentative hydrogen production by mixed cultures and further research is required to determine the full effect of furfural on microbial cultures.

Table 2.3 presents a summary of hydrogen fermentation studies carried out on various substrates in the presence of several inhibitors using both pure and mixed cultures. Generally, hydrogen production rates and yields were observed to decrease with increasing inhibitor concentrations while lag phases increased with increasing inhibitor concentrations. A batch fermentative hydrogen production study by Siqueira and Reginatto (2015) using mixed cultures grown on 40 g/L glucose in the presence of furfural revealed that no hydrogen was produced at 2 g/L furfural indicating 100 % inhibition from the control (0 g/L). The aforementioned authors further reported furfural inhibition threshold limits of less than 2 g/L with an IC 50 of 0.62 g/L. A study by Veeravalli et al. (2013) was carried out to examine the inhibitory effect of furfural, HMF, and linoleic acid on

fermentative hydrogen production from 5 g/L glucose using mixed cultures in a fed-batch system. A positive synergistic effect was observed in the presence of 0.75 g/L furfural, 0.25 g/L HMF and 2 g/L linoleic acids which gave the highest yield of 1.89 ± 0.27 mol H_2/mol glucose. The aforementioned authors also stated that furfural and/or HMF concentrations above 0.75 – 0.8 g/L lowered hydrogen yields.

Table 2:3: Batch studies showing hydrogen yields from various substrates with IC50 and threshold furfural concentration

Substrate	Culture	Inhibitor & Concentration range tested	[a] Max. hydrogen yield (mol H_2/mol substrate consumed)	[x] IC 50 (g/L)	Inibition threshold	References
40 g/L glucose	Mixed	Furfural (0.25 to 2 g/L)	0.58	0.62	1-2 g/L	Sequeira and Reginatto, 2015
		HMF (0.1 to 1 g/L)	0.39	0.48	> 1 g/L	
		Vanillin (0.25 to 2 g/L)	0.60	0.71	1-2 gL	
		Syringaldehyde (0.25 to 2 g/L)	0.72	1.05	1.5 -2 g/L	
		HBA (0.15 to 1 g/L)	0.24	0.38	0.5 - 1 g/L	
		Acetic acid (0.5 to 10 g/L)	1.13	5.14	5-10 g/L	
[b] 5 g/L glucose	Mixed	Furfural (1 g/L)	-	-	[c] 0.75 -0.8 g/L	Veeravalli et al., 2013
		HMF (1 g/L)	-	-		
22 g/L sucrose -rich synthetic wastewater	Mixed	Acetate (5 to 50 g/L)	1.04	11.05	> 5 g/L	Wang et al., 2008
5 g/L xylose	Mixed	Furfural (1 g/L)	0.51	-	-	Quéméneur et al., 2012
		HMF (1 g/L)	0.4	-	-	
		Phenol (1 g/L)	1.28	-	-	
		Syringaldehyde (1 g/L)	1.39	-	-	
		Vanillin (1 g/L)	1.3	-	-	
		Kraft lignin (1 g/L)	0.67	-	-	
		Organosolv lignin (1 g/L)	0.34	-	-	
5 gVS/L glucose + varying concentrations of dilute acid-pretreated sunflower stalks	Pure culture (*Clostridium sp*.)	[d] 3.75 % (v/v)	1.83	-	-	Monlau et al., 2013b
		[d] 7.5 % (v/v)	0.24	-	-	
		[d] 15 % (v/v)	0	-	-	
		[d] 35 % (v/v)	0	-	-	
5 g/L glucose	*Clostridium butyricum* isolate from sludge	Phenol (0.2 to 1.5 g/L)	1.32	-	> 1 g/L	Tai et al., 2010

[a] Maximum hydrogen yields are at the least furfural concentration tested; [b] treated with 2 g/L linoleic acid; [c] combined and/or individual threshold inhibition with 1 g/L HMF; [d] inhibitor composition: formate (0.6 g/L); acetate (0.81 g/L); furfural (1.15 g/L), HMF (0.13 g/L); [x] IC50 values based on maximum hydrogen production rate

2.9 Challenges associated with biohydrogen production

Hydrogen production has shown great potential to be the key fuel for the future (Gupta et al., 2013). Biological hydrogen production processes are gaining widespread attention as they can be operated at atmospheric temperature and pressure and can utilize renewable energy resources (Cai et al., 2004). However, the reported biohydrogen production rates, stabilities and efficiencies of these processes are still inadequate to make them commercially viable. It is therefore necessary to overcome the major challenges involved with biohydrogen production so as to effectively and efficiently scale up the process from laboratory to industrial or full scale (Das et al., 2008; Kotay and Das, 2008). These challenges include:

- Low hydrogen production rates and yields
- Insufficient knowledge of the metabolism of hydrogen-producing bacteria
- Hydrogen separation, purification, and storage

2.10 Single-stage versus two-stage anaerobic digestion processes

This thesis focuses mainly on biohydrogen production but a two-stage anaerobic digestion process which involves coupling a first-stage biohydrogen production process with a second-stage biomethane production process was also investigated. Biomethane can be produced from organic matter through anaerobic digestion. Methane production requires neutral pH (6.5 - 7.5), longer retention times, without the need to inhibit methanogens through preheating the sludge (Monlau et al., 2013a). Several lignocellulosic substrates such as wheat straw, rice straw, sugarcane bagasse, poplar wood etc, have been tested for their biomethane potential. Zheng et al. (2010) reported a methane yield of 233 mL/g VS from corn stover after pretreatment with sodium hydroxide at 20 °C for 3 days. Dinuccio et al. (2010) reported specific methane yields of 501, 317, 229, and 195 L

CH_4/kgVS from whey, maize, barley straw, and rice straw respectively. As in the case of hydrogen production, complex carbohydrates need to be pretreated in order to allow for easy conversion to methane. Theoretical methane yield from lignocellulosic biomass ($C_5H_9O_{2.5}NS_{0.025}$) was reported to be 475 L CH_4/kgVS but actual or experimental yields generally do not exceed 60 % due to poorly biodegradable compounds or non-biodegradable polymers (such as lignin) that are difficult to solubilize (Frigon and Guiot, 2010).

Biohydrogen production from carbon-rich substrates through fermentation produces volatile fatty acids (such as acetic, butyric, propionic acids) and alcohols (such as ethanol) as by-products of the process (Nasr et al., 2012). These metabolites are present in the effluent from this process which can be fed into an anaerobic digester as substrate for methane production. This two-stage anaerobic digestion process separates the acidogenic from methanogenic steps so as to enhance overall process performance, stability and efficiency (Li et al., 2015). The aim of a two-stage anaerobic digestion process is to produce VFAs in the first stage (acidification) which are converted to bioenergy in the form of methane in the second stage from the effluent of the first stage (thus extracting more net energy) while also reducing final COD concentration in effluent which is necessary for discharge (leading to further degradation of the waste) (Park et al., 2010). As resource recovery, hydrogen produced in the first stage, can be purified for use in fuel cells or liquefied and sold as industrial gas while methane from the second stage can be used to generate electricity and heat.

This process was first proposed by Pohland and Ghosh (1971) where both stages were physically separated in two reactors and since then, a number of researchers have studied this process using various combinations of different types of reactors (one for the acidogenic and the other for the methanogenic stage). Several studies have reported (using different substrates) enhancements in

methane yields, production rates and maximum energy recovery rates in a two-stage anaerobic digestion process compared with a single-stage process. Table 2.4 compares yields between a single-stage BMP process and a two-stage anaerobic digestion process from literature studies while Chapter 5 of this report presents a study on single-stage BMP and two-stage anaerobic digestion processes using extruded poplar wood hydrolysates.

Table 2.4: Comparison between a single-stage BMP process and a two-stage anaerobic digestion process

				Two- stage-anaerobic digestion		
			Single stage CH_4	First stage H_2	Second stage CH_4	Reference
Substrate		**System**	**mL CH_4/gVS $_{added}$**	**mL H_2/gVS $_{added}$**	**mL CH_4/gVS $_{added}$**	
Sweet sorghum	Solid fraction	Batch	[a] 78	-	-	Antonopoulou et al., 2008a
	Hydrolysate fraction	Continuous	-	[a] 10.4	[a] 29	
*Thermo-mechanical pulp wastewater		Continuous	[b] 300	[b] 340		Viñas et al., 1993
Potato waste		Continuous	-	[c] 71	513	Zhu et al., 2008
Cheese whey		Continuous	[d] 310	[d] 41	[d] 364	Antonopoulou et al., 2008b
Molasses		Continuous	-	1.4	17.7	Park et al., 2010
Food waste		Batch	-	290	240	Han et al., 2005
Grass silage	Grass silage	Batch	431	5.6	467	Pakarinen et al., 2009
	Solid fraction		299	3.4	490	
	Liquid fraction		703	31.1	520	
Potato		Batch	-	[e] 271	[e] 158	Xie et al., 2008
Thin stillage		Batch	[f] 490	[f] 117	[f] 621	Nasr et al., 2011; Nasr et al., 2012

* Wastewater from thermo-mechanically treated eucalyptus wood; [a] mL biogas/g Dry matter; [b] mL biogas/gCOD $_{removed}$; [c] converted from mL/g TS in study; [d] mL biogas/gCOD $_{added}$; [e] maximum biogas yields; [f] values obtained from experiments using anaerobic digester sludge (ADS)

2.11 Microbial kinetic modeling

Mathematical modeling is a tool for quantitative and qualitative analysis and is important in the simulation and analysis of technologies (Quarteroni, 2009). The most common empirical model describing the relationship between microbial growth rate and substrate concentration is the Monod model (Lobry et al., 1992).

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad 2.10$$

where μ_{max} (h^{-1}) is the maximum specific growth rate, Ks (g/L) is the saturation or half-velocity constant which is the concentration of the rate-limiting substrate at half the maximum specific growth rate, and S is the substrate concentration.

μ increases as S rises till it reaches μ_{max}. Also, at lower S, μ is approximately proportional to S (first order in S) while at higher S, μ is independent of S (zero order in S). Several models have been used to describe the effect of substrate concentration on the rates of substrate utilization, microbial growth and hydrogen production (Wang and Wan, 2009). When substrate inhibits a fermentative hydrogen production process at much higher concentrations, the simple Monod model becomes unsatisfactory. In this case, modified models of the Monod model with a substrate inhibition term will be employed to describe the effects of substrate inhibition on hydrogen production rate and specific microbial growth rate. The most widely used model for substrate inhibition is the Haldane Model, also called Andrews Model (Andrews,1968).

$$\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_i}} \qquad 2.11$$

where K_i is the inhibition constant.

Another substrate inhibition model, the Han-Levenspiel model (Wang and Wan, 2008) (Eq. 2.12), which is an extended Monod model, has also been used to describe the effects of glucose on

hydrogen production in batches and the authors concluded that this model better described the effects of glucose concentrations on fermentative hydrogen production than the Andrew model.

$$\mu = \frac{\mu_{max} S\,(1-\frac{S}{S_{max}})^{m}}{S+ K_S\,(1-\frac{S}{S_{max}})^{n}} \qquad 2.12$$

where S_{max} is the maximum substrate concentration above which the fermentative hydrogen production stops and m and n are exponent constants.

When low pH or biomass decay affects microbial growth and their ability to degrade substrate, a modified Monod model incorporating a pH term or biomass decay term is usually used. Ntaikou et al. (2008) used a modified Monod model to describe the effects of glucose concentration on its degradation rate (Equation 2.13)

$$\frac{dX}{dt} = \frac{\mu_{max} S}{K_s + S} * X * I_{pH} - k_{d*}\, X \qquad 2.13$$

where X is biomass concentration (g/L), k_d is decay constant (h^{-1}) and I_{pH} is the pH inhibition constant.

Other kinetic models have also been employed to describe the effect of inhibitor concentration on hydrogen production and microbial growth. One of such models is the modified Han-Levenspiel model shown in Equation 2.14 (Wang and Wan, 2009).

$$\mu = \mu_{max}\,(1 - \frac{I}{I_{max}})^{m} \qquad 2.14$$

where I is inhibitor concentration (g/L) and I_{max} is the maximum or critical inhibitor concentration Wang et al. (2008) used Eq. 2.15 to model the inhibitory effects of sodium acetate concentrations on batch hydrogen production and specific rates of sucrose degradation while Liu et al. (2006) used Eq. 2.16 to describe the inhibitory effects of butyrate concentration on specific growth rates of wild *Clostridium tyrobutyricum* in fed-batches.

$$\mu = \frac{\mu_{max}}{1 + (\frac{I}{K_c})^m} \quad 2.15$$

$$\mu = \frac{\mu_{max} K_c}{K_c + I} \quad 2.16$$

where K_c is the apparent specific growth rate, I is the inhibitor concentration, and m is an exponent constant.

In the present study, the modified Monod models (Eq. 2.17 and 2.18) that describe microbial growth and substrate degradation incorporating biomass decay (Shuler and Kargi, 2002), will be used to determine the kinetics of both mesophilic and thermophilic cultures grown on lignocellulosic hydrolysate in the presence of furfural.

$$\frac{dX}{dt} = -\frac{\mu_{max}(S)X}{[K_S + (S)]} - K_d X \quad 2.17$$

$$\frac{dS}{dt} = -\frac{-\mu_{max}(S)X}{Y_{X/S}[K_S + (S)]} \quad 2.18$$

where $Y_{x/s}$ (gVSS/g substrate consumed) is the biomass yield.

2.12 Synopsis

Biohydrogen production from lignocellulosic wastes using mixed cultures is fast gaining widespread interest. It has been established that pretreating this substrate to yield simple sugars which micro-organisms can easily break down to produce hydrogen, also releases compounds which are inhibitory to the fermentation process. Since furfural is considered to be one of the main inhibitors of this process, the knowledge of furfural's threshold limit is important in order to reduce its inhibitory effect as well as facilitate the economical and practical conversion of lignocellulosic biomass. Literature on fermentative hydrogen production lack detailed information on the inhibitory effects of furfural and furfural's threshold concentrations. Chapter 3 of this research

therefore investigates the impact of furfural on biohydrogen production rates and yields at mesophilic and thermophilic temperatures while Chapter 4 provides an insight into the effects of furfural on the microbial kinetics of mixed cultures as well as end-product yields. This kinetic information is valuable in designing and operating bioreactor systems.

While only a few studies on biomethane production from extruded agricultural products have been conducted, there is no information on biohydrogen production from extruded lignocellulosic biomass in literature reports. Furthermore, the impact of acidification on biomethane production from lignocellulosic biomass has not been evaluated. Chapter 5 of this research therefore assesses a single-stage BMP test and the two-stage anaerobic digestion process of real waste hydrolysates from poplar wood biomass pretreated using twin-screw extrusion technology.

2.13 References

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CHAPTER 3

Biological hydrogen production from synthetic lignocellulosic hydrolysate using mesophilic anaerobic digester sludge: Impact of furfural[1]

3.1. Introduction

Current global technologies for energy production and supply rely heavily on fossil fuels causing rapid depletion of these resources and increasing carbon dioxide emissions as energy consumption continues to increase. The biological production of hydrogen, which is a renewable energy carrier, can mitigate this trend and alleviate concerns involved with fossil fuel use. Biohydrogen production methods are more sustainable, environmentally-friendly, and less energy intensive compared to current methods of energy production (Gomez-flores et al., 2015; Kapdan and Kargi, 2005; Lay 2001).

The main light-independent process for biohydrogen production is dark fermentation. Biomasses that are rich in carbohydrates are the most-suitable feedstocks for biohydrogen production using fermentative anaerobic bacteria (Chen et al., 2006; Ntaikou et al., 2010;). The use of low-cost feedstock is necessary in establishing a cost-effective technology. Lignocellulosic materials such as agricultural residues (e.g corn stalks, corn cobs, sugar cane bagasse, rice straw), hardwood (e.g poplar wood, aspen wood) and softwood (e.g red cedar, red oak) are generally found in abundance as agricultural or industrial by-products with little or no commercial value (Cantarella et al., 2004; Costa Lopes et al., 2013; Du et al., 2010; Fenske et al., 1998; Lynd et al., 1996; Polman, 1994). In agriculture, most of these wastes are left unused on the fields after harvest, thus creating environmental problems and a waste of potential renewable resource (Pan et al., 2010).

[1] This chapter has been submitted to the *Bioresource Technology* journal for publication

Carbohydrates in lignocellulosic biomass are usually complex and not just simple hexose or pentose sugars (Galbe and Zacchi, 2012). It is relatively difficult to access the cellulosic and hemicellulosic polymers of lignocellulosic materials to yield sugars. These complex compounds need to be broken down into simpler forms for easy conversion to hydrogen gas (Cao et al., 2010). In order to enhance the yield and rate of biohydrogen production, lignocellulosic biomass must therefore undergo pretreatment.

Several pretreatment methods using steam explosion, ammonia fiber explosion (AFEX), acid, alkali, liquid hot water and many others have been employed (Cantarella et al., 2004; Du et al., 2010; Siqueira and Reginatto, 2015). Acids, particularly dilute acid pretreatment, have been widely used with respect to biohydrogen production as it is considered the easiest, most efficient, and cost-effective method that produces high sugar yields and favorably changes the structure of the substrate to facilitate fermentation (Cao et al., 2010; Chang et al., 2011; Cui et al., 2009; Mosier et al., 2005; Panagiotopoulos et al., 2009; Pattra et al., 2008). However, this process generates fermentation inhibitors such as furan derivatives (aldehydes including furfural and hydroxylmethylfurfural), ketones, phenols (such as vanillin, syringaldeyde) and organic acids (such as acetic acid) (Allen et al., 2010; Klinke et al., 2004). The hemicellulosic fraction of lignocellulose undergoes hydrolysis at high temperatures and pressures; and in the presence of dilute acids, yield monomeric sugars (mainly pentoses) in a reaction known as the Maillard reaction (Cantarella et al., 2004; Navarro, 1994) Under these conditions, the inhibitor furfural, is released as a by-product when the pentose sugars undergo dehydration. This inhibitor has been shown to have toxic effects on cells causing damage by inhibiting enzymes produced by micro-organisms during hydrolysis and fermentation of sugars resulting in low biohydrogen production rates and yields (Allen et al., 2010; Cantarella et al., 2004). Furfural also alters the growth of micro-

organisms by impeding enzymes responsible for fermentation, thus affecting their membrane integrity (Mills et al., 2009; Quéméneur.et al., 2012). Due to these inhibitory effects, furfural is considered to be a limiting factor in the biological conversion of lignocellulosic materials.

Microorganisms have the ability to minimize the effects of furfural as an inhibitor by metabolic switch between pathways where furfural is converted to less toxic compounds such as furfuryl alcohol or furoic acid, if its concentration does not exceed levels that the microorganisms can tolerate (Boopathy et al., 1993; Boyer et al., 1992; Liu et al., 2004; Liu et al., 2005). Hydrolysates can also be detoxified using charcoal, diethyl ether, ion exchange resin, activated carbon, $Ca(OH)_2$ (overliming) or with yeasts in order to increase hydrogen yields (Mateo et al., 2013; Zhao et al., 2011).

It is, however, important to determine the inhibition threshold levels of furfural prior to fermentation so as to maximize biohydrogen production rates and yields as well as reduce toxicity to tolerable levels in order to preserve microbial activity. The inhibitory effects of furfural on fermentative hydrogen production using mixed cultures have not been thoroughly studied. Literature on this subject are few in number with little or no information regarding furfural's inhibitory concentrations (Siqueira and Reginatto, 2015). Some studies have been done with furfural using pure substrates such as glucose (Siqueira and Reginatto, 2015) and xylose (Quéméneur et al., 2012); real hydrolysates (Cantarella et al., 2004; Cao et al., 2010); and pure cultures (Cao et al., 2010; Monlau et al., 2013); and other studies have been done to ascertain the effects of pH, substrate concentration and other intermediate products like acetate, butyrate etc. (Ginkel et al., 2001, Khanal et al., 2004). The present study employs the use of a mix of pure sugars and volatile fatty acids as substrate, simulating the composition of a typical real waste hydrolysate at different food –to-microorganisms ($S°/X°$) ratios.

Therefore, the main objectives of this study were to assess the impact of furfural on lignocellulosic hydrolysate using mixed cultures to evaluate hydrogen production potential and ascertain the threshold furfural concentrations that resulted in the maximum hydrogen production rates and yields.

3.2 Materials and Methods

3.2.1. Seed Sludge and Substrate

Mesophilic anaerobic digester sludge was collected from St. Marys Wastewater Treatment Plant, Ontario, Canada and preheated at 70 °C for 30 min prior to use so as to inactivate non-hydrogen producers (Hafez et al., 2010; Kumar et al., 2014). The pH, total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the sludge were 6.97, 16.2 g/L and 12.2 g/L respectively. Synthetic hydrolysate was prepared in the laboratory using substrate characteristics simulating the composition of a typical pretreated lignocellulosic hydrolysate. The substrate comprised mainly sugars and volatile fatty acids (VFAs) and included (per liter of distilled water) arabinose, 5.9 g; xylose, 50 g; mannose, 0.3 g; galactose, 2.5 g; glucose, 6.7 g; formate, 1.2 g and acetate, 1.8 g.

3.2.2 Batch Setup

Experiments were conducted in batches using 250 mL Wheaton glass serum bottles with working volumes of 200 mL under anaerobic conditions. 40 mL of seed was added per bottle and the volume of substrate ($V_{substrate}$) added to each bottle was calculated using the substrate-to-biomass (S°/X°) ratio equation as described by Nasr et al. (2014).

$$S°/X° \text{ (gCOD/gVSS)} = \frac{V_{substrate} * TCOD_{substrate}}{V_{seed} * VSS_{seed}} \quad 3.1$$

where $V_{substrate}$ and V_{seed} are the volumes of substrate and seed respectively in L, $TCOD_{substrate}$ is the total chemical oxygen demand of the substrate in g/L and VSS_{seed} is the volatile suspended solids content of the seed also in g/L. Four furfural concentrations (4, 2, 1, and 0.5 g/L) were tested at S°/X° of 4, 2, 1, and 0.5 $gCOD_{substrate}/gVSS_{seed}$ in triplicates. Batch controls consisted of substrate and seed sludge without furfural while blanks were run with seed sludge only. The composition of nutrient media added to each bottle in mg/L included: $CaCl_2$, 140; $MgCl_2.6H_2O$, 160; $MgSO_4.7H_2O$, 160; Urea, 1500; Na_2CO_3, 200; $KHCO_3$, 200; K_2HPO_4, 15; H_3PO_4, 500; trace metal solution (TMS), 500 (Hafez et al., 2010). The initial pH of the mixture was adjusted to 5.5 ± 0.04 using HCl but was not controlled during the experiment. Buffering capacity was however provided by adding 5 g/L $NaHCO_3$ to each bottle. Ten-mL samples were collected from each bottle for initial analysis before the bottles were purged with nitrogen gas to create an anaerobic condition. The batch was operated at a temperature of 37 °C in a swirling shaker (MaxQ 4000 Thermo Scientific CA benchtop shaker) at a speed of 180 rpm. At the end of the batch, final samples were taken for analysis.

3.2.3 Analytical methods

Glass syringes in the range 5 – 100 mL were used at regular intervals to release the gas in the bottles in order to equilibrate with ambient pressure (plunger displacement method) (Chen et al., 2006; Gupta et al., 2015). Hydrogen was analyzed using a gas chromatograph (Model 310 SRI Instruments, Torrance, CA) complete with a thermal conductivity detector (TCD) and a molecular sieve column (Mole sieve 5 Å, mesh 80/100, 6ft x 1/8 in). Argon gas was used as carrier gas at a flow rate of 30 mL/min and the temperature of column and TCD were 90 °C and 105 °C respectively. Chemical Oxygen Demand (COD) was measured using HACH methods and test kits (HACH DRB 200 COD reactor and HACH Odyssey DR 2800 spectrophotometer) (Gomez-flores

et al., 2015; Nasr et al., 2014). TSS and VSS were analyzed using standard methods (APHA, 1998). Soluble samples (filtered through 0.45 µm filter paper) were used to analyze monomeric sugars, VFAs and furfural using a Dionex IC20 Ion Chromatograph equipped with a refractive index detector (RID) (Perkin Elmer Series 200, PerkinElmer Instruments Inc., USA) and an Aminex® HPX-87H column (BIO-RAD laboratories, USA). The following parameters were used: pump flow rate – 0.6 mL/min; mobile phase - 9 mM H_2SO_4, column temperature- 30 °C and injection volume of 0.5 mL. Statistical analysis using a two-way ANOVA (Analysis of Variance) with post-hoc tests were done with an IBM Corp. Statistical Package for the Social Sciences (SPSS) software.

3.3 Results and Discussion

3.3.1 Biohydrogen production

Batches were set up as detailed in Section 3.2.2 and run until the daily hydrogen production was less than 1 % of the cumulative hydrogen volume, at which point the fermentation was assumed to be complete (Elbeshbishy et al., 2012). Figure 3.1 presents the cumulative hydrogen profiles plotted against time (h) while Figure 3.2 shows the cumulative hydrogen production (mL) and hydrogen yield (ml H_2/g sugars $_{initial}$) plotted against furfural concentration and S°/X°, after subtracting the volume of hydrogen produced from the blank. All values are averages of triplicate experiments.

Upon examination of the hydrogen profiles at all furfural concentrations (0 – 4 g/L), the S°/X° of 4 gCOD/gVSS showed two lag phases which could possibly be due to sequential utilization of the substrate by the culture with the simpler sugars (pentoses) being degraded first and the more complex sugars (hexoses) being degraded much later after an adaptation period. This trend was not observed at other S°/X° potentially due to the much lower concentrations of sugars as compared with an S°/X° of 4 g COD/gVSS. At S°/X° of 4 and 2 gCOD/gVSS, hydrogen production were

higher at both 0.5 g/L and 1 g/L furfural than even the controls (without furfural) while at the lower S°/X° (1 and 0.5 gCOD/gVSS), this trend occurred at only 0.5 g/L furfural. It can also be observed that fermentation contact time decreased with decreasing S°/X° indicating that the higher the substrate-to-biomass ratio, the longer it takes the mircoorganisms to degrade the substrate to produce hydrogen.

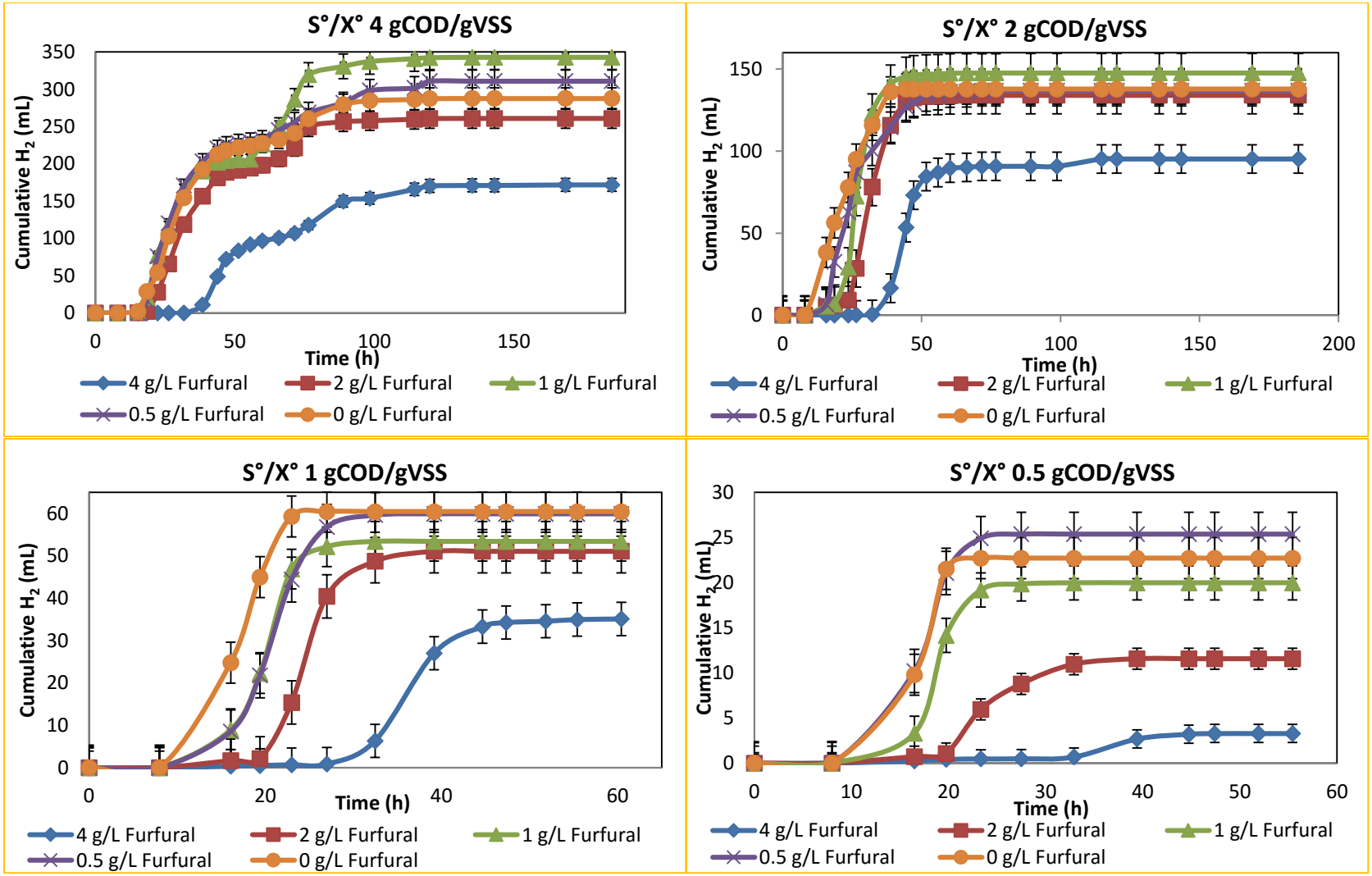

Figure 3.1: Cumulative hydrogen profiles at S°/X° 4, 2, 1, and 0.5 gCOD/gVSS

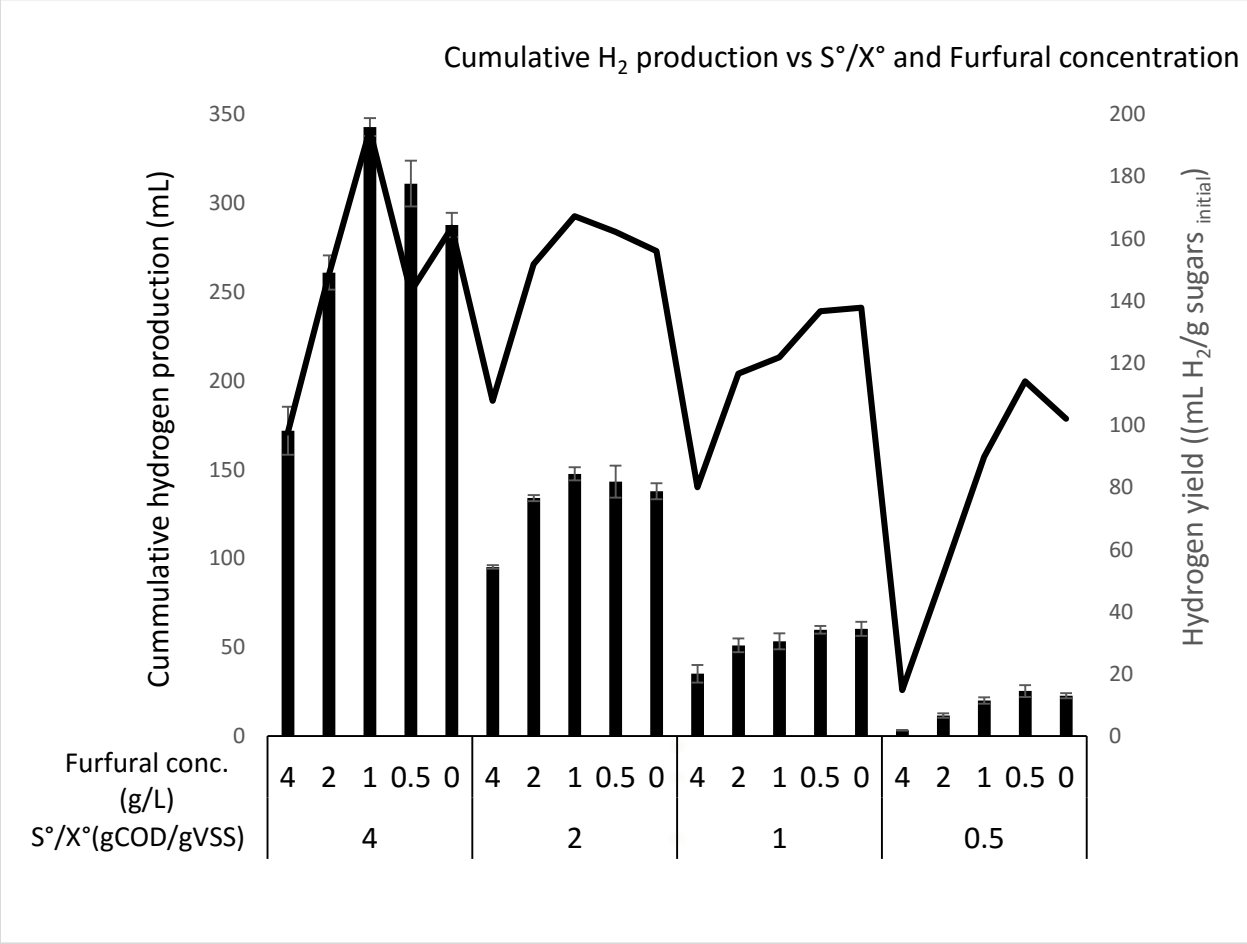

Figure 3.2: Cumulative H_2 production and yields vs furfural concentration and S°/X°

(Bar chart shows cumulative hydrogen production (mL) and line graph shows hydrogen yield (mL H_2/g sugars initial)

3.3.2 Gompertz parameters

The kinetic parameters such as hydrogen production potential (R), hydrogen production rate (H) and lag phase (λ) were obtained at the various test conditions using the modified Gompertz model (Chen et al., 2006).

$$H(t) = H.\exp\{-\exp\left[\frac{R_{max} \cdot e}{H}(\lambda - t) + 1\right]\} \qquad 3.2$$

where H *(t)* is the cumulative hydrogen production (mL) at time *t*; H is the hydrogen production potential (mL); R_{max} is the maximum hydrogen production rate (mL/h); λ is the lag phase (h) and

e is 2.71828. Maximum specific hydrogen production rates (max SHPR) in mL/gVSS $_{initial}$/h, were obtained by dividing R_{max} values by the initial mass of seed added per bottle. These parameters (shown in Table 3.1) were estimated by minimizing the sum of square errors (SSE) between experimental and estimated modeled data carried out on Microsoft Excel.

Table 3.1: Estimated Gompertz and calculated parameters for hydrogen production

			Gompertz parameters					Calculated parameters	
S°/X° (gCOD/gVSS)	**Sugar concentration (g/L)**	**Furfural concentration (g/L)**	**H (mL)**	**R_{max} (mL/h)**	**max SHPR (mL/gVSS$_{initial}$/h)**	**λ (h)**	**R^2**	**Cumulative H_2 (mL)**	**Hydrogen yield (mol H_2/mol sugars$_{initial}$)**
4	8.8	4	171	3.1	6.4	29.4	0.9996	172 ± 14	0.59 ± 0.05
		2	256	6.3	12.9	16.2	0.9998	261 ± 10	0.90 ± 0.03
		1	346	5.6	11.5	9.9	0.9997	343 ± 5	1.18 ± 0.02
		0.5	298	6.9	14.2	11.7	0.9999	311 ± 13	1.07 ± 0.04
		0	279	7.1	14.6	13.3	0.9999	288 ± 7	0.99 ± 0.02
2	4.4	4	93	7.5	15.4	36.8	0.9999	95 ± 1	0.65 ± 0.19
		2	134	9.5	19.5	23.7	0.9999	134 ± 2	0.92 ± 0.01
		1	148	13.7	28.2	21.4	0.9999	148 ± 4	1.02 ± 0.03
		0.5	144	6.2	12.7	13.1	0.9999	143 ± 9	0.99 ± 0.06
		0	139	6.4	13.2	10.3	0.9999	138 ± 5	0.95 ± 0.03
1	2.2	4	32	4.1	8.4	32.6	0.9999	35 ± 5	0.49 ± 0.07
		2	51	7.3	15.0	20.8	0.9999	51 ± 4	0.71 ± 0.05
		1	54	6.7	13.8	15.5	0.9999	53 ± 5	0.73 ± 0.06
		0.5	60	6.4	13.2	15.6	0.9999	60 ± 4	0.83 ± 0.03
		0	61	8.4	17.3	13.2	0.9999	60 ± 2	0.84 ± 0.05
0.5	1.1	4	3	0.4	0.8	31.1	0.9995	3 ± 0	0.08 ± 0.01
		2	12	1.2	2.5	18.7	0.9999	12 ± 1	0.33 ± 0.03
		1	20	3.8	7.8	15.7	0.9999	20 ± 2	0.55 ± 0.05
		0.5	25	4.6	9.5	15.1	0.9999	25 ± 3	0.68 ± 0.09
		0	23	7.1	14.6	14.3	0.9999	23 ± 1	0.63 ± 0.04

*Values of calculated parameters are averages of triplicate results ± standard deviation

Table 3.1 also shows cumulative hydrogen (mL) and hydrogen yields calculated from cumulative hydrogen figures in mol H_2/mol sugars $_{initial}$ by converting mL hydrogen to mole hydrogen and dividing by the number of moles of initial sugars present in the substrate.

Hydrogen production correlated well with the modified Gompertz equation with $R^2 > 0.99$. The overall maximum SHPR of 28.2 mL/g$VSS_{initial}$/h was observed at 1 g/L furfural at an S°/X° of 2. The least SHPR within each S°/X° corresponded to the least maximum hydrogen yield. The maximum hydrogen production rate (R_{max}) for the controls averaged 7.25 ± 0.8 mL/h clearly emphasizing that the substrate (sugars) concentration were not limiting across the different S°/X° ratios. The least R_{max} within each S°/X° was observed at 4 g/L furfural. The lag phase increased on average with increasing furfural concentrations but was lowest at the furfural concentrations that gave the most yield across all S°/X°, i.e., S°/X° of 4 at 1g/L furfural which had the shortest lag phase (9.9 h) also showed the overall maximum hydrogen production of 346 mL. In general, the shorter the lag phase, the higher the hydrogen yield. There was no definite correlation or trend in R_{max} and max SHPR within each S°/X° with respect to increasing furfural concentration.

The overall maximum H_2 yield of 1.18 mol H_2/mol sugars was observed at a furfural concentration of 1 g/L. The least overall hydrogen yield of 0.08 mol H_2/mol sugars was observed at an S°/X° of 0.5 gCOD/gVSS at 4 g/L furfural. Four g/L was the most inhibitory furfural concentration at all S°/X° showing the lowest yields. Within each S°/X°, there was no uniform trend with respect to increasing or decreasing H_2 yields whereas, in general, literature results show decreasing volumes of hydrogen produced with increasing furfural or inhibitor concentrations (Siqueira and Reginatto, 2015). But it can be established that at all S°/X°, furfural concentrations greater than 1 g/L were definitely inhibitory as shown by the increase in lag phases and decrease in hydrogen yields.

3.3.3 Volatile Fatty Acids

Acetate (HAc) and butyrate (HBu) were the main VFAs observed at all conditions at the end of the batch. Table 3.2 shows the ratios of the sum of final total volatile fatty acids ($TVFA_f$) and residual sugars (RS) to final soluble chemical oxygen demand (SCOD). Values ranged from 41 % to 93 % across all test conditions and were least at 4 g/L furfural at all S°/X° indicating the difficulty in acidification in the presence of furfural at this concentration. Sugars were observed to be completely degraded at the lower S°/X° of 0.5 and 1 gCOD/gVSS.

VFAs contributed on average, at all experimental conditions, about 67 % of the final SCOD indicating that other intermediates such as lactate or alcohols, may have been produced. Neither methane, ethanol nor formate were observed in the biogas produced. Over 90 % of the furfural was degraded after fermentation at all test conditions. The TVFAs did not clearly increase with an increase in furfural concentration across all S°/X° which is a similar trend to the hydrogen yield. However, over 98 % degradation of sugars in all samples was observed.

COD mass balances were calculated based on initial and final TCOD values as well as an equivalent COD for hydrogen produced (8 gCOD/ gH_2) using the following equation (Gupta et al., 2014).

$$\text{COD mass balance } (\%) = \frac{\text{TCOD}_{H_2} + \text{TCOD}_f}{\text{TCOD}_i} * 100 \qquad 3.3$$

COD balance across all experimental conditions were closed at an average of 94 ± 5% thus confirming the reliability of the data.

Table 3.2: Final products analysis showing ($TVFA_f$ + RS)/ $SCOD_f$

S°/X° (gCOD/gVSS)	Sugar conc (g/L)	Furfural conc. (g/L)	$TVFA_f$ (g/L)	$SCOD_f$ (g/L)	$TVFA_f/SCOD_f$	Residual sugars (RS) (g/L)	($TVFA_f$ + RS)/$SCOD_f$
4	8.8	4	6.1	10.5	0.58	0.8	0.65
		2	6.7	9.0	0.74	0.7	0.81
		1	6.0	9.2	0.65	0.5	0.70
		0.5	5.1	7.8	0.65	0.8	0.75
		0	5.0	7.0	0.71	0.6	0.80
2	4.4	4	3.3	7.2	0.46	0.1	0.48
		2	3.2	5.1	0.63	0.6	0.75
		1	3.1	4.5	0.68	0.3	0.74
		0.5	3.4	4.3	0.79	0.4	0.88
		0	2.8	4.1	0.68	0.2	0.73
1	2.2	4	2.0	4.9	0.41	ND	0.41
		2	1.8	3.2	0.57	ND	0.57
		1	1.7	2.9	0.59	ND	0.59
		0.5	1.7	2.5	0.67	ND	0.67
		0	1.6	2.0	0.82	ND	0.82
0.5	1.1	4	1.6	3.9	0.41	ND	0.41
		2	2.1	2.4	0.89	ND	0.89
		1	1.3	1.7	0.76	ND	0.76
		0.5	1.9	2.0	0.93	ND	0.93
		0	1.0	1.2	0.85	ND	0.85

ND - Not detected (detection limit of < 0.05 g/L)

3.3.4 Impact of furfural

A comparison between hydrogen yields and furfural concentrations was made by looking at the initial g furfural/g TSS ratio at all experimental conditions. Ideally, the lower the g furfural to g TSS ratio, the higher the hydrogen yield. A plot of hydrogen yield (mol H_2/mol sugars) against furfural/g TSS $_{initial}$ shown in Fig. 3.3 emphasizes the negative linear correlation observed with relatively high R^2 values. Also, irrespective of the S°/X°, pooled hydrogen yields data showed a negative linear correlation (R^2 = 0.78) to g furfural/g sugars $_{initial}$ (Fig. 3.4), indicating that both parameters influence hydrogen production.

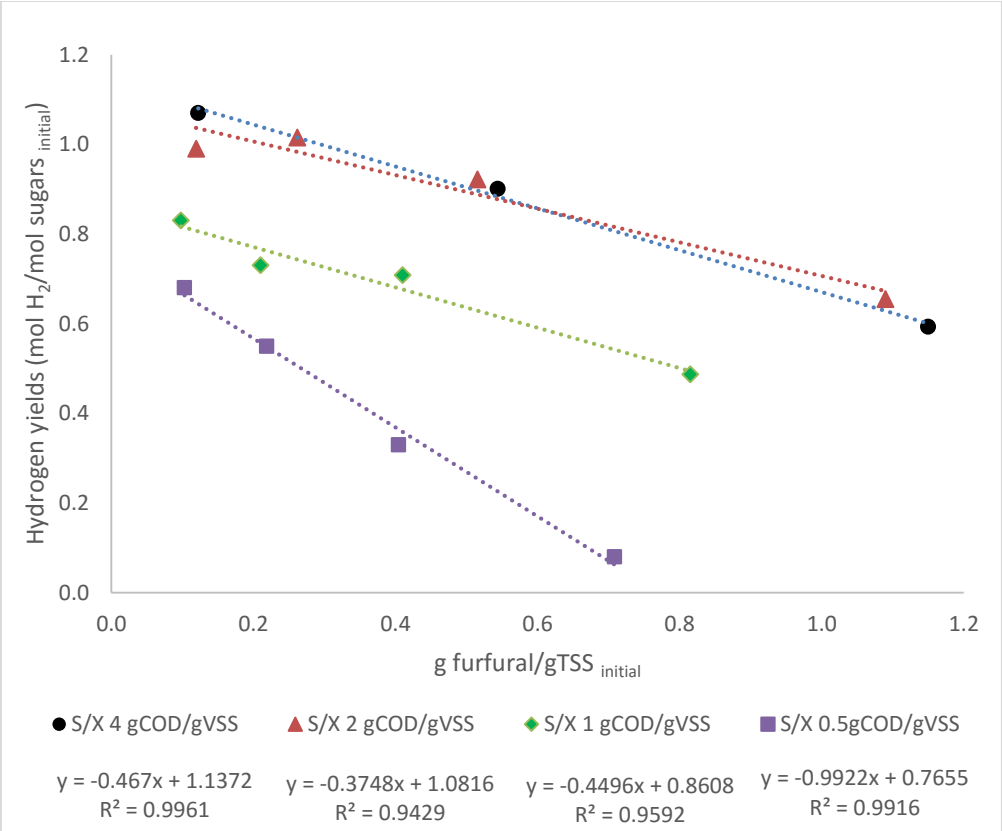

Figure 3.3: Hydrogen yields plotted against g furfural/g TSS $_{initial}$ at all S°/X°

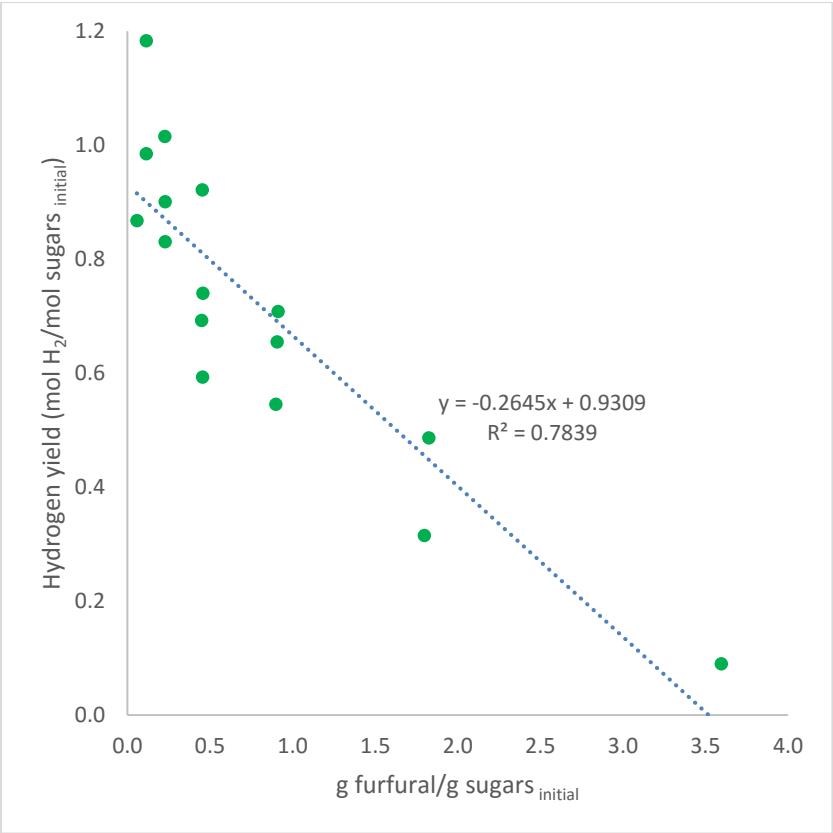

Figure 3.4: Pooled hydrogen yields data plotted against g furfural/g sugars $_{initial}$

A comparison of the results in the present study with similar literature studies operated batch systems (Table 3.3) show that for each study using the same microbial culture, the lower the g furfural/g sugars $_{initial}$ and g furfural/g VSS $_{initial}$, the higher the hydrogen yield. Also, irrespective of the S°/X°, the hydrogen yields decreased with increasing furfural concentrations. Again, this is true in all cases except at the conditions in this study where hydrogen yields were enhanced. Monlau et al. (2013) reported that the hydrogen yield from glucose decreased from 1.83 mol H_2/mol sugar $_{initial}$ at 0.17 g furfural/g VSS $_{initial}$ and 0.01 g furfural/g sugar $_{initial}$ to 0 mol H_2/mol sugar $_{initial}$ at 0.69 g furfural/g VSS $_{initial}$ and 0.031 g furfural/g sugar $_{initial}$ despite a relatively constant S°/X° of about 21 g sugars/gVSS $_{initial}$. The same trend was also observed by Siqueira and Reginatto (2015) despite operating at a lower S°/X° of 7.44 g sugars/gVSS $_{initial}$. On the other hand,

at the lower range of S°/X° values of 0.5 – 3.6 g sugars/g $VSS_{initial}$ used in this study, the hydrogen yields were relatively higher than yields from other studies carried out at about the same furfural concentration. Furthermore, as evident from the data in Table 3.3, lag phase generally increased with increasing furfural concentrations indicating the inhibitory effect of furfural.

Table 3.3: Comparison of fermentation parameters (Hydrogen yield, S°/X°, g Furfural/g sugars $_{initial}$ and g furfural/g biomass $_{initial}$)

Substrate	Sugar concentration	Furfural conc.	Hydrogen yield	S°/X°	g fur/g sugars $_{initial}$	g fur/g VSS $_{initial}$	Lag phase	Reference
	g/L	g/L	mol H_2/mol sugars $_{initial}$	g sugars/g VSS $_{initial}$			day	
Xylose	5	1	0.51	28.4	0.2	5.68	18.5	Quéméneur et al, 2012
Glucose	5	0.17	0	22.1	0.031	0.69	> 30	Monlau et al. 2013
		0.09	0.45	21	0.016	0.34	5.82	
		0.04	1.83	20.5	0.008	0.172	2.24	
Glucose	40	2	0	7.44	0.05	0.372	*Not reported	Siqueira and Reginatto, 2015
		1	0.1		0.025	0.186		
		0.5	0.15		0.013	0.093		
		0.25	0.18		0.006	0.047		
Synthetic hydrolysate (mix of xylose, mannose, glucose, galactose, arabinose, acetic and formic acids)	8.8	4	0.59	3.6	0.45	1.64	1.23	This study
		2	0.9		0.23	0.82	0.68	
		1	1.18		0.11	0.41	0.41	
		0.5	0.87		0.06	0.2	0.43	
	4.4	4	0.65	1.8	0.91	1.64	1.53	
		2	0.92		0.45	0.82	0.99	
		1	1.02		0.23	0.41	0.89	
		0.5	0.93		0.11	0.2	0.56	
	2.2	4	0.49	0.9	1.83	1.64	1.36	
		2	0.71		0.91	0.82	0.87	
		1	0.74		0.46	0.41	0.65	
		0.5	0.84		0.23	0.2	0.65	
	1.1	4	0.09	0.46	3.6	1.64	1.3	
		2	0.31		1.8	0.82	0.78	
		1	0.55		0.9	0.41	0.65	
		0.5	0.69		0.45	0.2	0.63	

Enhanced hydrogen yields were observed at S°/X° of 4 and 2 gCOD/gVSS at both 0.5 g/L and 1 g/L furfural with 19 % and 8 % increase respectively above their respective controls and at S°/X° of 1 and 0.5 gCOD/gVSS at 0.5 g/L furfural with 7 % and 4 % increase above their respective controls (0 g/L furfural). Note that both acetic and formic acids which were components of the substrate are inhibitory compounds (Cantarella et al., 2004; Cao et al., 2010; Kumar et al., 2014; Siqueira and Reginatto, 2015). It is possible that the synthetic hydrolysate in the presence of furfural and seed sludge used in this study, had an enhancory effect on hydrogen production at low furfural concentrations. In comparison to these results, a study by Cao et al. (2010) which examined hydrogen production and cell growth rate of *Thermoanaerobacterium thermosaccharolyticum W16* (4% v/v) on acid pretreated corn stover hydrolysate in batches, showed that hydrogen production started to decrease significantly at 1 g/L furfural (50.2 % inhibition) with little or no hydrogen observed at 1.8 g/L furfural. Another study by Nasr et al. (2014) showed that furfural concentrations of <1.09 g/L had no impact on hydrogen production from various streams of pretreated corn cobs using mesophilic anaerobic digester sludge. Batch tests using 40 g/L glucose and mixed sludge were set up for fermentative hydrogen production in the presence of furfural (0.25 – 2 g/L) and showed decreasing hydrogen yields with increasing furfural concentrations and no hydrogen was produced at 2 g/L furfural (Siqueira and Reginatto, 2015). Monlau et al. (2013) observed a similar trend but with no hydrogen production at 0.4 g/L furfural from 5 gVS/L glucose in batch tests using mixed cultures. Another batch study using anaerobic digester sludge (ADS) with 5 g/L xylose as substrate, reported a 69 % decrease in hydrogen yield upon addition of 1 g/L furfural (Quéméneur et al., 2012). While generally, all the aforementioned studies observed furfural inhibition, the inhibition threshold levels varied due to changes in furfural-to-sugar and furfural-to-biomass ratios as explained above.

The observation that at the higher S°/X° ratios (4 and 2 gCOD/gVSS), furfural enhanced hydrogen yields at 1 g/L and not at 0.5 g/L and at the lower S°/X°, yield was enhanced only at 0.5 g/L furfural from their respective controls is not well understood. However, scrutiny of the data in Table 3.3 reveals that at any given S°/X°, the yields were high and close to optimum at or below furfural-to-sugar ratios of 0.23 and furfural-to-biomass ratios of 0.41. Equation 3.4 presents a thermodynamically favorable reaction proposed by Haroun et al. (2016) where furfural was broken down into acetic acid and hydrogen at low concentrations.

$$C_5H_4O_2 + 6H_2O \longrightarrow CH_3COOH + 3CO_2 + 6H_2 \quad \Delta G = -152 \text{ KJ/mol} \qquad 3.4$$

In fermentation, acetic acid production is a hydrogen-producing pathway (Guo et al., 2010). This observation rationalizes the results obtained in this study as enhancement in hydrogen production and yields were observed at low concentrations of up to 1 g/L furfural. Also, a confirmatory test using mesophilic seed only and 1 g/L furfural without any substrate was tested and indeed hydrogen was produced thus confirming that mesophilic cultures were able to anaerobically degrade furfural.

Liu et al. (2015) reported no hydrogen production at concentrations between 0 – 1 g/L furfural but their findings with hydroxylmethyl furfural (HMF) are similar to those in this study. They observed a stimulatory effect on hydrogen production at HMF concentrations of up to 1 g/L from steam-exploded corn stalk. They further explained that probably HMF affected the activity of seed sludge, and that the mechanism needs to be further studied.

3.3.5 Statistical analysis

Statistical analysis of the data obtained at various S°/X° and furfural concentrations was performed. Treatment of the experimental results was based on the average of the triplicate cumulative

hydrogen values and the two-way ANOVA at a 95 % confidence level. The differences between test conditions were considered significant at $P < 0.05$.

Results determine whether the variables (S°/X° and furfural concentrations) and their interaction (SX*Furfural) had statistically significant effects on hydrogen yield suggesting that S°/X° and furfural concentrations were dependent on each other. Results shown in Table 3.4 indicate a statistically significant interaction at p= 0.000 (since p < 0.05) at all S°/X° and furfural concentrations. Maximum hydrogen production rates (R) (mL/hr) were analyzed and the same degree of significance was observed.

Another test i.e. the Tukey's Honestly Significant Difference (HSD) test (a post-hoc test for multiple comparisons) was carried out following the ANOVA test in order to further investigate which pairs of S°/X° levels (and furfural concentrations) yielded significantly different cumulative hydrogen profiles by comparing two means (Abdi and Williams, 2010). Table 3.5a presents pairwise comparisons between all the furfural concentrations tested while Table 3.5b presents comparisons of the means between S°/X°. These results suggest that all pairs of furfural concentrations and S°/X° gave statistically significantly different cumulative hydrogen profiles (p-value < 0.05).

Table 3.4: Two-way ANOVA analysis results

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	591705.754[a]	19	31142.408	1508.347	0.000
Intercept	804244.931	1	804244.931	38952.69	0.000
S°/X°	537768.055	3	179256.018	8682.061	0.000
Furfural	28202.18	4	7050.545	341.485	0.000
S°/X° * Furfural	25735.519	12	2144.627	103.873	0.000
Error	825.868	40	20.647		
Total	1396776.553	60			
Corrected Total	592531.623	59			

[a]: R squared =0.999; df: degree of freedom; F: is the ratio of two different measures of variance for a set of data

Table 3.5a: Tukey's HSD test for multiple comparisons between furfural concentrations

				95 % Confidence interval	
Furfural (I)	Furfural (J)	Mean difference (I-J)	Sig.	Lower Bound	Upper Bound
0	0	7.1433	0.004	1.8452	12.4415
	1	-13.8542	0	-19.1523	-8.556
	2	12.7567	0	7.4585	18.0548
	4	50.9283	0	45.6302	56.2265
0.5	0	-7.1433	0.004	-12.4415	-1.8452
	1	-20.9975	0	-26.2956	-15.6994
	2	5.6133	0.033	0.3152	10.9115
	4	43.785	0	38.4869	49.0831
1	0	13.8542	0	8.556	19.1523
	0.5	20.9975	0	16.6994	26.2956
	2	26.6108	0	21.3127	30.909
	4	64.7825	0	59.4844	70.0806
2	0	-12.7567	0	-18.0548	-7.4585
	0.5	-5.6133	0.033	-10.9115	-0.3152
	1	-26.6108	0	-31.909	-21.3127
	4	38.1717	0	32.8735	43.4698
4	0	-50.9283	0	-56.2265	-45.6302
	0.5	-43.785	0	-59.0831	-38.4869
	1	-64.7825	0	-70.0806	-59.4844
	2	-38.1717	0	-43.4698	-32.8735

Table 3.5b: Tukey's HSD test for multiple comparisons between S°/X°

				95 % Confidence interval	
S°/X° (I)	S°/X° (J)	`Mean difference (I-J)	Sig.	Lower Bound	Upper Bound
0.5	1	-35.4	0	-39.8	-31.0
	2	-114.9	0	-119.4	-110.5
	4	-246.5	0	-250.9	-242.0
1	0.5	35.4	0	31.0	39.8
	2	-79.5	0	-84.0	-75.1
	4	-211.1	0	-215.5	-206.6
2	0.5	114.9	0	110.5	119.4
	1	79.5	0	75.1	84.0
	4	-131.5	0	-136.0	-127.1
4	0.5	246.5	0	242.0	250.9
	1	211.1	0	206.6	215.5
	2	131.5	0	127.1	136.0

3.4 Summary and Conclusions

This work studied the effects of furfural on biohydrogen production using mesophilic anaerobic digester sludge with synthetic hydrolysate as substrate. The major conclusions that can be deduced from this work include:

- An S°/X° of 4 was observed to be optimal of all the ratios tested as it produced the highest hydrogen yields under the given test conditions
- Hydrogen yields were enhanced at all S°/X° ratios tested at low furfural concentrations of ≤ 1 g/L with increase as high as 19 % from that of the control
- Furfural concentrations greater than the threshold concentration of 1 g/L (i. e. 2 g/L and 4 g/L) were inhibitory as indicated by longer lag phases and lower yields
- Furfural-to-sugar and furfural-to-biomass ratios are important parameters that influence fermentative hydrogen production from lignocellulosic biomass

3.5 References

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CHAPTER 4

Impact of furfural on biological hydrogen production kinetics from synthetic lignocellulosic hydrolysate using mesophilic and thermophilic mixed cultures[2]

4.1 Introduction

As the world strives towards a low-carbon future, the need to zero down on a fuel that is emissions-free, cheap and reliable cannot be over-emphasized. Hydrogen has been described as a key fuel for the future as it burns clean (zero CO_2 emissions) (Ntaikou et al, 2009), has a high heating value (142 MJ/kg) (Tuna et al., 2009) and can be produced from waste biomass (Nath and Das, 2011). Hydrogen can be generated through several means, most of which are fossil-fuel reliant, energy intensive and expensive but biological hydrogen production is fast gaining widespread attention as a viable and sustainable substitute to the current traditional methods of hydrogen production (Rajhi et al., 2013). The most favorable method of biological hydrogen production is dark fermentation which is a process where micro-organisms convert sugars to hydrogen, carbon dioxide and organic acids (Show et al., 2012). It is a light-independent process and is considered the most beneficial method of hydrogen production since it can be carried out in simple reactors, can be used on a wide range of substrates at non-sterile conditions and produces hydrogen at high rates and costs (Hallenbeck *et al.*, 2012, Show et al., 2012; Valdez-Vazquez *et al.*, 2005; Wang and Wan, 2008).

Lignocellulosic wastes have been identified as ideal substrates for hydrogen production as they are carbohydrate–rich, abundant in nature, cheap, do not compete for land with food and their use could help alleviate land pollution (Pan et al., 2010; Procentese et al., 2014). Examples of lignocellulosic wastes include agricultural and food processing wastes such as corn stover, sugarcane bagasse, rice straw etc; municipal solid wastes such as paper, plastics, cloth etc; and forestry wastes such as

[2] A version of this chapter has been submitted to the *Renewable Energy* journal for publication

poplar wood (Adapa et al., 2011; Show et al., 2012). These wastes, however, are made up of complex carbohydrates (mainly cellulose and hemicellulose) which need to be broken down into simpler sugars for easy conversion to hydrogen. This breakdown is done using pretreatment processes which produce several by-products in addition to simple sugars. One of these by-products is furfural, which is formed when pentoses present mainly in the hemicellulosic component of lignocellulosic biomasses are broken down during acid or alkaline pretreatment processes (Aguilar et al., 2002; Cantarella et al., 2004). Furfural is thought to adversely affect the membrane growth, integrity and permeability of hydrogen-producing bacteria by reducing biological and enzymatic functions, destroying DNA and inhibiting protein synthesis, which lead to decreased hydrogen production rates and yields (Liu et al., 2004). For these reasons, furfural is described as an inhibitor and a limiting factor in the fermentative hydrogen production process.

The impact of furfural on the kinetic parameters of hydrogen production in a mixed culture environment is not available in the literature. To the best of the authors' knowledge, no kinetic model has been used to describe the impact of furfural on biohydrogen production from lignocellulosic wastes using mixed cultures. Kinetic studies of simple substrates such as glucose, cellobiose, sucrose etc. using pure and mixed cultures are presented in Table 4.1a and 4.1b respectively. The simple Monod model as well as modifications of this model were employed in describing their kinetic parameters. A batch fermentative hydrogen production study by Siqueira and Reginatto (2015) using mixed cultures grown on 40 g/L glucose in the presence of furfural concentrations in the range 0 g/L to 2 g/L discussed furfural inhibition in terms of the Gompertz model. The aforementioned authors revealed a decrease in the hydrogen yields, maximum hydrogen production potential and maximum hydrogen production rate with increasing furfural concentration. Also, the lag phase duration increased with increasing furfural concentration.

Mesophilic mixed cultures are mostly used for biogas production but mixed cultures at thermophilic conditions are gaining wide-spread interest as they have been reported to produce very high hydrogen yields (Yokoyama et al., 2009). While progress has been made in optimizing pretreatment methods of lignocellulosic biomass to achieve higher sugar yields, potential inhibition by furfural as well as other industrially important fermentation products needs to be thoroughly studied in order to reduce their inhibitory effects and enable the cost-effective and practical conversion of this biomass. The aim of this study, therefore, was to investigate the impact of furfural on hydrogen production and microbial kinetics, from lignocellulosic biomass using mixed cultures (both mesophilic and thermophilic anaerobic digester sludge). The knowledge of these biokinetic parameters through modeling will enhance the engineering of mechanisms, processes, design and optimization of biohydrogen production and its applications and for effective scale-up and design of bioreactors.

Table 4.1 a: Kinetic parameters using various modifications of the Monod model for hydrogen production from pure cultures

Reactor	Temp. (°C)	Model used	Culture	Substrate	μ_{max} (h^{-1})	K_s (g/L)	k_d (h^{-1})	$Y_{x/s}$ (g biomass/g substrate)	Reference
Batch	37	Modified Monod using pH and substrate inhibition	*Ruminococcus albus* DSMZ 20455	Glucose	0.654 ± 0.039	0.765 ± 0.029	-	0.139 ± 0.012	Ntaikou et al., 2009
	37	Simple Monod	*Enterobacter cloacae*	Glucose	0.568	3.658	-	0.084	Kumar et al., 2000
	37	Modified Monod with substrate inhibition term	*Enterobacter cloacae* DM 11	Glucose	0.398	5.509	-	-	Nath et al., 2008
	35	Monod with lower pH inhibition	*Clostridium acetobutyricum* M121	Glucose	-	0.18[a]	-	0.20[b]	Lin et al, 2007
			Clostridium butyricum ATCC 19398		-	0.78[a]	-	0.34[b]	
			Clostridium tyrobutyricum FYa102		-	0.72[a]	-	0.46[b]	
			Clostridium beijerinckii L9		-	0.47[a]	-	0.23[b]	
	37	Monod	*Clostridium termitidis CT1112 (ATCC 51846)*	Glucose	0.30	0.87	0.003	0.21[c]	Gomez-flores et al., 2015
				Cellobiose	0.34	0.37	0.004	0.30[c]	
	58	Monod	*Clostridium thermocellum* wild type	Cellobiose	0.571	0.915	-	0.234	Linville et al., 2013
		Monod with general unitless inhibition factor	*Clostridium thermocellum* mutant strain	Cellobiose	1.223	2.217	-	0.244	
	60	Simple Monod	*Thermoanaerobacterium thermosaccharolyticum* PSU-2	Sucrose	0.31	1.47	-	-	O-thong et al., 2008
	ND	Modified Monod using pH inhibition and decay constant	*Ruminococcus albus*	Glucose	0.603 ± 0.011	276 ± 33.38	-	0.147 ± 0.01	Nath and Das, 2011
Continuous	37	ND	*Citrobacter intermedius*	Glucose	0.22	-	-	0.114[d]	Chen et al., 2006

[a] converted from mmol/L in study to g/L; [b] converted from mmol/mmol in study to g biomass/g substrate; [c] g dry wt/g substrate; [d] converted from g biomass/mole substrate in study to g biomass /g substrate; ND-Not Defined

Table 4.1 b: Kinetic parameters using various modifications of the Monod model for hydrogen production from mixed cultures

Reactor	Temp. (°C)	Model used	Culture	Substrate	μ (h^{-1})	K_s (g/L)	$Y_{x/s}$ (g biomass/g substrate)	Reference
Batch	30	Simple Monod	Mixed microflora from organic farm soil	Glucose	[a]0.001	[b]15.1	-	Sharma and Li, 2009
	35	Michaelis-Menten	Mixed microflora from anaerobic digestor	Sucrose	-	[b]1.29	-	Chen et al., 2006
	37	Monod	Anaerobic digester sludge	Starch	0.048	0.2	0.085[c]	Gupta et al., 2015
				Cellulose	0.05	2.1	0.085[c]	
				Starch-cellulose	0.072	0.1	0.085[c]	
	60	Monod	Anaerobic digester sludge	Starch	0.029	4	0.085[c]	
				Cellulose	0.053	1.7	0.085[c]	
				Starch-cellulose	0.077	3.9	0.085[c]	
	ND	First order	Mixed cultures	Sucrose	0.1	-	-	Sung et al., 2003
				Non-fat dried milk	0.176	-	-	
				Food waste	0.215	-	-	
Continuous	35	Monod with Dilution rate (D) term	Mixed cultures	Sucrose	0.172	[b]0.061	0.1[d]	Chen et al., 2001
	ND	Monod with D and endogenous rate constant (K_e) term	Mixed anaerobic microflora from food processing waste water	Glucose	1	0.178	0.45	Nath and Das, 2011
		Monod with Dilution rate (D) term	Mixed anaerobic microflora from UASB reactor treating food processing waste water	Glucose	0.75	0.2	0.3	

[a] converted from d^{-1} in study to h^{-1}; [b] converted from gCOD/L in study to g/L; [c] biomass yield converted from gVSS/gCOD in study to gVSS/g sugar using 1.067 gCOD/g sugars; [d] converted from gVSS/mole substrate in study to g VSS/ g sucrose, ND: Not defined

4.2. Materials and Methods

4.2.1. Microbial seed and Substrate

Mesophilic anaerobic digester sludge (ADS) was obtained from the Guelph Wastewater Treatment Plant, Guelph, Canada while thermophilic ADS was collected from the Ravensview Wastewater Treatment Facility, Kingston, Canada. Both mesophilic and thermophilic ADS used for biohydrogen production were preheated at 70 °C for 30 min prior to use to suppress the activity of hydrogen-consuming bacteria (Hafez et al., 2010). The substrate utilized was synthetic lignocellulosic hydrolysate prepared in the laboratory (utilizing analytical reagent grade chemicals obtained commercially) using the same composition but half the concentrations of the substrate described in Chapter 3 of this work. The substrate comprised on a gCOD basis, 96 % sugars of which 85 % are pentoses (C5 sugars). The characteristics of the ADS and substrate used for this study are presented in Tables 4.2a and 4.2b respectively.

Table 4.2 a: ADS characterization

ADS	pH	*TSS (g/L)	**VSS (g/L)
Mesophilic	7.33 ± 0.01	18.7 ± 0.24	12.7 ± 0.1
Thermophilic	8.06 ± 0.03	19.4 ± 0.43	11.4 ± 0.1

*TSS – Total Suspended Solids; **VSS – Volatile Suspended Solids

Table 4.2 b: Substrate composition

Sugars	Conc in g/L
Arabinose	2.95
Xylose	25
Mannose	0.15
Galactose	1.25
Glucose	3.35
***VFAs**	
Formate	0.62
Acetate	0.91

*VFAs- Volatile Fatty Acids

4.2.2 Experimental Setup

Furfural concentrations of 0 g/L, 1 g/L and 4 g/L were tested in parallel at both mesophilic and thermophilic conditions. Continuously-stirred tank batch bioreactors were operated at an initial (S°/X°) of 4 gCOD/gVSS. All experiments were conducted using 8 L of sludge and a total sugars concentration of 32.7 g/L with a reactor working volume of 11 L. Reactors were purged with nitrogen gas for a few minutes in order to ensure anaerobic conditions throughout the experiment. Reactors were also equipped with thermometers and pH probes for continuous monitoring of temperature - 37 ± 2 ° C (mesophilic) and 55 ± 2 ° C (thermophilic) - and pH at 5.5 ± 0.2 respectively. Temperature was maintained by wrapping the reactors with masterflex L/S 35 pump tubings connected to a thermostatic water bath (Thermo Electron Corporation, 180 Series Precision water bath, Model 2835, USA) and enclosed with insulation jackets. The reactors were fitted with mixing rods for continuous stirring and chemical feed pumps were connected to pH controllers to automatically dose acid (2N HCl) and base (2N NaOH) when required. Liquid samples were taken with time throughout the experiment so as to monitor sugars degradation and products formation with time while gas samples were analyzed every few hours to ascertain hydrogen composition.

4.2.3 Analytical procedures

Hydrogen was measured using a gas chromatograph (Model 310 SRI Instruments, Torrance, CA) complete with a thermal conductivity detector (TCD) and a molecular sieve column (Mole sieve 5 Å, mesh 80/100, 6ft x 1/8 in). Argon gas was used as carrier gas at a flow rate of 30 mL/min and the temperature of column and TCD were 90 °C and 105 °C respectively. Head space gas measurements were calculated using mass balance equations as described by López et al. (2007).

COD was measured using HACH methods and test kits (HACH DRB 200 COD reactor and HACH Odyssey DR 2800 spectrophotometer). TSS and VSS were analyzed using standard methods (APHA, 1998) . Soluble fermentation products (monomeric sugars, furfural, lactic and formic acids) were analyzed using a Dionex IC20 Ion Chromatograph equipped with a refractive index detector (RID) (Perkin Elmer Series 200, PerkinElmer Instruments Inc., USA) and an Aminex® HPX-87H column (BIO-RAD laboratories, USA) with the following parameters: pump flow rate – 0.6 mL/min; mobile phase - 9 mM H_2SO_4, column temperature- 30 °C and injection volume of 0.5 mL. Other VFAs (acetic, propionic, butyric and valeric acids) were analyzed using a gas chromatograph (Varian 8500, Varian Inc., Toronto, Canada) with a flame ionization detector (FID) equipped with a fused silica column (30 m x 0.32 mm) with the following parameters: carrier gas – helium, flow rate - 5 mL/min, column temperature-110 °C, detector temperature – 250 °.

4.2.4 Monod Model development

The Monod model was employed in this study as it integrates microbial growth and substrate consumption. The Monod kinetics parameters- μ_{max}, maximum specific growth rate; K_s, half-saturation constant; k_d, decay coefficient and $Y_{X/S}$, microbial biomass yield of the mixed cultures- were obtained using a numerical model on MATLAB® (version R2015b). A non-linear least square fit, *lsqcurvefit*, was the objective function used.

The solver equation employed to estimate numerical integration of the ordinary differential equations for biomass growth and sugars consumption (Eq. 4.1 & 4.2 respectively) was *Ode45*, which applies fourth and fifth order Runge-kutta methods (Gomez-flores et al, 2015).

$$\frac{dX}{dt} = \frac{\mu_{max}(S)X}{[K_X + (S)]} - k_d X \qquad 4.1$$

$$\frac{dS}{dt} = -\frac{\mu_{max}(S)X}{Y_{X/S}[K_S + (S)]} \quad 4.2$$

Mathematical expressions for product yields were developed to accurately describe the kinetics of production or consumption by adequately modifying the above equations. Product yield equations are as follows:

$$\frac{dPY}{dt} = \frac{Y_{PY/S}}{Y_{X/S}} \frac{\mu_{max}(S)X}{[K_S + (S)]} \quad 4.3$$

where PY is the product yield for each product formed expressed as g product per g sugars.

Palmqvist and Hahn-Hagerdal (2000) reported that furfural degradation rate increased with increasing furfural concentrations and increasing specific growth rate of the micro-organisms. Therefore, furfural was modeled using a first order equation with respect to both inhibitor and biomass concentrations (Linville et al., 2013). Formate degradation had previously been modeled using first order kinetics as it breaks down into CO_2 and hydrogen only (Bagramyan and Trchounian, 2003; Ntaikou et al., 2009). For this study, both formate and furfural were therefore modeled using the following equation:

$$\frac{dW}{dt} = -K_F(F)X \quad 4.4$$

where F (g/L): formate or furfural concentrations; K_F (L g/VSS/h): formate or furfural consumption or degradation constants.

Since lactate was produced and consumed at 1 g/L furfural under mesophilic conditions, it was modeled as a combination of the production and consumption terms shown in eqs. 4.3 and 4.4 to give equation 4.5:

$$\frac{dL}{dt} = \frac{Y_{L/S}}{Y_{X/S}} \frac{\mu_{max}(S)X}{[K_S + (S)]} - K_L(L)X \quad 4.5$$

4.3 Results and Discussion

4.3.1 Effect of furfural on biohydrogen production

Fig. 4.1 presents the cumulative hydrogen production profiles for both mesophilic and thermophilic experiments. Gas volumes at thermophilic temperatures were normalized to 37 °C for comparison purposes. Thermophilic fermentation at 0 g/L furfural produced around 91 L of hydrogen versus 66 L from the mesophilic experiment which represents a 38 % increase in hydrogen production. Reports have shown that thermophilic conditions generally favour hydrogen production compared to mesophilic cultures as higher temperatures depress hydrogen-consuming reactions and favour the kinetics and thermodynamics of hydrogen production (Gupta et al., 2015; Shin et al., 2004; Valdez-Vazquez et al., 2005). It can be observed that the mesophilic experiment at 1 g/L furfural showed a 45 % increase in hydrogen production from the control. Thermophilic experiments at 1 g/L furfural did not show any enhancement but rather a 50 % decrease in hydrogen production from the control, possibly due to the presence of a different community of micro-organisms in the thermophilic cultures which were negatively affected by the presence of furfural. Differences in microbial structures, communities and populations have been reported between mesophilic and thermophilic temperatures. Thus, it can be deduced that at thermophilic conditions, furfural inhibition threshold level is below 1 g/L as compared with above 1 g/L at mesophilic conditions. Higher cumulative hydrogen production (171 mL), rates (3.46 mL H_2/L/h) and yields 0.1 L H_2/g total sugar were observed when hydrogen gas was produced from cheese whey powder using thermophilic mixed cultures compared to mesophilic cultures (Kargi et al., 2012. The aforementioned authors stated that it was probably due to the elimination of hydrogen-consuming bacteria at high temperatures which were active in mesophilic fermentation thus reducing hydrogen

yields and rates. Guo et al. (2010) observed through denaturing gradient gel electrophoresis analyses, differences in the microbial structures of thermophiles with *Clostridium thermocellum* and *Caldanaerobacter subterraneus* observed to be responsible for hydrogen production from cow waste slurry at 60 °C and 75 °C respectively.

Mesophilic and thermophilic yields can be compared in this study as tests were carried out under similar gFur/gVSS$_{initial}$ and gFur/g sugars $_{initial}$ values (as shown in Table 4.3) and hydrogen volume produced under thermophilic conditions were normalized to 37 °C. Hydrogen yields presented in Table 4.3 show a maximum overall hydrogen yield of 1.6 mol H_2/mol sugars at 1 g/L furfural for the mesophilic experiment and a hydrogen yield of 0.7 mol H_2/mol sugars at 1 g/L furfural under thermophilic conditions. 4 g/L furfural was the most inhibitory condition tested as shown by the low hydrogen yield of 0.3 mol H_2/mol sugars under mesophilic conditions with no hydrogen production at thermophilic temperatures indicating that the thermophilic hydrogen-producing community were completely inhibited most likely due to the extremely long contact time (of about 120 days) which might have resulted in the inactivity/death of the cells. Contact time was observed to be longer in the thermophilic than the mesophilic experiments again emphasizing the fact that the microbial communities in both cultures differ from each other.

As xylose was the main sugar present in the substrate, theoretical yields were calculated based on xylose in accordance with the following stoichiometric equation (Chaganti et al., 2012; Fangkum and Reungsang, 2011).

$$C_5H_{10}O_5 + 1.67\ H_2O \longrightarrow 1.67\ CH_3COO^- + 1.67\ CO_2 + 1.67\ H^+ + 3.33\ H_2 \qquad 4.6$$

Based on the above equation, the theoretical hydrogen yield for xylose, ignoring biomass synthesis is 3.33 mol H_2/mol sugar consumed. On a molar basis, mesophilic experiments at 1 g/L furfural

produced 48 % of the theoretical molar yield of hydrogen produced compared with 33 % and 9 % produced at 0 g/L furfural and 4 g/L furfural respectively. Thermophilic experiments showed 42 %, 21 % and 0 % of the theoretical molar hydrogen yield at 0 g/L, 1 g/L and 4 g/L furfural respectively. The theoretical hydrogen yields from xylose on a gCOD basis are 0.53 LH_2/gCOD $_{consumed}$ at 37 °C and 0.56 LH_2/gCOD $_{consumed}$ at 55 °C. Mesophilic experiments showed 96 %, 111 % and 34 % of the theoretical hydrogen yields at 0 g/L, 1 g/L and 4 g/L furfural while thermophilic experiments showed 93 %, 86 % and 0 % on a L H_2/gCOD $_{consumed}$ basis at 0 g/L, 1 g/L and 4 g/L furfural respectively. The increased ratio of experimental-to-theoretical hydrogen production observed at 1 g//L furfural under mesophilic conditions reveals that furfural was consumed as COD and converted to hydrogen as observed by the increased yield compared to the control. Although COD was consumed at 4 g/L furfural under thermophilic conditions, it was not converted to hydrogen. Biomass yield was calculated from initial and final VSS concentrations of each batch and was observed to be approx. 0.1 gVSS/g sugar in all batches except at 4 g/L furfural under thermophilic conditions which had a biomass yield of 0.2 gVSS/g sugar. Hafez et al. (2010) reported biomass yields of 0.09 to 0.21 gVSS/g glucose from biohydrogen production from glucose in a continuous-flow system. The hydrogen yields presented in Table 4.3, have been corrected for the biomass yields using 1.42 gCOD/gVSS as the theoretical conversion factor.

COD mass was calculated based on 8 gCOD/gH_2 using hydrogen densities of 0.079 gH_2/L and 0.0748 gH_2/L at 37 °C and 55 °C respectively. The mass balance closed on average at 84 ± 2 % and 85 ± 3 % in mesophilic and thermophilic experiments respectively showing the reliability of these data.

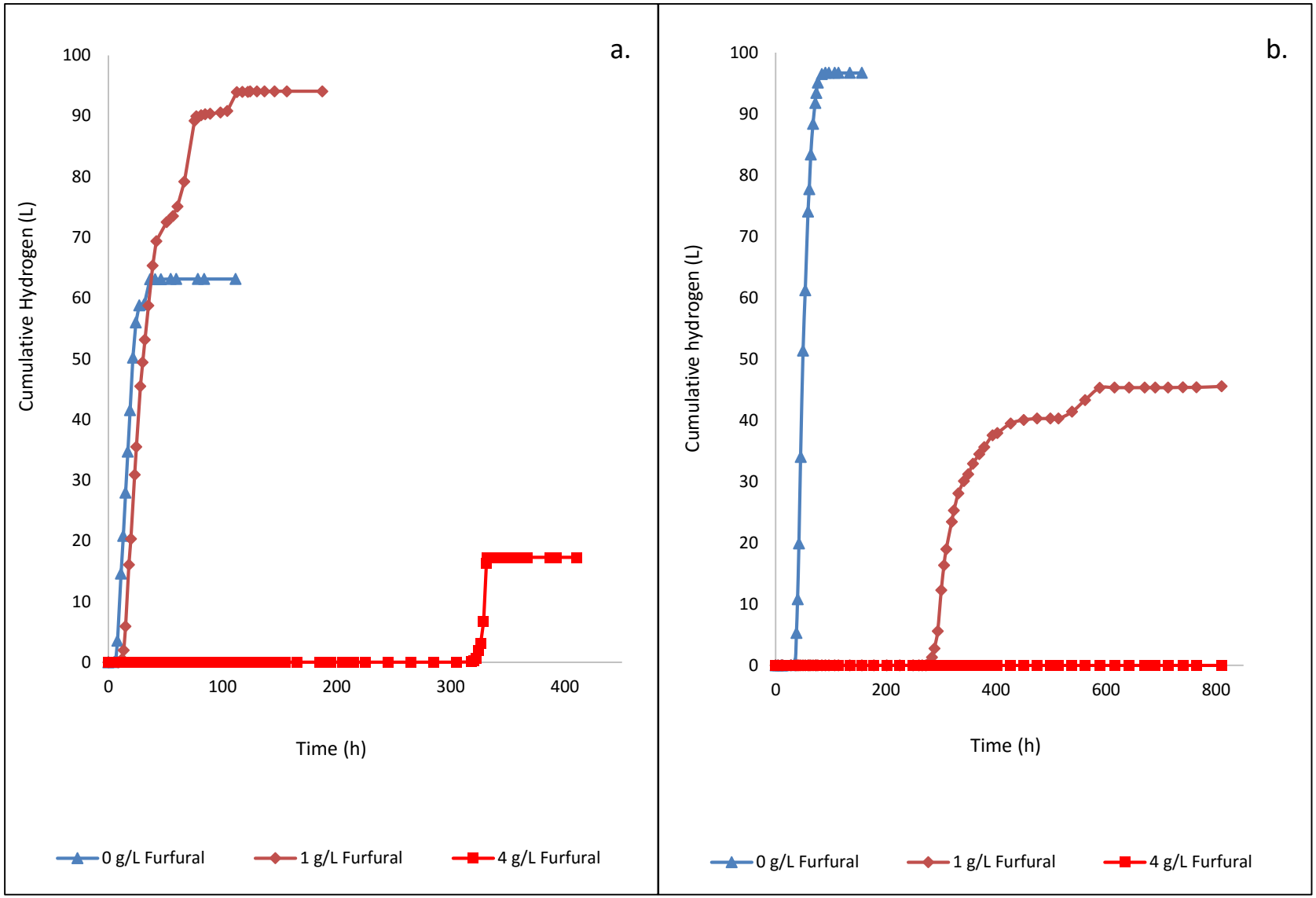


Figure 4.1: a) Mesophilic cumulative hydrogen production profile b) Thermophilic cumulative hydrogen production profile

Table 4.3: Hydrogen yields from mesophilic and thermophilic experiments

Batch experiment	Furfural conc	H_2 prod.	Experimental Yields					
	g/L	L H_2	mol H_2/mol sugars	gFur/gVSS $_{initial}$	g Fur/g sugars $_{initial}$	LH_2/gCOD $_{sugar\ added}$	LH_2/gCOD $_{consumed}$	**% of theoretical
Mesophilic (37 °C)	0	66	1.1	-	-	0.17	0.51	96
	1	96	1.6	0.11	0.03	0.25	0.59	111
	4	19	0.3	0.43	0.12	0.04	0.18	34
Thermophilic (55 °C)	0	*97	1.4	-	-	0.23	0.52	93
	1	*46	0.7	0.12	0.03	0.10	0.48	86
	4	*0	0	0.48	0.13	0	0	0

*Volumes of hydrogen at thermophilic temperatures were corrected to 37 °C (mesophilic temperatures) for comparison basis; **based on yields in LCH_4/gCOD consumed

4.3.2 Metabolite formation

Hydrogen production through anaerobic processes always occurs with VFA production. Soluble samples collected over time were analyzed for residual sugars and VFAs. All sugars were completely degraded in both mesophilic and thermophilic experiments. Fig. 4.2 presents the substrate degradation and metabolites (VFA) formation profiles for mesophilic and thermophilic experiments. Xylose, mannose and galactose were detected at the same retention time on the ion chromatograph used for analysis so they were measured together. Formate was completely broken down while acetate was shown to increase with time in all experiments, as expected when hydrogen is produced, except at 4 g/L furfural at thermophilic conditions where no hydrogen was observed and acetate concentration remained constant throughout the experiment. Lactate produced at 1 g/L furfural under mesophilic conditions was completely consumed at the same time as evidenced by a slight peak in hydrogen production (See Fig. 4.2a). This observation is confirmed as lactate has been reportedly utilized to produce hydrogen in addition to acetic acid, water and CO_2 (Costello et al., 1991; Grause et al., 2012). In the case of the thermophilic experiments, since formate was consumed from the start, Fig. 4.2b shows no formate profiles.

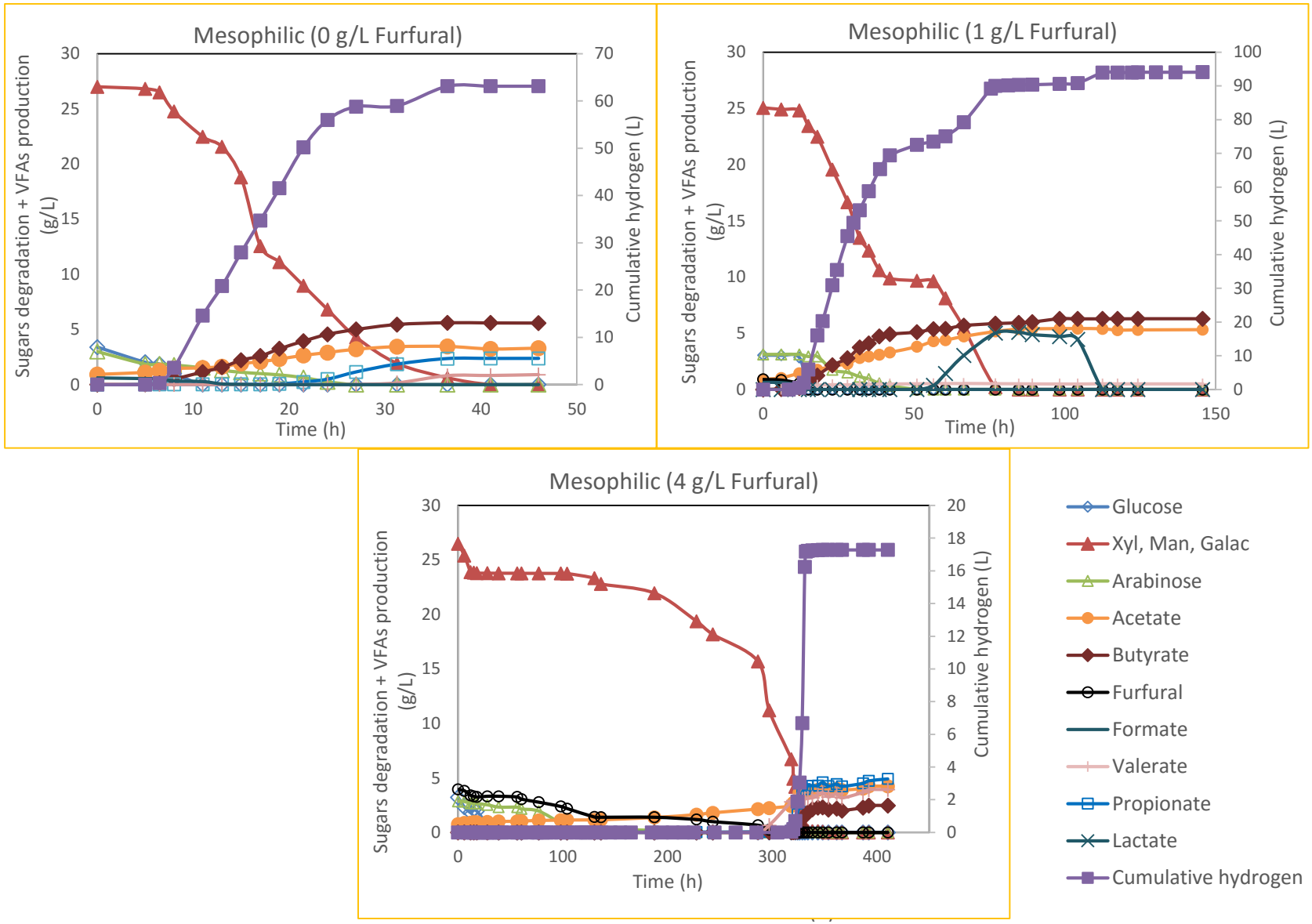


Figure 4.2 a: Cumulative hydrogen curves for mesophilic experiment at 0 g/L, 1 g/L and 4 g/L furfural

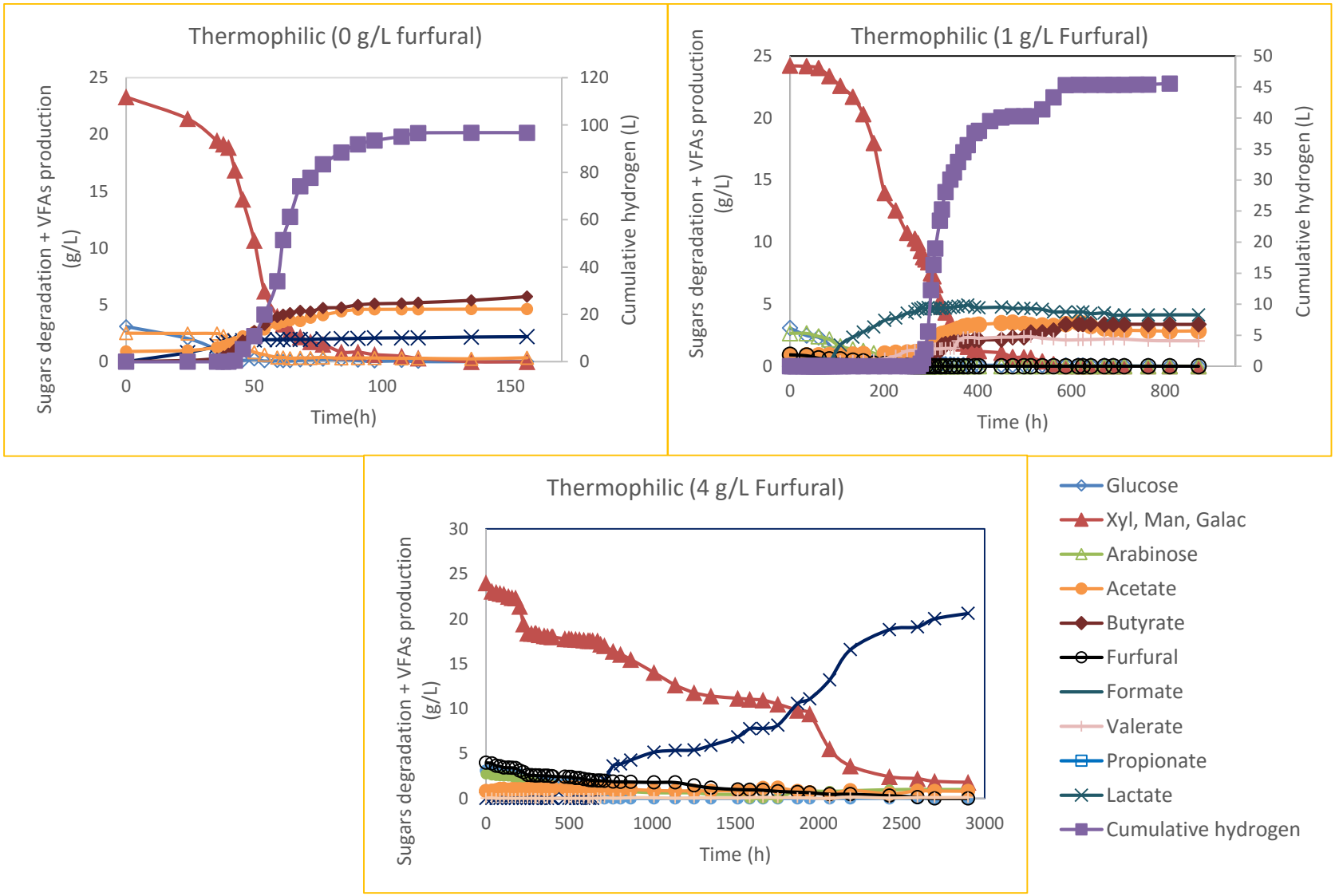


Figure 4.2 b: Cumulative hydrogen curves for thermophilic experiment at 0 g/L, 1 g/L and 4 g/L furfural

In both mesophilic and thermophilic controls, hydrogen was a direct product of substrate consumption but in the batches containing furfural, hydrogen was produced only after furfural was completely degraded. In both experiments, the higher furfural concentrations took longer to degrade as expected but although the sugars were observed to be broken down, hydrogen was not produced. The sugars were reduced into products that were not analyzed and which probably could not be further broken down to give hydrogen as the percentage of known final TVFAs to SCOD final, shown in Table 4.4, for both mesophilic and thermophilic experiments, reveal that a small percentage of TVFAs were unaccounted for. Fig. 4.3 presents plots of the temporal variation of the TVFA-to-SCOD ratio and cumulative hydrogen production against time for both mesophilic and thermophilic experiments. It can be observed that TVFA-to-SCOD ratio increases in the same rate in the exponential phase as hydrogen production in all cases except at 4 g/L furfural under thermophilic conditions were even though TVFA-to-SCOD ratio increased with time, no hydrogen was produced which explains that SCOD was definitely converted to other unknown compounds which were unaccounted for. This statement also holds true for the stationary phases in the mesophilic experiment at 0 g/L and 4 g/L furfural and the thermophilic experiment at 0 g/L furfural where TVFA-to-SCOD ratio kept increasing with time without any increase in hydrogen production. By the time furfural was completely reduced, the hydrogen producers in the cultures picked up and produced hydrogen from the sugars that were left in the reactor. It has been reported that furfural can be broken down to acetate at low concentrations and it is known that the acetic acid pathway is the most predominant pathway for hydrogen production. Boopathy and Daniels (1991) reported that *Desulfovibrio furfuralis* converted a maximum of 0.48 g/L furfural to 2 mol acetate/mol furfural while a sulphate-reducing bacterium isolate reduced a maximum of 1.1 g/L furfural to 1 mole acetate /mol furfural in batch studies. A 17 % and 6 % increase in hydrogen

yields were observed relative to the control, in the presence of 0.25 g/L furfural in 10 g/L glucose-fed and 10 g/L xylose-fed reactors respectively using acclimatized mixed cultures in continuous–flow systems (Haroun et al, 2016). It can also be asserted that furfural might have been degraded to intermediates such as furfuryl alcohol, furoic acid, furaldehyde etc. (Belay et al., 1997; Huber et al., 2010) which could have been converted to hydrogen, rationalizing the enhanced mesophilic yield observed at 1 g/L furfural. In the thermophilic experiment, lactate accounted for over 70 % of the SCOD at 4 g/L furfural, and the lactic acid pathway is a non-hydrogen producing pathway (Guo et al., 2010).

One of the most important factors to be considered in hydrogen production is pH as it affects metabolic pathways, thus regulating the distribution of end products and possibly influencing the length of the lag phase (Bartacek et al., 2007; Davilla-Vazquez et al., 2008; Hallenbeck and Ghosh, 2009; Saraphirom and Reungsang, 2010). The effect of pH in this study was eliminated by strict pH control within a very narrow range of 5.5 ± 0.2 as it has been reported that microbial cultures are sensitive to pH changes thus affecting the observed metabolites formed as well as the microbial community structure (Lee et al., 2008).

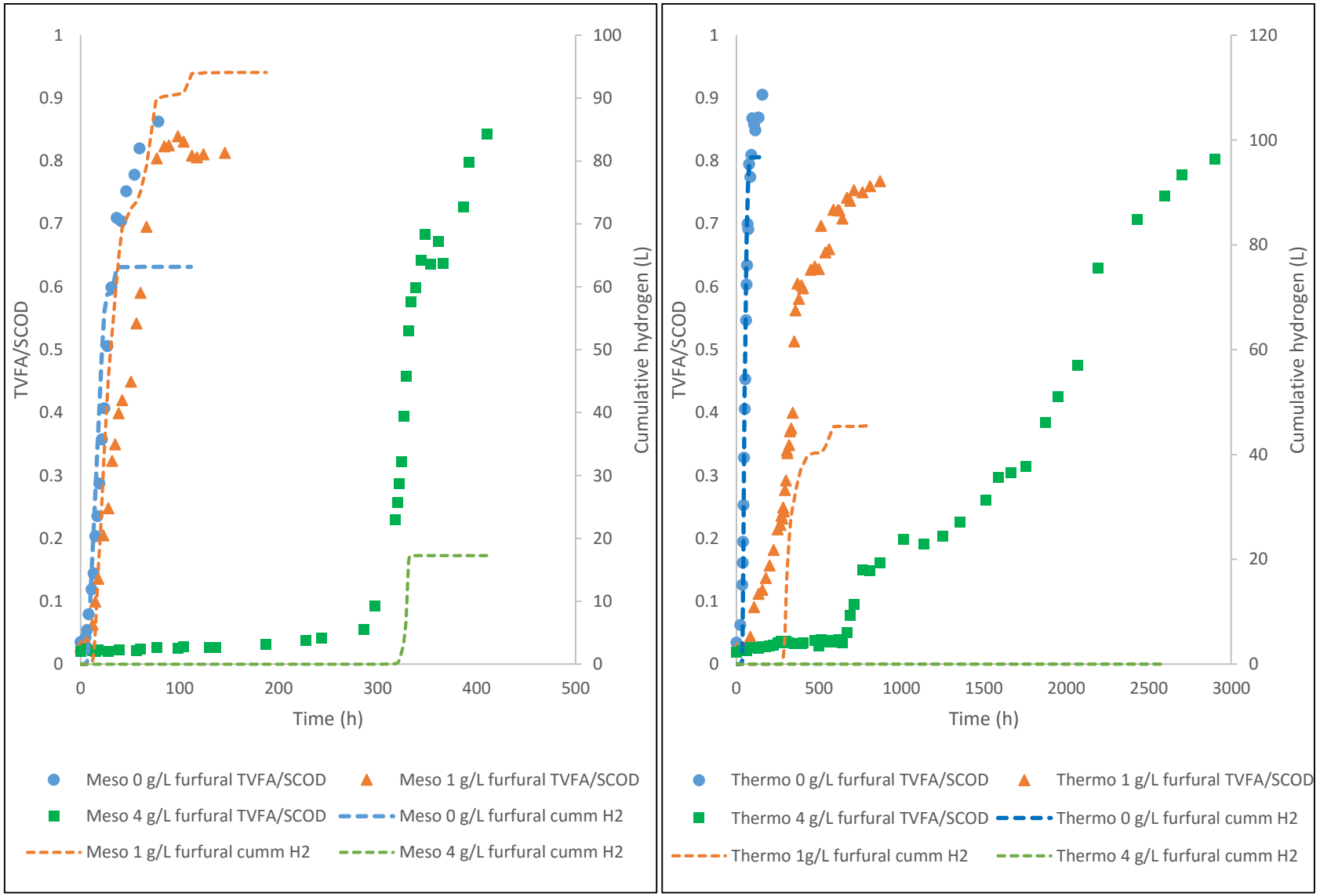


Figure 4.3: TVFA/SCOD and cumulative hydrogen production with time for mesophilic and thermophilic experiments

4.3.3 Final fermentation metabolites

Table 4.4 shows the VFA fractions observed at the end of the batch as well as (TVFA/SCOD) % for both mesophilic and thermophilic experiments.

4.3.3.1 Mesophilic experiments

VFA analysis revealed high concentrations of acetate and butyrate with butyrate being predominant (on a concentration basis) at 0 g/L and 1 g/L furfural while propionate was the main VFA observed at 4 g/L furfural which gave the least hydrogen production. About half the concentration of propionate observed at 4 g/L was produced at 0 g/L furfural but 1 g/L furfural which gave the highest hydrogen production, showed negligible propionate concentrations which is reasonable since the propionate pathway is associated with low hydrogen production as it is a hydrogen-consuming metabolite (Kongjan et al.; 2009, Shin et al., 2004). It can therefore be inferred that furfural at 1 g/L changed the biodegradation pathway of hydrogen production thus showing enhanced yields. Valerate was also observed at all cases but showed the highest concentration at 4 g/L furfural and least at 1 g/L furfural. Lactate was observed to be produced at only 1 g/L furfural but was subsequently consumed. Negligible ethanol concentrations were observed at 1 g/L and 4 g/L furfural. TVFAs were over 80 % of the SCOD observed across all furfural concentrations. Higher acetate concentrations are associated with increased hydrogen production (Gupta et al., 2015). This observation was also noticed in this study in the control which showed the highest acetate concentration and hydrogen production.

4.3.3.2 Thermophilic experiments

As apparent from Table 4.4, lactate was observed at all furfural concentrations including the control and concentration increased with increasing furfural concentration. Valerate and negligible

propionate were observed at 1 g/L furfural with neither product observed at 0 g/L furfural. No butyrate was observed at the 4 g/L furfural experiment while acetate concentration was constant from the start to the end of experiment (neither produced nor consumed) which is reasonable as no hydrogen was observed considering that acetate and butyrate are hydrogen-producing pathways. Acetate and butyrate concentrations decreased with increasing furfural concentration. Acid-forming pathways dominated the TVFA composition of both mesophilic and thermophilic experiments as the percentage of TVFAs to SCOD was greater than 75 % in all cases.

The relatively higher concentrations of butyrate compared to acetate at 0 g/L and 1 g/L furfural reveal that both mesophilic and thermophilic hydrogen production thermodynamically favoured the butyric acid pathway. Valdez-Vazquez et al., (2005) also reported that butyrate was the predominant VFA observed during the semi-continuous hydrogen production from the organic fraction of municipal solid waste at 37 ℃.

Table 4.4: Metabolites concentration and COD mass balance for mesophilic and thermophilic experiments

Mesophilic experiment		Metabolites							
Furfural conc. (g/L)	**Unit**	**$SCOD_f$**	**EtOH**	**HAc**	**HPr**	**Iso HBu**	**HBu**	**HVa**	**% TVFAs of SCOD**
0	g/L	23.4	0	3.5	2.4	0	5.6	1.3	86
	gCOD	257.4	0	40.6	40.0	0	112.4	29.0	
1	g/L	22.55	0.14	5.3	0.1	0.1	6.3	0.5	83
	gCOD	248.1	3.2	62.4	2.0	2.2	125.8	11.4	
4	g/L	29.0	0.07	4.2	4.9	0	2.5	3.9	85
	gCOD	319.0	1.4	49.3	81.8	0	50.0	88.1	

Thermophilic experiment		Metabolites							
Furfural conc. (g/L)	**Unit**	**$SCOD_f$**	**EtOH**	**HAc**	**HPr**	**HBu**	**HLa**	**HVa**	**% TVFAs of SCOD**
0	g/L	19.95	0	4.6	0	5.7	2.2	0	89
	gCOD	219.5	0	54.3	0	114.6	25.8	0	
1	g/L	23.25	0.14	2.9	0.05	3.4	4.2	2.1	78
	gCOD	255.8	3.2	33.5	0.8	67.4	48.7	46.0	
4	g/L	28.88	0.05	0.86	0.03	0	20.6	0.11	81
	gCOD	317.7	1.1	10.1	0.5	0	241.8	2.5	

$SCOD_f$ = Soluble COD final; TVFAs= Total Volatile Fatty Acids;

Metabolites COD accounts for the sum of ethanol (EtOH), acetate (HAc), propionate (HPr), butyrate (HBu), isobutyrate (Iso HBu), lactate (HLa), and valerate (HVa) as mg COD

% TVFAs = (sum of TVFA COD/$SCOD_f$) * 100

4.3.3.3 Comparison of experimental and theoretical hydrogen production

Based on the theoretical volumes of hydrogen that can be produced from acetate and butyrate (Chaganti et al., 2012) and consumed from propionate (Gupta et al., (2015), the theoretical hydrogen production from a combination of these VFAs were calculated using xylose as the ideal substrate (as it was the most abundant sugar in the substrate composition) and hydrogen densities of 0.079 g/L and 0.0748 g/L at both mesophilic and thermophilic temperatures respectively. The experimental hydrogen production values shown in Table 4.5 indicate that acetate, butyrate and propionate together account for 112 % and 108 % of the theoretical values at 0 g/L and 1 g/L respectively indicating that there were other less significant pathways that led to hydrogen production. But, interestingly, at 4 g/L furfural under mesophilic conditions, experimental hydrogen production could only account for 52 % of the theoretical hydrogen production.

At thermophilic conditions, experimental hydrogen production was 109 % of the theoretical at 0 g/L furfural and 85 % at 1 g/L furfural. Complete inhibition was however observed at 4 g/L under thermophilic conditions where no hydrogen was produced.

Table 4.5: Experimental versus theoretical hydrogen production based on acetate, butyrate and propionate concentrations

	Furfural concentration				Furfural concentration		
Mesophilic experiment	**0 g/L**	**1 g/L**	**4 g/L**	**Thermophilic experiment**	**0 g/L**	**1 g/L**	**4 g/L**
Acetate (g/L)	3.5	5.3	4.2	Acetate (g/L)	4.6	2.85	0.86
Butyrate (g/L)	5.6	6.3	2.5	Butyrate (g/L)	5.7	3.37	0
Propionate (g/L)	2.41	0.12	4.9	Propionate (g/L)	0	0.05	0.03
Theoretical LH_2 from HAc, HBu, HPr (L)	59	89	36	Theoretical LH_2 from HAc, HBu, HPr (L)	83	50	8
Experimental H_2 (L)	66	96	19	Experimental H_2 (L)	91	43	0
Experimental H_2/ Theoretical H_2 (%)	112	108	52	Experimental H_2/ Theoretical H_2 (%)	109	85	0

*Theoretical volume of hydrogen from HAc, HBu and HPr were calculated by converting the mass of hydrogen that can be obtained per gram of metabolite using (xylose as substrate) from the balanced equations shown below to L H_2 at the respective temperature.

Acetate (HAc): $C_5H_{10}O_5 + 1.67\ H_2O \longrightarrow 1.67\ CH_3COO^- + 1.67\ CO_2 + 3.33\ H_2 + 1.67H^+$ (0.841 L H_2/gHAc at 37 °C & 0.889 LH_2/gHAc at 55 °C)

Butyrate (HBu): $C_5H_{10}O_5 \longrightarrow 0.83\ CH_3CH_2CH_2COO^- + 0.83\ H^+ + 1.67\ CO_2 + 1.67\ H_2$ (0.579 L H_2/gHBu at 37 °C & 0.611 LH_2/gHBu at 55 °C)

Propionate (HPr): $C_5H_{10}O_5 + 1.67\ H_2 \longrightarrow 1.67\ CH_3CH_2COOH + 1.67\ H_2O$ (0.342 L H_2/gHPr at 37 °C & 0.361 LH_2/gHPr at 55 °C)

4.3.4 Kinetic Models

4.3.4.1 Gompertz parameters

The modified Gompertz equation as described by Chen et al., (2006) was employed in order to estimate P_{max} (hydrogen production potential (mL)); R_{max} (maximum hydrogen production rate (L/d) and λ (lag phase (h)). R_{max} was normalized to the initial mass of seed added (mL/gVSS $_{initial}$/d) which is the maximum specific hydrogen production rate (max SHPR). The parameters (shown in Table 4.6) were estimated by the solver function on Microsoft Excel 2013. The coefficient of determination (R^2) values of all fits were greater than 0.999 indicating that the modified Gompertz model adequately described the cumulative hydrogen production in these tests. Mesophilic experiments showed higher hydrogen production rates compared with thermophilic experiments at furfural concentrations of 1 g/L and 4 g/L while interestingly, the rates of the controls under both conditions were identical at around 1 L gVSS $_{initial}^{-1}d^{-1}$. Note that R_m and max SHPR at 4 g/L furfural under mesophilic experiment were much higher than the control under mesophilic conditions as the exponential phase occurred within a very short period of time (about 14 hours) thus showing high rates. The observed enhancement in hydrogen production and yield at mesophilic conditions at 1 g/L furfural were not reflected in the hydrogen production rates which were 40 % lower than the control. Lag phases increased while maximum hydrogen production rates decreased with increasing furfural concentration at both mesophilic and thermophilic conditions. An increase in lag phase in the presence of increasing inhibitor concentration indicates a delay in the activity and metabolism of hydrogen producers (Kumar et al., 2014). Thermophilic experiments showed considerably longer lag phases than the mesophilic experiments at the same furfural concentrations definitely due to differences in the microbial consortia.

Table 4.6: Gompertz parameters for mesophilic and thermophilic experiments

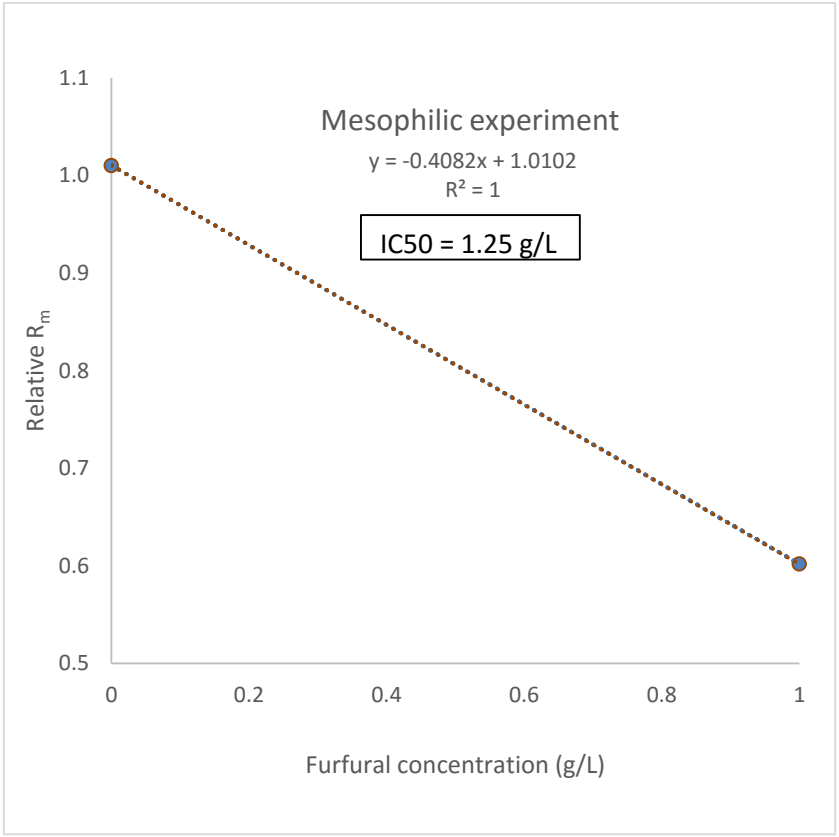
Batch experiment	Furfural conc.	P_{max} (max H_2)	R_{max} (maximum H_2 prod. rate)	Max SHPR (specific H_2 prod. rate)	λ (Lag phase)	R^2
	g/L	L	L/d	L/gVSS $_{initial}$-d	d	
Mesophilic	0	63	101	0.99	0.3	0.9999
	1	92	60	0.59	0.5	0.9998
	4	17	173	1.70	13.7	0.9999
Thermophilic	0	91	91	1.0	1.6	0.9999
	1	41	12	0.13	11.6	0.9998
	4	0	0	0	0	0

4.3.4.2 Half maximum inhibitory concentration (IC50)

IC50 is the inhibitor concentration required to decrease the maximum hydrogen production rate by 50 % and was calculated by dividing the normalized R_m values for the experiments with furfural by the maximum hydrogen production rate of the control at the respective temperatures as described by following equation (Siqueira and Reginatto, 2015):

$$\text{Relative } R_m = \frac{R_m \text{ furfural}}{R_m \text{ control}} \quad 4.7$$

Fig. 4.4 show linear plots between relative maximum hydrogen production rate and furfural concentration which correlated well for both mesophilic and thermophilic experiments with R^2 of 1. Both experiments at 4 g/L furfural were ignored in determining the IC50 as the mesophilic experiment showed much higher R_m values than the control with relative R_m greater than 1 while the thermophilic experiment produced no hydrogen and as such relative Rm is 0. Estimates of the IC50 values were fitted using Microsoft Excel and were observed to be 1.25 g/L and 0.6 g/L furfural for mesophilic and thermophilic cultures respectively. This shows that the inhibitory effect was greater on the thermophilic than mesophilic cultures. A study by Siqueira and Reginatto (2015) revealed an IC50 of 0.62 g/L for furfural during hydrogen production from 40 g/L glucose at mesophilic temperatures. The lesser inhibitory effect noticed in this study at mesophilic temperatures compared to the aforementioned study could be due to the presence of potentially different microbial cultures in both studies as the aforementaioned study employed mixed cultures from an unflow anaerobic sludge blanket (UASB) reactor that treated effluent from a sugar and ethanol mill while the present study used mixed cultures from a wastewater treatment plant.



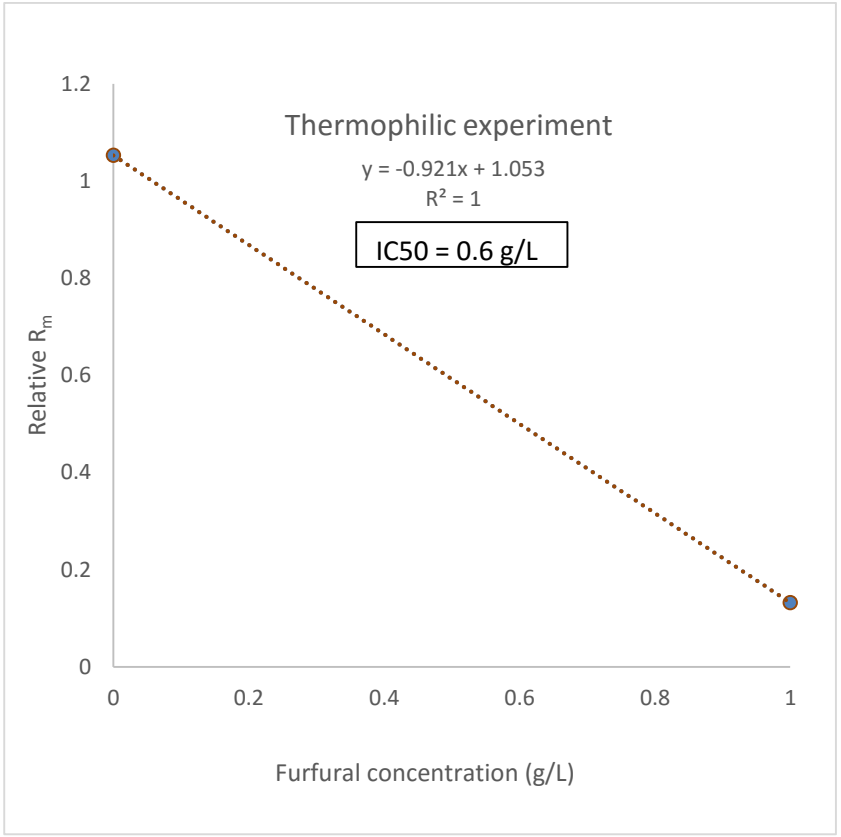


Figure 4.4: Relative hydrogen production rate versus furfural concentration

4.3.4.3 Monod kinetic parameters

Since only two furfural concentrations were studied (1 g/L and 4 g/L), there were not enough data points to establish a particular type of inhibition for furfural, be it competitive, uncompetitive or non-competitive. The equations described in section 4.2.4 were utilized to estimate the microbial kinetic constants and kinetic parameters for the metabolites generated using a simple Monod model without inhibition in MATLAB® and are presented in Table 4.7. Only the exponential growth phases were modeled. Fig. 4.5 presents the experimental versus modeled data profiles of substrate degradation and product formation with time for mesophilic and thermophilic experiments. It can be visually observed from these figures that the experimental and modeled data correlate reasonably. Tables 4.8a and 4.8b show the average percent errors (APEs) and root mean square errors (RMSE) for the substrate and each product at mesophilic and thermophilic conditions respectively. All APEs were below 10 % except for those of butyrate yield, formate and furfural consumption constants under mesophilic conditions and substrate degradation and furfural consumption constants under thermophilic conditions which were around 20 %. RSMEs in all cases were relatively low. The overall goodness of fit show that the predicted model was able to simulate the experimental data to a reasonable extent, although there were very few cases were the Monod model could not accurately predict the experimental data for sugars degradation and/or product formation. In the mesophilic experiments, the model was not ideal for predicting furfural degradation and acetate formation at 1 g/L furfural and sugars degradation at 4 g/L furfural. In the case of the thermophilic experiments, the model did not accurately predict hydrogen production, valerate formation and furfural degradation at 1 g/L furfural and lactate formation and fufural degradation at 4 g/L furfural. These anomalies were indicated by either high APEs or poor visual fit between the modeled lines and experimental data and the observations suggest that the formation

of acetate, lactate, and valerate cannot be simply related to the sugar and furfural degradation kinetics, implying that multiple intricate pathways contributed to the formation of these products.

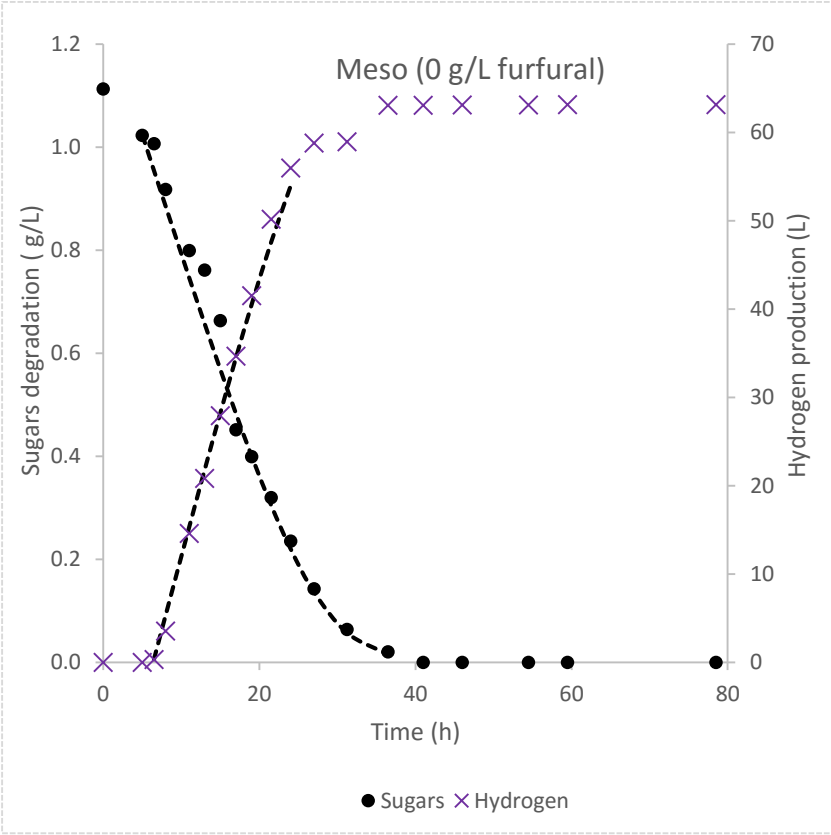
Since initial substrate composition comprised both C5 and C6 sugars, concentrations of glucose (C6), mannose (C6), galactose (C6), xylose (C5), and arabinose (C5) where normalized to mol carbon/L using their respective number of moles of carbon and molar mass. The sum of these sugars were modeled against time to give μ_{max} in h^{-1}, k_s in mol carbon/L, and $Y_{x/s}$ in gVSS/mol carbon as presented in Tables 4.7a and 4.7b for the mesophilic and thermophilic experiments respectively. 0.1 gVSS/gVSS-d (which corresponds to 0.0042 gVSS/gVSS-h) is reported to be the typical decay coefficient value for anaerobic mixed cultures and was employed in modeling at all cases in this study (Tchobanoglous et al., 2003). μ_{max} were similar at 0 g/L furfural for both mesophilic and thermophilic experiments (0.014 h^{-1}) which is logical as they both showed similar hydrogen production rates of around 1 L gVSS $initial^{-1}$ d^{-1}, K_s values were higher in the thermophilic than mesophilic experiments. Calculated biomass yields of 0.1 gVSS/gCOD for all except at 4 g/L furfural which was 0.2 gVSS/gCOD, were used for the model. This corresponds to 3 gVSS/mol Carbon and 6 gVSS/mol carbon respectively when converted assuming all the sugars present in the substrate were xylose (See footnote under Table 4.7 for conversion formula). K_s in this study ranged from 6.6 to 8.1 g sugars /L and 13.8 to 14.4 g sugars/L when converted (see footnote under Table 4.7 for conversion formula). These values are within the same order of magnitude reported in the literature. Gupta et al. (2015) reported μ_{max} and K_s values of 0.029 h^{-1} and 4 g/L respectively for mixed cultures at thermophilic temperatures using starch as substrate. Sharma and Li (2009) reported the kinetic parameters of mixed cultures grown on glucose at 30 °C with μ_{max} and K_s of 0.001 h^{-1} and 15.1 g/L respectively. However, it is noteworthy that the literature studies were carried out on substrates without any furfural.

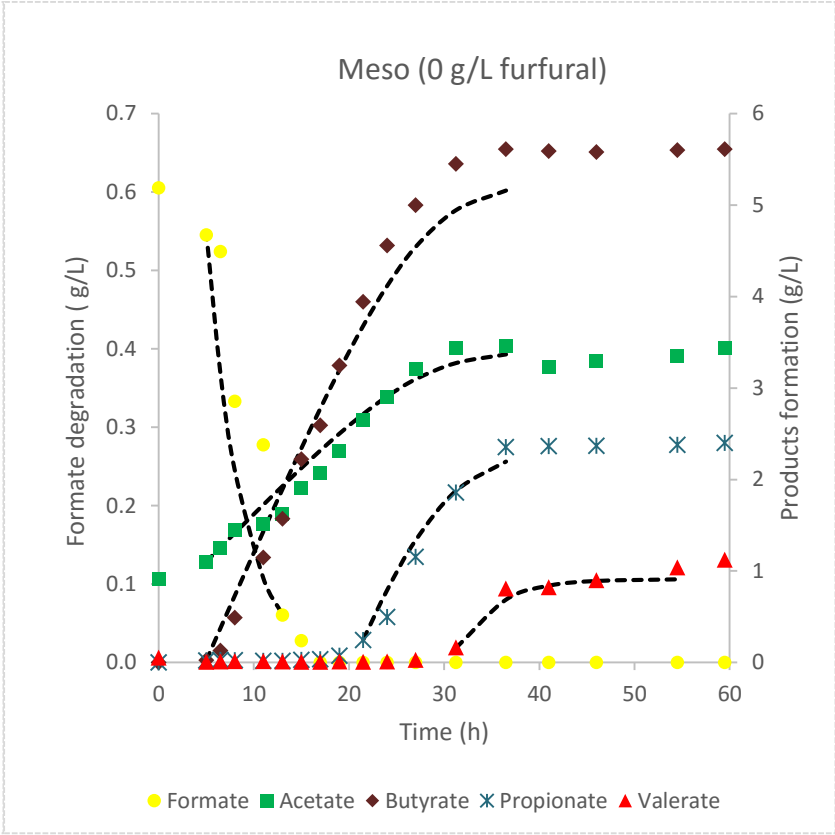
Hydrogen was modeled just as any other product using eq. 4.3. The modeled hydrogen yields under mesophilic conditions were observed to be greater at 1 g/L furfural compared to the control which further justifies the enhanced yields. Higher acetate and butyrate metabolite concentrations as well as lower valerate concentrations observed at 1 g/L furfural under mesophilic conditions relative to the control were also reflected in the modeled product yields. The products at 4 g/L furfural under mesophilic conditions could not be accurately modeled as the exponential phase was short with very few data points.

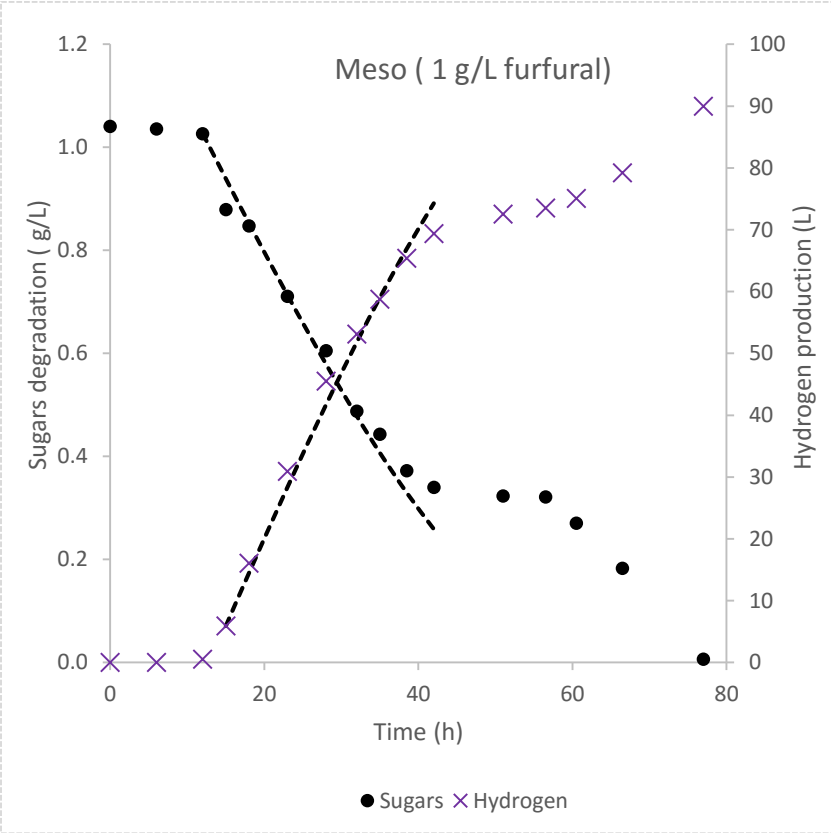
Under thermophilic conditions, the 50 % inhibition observed at 1 g//L furfural compared to 0 g/L furfural was reflected in the modeled hydrogen yields. Modeled lactate yields increased with increasing lactate concentration from 0 g/L to 4 g/L furfural. K_{fur} (furfural degradation constarnt) decreased with increasing furfural concentration between 1 g/L and 4 g/L furfural. As earlier stated, since formate was consumed almost immediately, it was no modeled under thermophilic conditions. Lactate was produced without consumption under thermophilic conditions, and as such there were no degradation constants.

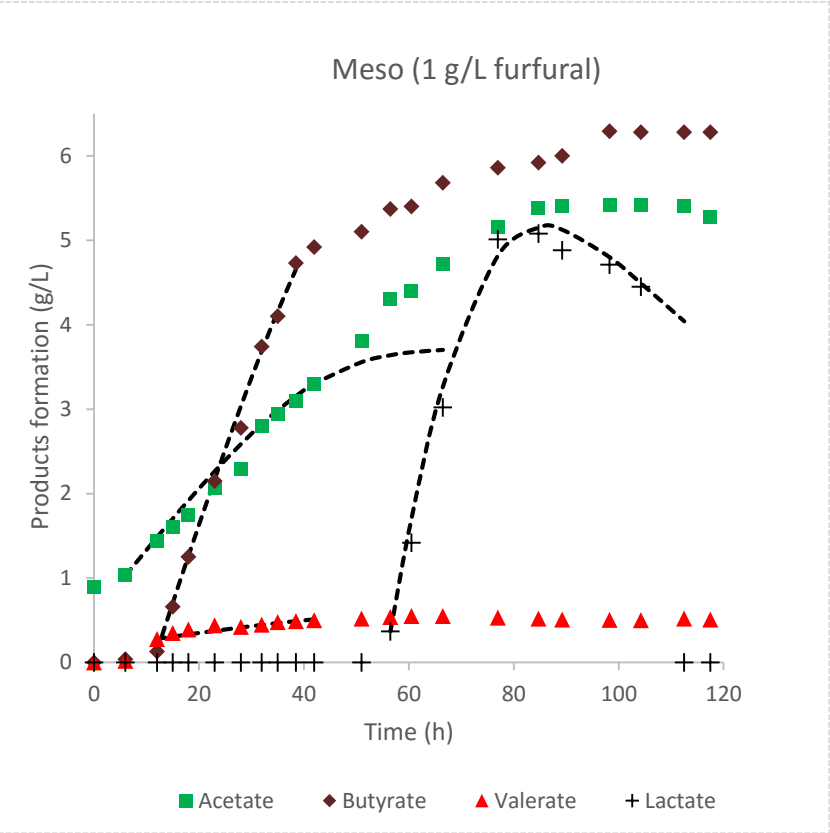
In both mesophilic and thermophilic experiments, the highest μ_{max} and lowest Ks were observed at 0 g/L furfural. It has been reported that higher μ_{max} and lower K_s indicate enhanced kinetics (Gupta et al., 2015). This observation is true at the condition that showed the highest hydrogen production rate. This shows that microbial kinetics are more favorable in the absence of furfural and the lower the furfural concentration, the better or more enhanced the microbial kinetics.

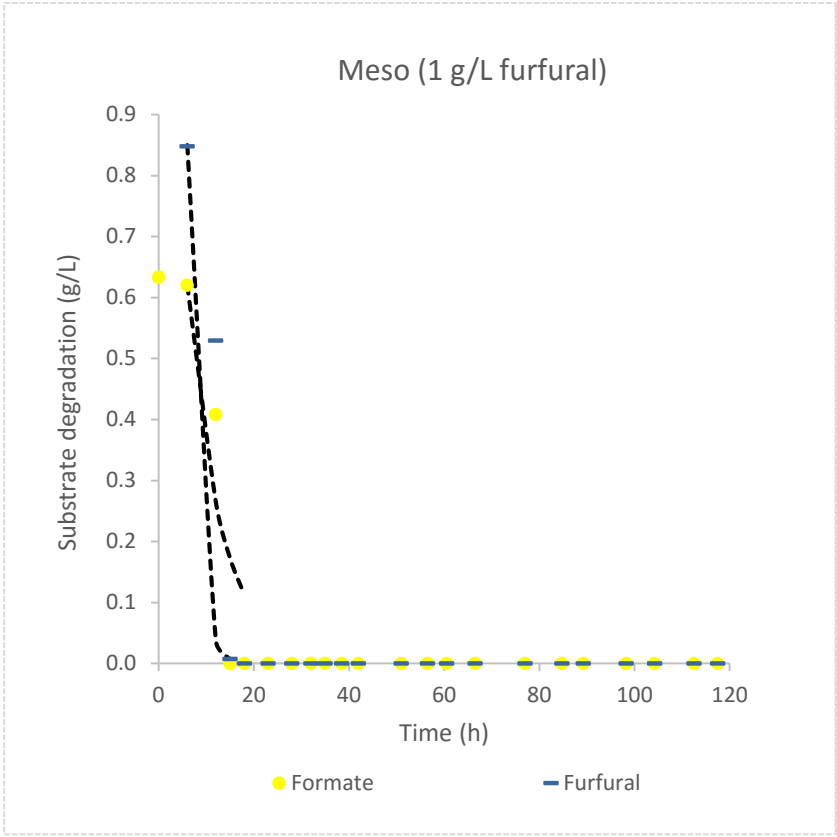
Furfural has been shown to negatively impact yields, rates and microbial kinetics except at 1 g/L furfural under mesophilic conditions where an enhancement in yield but not rate, was observed which could be due to the fact that this furfural concentration was below the threshold limit.

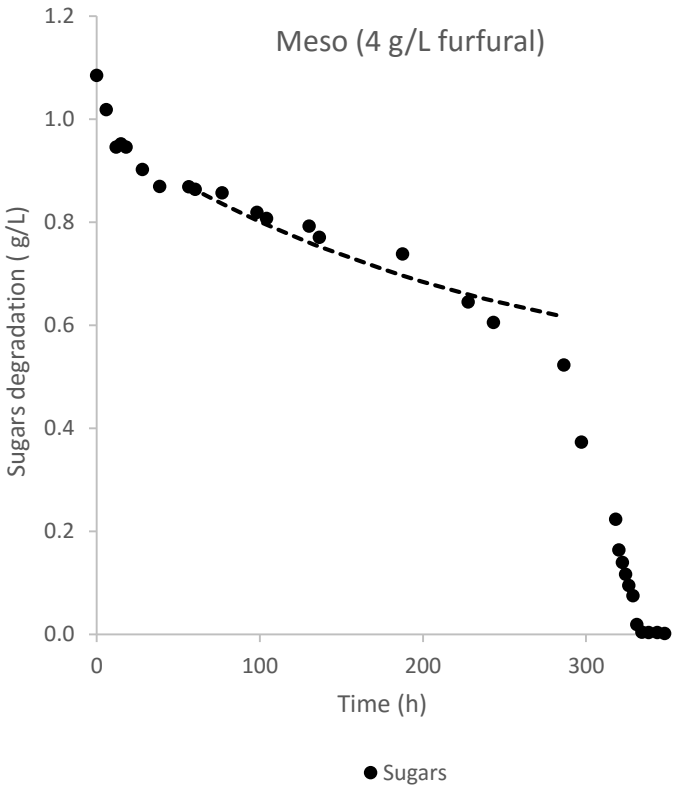

Meso (0 g/L furfural)
Sugars degradation (g/L)
Hydrogen production (L)
Time (h)
Sugars
Hydrogen

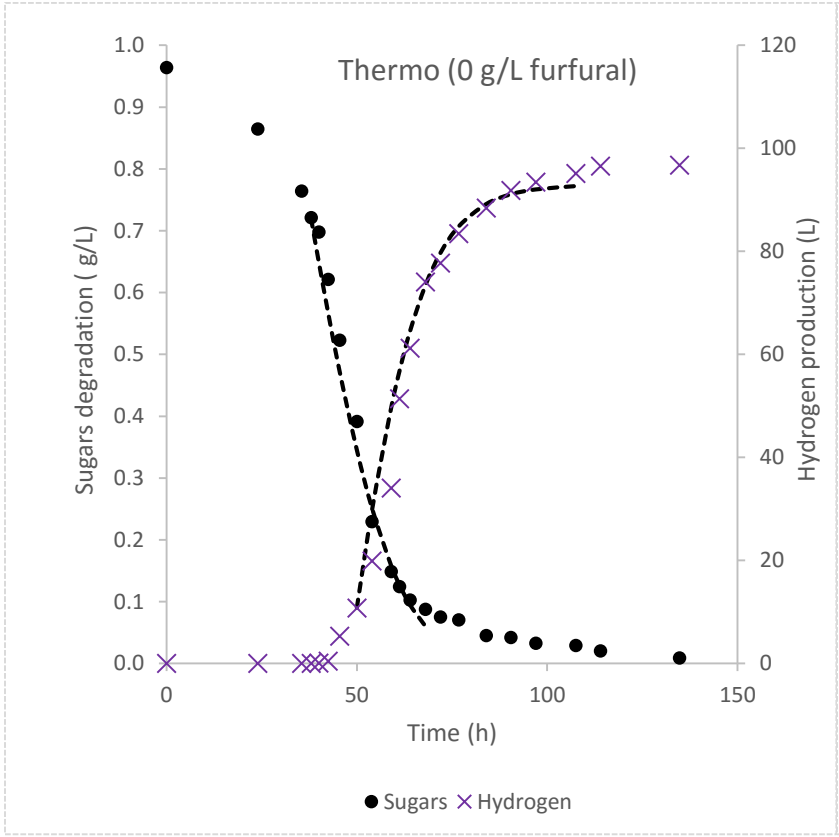

Meso (0 g/L furfural)
Formate degradation (g/L)
Products formation (g/L)
Time (h)
Formate
Acetate
Butyrate
Propionate
Valerate

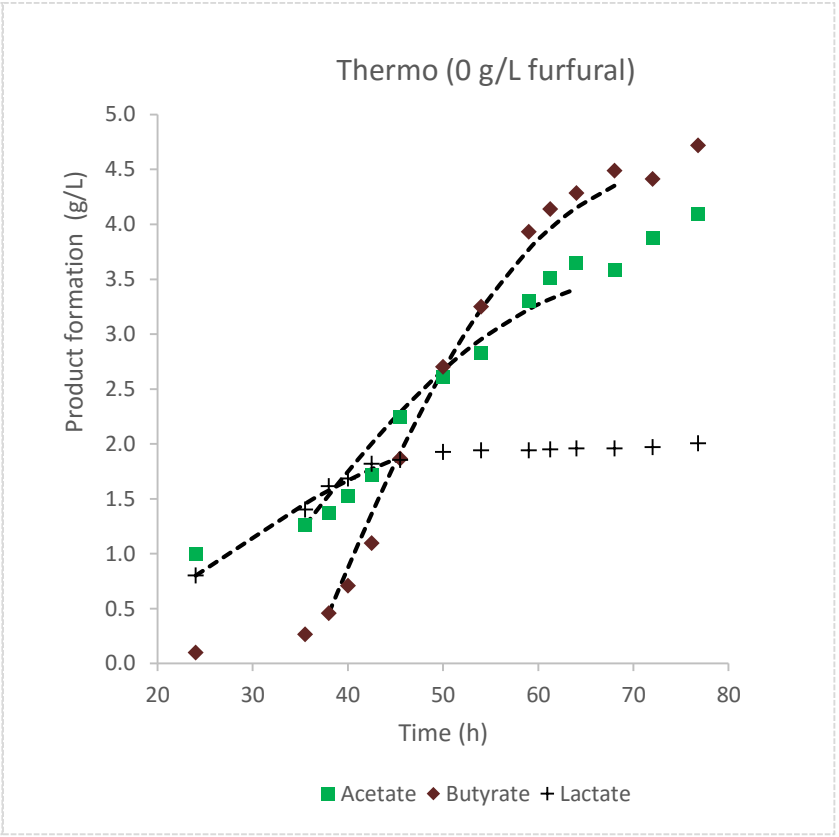

Meso (1 g/L furfural)
Sugars degradation (g/L)
Hydrogen production (L)
Time (h)
Sugars
Hydrogen

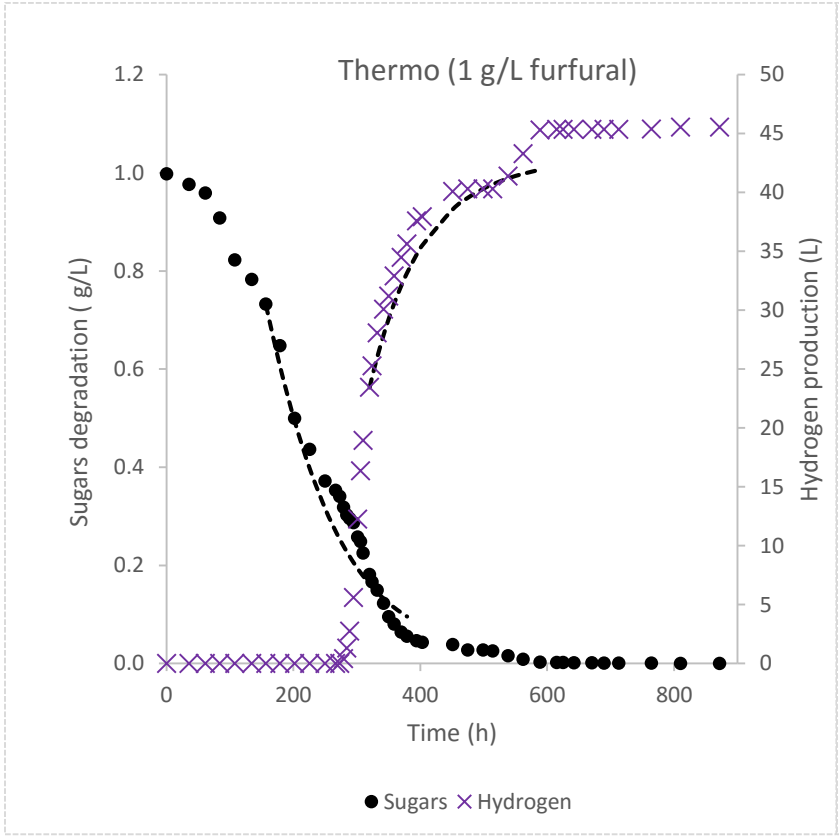

Meso (1 g/L furfural)
Products formation (g/L)
Time (h)
Acetate
Butyrate
Valerate
Lactate

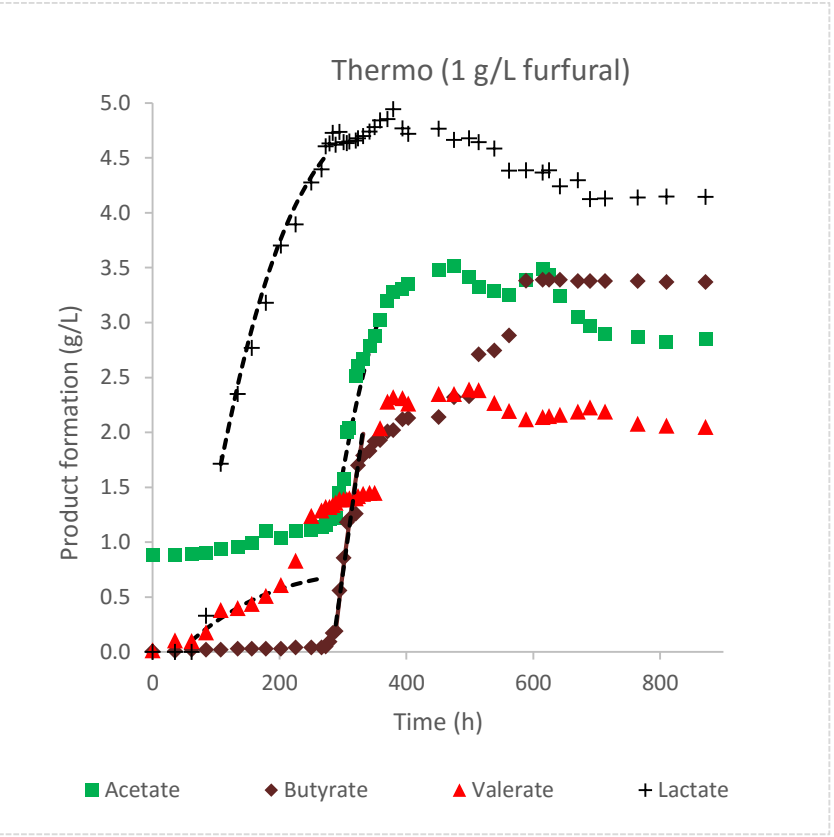

Meso (1 g/L furfural)
Substrate degradation (g/L)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
120
Time (h)
Formate
Furfural

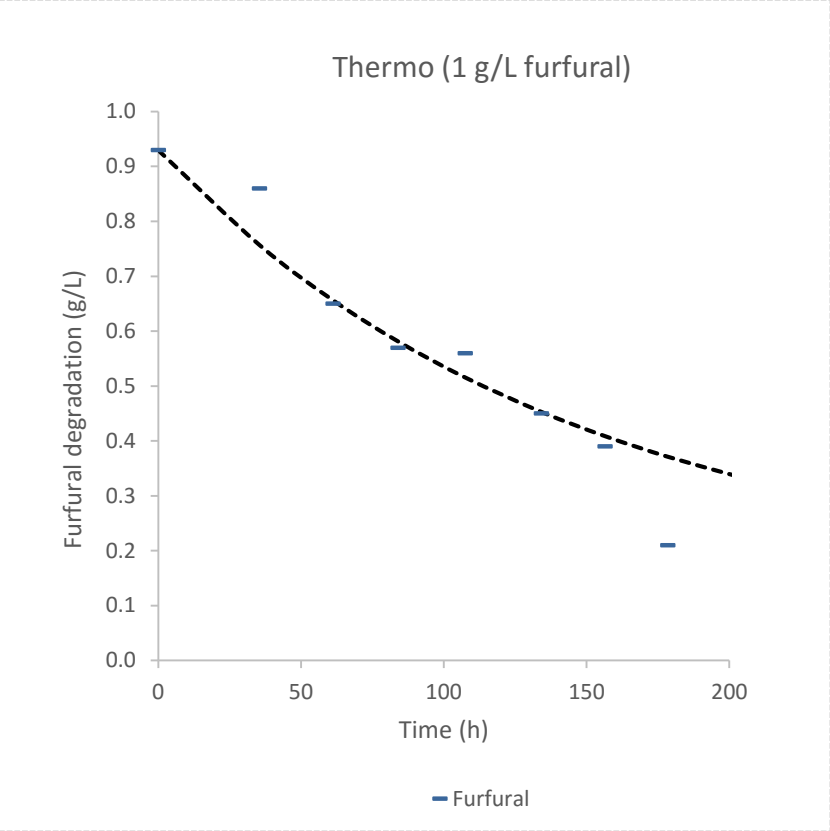

Meso (4 g/L furfural)
Sugars degradation (g/L)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (h)
Sugars

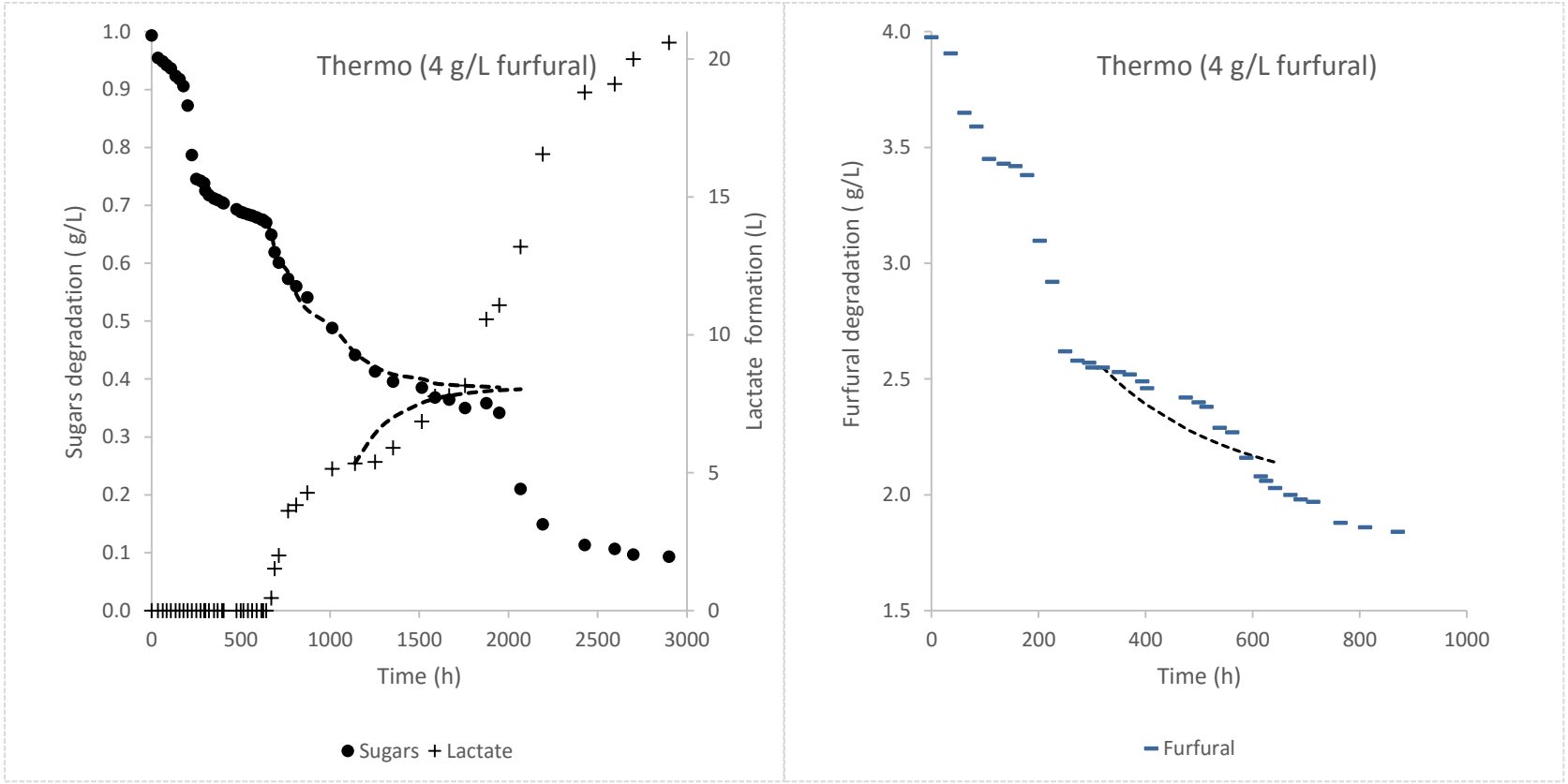

Thermo (0 g/L furfural)
Sugars degradation (g/L)
Hydrogen production (L)
Time (h)
Sugars
Hydrogen


Thermo (0 g/L furfural)
Product formation (g/L)
Time (h)
Acetate
Butyrate
Lactate


Thermo (1 g/L furfural)
Sugars degradation (g/L)
Hydrogen production (L)
Time (h)
Sugars
Hydrogen


Thermo (1 g/L furfural)
Product formation (g/L)
Time (h)
Acetate
Butyrate
Valerate
Lactate


Thermo (1 g/L furfural)
Furfural degradation (g/L)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
Time (h)
Furfural



Symbols= experimental data; Dotted lines= modeled data; Meso= mesophilic experiment; Thermo= thermophilic experiment

Figure 4.5: Modeled versus experimental data for substrate degradation and product formation with time for mesophilic and thermophilic experiments

Table 4.7 a: Kinetic parameters for mesophilic experiment at 0 g/L furfural, 1 g/L furfural and 4 g/L furfural obtained using the MONOD model on MATLAB®

			Mesophilic experiment		
	Kinetic parameters	**Unit**	**0 g/L furfural**	**1 g/L furfural**	**4 g/L furfural**
	μ_{max}	h^{-1}	0.014	0.007	0.0005
	K_s	mol Carbon/L	0.22	0.25	0.27
	$Y_{X/S}$	gVSS/mol Carbon	3	3	3
Product Yields	$Y_{H/S}$	L H_2 L/mol Carbon consumed	70.6	115	Short exponential phase with no good fit
	$Y_{A/S}$	g product/mol Carbon consumed	2.25	2.6	
	$Y_{B/S}$		5.1	6.5	
	$Y_{P/S}$		6.5	NA	
	$Y_{V/S}$		3.9	0.1	
	$Y_{L/S}$		NA	23.8	
Consumption constants	K_L	L g^{-1} VSS h^{-1}	NA	0.0015	
	K_{for}		0.022	0.009	
	K_{fur}		NA	0.033	

$$K_s \text{ (g sugars/L)} = \frac{\text{mol C}}{\text{L}} * \frac{\text{mol xylose}}{5 \text{ mol C}} * \frac{150 \text{ g xylose}}{\text{mol xylose}}$$

$$Y_{X/S} \text{ (gVSS/g sugars)} = \frac{\text{gVSS}}{\text{mol Carbon}} * \frac{5 \text{ mol C}}{\text{mol xylose}} * \frac{\text{mol xylose}}{150 \text{ g xylose}}$$

Table 4.7 b: Kinetic parameters for thermophilic experiment at 0 g/L furfural, 1 g/L furfural and 4 g/L furfural obtained using the MONOD model on MATLAB®

			Thermophilic experiment		
	Kinetic parameters	**Unit**	**0 g/L furfural**	**1 g/L furfural**	**4 g/L furfural**
	μ_{max}	h^{-1}	0.014	0.0024	0.0011
	K_s	mol Carbon/L	0.46	0.47	0.48
	$Y_{X/S}$	gVSS/mol Carbon	3	3	6
Product Yields	$Y_{H/S}$	L H_2 L/mol Carbon consumed	210	115	NA
	$Y_{A/S}$	g product/mol Carbon consumed	3.2	11.9	*-
	$Y_{B/S}$		5.9	16.5	NA
	$Y_{P/S}$		NA	NA	NA
	$Y_{V/S}$		NA	0.24	NA
	$Y_{L/S}$		1.7	4.3	13
Consumption constants	K_L	L g^{-1} VSS h^{-1}	Not consumed		
	K_{for}		Consumed too quickly		
	K_{fur}		NA	0.0007	0.000075

Table 4.8 a: APE and RSME for biomass, substrate and metabolites for mesophilic experiment at 0 g/L furfural, 1 g/L furfural and 4 g/L furfural

		Mesophilic experiment					
		0 g/L furfural		1 g/L furfural		4 g/L furfural	
	Kinetic parameters	APE (%)	RMSE (mol Carbon/L)	APE (%)	RMSE (mol Carbon/L)	APE (%)	RMSE (mol Carbon/L)
	μ_{max}						
	K_s	8	0.05	6	0.04	4	0.04
	$Y_{X/S}$						
Product Yields	$Y_{H/S}$	8	1.37	5	2.54	NA	
	$Y_{A/S}$	6	0.16	7	0.4		
	$Y_{B/S}$	27	0.31	3	1.81		
	$Y_{P/S}$	9	0.13	NA	NA		
	$Y_{V/S}$	7	0.08	6	0.03		
	$Y_{L/S}$	NA	NA	5	1.36		
Consumption constants	K_L	NA	NA				
	K_{for}	24	0.11	9	0.13		
	K_{fur}	NA	NA	24	0.25		

APE=Average Percent Error; RMSE= Root Mean Square Error; k_d = 0.1 gVSS/gVSS-d

Table 4.8 b: APE and RSME for biomass, substrate and metabolites for thermophilic experiment at 0 g/L furfural, 1 g/L furfural and 4 g/L furfural

		Thermophilic experiment					
		0 g/L furfural		1 g/L furfural		4 g/L furfural	
	Kinetic parameters	APE (%)	RMSE (mol Carbon/L)	APE (%)	RMSE (mol Carbon/L)	APE (%)	RMSE (mol Carbon/L)
	μ_{max} K_s $Y_{X/S}$	9	0.03	21	0.05	4	0.1
Product Yields	$Y_{H/S}$	11	5.74	5	1.93	NA	NA
	$Y_{A/S}$	7	0.17	5	0.16	*-	*-
	$Y_{B/S}$	7	0.14	9	0.15	NA	NA
	$Y_{P/S}$	NA	NA	NA	NA	NA	NA
	$Y_{V/S}$	NA	NA	18	0.28	NA	NA
	$Y_{L/S}$	2	0.04	3	0.12	14	2.1
Consumption constants	K_L K_{for}	NA					
	K_{fur}	NA		20	0.09	4	0.1

4.4 Summary and Conclusions

- The hydrogen yield at 1 g/L furfural was enhanced by around 45 % despite a 40 % decrease in rate relative to the control under mesophilic conditions
- Hydrogen enhancement was not observed in the presence of furfural under thermophilic conditions; rather hydrogen yields at 1 g/L furfural were 50 % less than the control
- Hydrogen producers in the mixed cultures were inhibited in the presence of furfural; furfural had to be degraded to undetectable limits before any hydrogen was observed
- 4 g/L furfural was severely inhibitory to the thermophilic cultures as no hydrogen was observed even after furfural was broken down following an extended contact time of about 120 days
- IC50 for furfural under mesophilic and thermophilic cultures were 1.25 g/L and 0.6 g/L respectively
- Hydrogen was not a product of the direct consumption of sugars in the presence of furfural.
- Enhanced microbial kinetics were observed in the absence of furfural in both mesophilic and thermophilic experiments emphasizing that furfural is indeed inhibitory

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CHAPTER 5

Single-stage and two-stage anaerobic digestion of extruded lignocellulosic biomass[3]

5.1 Introduction

The demand for developing sustainable energy has increased as a result of rapid population growth and depleting fossil fuel supplies. Hydrogen and methane have received significant attention as alternative and valuable energy carriers during the last decade and can be utilized for vehicle fuel, heat and electricity generation (Pakarinen et al., 2009). Lignocellulosic biomass found mostly in agricultural and food processing residues, municipal solid wastes, and forest residues, has a great potential for biogas production due to its high sugar content (Adapa et al., 2011). One of the major limitations in biogas production from lignocellulosic biomass is low biodegradability and production yield due to its complex crystalline structure and the presence of lignin thus necessitating pretreatment (Kratky et al., 2011).

Among the various pretreatment processes available, extrusion is a simple, cheap, and well-established method which can be used as a physicochemical method for the pretreatment of lignocellulosic biomass (Lin et al. 2012; Zheng and Rehmann, 2014). Twin Screw Extrusion (TSE) is a pretreatment technology that allows the continuous production of highly homogenous and finely-structured products through physico-chemical means. The extruder consists of intermeshing, co-rotating screws mounted on grooved shafts in a closed barrel (Martin, 2013; Zheng and Rehmann, 2014). This technology has proven to be viable and has great flexibility and adaptability with reference to scale up and process modifications (Zheng and Rehmann, 2014).

[3] This chapter has been submitted to the *Applied Energy* journal for publication

While chemical and biological pretreatment methods change the physical and chemical properties of biomass, the extrusion pretreatment method does not affect the chemical composition of biomass but changes its physical properties such as specific surface area, bulk density, and specific porosity (Karunanithy and Muthukumarappan, 2011; Karaunanithy et al., 2012; Zheng and Rehmann, 2014). Particle size and crystallinity of lignocellulosic biomass are reduced and the surface area of biomass and sugar availability are also increased by extrusion (Jurisic et al., 2009; Karunanithy and Muthukumarappan, 2011; Zhang et al., 2012). Extrusion pretreatment has also been known to improve biogas yields (Angelidaki and Ahring, 2000; Hjorth et al., 2011). Compared to other physical pretreatment processes such as hydrothermal or steam explosion methods, the extruder is operated at a lower temperature which reduces energy consumption and operating costs and prevents lignin oxidation and carbohydrate degradation (Lin et al., 2012). In addition, it has the ability to pretreat a wide range of biomass including forest, agricultural and energy crops.

There is limited information on biogas production from extruded biomass, since most researches have focused on bioethanol production. Karunanithy and Mathukumarappan (2013) reported that fermentation results of feedstocks pretreated using the extrusion technology were limited. A good number of different biomasses have been reportedly treated using the extruder with limited studies on poplar wood (See Tables 5.1 and 5.2). Few studies reported the enhancement of methane production using extrusion pretreatment of other types of biomass and enhancement of hydrogen yields from poplar wood pretreated using other technologies, but there is no publication on hydrogen production from extruded poplar wood biomass. Using a twin-screw extruder, the degradability of organic matter was promoted and methane production yields from different biomasses (straw, grass, treated manure and deep litter) in batch tests were boosted from 18 % to 70 % after 28 days and 9 % to 28 % after 90 days (Hjorth et al., 2011). Chen et al. (2014) compared

the particle size reduction of rice straw by extrusion or milling pretreatment and observed that extrusion significantly reduced the particle size by around 25 %. The aforementioned authors further reported that methane production of extruded rice straw was 1.5 and 2 times that of milled and untreated rice straw, respectively. Methane production from a mixture of rice straw silage, maize silage, and triticale silage in a continuously mixed digester increased by up to 16 % and volatile solids (VS) degradation was accelerated by around 15 % through extrusion (Menardo et al., 2015). Wahid et al., (2015) reported that extrusion increased sugar availability from 21 % to 42 % for wheat straw and from 7 % to 26 % for deep litter and methane yields from 12 % to 29 % (wheat straw) and 4 % to 11 % (deep litter). Most recently, extrusion combined with sodium hydroxide pretreatment of rice straw was reported to enhance methane production by 54 % and energy recovery increased from 39 % to 60 % (Zhang et al., 2015).

In the last decade, several studies of two-stage anaerobic digestion were carried out using thermomechanical pulp (TMP) (wastewater from thermo-mechanically treated eucalyptus wood) (Viñas et al., 1993); food waste (Han et al., 2005); olive pulp (Gavala et al., 2005); potato waste (Zhu et al., 2008); cheese whey (Antonopoulou et al., 2008); molasses (Park et al., 2010); and thin stillage (Nasr et al., 2012). Viñas et al. (1993) reported that methane yield from TMP wastewater in a two-stage anaerobic digestion was 0.34 L CH_4/gCOD $_{removed}$ with a 90 % COD removal efficiency corresponding to a 12 % and over 16 % increase compared to a single-stage process respectively. Han et al. (2005) optimized both acidogenic hydrogenesis and methanogenesis of the two-stage anaerobic digestion of food waste and achieved an overall COD removal efficiency of 95 %. Thermophilic BHP and BMP production from olive pulp were investigated in both batch and continuous studies with maximum hydrogen and methane potentials of 36 mL H_2/ g TS and 426 mL CH_4/ g TS respectively (Gavala et al., 2005). Zhu et al., (2008) studied two-stage anaerobic

digestion using potato waste and observed maximum hydrogen, methane and total energy yields of 68 L/kg TS, 225 L/kg TS and 2.74 kW h/kg TS respectively with overall VS and COD removal efficiencies of 70 % and 64 % respectively. Production rates of 7.53 L H_2/d and 75.6 L CH_4/d were observed in the two-stage anaerobic digestion of cheese-whey in a continuous system while 17.9 L CH_4/L cheese whey was observed in batch studies of a single-stage BMP process (Antonopoulou et al., 2008). Park et al., (2010) also carried out a comprehensive study of the two-stage anaerobic digestion process from molasses with the highest hydrogen and methane production rates of 2.8 LH_2/L- reactor/d and 1.48 LCH_4/L-reactor/d and an overall COD removal efficiency of about 80 %. Furthermore, Nasr et al. (2012) achieved maximum methane yields of 0.26 and 0.33 LCH_4/gCOD_{added} in a single-stage and two-stage anaerobic digestion process respectively, using thin stillage and the total energy yield in a two-stage process increased by 18.5 % over the single-stage process. The aforementioned authors further reported that separating the acidogenic and methanogenic stages of anaerobic digestion increased the TVFAs to TCOD ratio from 10 % in the raw thin stillage to 54 % in the first step of the two-stage anaerobic digestion process.

It is thus evident that while there are a handful of studies on biomethane production from extruded agricultural products, there is no information on biohydrogen production from extruded lignocellulosic biomass. The objective of this study, therefore, was to investigate the potential of renewable energy production from extruded lignocellulosic biomass using mixed cultures. This was achieved by assessing the hydrogen and methane production potentials, production rates and yields from poplar wood pretreated using the twin-screw extrusion technology. Focus was on batch studies of a single-stage methane production process from poplar wood hydrolysates and a two-stage anaerobic digestion process (first-stage biohydrogen fermentation from poplar wood hydrolysates sequentially followed by a second stage biomethane production from the hydrogen-

effluent from the first stage). Comparisons between the single-stage methane production process and the second-stage methane production process were also evaluated.

Table 5.1: Poplar wood treated with various pretreatment methods

Feedstock	Pretreatment	Biogas yields			Reference
		Untreated	Pretreated	Increase (%)	
Poplar wood	None	178 LCH_4/kgDM	N/A		Dubrovskis and Putnins (2014)
Poplar processing residues	NaOH (3%, 5%, 7%)	*111 LCH_4/kgDM	*224 LCH_4/kgDM *237 LCH_4/kgDM *215 LCH_4/kgDM	102 114 94	Yao et al. (2013)
Poplar leaves	HCl (0.5%, 1%, 2%, 4%)	15.1 LH_2/kgDM	27.6 LH_2/kgDM 28.4 LH_2/kgDM 29.1 LH_2/kgDM 33.5 LH2/kgDM	83 88 93 122	Cui et al. (2010)

DM: dry matter; *Calculated values using given parameters from study

Table 5.2: Feedstock treated using extrusion technology

Feedstock	Extrusion conditions	Biogas yields			Reference
		Untreated	Extruded	Increase (%)	
Rice straw	Two counter-rotating screw	153 LCH_4/kgVS-d	177 LCH_4/kgVS-d	16	Menardo et al. (2015)
Hay	Single screw		212 LCH_4/kg-DM		Maroušek (2012)
Wheat straw	Co-rotating twin screw	278 - 300 LCH_4/kgVS	303 - 327 LCH_4/kgVS		Wahid et al. (2015)
Deep litter	Co-rotating twin screw	271 - 306 LCH_4/kgVS	292 - 307 LCH_4/kgVS		Wahid et al. (2015)
Rice straw	Twin-screw	132 LCH_4/kgVS	227 LCH_4/kgVS	72	Chen et al. (2014)
Barley straw	Two counter-rotating screws			68 10	Hjorth et al. (2011)
Grass	Two counter-rotating screws			47 6	Hjorth et al. (2011)
Deep litter	Two counter-rotating screws			34 26	Hjorth et al. (2011)

DM: dry matter; VS: Volatile Solids

5.2 Materials and Methods

5.2.1 Seed Sludge

All tests were conducted using mesophilic anaerobic digester sludge (mixed cultures) obtained from the Guelph Wastewater Treatment Plant, Guelph, Ontario, Canada, as seed. The characteristics of the seed used were as follows: pH: 6.9 ± 0.03; Total Suspended Solids (TSS): 17.9 ± 0.53 g/L, Volatile Suspended Solids (VSS): 11.2 ± 0.27 g/L, Total Phosphorus (TP): 1.45 ± 0.06 g/L and Total Nitrogen (TN): 2.43 ± 0.04 g/L. Since TN and TP concentrations in the seed were sufficient, no extra nutrients were added to the batch.

5.2.2 Experimental design and batch setup

The volumes of substrate and seed required to maintain S°/X° ratios of 0.5 and 1 for BHP first-stage, BMP single-stage and BMP second-stage tests, were calculated on a gCOD/gVSS basis using the following equation:

$$\frac{S^\circ}{X^\circ}\left(\frac{\text{gCOD}}{\text{gVSS}}\right) = \frac{\text{V}_{\text{substrate}}\,(\text{L}) * \text{TCOD}_{\text{substrate}}\,(\frac{\text{g}}{\text{L}})}{\text{V}_{\text{seed}}\,(\text{L}) * \text{VSS}_{\text{seed}}\,(\frac{\text{g}}{\text{L}})} \qquad 5.1$$

Where $V_{substrate}$ is the volume of substrate; V_{seed} is the volume of seed, $TCOD_{substrate}$ is the total chemical oxygen demand (TCOD) of substrate and VSS_{seed} is the volatile suspended solids content of seed (Gupta et al., 2015).

Blank experiment (seed only) was run alongside the substrates to determine its hydrogen and methane potential, which were subtracted from the substrates plus seed biogas potential in order to correct for blank biogas production. All batch tests were conducted in duplicates using 250 mL Wheaton glass serum bottles with a working volume of 200 mL.

For BHP tests, the seed sludge was preheated at 70 ^{o}C for 30 min prior to starting the experiments so as to inhibit methanogens. The initial pH was adjusted to 5.5 ± 0.2 using 1 M HCl or 1 N NaOH

as required. Glucose was used as substrate in the control bottles to ensure the quality of the seed. Since pH was not controlled throughout the experiment, 5 g/L $NaHCO_3$ was added to provide buffering capacity. In order to maintain anaerobic conditions, the headspace of the bottles was flushed with nitrogen gas (99.999% N_2, PraxAir, London, ON, Canada) for a few minutes after which the bottles were placed in a swirling-action shaker operating at 180 rpm and maintained at a temperature of 37 ºC. The same methodology was incorporated in the setup of the BMP batches except that initial pH was adjusted to 7.2 ± 0.2 and acetic acid was used as substrate in the control bottles.

For the second-stage BMP tests of the two-stage anaerobic digestion process, final samples from the BHP tests (first stage) were centrifuged at 8000 rpm for 20 min and the supernatant was used as substrate for a second-stage BMP test. The supernatant samples for each bottle were fully characterized and the volumes of samples required to maintain the desired S°/X° of 0.5 and 1 gCOD/gVSS were calculated using Equation 5.1 above.

5.2.3 Analytical Methods

5.2.3.1 Gas measurement

Biogas composition were measured using suitably-sized glass syringes in the range of 20 to 100 mL where gas was released from the headspace of the serum bottles to equilibrate with ambient pressure. Hydrogen and methane content were determined using a gas chromatograph (SRI 310, SRI instruments, Torrance, CA) with a thermal conductivity detector (TCD) temperature of 90 °C and a molecular sieve column (mole sieve 5 Å, mesh 80/100, 6 ft * 1/8 in) at a temperature of 105 °C. Argon was used as carrier gas at a flow rate of 30 mL/min. Gas composition was analyzed every 4 h for the first 2 days and then every 12 h thereafter for the BHP tests and every 12 h for the

first 4 days and 2 to 3 days thereafter for the BMP tests. Tests were deemed to be completed when biogas production was observed to be less than 1 % of the cumulative (Elbeshbishy et al., 2012).

5.2.3.2 General water quality analysis

Total and volatile solids (TS and VS) in liquid and solid samples as well as TSS and VSS of liquid samples were measured according to standard methods (APHA, 1998). HACH methods and testing kits (HACH Odyssey DR/2500) were employed to measure the TCOD and soluble COD (SCOD), TN and TP.

5.2.3.3 Measurement of organics

Carbohydrates were analyzed using the phenol sulphuric acid method which is a colorimetric method for the determination of polysaccharides (Dubois et al., 1956). The lignin, cellulose and hemicellulose contents of the solid samples were determined using analytical procedures for standard biomass analysis as described by Sluiter et al., (2008) which involved hydrolyzing samples at 30 ºC for 2 h using 72 (%) w/w sulphuric acid. Lignin was measured gravimetrically after acid hydrolysis of biomass as it is insoluble in sulfuric acid. Structural carbohydrates (cellulose and hemicellulose) are however depolymerized during acid hydrolysis of biomass and were quantified using HPLC. Soluble samples from the liquid stream (filtered through 0.45 µm membrane filter) were analyzed to determine the monomeric sugars, volatile fatty acids (VFAs), HMF (5-Hydroxymethyl furfural) and furfural concentrations using a Dionex IC20 Ion Chromatograph equipped with a refractive index detector (RID) (Perkin Elmer Series 200, PerkinElmer Instruments Inc., USA) and an Aminex® HPX-87H column (BIO-RAD laboratories, USA). The following parameters were used: pump flow rate - 0.6 mL/min; mobile phase - 9 mM H_2SO_4, column temperature - 50 °C and injection volume of 0.5 mL. For solid stream analysis,

soluble leachable organics content were determined by dissolving various masses of each original sample (e.g 0.5 g, 1 g, 2 g, 4 g) into separate 1 L measuring cylinders filled with distilled water and mixed thoroughly to allow the soluble components leach into the water, before filtering through 0.45 µm membrane filters. The filtered samples were then analyzed for COD and carbohydrates and linear correlations between the leached concentrations and initial dry mass were developed to determine the feedstock-specific leachable COD and carbohydrates. Unfiltered samples were used for TCOD and total carbohydrate determination.

5.2.3.4 Measurement of Ash and other inorganics

Ash was the residue that remained after the total solids present in the sample were ignited at 575 ± 25 °C for about 4 h to eliminate all carbon present in the sample (Sluiter et al., 2005). Other inorganics were determined by subtracting the sum of weights of lignin, cellulose, hemicellulose and ash from 100 %.

Solid sample characteristics were measured based on the mass of wet samples except for lignin, cellulose, hemicellulose and ash which were analyzed on a dry sample basis.

5.2.4 Computational methods

5.2.4.1 Headspace gas measurement

Headspace gas volumes were included in the total volume of gas measured at each time interval using the following mass balance equation:

$$V_{H_{2,i}} = V_{H_{2,i-1}} + C_{H_{2,i}} \times V_{G,i} + V_{h,i}\,(C_{H_{2,i}} - C_{H_{2,i-1}}) \qquad 5.2$$

where $V_{H_{2,i}}$ and $V_{H_{2,i-1}}$ are cumulative gas volumes at the present (i) and preceding (i -1) time intervals; $C_{H_{2,i}}$ *and* $C_{H_{2,i-1}}$ are the fractions of gas in the headspace of the reactor in the present

and preceding intervals while $V_{G,i}$ and $V_{h,i}$ are the total gas volumes accumulated between the preceding and present intervals and the total volume of reactor headspace in the present interval respectively (Gomez-Florez et al., 2015).

This calculation assumes that the sample of gas taken from the headspace after measuring total gas production has the same gas composition as the gas remaining in the headspace. This is thought to be true in a homogeneously mixed system (López et al., 2007).

5.2.4.2 Gompertz model

The modified Gompertz equation (Equation 5.3) as described by Chen et al. (2006) was employed, with the parameters estimated using the solver function on Microsoft Excel 2013.

$$P(t) = P \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad 5.3$$

Where *P* (t) is the cumulative biogas potential (mL) at time (t); λ is the lag phase (d), *P* is the biogas production potential (mL); R_m is the biogas production rate (mL/d); and e is exp (1) which is approx. 2.7. In this study, *P* was expressed as milliliters, R_m was expressed as milliliters per gram initial VSS of seed per day, and λ was expressed in day.

5.2.4.3 BMP parameter estimation model

The BMP parameter estimation model as described by Gunaseelan (2004) was employed to estimate the coefficients B_o and k which is a first order rate used to compare extents and rates of biomass conversion to methane. This rate is described using the following equation:

$$B = B_o(1 - e^{-kt}) \quad 5.4$$

where B is the cumulative methane yield (mL/gVSS $_{initial}$) at time t; B_o is the ultimate methane yield in mL/gVSS $_{initial}$ at the end of the fermentation period, and k is the methane production rate constant in d^{-1}.

5.2.4.4 Biogas yields and COD mass balance calculations

Normalized biogas yields in gCOD $_{added}$ were calculated based on initial feedstock added while yields in gCOD $_{consumed}$ were calculated based on the difference between the initial and final COD values of the bottle after correcting for the blank.

COD mass balance for the biohydrogen and biomethane tests were calculated (after correcting for the volume of the gas produced by the blank) using the following equation:

$$\text{COD mass balance (\%)} = \frac{(0.2*\text{TCOD}_{\text{final}} + \text{COD}_{\text{biogas}})* 100}{(\text{TCOD}_{\text{initial}})*0.2} \quad 5.5$$

where: 0.2 L =working volume per bottle; biogas= hydrogen or methane

5.3 Results and Discussion

5.3.1. Material characterization

Eight hydrolysate samples from the poplar wood biomass were obtained from the TSE operated at GreenField Specialty Alcohols Inc., Chatham-Kent, Ontario, Canada. Two sample streams were received – three samples from a liquid steam (L1 to L3) and five samples from a solid stream (S1 to S5). Sample streams were collected over a 5-day period from two different sections of the extruder operating at the following conditions: liquid stream: 170 ºC and 100 psig and solid stream: 190 ºC and 160 psig. The liquid and solid streams were run separately as substrates.

TSE sample characteristics were determined as described under Analytical methods (Section 5.2.3) and based on three replicates (average percent error (APE) of less than 10 %) prior to conducting

the BHP and BMP tests, are shown in Tables 5.3a and 5.3b. The composition of the liquid streams was observed to contain significant amounts of simple sugars, VFAs and refractory compounds such as furfural and hydroxyl methyl furfural (HMF). Furfural, 5-hydroxymethylfurfural (HMF), vanillin, syringaldehyde and acetic acid are usually formed during the pretreatment of feedstock and the greater the severity of degradation (such as pretreatment temperature, acid concentration, residence time, etc.), the greater the concentrations of these recalcitrant compounds in the pretreated material (Lin et al., 2015; Siqueira and Reginatto, 2015). Karunanithy and Mathukumarappan (2013) observed no furfural and HMF in any of the extrusion pretreatment employed on feedstocks such as grass, corn stover, miscanthus and straw with or without alkalis. Chen et al. (2014), however, observed 0.3 to 1 g/L of furfural when dilute sulfuric acid was added to rice straw in a twin screw extruder. The wide variations in sample characteristics depicted in Tables 5.3a and 5.3b could be due to lack of homogeneity of the hydrolysates, changes in extruder configuration and/or operational conditions.

Generally, lignocellulosic materials are composed of cellulose, hemicellulose and lignin with cellulose being the most abundant component representing about 30 % to 70 % of the biomass, while hemicellulose and lignin represent around 15 % to 30 % and 10 % to 25 % of the biomass respectively (Monlau et al., 2013). The lignin content of the solid samples in this study was 42 % (on average) of the organic matter content, with cellulose and hemicellulose accounting for about 45 % and 13 % respectively. The balance is made up of ash and other extractives. Sannigrahi and Ragauskas (2010) reported, poplar wood lignin content ranging from 21 % to 29 %, cellulose from 42 % to 49 % and hemicellulose from 16 % to 23 % (pretreatment method not specified). The aforementioned authors further stated that poplar wood had higher cellulose and lignin content than other lignocellulosic biomass such as corn stover and switch grass. Monlau et al. (2013) reported

lignin, hemicellulose, and cellulose contents of poplar wood to be 19.5 %, 22.5 % and 44.5 % respectively (pretreatment method also not specified). Another study reported organic matter content of poplar wood as lignin (21.8 %), hemicellulose (10.6 %) and cellulose (40.8 %) after alkaline pretreatment followed by purification with an acid mixture (Sun et al., 2005). The high lignin content recorded in this report could be due to the type of pretreament method employed (extrusion) and/or the nature of the wood.

The carbohydrate content of the solid samples was about 65 % of the TCOD with an average moisture content of approximately 70 % on average. SCOD/VS ratio for the solid samples ranged between 0.26 to 0.47. Pakarinen et al. (2009) reported a SCOD/VS ratio of 0.2 for grass silage. Sample characteristics show that both the liquid and solid streams are rich in organic matter that can be broken down to produce biogas.

Table 5.3 a: Characterization of TSE samples for liquid streams

Parameter	Unit	L1	L2	L3
pH		3.67 ± 0.01	3.80 ± 0.02	3.46 ± 0.01
TCOD	g/L	42.8 ± 0.35	32 ± 0.21	139.2 ± 0.21
SCOD	g/L	39.5 ± 0.21	28.7 ± 0.14	138.2 ± 0.14
Total carbohydrate	g/L	32.2 ± 0.07	25.5 ± 0.11	120.9 ± 0.38
Soluble carbohydrate	g/L	28.1 ± 0.01	22.4 ± 0.02	110.1 ± 0.06
TS	g/L	32.1 ± 1.1	22.4 ± 0.18	103.2 ± 1.26
VS	g/L	31.5 ±1.14	22 ± 0.15	100.4 ± 0.57
TSS	g/L	3.9 ± 0.44	3.1 ± 0.47	7.5 ± 0.33
VSS	g/L	3.4 ± 0.08	3.0 ± 0.5	7.3 ± 0.42
TP	g/L	0.9 ± 0.02	0.6 ± 0.02	0.5 ± 0.02
TN	g/L	0.1 ± 0.01	0.1 ± 0.01	0.3 ± 0.01
Glucose	g/L	0.27	0.21	0.31
Xylose	g/L	4.29	4.1	9.11
Arabinose	g/L	2.91	0.26	0.23
Formate	g/L	0	0	0
Acetate	g/L	2.91	3	3.72
HMF	g/L	0.18	0.38	0.31
Furfural	g/L	1.01	2.45	1.36

*Values are mean of triplicates ± standard deviation

Table 5.3 b: Characterization of TSE samples for solid streams

Parameter	Unit	S1	S2	S3	S4	S5
[a]TCOD	mg/g sample	363 ± 29	318 ± 38	380 ± 81	235 ± 25	289 ± 71
[a]Leachable COD	mg/g sample	115 ± 0	91 ± 0	90 ± 0	62 ± 0	95 ± 0
[a]Total carbohydrate	mg/g sample	217 ± 3	208 ± 1	251 ± 2	174 ± 3	168 ± 9
[a]Leachable carbohydrate	mg/g sample	66 ± 2	47 ± 0	35 ± 0	51 ± 0	63 ± 0
[a]TS	mg/g sample	449 ± 8	324 ± 1	315 ± 5	165 ± 6	204 ± 16
[a]VS	mg/g sample	446 ± 6	322 ± 1	313 ± 3	163 ± 8	201 ± 11
[a]TP	mg/g sample	45 ± 0.2	3 ± 0.3	15 ± 1.1	6 ± 0.4	8 ± 0.5
[a]TN	mg/g sample	2 ± 0.4	1 ± 0	3 ± 0	3 ± 0.2	2 ± 0.4
*[b]Lignin	wt%	34.6 ± 1.9	33.1 ± 2.3	35.7 ± 2.7	36.2 ± 1.6	34.8 ± 1.3
[b]Cellulose	wt%	38.2 ± 2.8	36.9 ± 1.7	36.9 ± 1.4	37.8 ± 3.6	38.2 ± 2.7
[b]Hemicellulose	wt%	11.5 ± 0.9	10.2 ± 0.6	11.8 ± 0.3	10.8 ± 1.1	11.0 ± 1.1
[b]Ash	wt%	9.8 ± 1.0	11.6 ± 0.8	10.1 ± 0.7	10.4 ± 0.8	10.2 ± 0.6
[b]Others	wt%	5.9 ± 0.4	8.2 ± 0.7	5.5 ± 0.4	4.8 ± 0.6	5.8 ± 0.5

*Values are mean of triplicates ± standard deviation; [a] wet mass basis; [b] dry mass basis; * sum of acid soluble and acid-insoluble lignin

5.3.2 First-stage BHP tests

Batches were set up as described in Section 5.2.2. No methane gas was detected in all BHP experiments as seed was preheated before use which efficiently suppressed the activity of methanogens. Fig. 5.1 illustrates the cumulative average hydrogen production from the different streams of extruded poplar wood samples. It shows that hydrogen production from the liquid fractions were higher than the solid streams. This is a logical observation as the liquid streams consist of readily fermentable sugars (see Table 5.3a). The highest cumulative hydrogen production observed at an S°/X° of 0.5 gCOD/gVSS were: liquid fractions (L3: 183 mL) and solid fractions (S1: 60 mL) and at an S°/X° of 1 gCOD/gVSS were: liquid fractions (L3: 338 mL) and solid fractions (S1: 123 mL). These samples (L3 and S1) showed the highest SCOD and leachable COD concentrations respectively compared to the other samples. It can be observed that the hydrogen production at an S°/X° of 1 gCOD/gVSS were higher than at an S°/X° of 0.5 gCOD/gVSS due to an increase in substrate mass. Table 5.4 presents a summary of the biohydrogen yields and Gompertz rates data. Average biohydrogen yields of 0.469 $LH_2/gCOD_{consumed}$ and 0.452 $LH_2/gCOD_{consumed}$ were obtained from the liquid and solid streams respectively. A linear plot of yields at an S°/X° of 0.5 gCOD/gVSS against an S°/X° of 1 gCOD/gVSS (figures not shown), R^2 of > 0.91 revealed that hydrogen yields were independent of S°/X°. Thus, averages of yields obtained at both S°/X° of 0.5 and 1 gCOD/gVSS will be reported for simplicity except otherwise stated.

Maximum hydrogen production rates were normalized to initial VSS of seed in all cases to give the specific hydrogen production rates (SHPR). As was the case with the cumulative hydrogen production, *P* and SHPR of the liquid streams were significantly higher than the solid samples (206 mL and 45.8 mL $H_2/gVSS_{initial}/d$ for the liquid streams and 72 mL and 19.8 mL $H_2/gVSS_{initial}/d$ for

the solid streams). This could be due to the presence of lignin, cellulose and hemicellulose in the solid samples which are not easily broken down by the hydrogen-producing bacteria. *P* and SHPR were generally greater at an S°/X° of 1 gCOD/gVSS than at 0.5 gCOD/gVSS with the hydrogen potential observed to be about 2 times at the former than the latter S°/X°. The same trends of higher hydrogen production potential and rates with increasing S°/X° in the range of 4 to 6 gCOD/gVSS were also observed by Nasr et al. (2012). Lag phases (λ) for both liquid and solid samples were relatively similar. R^2 for the Gompertz model across all samples were around 0.999.

Incorporating the ratios of the sum of final COD and biohydrogen COD to initial COD, mass balance calculations were closed between 90 % and 97 % confirming the reliability of the data. Furthermore, results of the duplicates (plots not shown), indicate that the biohydrogen data were reproducible with APEs of less than 10 %.

Although, furfural and HMF have been reported to adversely affect hydrogen production, this study showed no direct correlation between hydrogen yields and rates with the initial concentrations of both furfural and HMF concentration which indicate that there is indeed a threshold concentration for these compounds which the cultures responsible for fermentation can tolerate, and possibly breakdown to produce biogas. This same trend was also reported by Nasr et al. (2014). Analysis of final samples revealed no refractory compounds implying that they were completely broken down during the fermentation process.

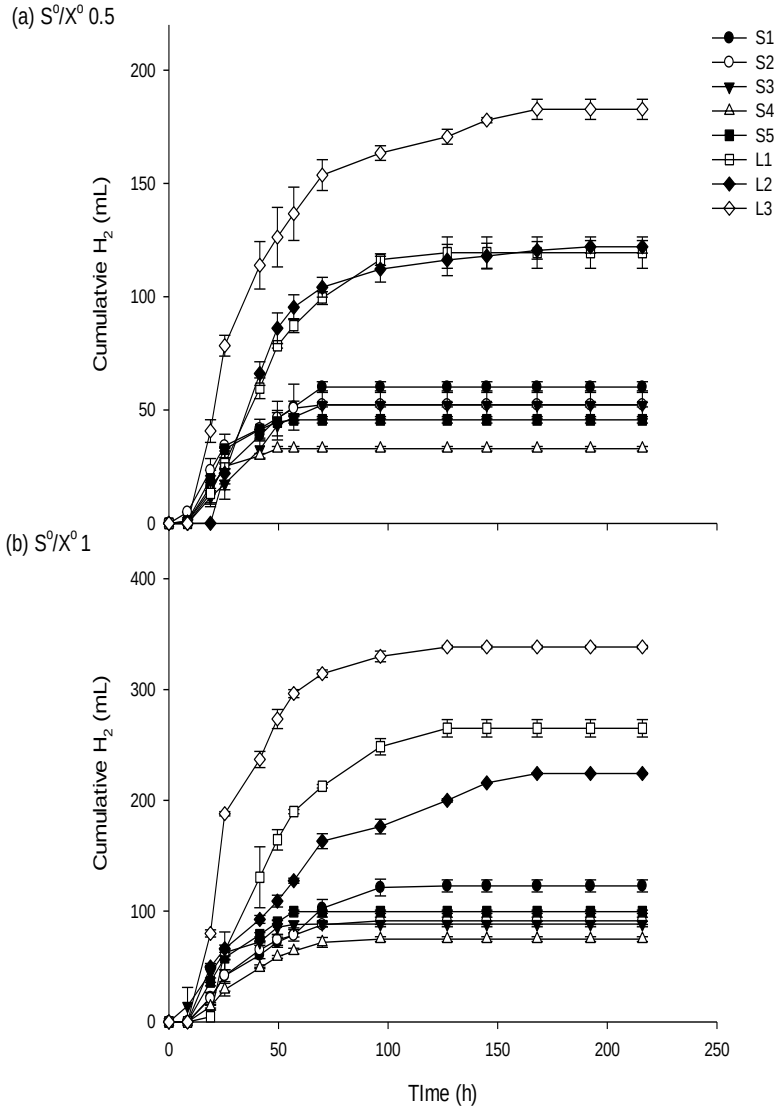


Figure 5.1: Cumulative hydrogen production curves

Table 5.4: Summary table showing first-stage biohydrogen yields and rates data

	[a]Samples	Unit	First stage BHP
Biohydrogen yields	L	L H_2/gCOD $_{added}$	0.131 ± 0.02
	S		0.038 ± 0.01
	L	L H_2/gCOD $_{consumed}$	0.469 ± 0.03
	S		0.452 ± 0.02
Gompertz parameters	L	P (mL)	206 ± 85
		SHPR (mL H_2/gVSS $_{initial}$/d)	45.8 ± 24
		λ (d)	0.5 ± 0.2
	S	P (mL)	72 ± 28
		SHPR (mL H_2/gVSS $_{initial}$/d)	19.8 ± 6.4
		λ (d)	0.4 ± 0.1
Feedstock COD removal efficiency	L	%	8
	S		2

[a] L: Liquid stream; S: Solid stream
All values are averages ± standard deviation of sample duplicates of the respective streams at both S°/X° of 0.5 and 1 gCOD/gVSS except otherwise stated

5.3.3 Single and Second-stage BMP tests

5.3.3.1 Methane production

Single-stage and second-stage BMP tests were performed in order to assess the impact of separating the acidogenic and methanogenic stages of anaerobic digestion as well as to investigate the extent of acidification of the first stage biohydrogen production process. Operating conditions of acidic pH and short retention times in the first stage of a two-stage anaerobic digestion process favour fermentation of substrates to hydrogen and the accumulation of VFAs. In the second stage, neutral pH and longer retention times favour methane production from VFAs from the effluent of the first stage (Monlau et al., 2013).

The effluent pH of the first-stage hydrogen production step was around 4.7 on average and the second-stage BMP test was set up as described in Section 5.2.2 Cumulative methane production profiles of both single and two-stage BMP tests are shown in Fig. 5.2. As seen with the BHP tests, no direct correlation was observed between the potential refractory compounds present in the initial liquid streams of all samples with methane yields and rates.

Second-stage BMP yields presented in Table 5 were calculated based on the COD of the substrate obtained after centrifugation of the first-stage BHP effluent. Methane yields of 0.369 L CH_4/gCOD $_{consumed}$ and 0.353 L CH_4/gCOD $_{consumed}$ were obtained from liquid and solid streams of the second-stage BMP which are an 11 % and 7 % increase from the single-stage BMP process. This same trend of higher yields in the second-stage BMP compared to a single-stage BMP process was also reported by Nasr et al. (2012), Rincon et al. (2009) and Viñas et al. (1993). Statistical analysis using T-test was employed to determine the degree of significance between the yields of a single-stage and two-stage anaerobic digestion process based on both COD added and consumed. As expected, since methane production per mass COD converted is a stoichiometric parameter, the

differences between single-stage and second-stage BMP yields normalized to COD consumption were not statistically significant at the 95 % confidence limit ($p < 0.05$). The T-test confirmed that while at an S°/X° of 0.5 gCOD/gVSS, the differences in yields based on gCOD added between single-stage and second-stage BMPs were not significant at $p < 0.05$, the differences were significant at an S°/X° of 1 gCOD/gVSS. This observation is reasonable as the conversion efficiencies (see Table 5.5) on a L CH_4/gCOD added basis in the single and second-stage BMP processes, were similar (76 % and 70 % in the single-stage BMP process compared to 78 % and 71 % in the second-stage BMP process for the liquid and solid streams respectively) at an S°/X° of 0.5 gCOD/gVSS. On the other hand, at an S°/X° of 1 gCOD/gVSS, conversion efficiencies were much higher in the second-stage process compared to the single-stage process which explains why differences in yields based on COD added between both processes were statistically significant at an S°/X° of 1 gCOD/gVSS and not at 0.5 gCOD/gVSS.

Methane production potential from liquid and solid streams for both single and second-stage BMP tests were comparable with no lag phase implying that methanogens could better degrade the complex carbohydrates present in the solid samples than the hydrogen producers. Specific methane production rates (SMPR) values were comparable between the two S°/X° conditions in the single stage but generally higher rates were observed at the higher S°/X° in the second-stage BMP process (data not shown). SMPRs were higher in the liquid stream than the solid stream in both single and second stage BMP tests. Even though cumulative methane production was generally lower in the second-stage compared with the single-stage BMP process due to partial conversion in the first stage, production rates were faster in the second-stage than the single-stage BMP process. Data analysis showed that it took on generally less time to reach 75 % of the cumulative methane production in the second-stage than the single-stage BMP process (see Table 5.5) which results in

the enhancement of the overall performance of the two-stage process. Shorter solids retention time (SRT) was also reported in terms of COD degradation in the two-stage anaerobic digestion of thin stillage when compared with the single-stage anaerobic digestion process by Nasr et al. (2012). COD mass balance calculations closed between 92 % to 97 % showing the reliability of these data. As with the BHP results, BMP data were reproducible with APEs of less than 10 %.

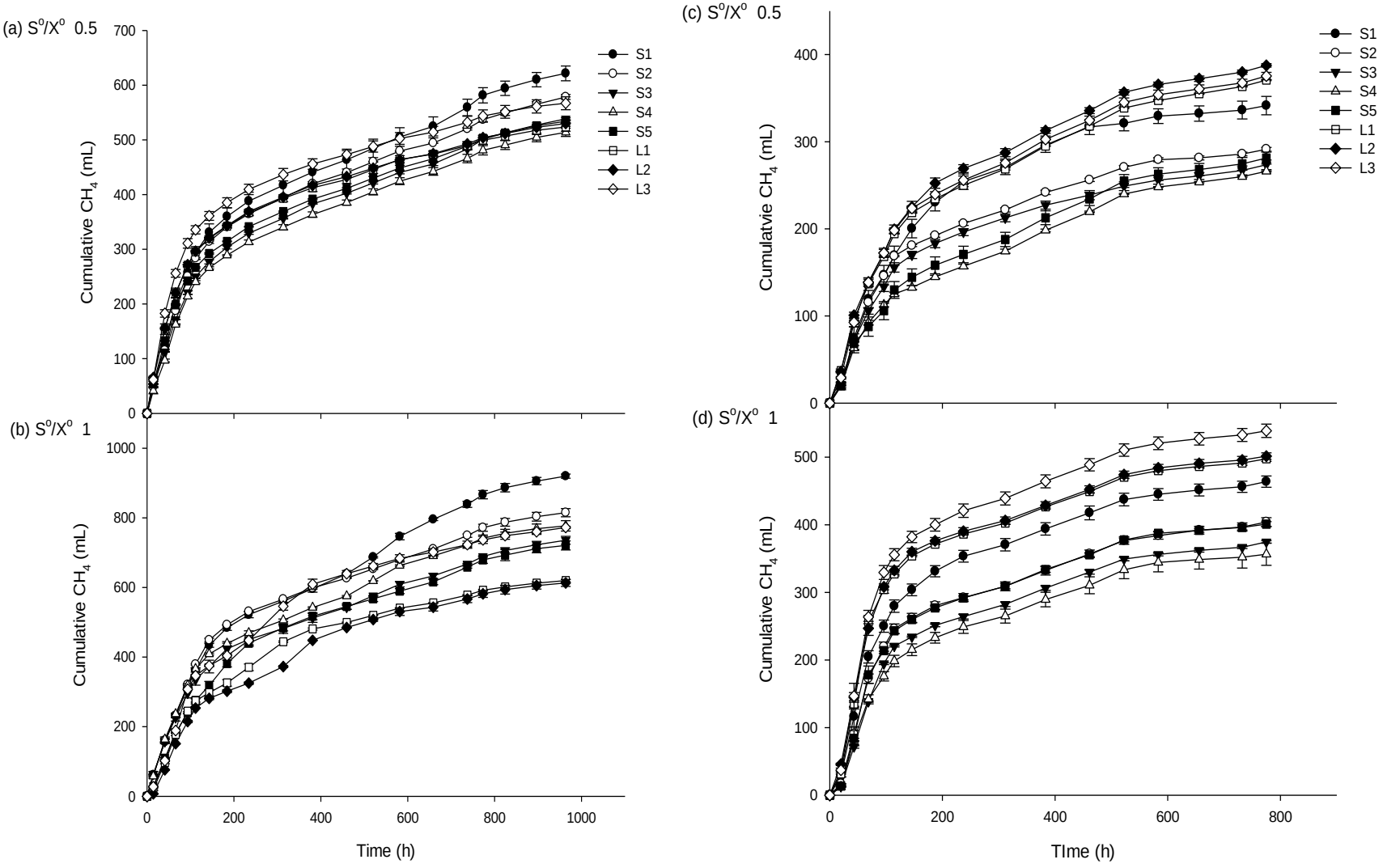


Figure 5.2: Cumulative methane production curves. (a) and (b): single-stage BMP, (c) and (d) 2-stage BMP

Table 5.5: Summary of single-stage and second-stage biomethane production data

	[a] Samples		Unit	Single-stage BMP	Second-stage BMP
Biomethane yields	L		L CH_4/gCOD $_{added}$	0.276 ± 0.03	0.320 ± 0.02
	S			0.263 ± 0.04	0.299 ± 0.04
	L		L CH_4/gCOD $_{consumed}$	0.330 ± 0.01	0.369 ± 0.01
	S			0.327 ± 0.01	0.353 ± 0.01
Gompertz parameters	L		P (mL)	562 ± 96	411 ± 66
			SMPR (mL CH_4/$gVSS_{initial}$/d)	19.9 ± 2.3	40.7 ± 23.0
			λ (d)	0	0
	S		P (mL)	624 ± 140	322 ± 58
			SMPR (mL CH_4/$gVSS_{initial}$/d)	14.3 ± 2.9	31.6 ± 14.4
			λ (d)	0	0
[b] COD conversion efficiency	L	0.5 gCOD/gVSS	%	76 ± 2	78 ± 1
	S			70 ± 3	71 ± 3
	L	1 gCOD/gVSS		62 ± 1	83 ± 1
	S			61 ± 4	79 ± 2
Time required to reach 75% of cumulative methane	L	0.5 gCOD/gVSS	h	329 ± 20	304 ± 7
	S			449 ± 22	316 ± 63
	L	1 gCOD/gVSS		369 ± 32	193 ± 5
	S			467 ± 41	283 ± 37

[a] L: Liquid stream; S: Solid stream; [b] based on methane yields per gCOD added using theoretical maximum methane yield of 0.4 LCH_4/gCOD $_{consumed}$ at 37ºC
All values are averages ± standard deviation of sample duplicates of the respective streams at both S°/X° of 0.5 and 1 gCOD/gVSS except otherwise stated

5.3.3.2 Anaerobic biodegradability

Methane yields can be predicated using first-order kinetic models which are mostly applied to anaerobic digestion systems. One of such models is the biomethane estimation parameter model (see Section 5.2.4.3). Ultimate methane yields (B_o) were normalized to initial VSS of seed ($B_{o\text{-}exp}$) by dividing the net methane production under operating conditions by the weight of seed added on a gVSS basis. $B_{o\text{-}exp}$ and k for both liquid and solid streams were similar (within each stage) (see Table 5.6) and this is reasonable as methane yields from both streams were comparable within each S°/X°. Higher kinetics usually indicate higher conversion efficiencies with the two-stage anaerobic digestion process showing, on average, around 20 % higher parameter values than that of the single-stage BMP process. Modeled BMP yields were observed to be very similar to the calculated yields (data not shown) with overall APE of less than 5 %.

Another first order model describes the rate and extent of biodegradation by ignoring the accumulation of intermediary compounds formed during the anaerobic digestion process thereby relating methane production to hydrolysis rate (Raposo et al., 2011). Taking into consideration the theoretical methane yield of 0.35 L/gCOD at STP which corresponds to 0.4 L/gCOD at 37 ºC, the extent of anaerobic biodegradability (BD_{CH4}) of extruded poplar wood hydrolysates was estimated from the experimental methane yields using the following equation:

$$BD_{CH4}\ (\%) = (B_{o\ exp}/B_{o\ th}) * 100 \qquad 5.6$$

Where $B_{o\ exp}$ and $B_{o\ th}$ are the experimental and theoretical methane potential (L) based on initial COD of the original hydrolysate samples. From Table 5.6, it can be observed that on average, the anaerobic biodegradability of the poplar wood hydrolysates in the single-stage BMP process was 48 % and 40 % in the liquid and solid samples respectively. For the second-stage BMP process,

these values on average were 84 % and 97 % for the liquid and solid respectively, implying that the initial acidogenesis in the two-stage anaerobic digestion process enhanced the methanogenic step. Nasr et al. (2012), reported the anaerobic biodegradability of thin stillage to be 88 % and 99 % in a single-stage and two-stage anaerobic digestion process respectively. The low biodegradability values observed in the single-stage BMP process can be attributed to the low COD conversion efficiencies observed in this stage especially at an S°/X° of 1 gCOD/gVSS as explained earlier.

Table 5.6: Anaerobic biodegradability of poplar wood hydrolysates

	[a] Samples		Unit	Single-stage BMP	Second-stage BMP
BMP estimation parameters	L	$B_{o\text{-}exp}$	mL CH_4/gVSS $_{initial}$	272 ± 53	329 ± 89
		k	d^{-1}	0.14 ± 0.05	0.18 ± 0.04
	S	$B_{o\text{-}exp}$	mL CH_4/gVSS $_{initial}$	257 ± 58	320 ± 86
		k	d^{-1}	0.12 ± 0.02	0.15 ± 0.03
Anaerobic degradability	L		%	48 ± 18	84 ± 18
	S			40 ± 17	97 ± 46

[a] L: Liquid stream; S: Solid stream

All values are averages ± standard deviation of sample duplicates of the respective streams at both S°/X° of 0.5 and 1 gCOD/gVSS except otherwise stated

5.3.3.3 VFAs and feedstock COD removal efficiencies

As mentioned earlier, the biohydrogen production step, which is the first stage in a two-stage anaerobic digestion process, is an acidification process where the sugars are broken down into hydrogen as well as metabolic products predominantly volatile fatty acids (acetic, butyric, and propionic acids) and alcohols (ethanol and butanol) depending on the microbial communities present as well as operating conditions with acetic and butyric acids production favouring concurrent hydrogen production (Antonopoulou et al., 2008). Acetate and butyrate were the main VFAs observed in the final samples, accounting on average for over 80 % of the total VFAs with negligible ethanol production. Table 5.7 presents the TVFA/SCOD $_{initial}$ ratios for all tests as well as COD removal efficiencies. It can be observed that S°/X° had no impact on TVFA/SCOD $_{initial}$ and as such no impact on acidification. However, the ratios were around 50 % higher on average in the second-stage compared with the single-stage BMP process thus emphasizing the importance of separating the acidification stage from the methanogenic stage as there is increased VFAs for the second stage which will enhance methane production.

Average feedstock COD removal efficiency for the BHP stage were 8 % and 2 % for the liquid and solid streams respectively but 69 % and 66 % were observed for the liquid and solid streams respectively in the single-stage compared with 82 % and 75 % overall in the two-stage anaerobic digestion process. Viñas et al. (1993) reported a 20 % COD removal in the first stage hydrogen production process treating TMP wastewater in a UASB reactor while Antonopoulou et al. (2008) reported a 5 % COD reduction in the continuous production of hydrogen from cheese whey. Elbeshbishy and Nakhla (2011) reported a COD destruction efficiency of 80 % in a single-stage anaerobic digestion process utilizing food waste as substrate while an overall COD destruction efficiency of 90 % was both reported by Blonskaja et al. (2003) in the treatment of distillery waste

and Hafez et al. (2010) in the treatment of synthetic wastewater/leachate solution in a two-stage anaerobic digestion process.

Table 5.7: TVFA $_{initial}$/SCOD $_{initial}$ and COD removal efficiencies data for single-stage BMP and second-stage BMP processes

		[a] Samples	Unit	Single-stage BMP	Second-stage BMP
TVFA $_{initial}$/SCOD $_{initial}$	L	0.5 gCOD/gVSS	-	0.16 ± 0.04	0.25 ± 0.03
	S			0.27 ± 0.12	0.44 ± 0.01
	L	1 gCOD/gVSS		0.15 ± 0.04	0.23 ± 0.02
	S			0.30 ± 0.14	0.41 ± 0.02
Feedstock COD removal efficiency		L	%	69 ± 2	80 ± 1
		S		66 ± 3	75 ± 2

[a] L: Liquid stream; S: Solid stream

All values are averages ± standard deviation of sample duplicates of the respective streams at both S°/X° of 0.5 and 1 gCOD/gVSS except otherwise stated

5.3.3.4 Fate of Lignin

Lignin is the major non-carbohydrate component of lignocellulosic materials. It is closely attached to cellulose and hemicellulose through a variety of chemical bonds and is responsible for the remarkable strength of plants. It is a cross-linked hydrophobic polymer, insoluble and resistant to anaerobic breakdown and its presence affects the degradability of the lignocellulosic biomass (Monlau et al., 2013). Irrespective of lignin's resistance to microbial biodegradation, some organisms, particularly fungi, have developed the necessary enzymes to break it. White-rot fungi produce manganese peroxidases which degrade lignin (Kirk and Farrell, 1987). Lignin is the most recalcitrant of all the plant cell components, and as such the higher the proportion of lignin the lower the bioavailability of the substrate. Lignin molecules reduce the surface area available to enzymatic penetration and activity (Haug, 1993).

This study reveals that it was difficult for the hydrogen-producing community to degrade the lignin component of the hydrolysate as shown in Table 5.8a with only 2 % of lignin degraded on average at both S°/X° values. This also explains the relatively low hydrogen yields obtained from the solid samples compared to the liquid samples. In contrast, methanogens were able to degrade lignin in the solid samples to produce methane with yields comparable to those obtained from the liquid stream. Table 5.8b presents analysis of the initial and final samples for the single-stage methane production process which showed on average around 77 % degradation of lignin from the initial mass of lignin present in the solid samples at both S°/X° of 0.5 and 1 gCOD/gVSS in about 40 days.

Table 5.8 a: Lignin degradation for the BHP process

	BHP process									
	Initial setup				After batch completion					
	Sample #	Mass of sample added	Lignin content		TSS	Lignin content				% Lignin degraded
gCOD/gVSS		g	wt %	g	g/L	wt %	g/L	g	g	%
S°/X° 0.5	S1	3.4	34.6	1.2	20.4	37	7.6	1.66	1.17	1
	S2	3.9	33.1	1.3	22.2	36	8.0	1.76	1.26	2
	S3	3.2	35.7	1.1	21.7	34	7.4	1.62	1.13	1
	S4	5.2	36.2	1.9	19.8	53	10.5	2.31	1.82	3
	S5	4.2	34.8	1.5	18.3	48	8.8	1.94	1.44	1
									Average lignin degradation	2
S°/X° 1	S1	6.8	34.6	2.4	23.2	55	12.8	2.81	2.32	2
	S2	7.8	33.1	2.6	23.3	59	13.7	3.02	2.52	2
	S3	6.4	35.7	2.3	22.0	57	12.5	2.76	2.26	1
	S4	10.4	36.2	3.8	22.7	83	18.9	4.15	3.65	3
	S5	8.4	34.8	2.9	21.0	72	15.1	3.33	2.84	3
	Seed	0	0	0	23.0	10	2.3	0.50		
									Average lignin degradation	2
									Overall average lignin degradation (%)	2

*g =mass after correcting for the blank

Table 5.8 b: Lignin degradation for the single-stage BMP process

	Single -stage BMP process										
	Initial setup				After batch completion						
	Sample #	Mass of sample added	Lignin content		TSS	Lignin content				% Lignin degraded	
gCOD/gVSS		g	wt %	g	g/L	wt %	g/L	g	g	%	
S°/X° 0.5	S1	3.0	34.6	1.0	18.6	22	4.1	0.90	0.35	67	
	S2	3.4	33.1	1.1	17.8	21	3.8	0.83	0.28	75	
	S3	2.9	35.7	1.0	19.6	21	4.1	0.90	0.35	66	
	S4	4.7	36.2	1.7	17.4	22	3.8	0.83	0.28	84	
	S5	3.8	34.8	1.3	17.2	17	2.9	0.63	0.08	94	
									Average lignin degradation	77	
S°/X° 1	S1	6.0	34.6	2.1	21.5	25	5.3	1.16	0.61	71	
	S2	6.9	33.1	2.3	20.8	27	5.7	1.25	0.70	69	
	S3	5.8	35.7	2.1	19.6	23	4.6	1.01	0.46	78	
	S4	9.3	36.2	3.4	18.6	22	4.1	0.90	0.34	90	
	S5	7.6	34.8	2.6	18.5	28	5.2	1.14	0.59	78	
	Seed	0	0	0	18.6	14	2.5	0.55			
									Average lignin degradation	77	
									Overall average lignin degradation (%)	77	

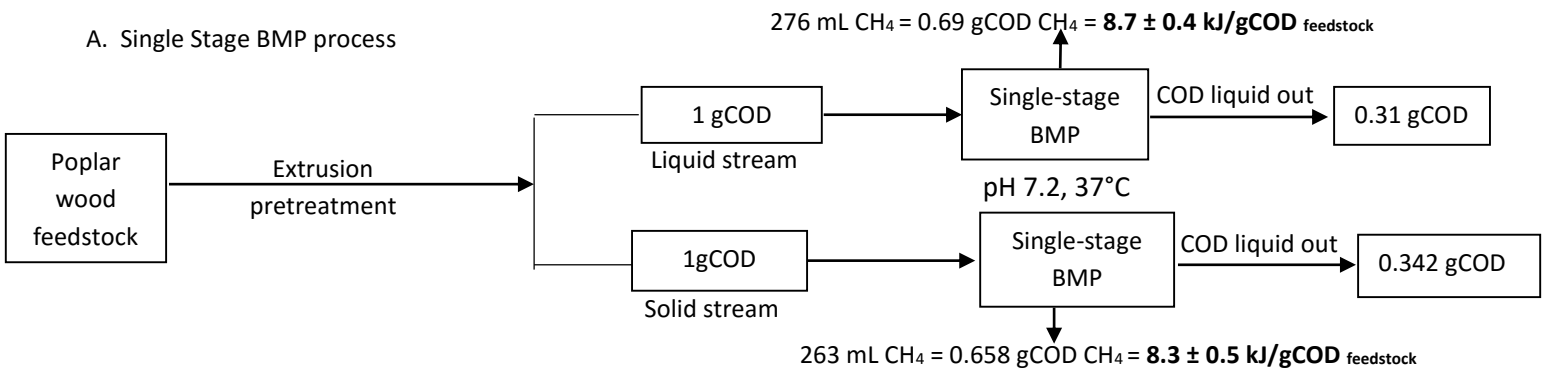
*g =mass after correcting for the blank

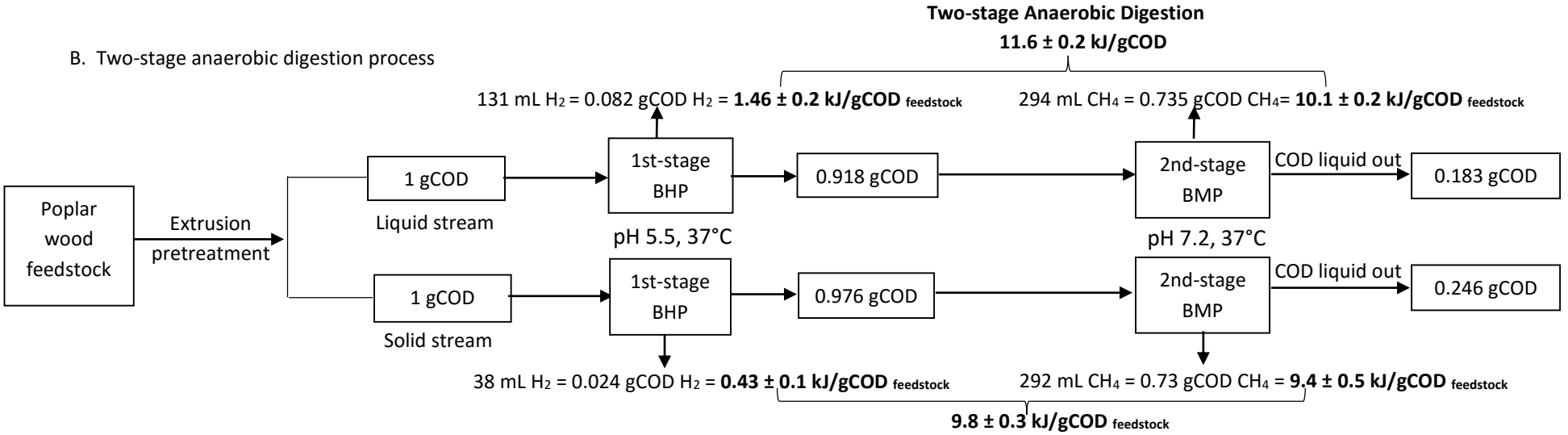
5.3.3.5 Biogas energy yields

In order to compare the performance of the single and second-stage BMP processes as well as assess the overall performance of a two-stage anaerobic digestion process, energy recovery was calculated. Table 5.9 summarizes the biogas yields for the BHP, single and second-stage BMP processes as well as their feedstock removal efficiencies while Fig. 5.3 presents a schematic of the energy yields per gram of $COD_{feedstock}$ for the liquid and solid streams of all processes. Assuming the energy yields of hydrogen and methane at STP are 142 kJ/g or 284 kJ/mol (Shi et al., 2010) and 50 kJ/g or 801 kJ/mol (Xie et al., 2008) respectively, 11.2 kJ/L H_2 and 31.5 kJ/L CH_4 at 37 °C were employed in this study for the estimation of energy content. Energy yields obtained from the single-stage BMP process were 8.7 $kJ/gCOD_{feedstock}$ and 8.3 $kJ/gCOD_{feedstock}$ from the liquid and solid streams respectively. On the other hand, the second-stage BMP process showed energy yields of 10.1 $kJ/gCOD_{feedstock}$ and 9.4 $kJ/gCOD_{feedstock}$ from the liquid and solid streams respectively while 1.46 $kJ/gCOD_{feedstock}$ and 0.43 $kJ/gCOD_{feedstock}$ were obtained from the first-stage BHP tests for the liquid and solid streams respectively. Therefore, the overall energy output obtained from the two-stage anaerobic digestion process, including both hydrogen and methane, were 11.6 $kJ/gCOD_{feedstock}$ and 9.8 $CH_4/gCOD_{feedstock}$ which represent a 33 % and 18 % increase in energy yields compared to the single-stage digestion for the liquid and solid samples respectively. Nasr et al., (2012) reported an 18.5 % increase in energy yields between the single and two-stage anaerobic digestion processes. This proves the advantages of the two-stage over a single stage process which includes enhancement in overall yields, performance, and efficiency.

Table 5.9: Summary table of biogas yields in L biogas/gCOD added and feedstock COD removal efficiencies (shown in brackets)

			Two-stage anaerobic digestion	
Samples	**Unit**	**Single-stage BMP**	**First-stage BHP**	**Second-stage BMP**
L	L biogas/gCOD $_{added}$	0.276 ± 0.03 (69%)	0.13 ± 0.02 (8 %)	0.320 ± 0.02 (80 %)
S		0.263 ± 0.04 (66 %)	0.04 ± 0.01 (2 %)	0.299 ± 0.04 (75 %)





Energy yield (kJ/gCOD $_{feedstock}$) = kJ/L biogas* L biogas/gCOD $_{feedstock}$; Energy yields for the second-stage BMP ignored COD lost during centrifugation; Volume of hydrogen or methane gas at 37 °C is 25.4 L/mol; 16 gCOD H_2 and 64 gCOD CH_4 are the gCOD equivalents of hydrogen and methane gas respectively.

Figure 5.3: Schematic of process and biogas yields from single and two–stage anaerobic digestion processes

5.4 Conclusions

The feasibility of hydrogen production using extruded poplar wood as substrate, was demonstrated in this study with average hydrogen yields of 0.469 LH_2/gCOD $_{consumed}$ and 0.452 LH_2/gCOD $_{consumed}$ from the liquid and solid streams respectively. The liquid stream however, showed higher hydrogen production potential and rates compared to the solid streams. Biomethane yields in L CH_4/gCOD $_{consumed}$ were comparable between the liquid and solid streams for both single and second-stage BMP tests. Even though the Gompertz parameters showed lower maximum methane production potential in the second-stage compared to the single-stage BMP tests, maximum methane production rates were observed to be higher in the second–stage process compared to single stage process (40.7 mL CH_4/gVSS initial/d vs 19.9 mL CH_4/gVSS initial/d for the liquid samples and 31.6 mL CH_4/gVSS initial/d vs 14.3 mL CH_4/gVSS initial/d for the solid samples).

The benefits of two-stage over single stage anaerobic digestion from this study included higher biomethane rates and efficiencies, increased net energy production and overall enhancement of the process. The impact of separating the acidogenic and methanogenic stages of anaerobic digestion was indicated by the extent of acidification after the first stage biohydrogen production process (around 50 % increase on average) which improved the performance of the second-stage BMP process. Also, feedstock COD removal efficiency was enhanced in the second-stage BMP process after acidification by 16 % and 14 % for the liquid and solid streams respectively compared to the single-stage BMP process. Furthermore, the two-stage anaerobic digestion process showed a 33 % and 18 % increase in energy yields in the liquid and solid samples respectively from the single stage anaerobic digestion process.

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CHAPTER 6

6 General conclusions & Recommendations for future work

6.1 General Conclusions

- The feasibility of hydrogen production using extruded poplar wood as substrate, was demonstrated. Poplar wood is an effective carbon source for hydrogen production as well as the sequential hydrogen and methane production by a two-stage anaerobic digestion process.
- The significance of acidification after the first stage biohydrogen production process was indicated by increased anaerobic biodegradability, energy yields and feedstock COD removal efficiencies in the second-stage BMP process compared to the single-stage BMP process.
- Of all ratios tested, an S°/X° of 4 gCOD/gVSS was observed to be optimal for biohydrogen production from synthetic lignocellulosic hydrolysates using mesophilic mixed cultures under the given test conditions
- At low concentrations of ≤ 1 g/L, furfural was degraded to produce hydrogen under mesophilic conditions and favorably changed the biodegradation pathway causing an increase in hydrogen yields
- Furfural-to-sugar and furfural-to-biomass ratios are important parameters that influence fermentative hydrogen production from lignocellulosic biomass
- IC50 for furfural under mesophilic and thermophilic cultures were 1.25 g/L and 0.6 g/L respectively
- Hydrogen producers in the mixed cultures were inhibited in the presence of furfural; furfural had to be degraded to undetectable limits before any hydrogen was observed

- Enhanced microbial kinetics were observed in the absence of furfural in both mesophilic and thermophilic experiments

6.2 Recommendations for future work

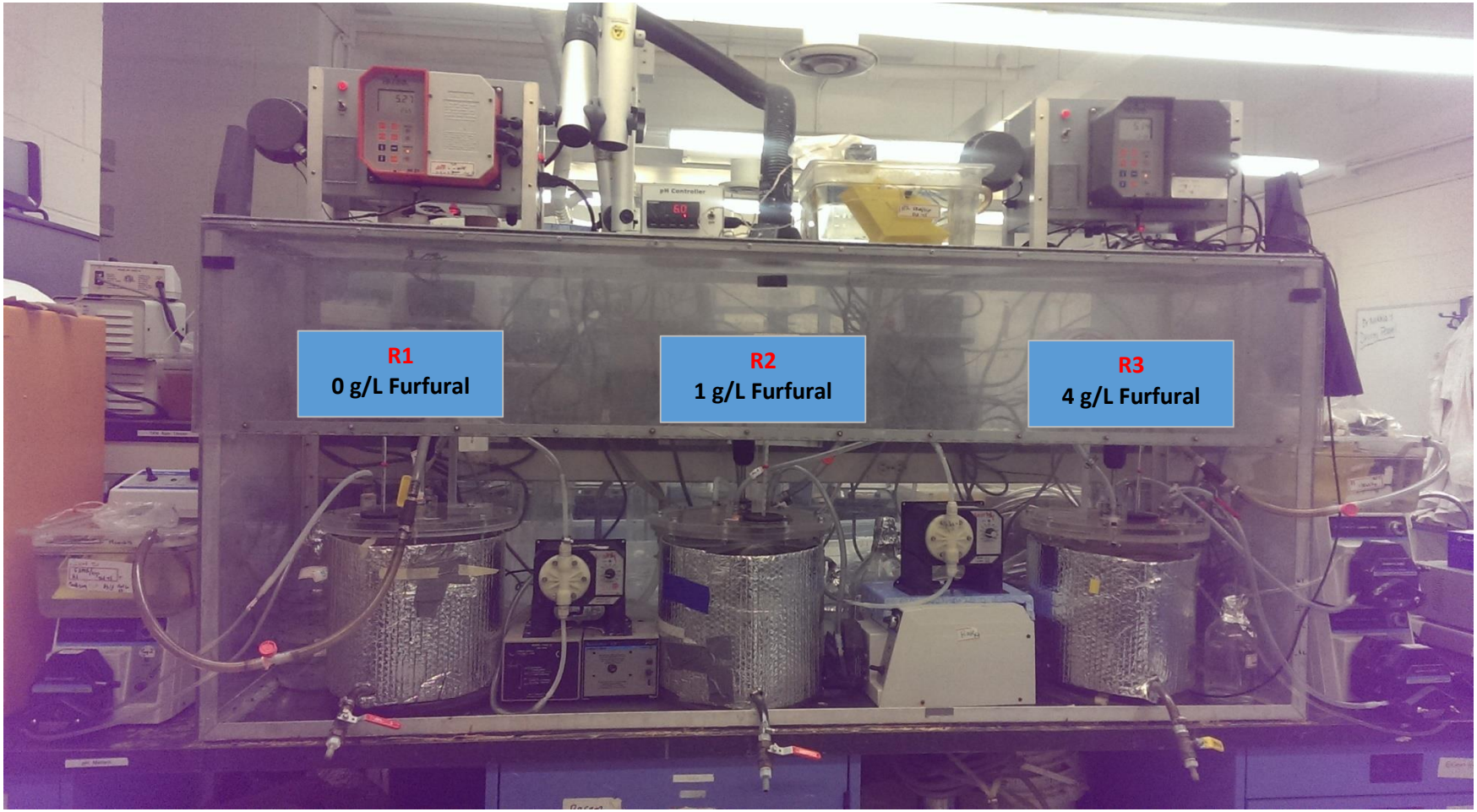
Based on the findings of this work, future research should address the following areas:

- Investigation of biohydrogen and biomethane production at a wider range of S°/X°
- Investigation of other inhibitors (such as HMF, HBA, syringaldehyde, vanillin, and acetic acid) of the fermentative hydrogen production process as well as any synergism between them
- Further research and development aimed at increasing biogas synthesis rates and yields
- Development of a pilot-scale process testing real lignocellulosic hydrolysates in order to validate the kinetic parameters obtained
- Microbial community identification and quantification studies will provide insight into the communities present in the mixed cultures

Appendices

Appendix A

Photo of reactor setup for Chapter 4



Appendix B

Appendix B1: Sugars degradation and metabolite formation data with time at 0 g/L furfural under mesophilic conditions for Chapter 4

Mesophilic experiment at 0 g/L Furfural

	Sugars			VFAs						
Time (h)	**Glucose**	***Xyl, Man & Galac**	**Arabinose**	**Lactate**	**Formate**	**Acetate**	**Propionate**	**Butyrate**	**Valerate**	**Furfural**
0	3.40	27.00	3.00	0	0.61	0.92	0.00	0.00	0.05	0
5	2.02	26.81	1.87	0	0.55	1.10	0.02	0.02	0.01	0
7	1.85	26.50	1.87	0	0.52	1.25	0.03	0.13	0.01	0
8	1.00	24.76	1.79	0	0.33	1.45	0.03	0.49	0.02	0
11	0	22.47	1.53	0	0.28	1.51	0.02	1.15	0.02	0
13	0	21.55	1.30	0	0.06	1.63	0.02	1.57	0.01	0
15	0	18.78	1.13	0	0.03	1.91	0.03	2.22	0.01	0
17	0	12.55	1.00	0	0	2.07	0.04	2.59	0.01	0
19	0	11.11	0.88	0	0	2.32	0.08	3.25	0.01	0
22	0	8.96	0.64	0	0	2.65	0.25	3.94	0.01	0
24	0	6.81	0.27	0	0	2.90	0.50	4.56	0.01	0
27	0	4.29	0	0	0	3.21	1.15	5.00	0.03	0
31	0	1.92	0	0	0	3.44	1.86	5.45	0.16	0
37	0	0.62	0	0	0	3.46	2.36	5.61	0.81	0
41	0	0.01	0	0	0	3.23	2.37	5.59	0.82	0
46	0	0	0	0	0	3.30	2.37	5.58	0.90	0
55	0	0	0	0	0	3.35	2.38	5.60	1.04	0
60	0	0	0	0	0	3.44	2.40	5.61	1.13	0
79	0	0	0	0	0	3.46	2.41	5.62	1.29	0

*Xylose, Mannose & Galactose

Appendix B2: Sugars degradation and metabolite formation data with time at 1 g/L furfural under mesophilic conditions for Chapter 4

Mesophilic experiment at 1 g/L Furfural

Time (h)	Sugars (g/L)			VFAs (g/L)						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
0	3.05	25.05	3.13	0	0.63	0.89	0	0	0	0.90
6	3.04	24.91	3.13	0	0.62	1.03	0.02	0.04	0.02	0.85
12	2.85	24.82	3.12	0	0.41	1.44	0.02	0.13	0.28	0.53
15	0	23.44	2.94	0	0	1.60	0.01	0.66	0.35	0.01
18	0	22.48	2.94	0	0	1.75	0.01	1.25	0.39	0
23	0	19.55	1.76	0	0	2.07	0.01	2.15	0.44	0
28	0	16.65	1.52	0	0	2.30	0.02	2.78	0.42	0
32	0	13.49	1.14	0	0	2.80	0.01	3.74	0.45	0
35	0	12.34	0.94	0	0	2.94	0.01	4.10	0.48	0
39	0	10.61	0.55	0	0	3.10	0.03	4.73	0.49	0
42	0	9.87	0.32	0	0	3.30	0.03	4.92	0.50	0
51	0	9.68	0.01	0	0	3.80	0.03	5.10	0.52	0
57	0	9.63	0	0.37	0	4.30	0.03	5.37	0.54	0
61	0	8.11	0	1.42	0	4.40	0.04	5.40	0.55	0
67	0	5.48	0	3.02	0	4.72	0.07	5.68	0.55	0
77	0	0.20	0	5.01	0	5.16	0.09	5.86	0.53	0
85	0	0	0	5.08	0	5.38	0.07	5.92	0.52	0
89	0	0	0	4.88	0	5.40	0.08	6.00	0.51	0
98	0	0	0	4.71	0	5.41	0.11	6.29	0.50	0
104	0	0	0	4.45	0	5.42	0.13	6.28	0.50	0
113	0	0	0	0	0	5.40	0.13	6.28	0.52	0
118	0	0	0	0	0	5.28	0.13	6.28	0.51	0
124	0	0	0	0	0	5.30	0.12	6.29	0.51	0
146	0	0	0	0	0	5.32	0.12	6.29	0.51	0

Appendix B3: Sugars degradation and metabolite formation data with time at 4 g/L furfural under mesophilic conditions for Chapter 4

Mesophilic experiment at 4 g/L furfural

Time (h)	Sugars (g/L)			VFAs (g/L)						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
0	3.2	26.48	2.85	0	0.63	0.75	0	0	0	3.94
6	2.3	25.40	2.83	0	0.62	0.88	0	0	0	3.83
12	1.8	23.87	2.68	0	0.41	0.89	0	0	0	3.40
15	2.1	23.83	2.67	0	0	0.93	0	0	0	3.32
18	1.9	23.78	2.65	0	0	0.93	0	0	0	3.25
28	0.8	23.78	2.53	0	0	0.96	0	0	0	3.32
39	0	23.77	2.32	0	0	1.01	0	0	0	3.32
57	0	23.77	2.31	0	0	1.02	0	0	0	3.26
61	0	23.77	2.15	0	0	1.04	0.01	0	0	3.06
77	0	23.76	1.96	0	0	1.10	0.01	0	0	2.78
98	0	23.76	0.84	0	0	1.13	0.01	0	0	2.39
104	0	23.75	0.49	0	0	1.14	0.03	0	0	2.19
130	0	23.32	0.46	0	0	1.15	0.03	0	0	1.42
137	0	22.79	0.33	0	0	1.16	0.01	0	0	1.40
188	0	21.96	0.20	0	0	1.37	0.01	0	0	1.40
228	0	19.37	0	0	0	1.63	0.03	0	0	1.19
243	0	18.18	0	0	0	1.80	0.01	0	0	1.00
286	0	15.69	0	0	0	2.14	0.04	0.01	0	0.67
297	0	11.21	0	0	0	2.23	0.04	0.02	0.65	0.26
318	0	6.72	0	0	0	2.42	0.15	0.02	2.80	0.02
320	0	4.93	0	0	0	2.69	0.42	0.03	2.92	0
322	0	4.20	0	0	0	2.84	0.94	0.04	2.92	0
324	0	3.51	0	0	0	3.02	1.67	0.06	3	0
326	0	2.86	0	0	0	3.62	2.70	0.11	3	0

Mesophilic experiment at 4 g/L furfural cont'd

Time (h)	Sugars (g/L)			VFAs (g/L)						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
329	0	2.26	0	0	0	3.64	3.66	0.44	3.05	0
331	0	0.58	0	0	0	3.65	3.97	1.49	3.10	0
334	0	0.13	0	0	0	3.78	4.27	1.83	3.15	0
339	0	0.11	0	0	0	3.81	4.28	2.11	3.17	0
344	0	0.11	0	0	0	3.92	4.30	2.28	3.45	0
348	0	0.06	0	0	0	4.03	4.59	2.30	3.65	0
354	0	0	0	0	0	3.91	4.25	2.10	3.25	0
362	0	0	0	0	0	4.01	4.44	2.18	3.54	0
367	0	0	0	0	0	3.88	4.22	2.01	3.16	0
387	0	0	0	0	0	4.02	4.50	2.26	3.66	0
392	0	0	0	0	0	4.22	4.73	2.46	3.92	0
411	0	0	0	0	0	4.23	4.91	2.47	3.93	0

Appendix B4: Sugars degradation and metabolite formation data with time at 0 g/L furfural under thermophilic conditions for Chapter 4

Thermophilic experiment at 0 g/L furfural

	Sugars			VFAs						
Time (h)	**Glucose**	**Xyl, Man & Galac**	**Arabinose**	**Lactate**	**Formate**	**Acetate**	**Propionate**	**Butyrate**	**Valerate**	**Furfural**
0	3.11	23.31	2.51	0	0.61	0.91	0.04	0.01	0.01	0
24	2.05	21.40	2.49	0.8	0.0	1.00	0.04	0.10	0.01	0
36	0.99	19.46	2.48	1.4	0.0	1.26	0.03	0.27	0.01	0
38	0.23	19.15	2.26	1.6	0.0	1.37	0.03	0.46	0.03	0
40	0.12	18.87	1.94	1.7	0.0	1.53	0.04	0.71	0.02	0
43	0.14	16.86	1.64	1.8	0.0	1.72	0.04	1.10	0.02	0
46	0.10	14.30	1.28	1.9	0.0	2.24	0.08	1.87	0.04	0
50	0.21	10.69	0.85	1.9	0.0	2.61	0.07	2.70	0.04	0
54	0.06	6.20	0.62	1.9	0.0	2.83	0.10	3.25	0.05	0
59	0.07	4.02	0.38	2.0	0.0	3.30	0.10	3.93	0.06	0
61	0.04	3.32	0.38	2.0	0.0	3.51	0.11	4.14	0.04	0
64	0.05	2.72	0.30	2.0	0.0	3.64	0.11	4.29	0.05	0
68	0.11	2.18	0.34	2.0	0.0	3.58	0.11	4.49	0.05	0
72	0.17	1.75	0.33	2.0	0.0	3.88	0.14	4.41	0.05	0
77	0.19	1.55	0.38	2.0	0.0	4.10	0.13	4.72	0.04	0
84	0.19	0.86	0.30	2.0	0.0	4.44	0.05	4.78	0.04	0
91	0.09	0.85	0.32	2.0	0.0	4.60	0.15	5.00	0.00	0
97	0.02	0.64	0.33	2.1	0.0	4.61	0.19	5.10	0.00	0
108	0.07	0.50	0.30	2.1	0.0	4.62	0.17	5.14	0.03	0
114	0	0.31	0.30	2.1	0.0	4.62	0.14	5.20	0.03	0
135	0	0.01	0.25	2.2	0.0	4.63	0.17	5.40	0.04	0
157	0	0.0	0.35	2.2	0.0	4.63	0.12	5.73	0.09	0

Appendix B5: Sugars degradation and metabolite formation data with time at 1 g/L furfural under thermophilic conditions for Chapter 4

Thermophilic experiment at 1 g/L furfural

Time (h)	Sugars			VFAs						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
0	3.08	24.20	2.66	0	0	0.88	0.03	0.01	0.01	0.93
36	2.48	24.16	2.65	0	0	0.88	0.02	0.01	0.11	0.86
61	2.33	24.02	2.41	0	0	0.89	0.02	0.02	0.10	0.65
84	1.72	23.36	2.16	0.33	0	0.90	0.02	0.02	0.18	0.57
108	0.47	22.59	1.61	1.71	0	0.94	0.02	0.02	0.38	0.56
134	0.44	21.69	1.36	2.35	0	0.96	0.03	0.03	0.40	0.45
157	0.43	20.31	1.23	2.77	0	0.99	0.02	0.03	0.44	0.39
179	0.40	17.97	1.06	3.18	0	1.10	0.01	0.03	0.51	0.21
202	0.38	13.95	0.66	3.70	0	1.04	0.01	0.03	0.61	0.18
226	0.36	12.54	0.18	3.89	0	1.10	0.03	0.04	0.83	0.03
250	0.34	10.73	0.09	4.28	0	1.11	0.03	0.04	1.24	0.02
267	0.32	10.26	0	4.40	0	1.14	0.03	0.04	1.29	0.01
273	0.30	9.92	0	4.61	0	1.16	0.02	0.05	1.32	0
279	0.29	9.27	0	4.63	0	1.21	0.02	0.10	1.32	0
284	0.29	8.79	0	4.73	0	1.22	0.02	0.17	1.34	0
289	0.25	8.61	0	4.62	0	1.22	0.02	0.19	1.36	0
295	0.24	8.38	0	4.74	0	1.45	0.02	0.56	1.39	0
301	0.24	7.50	0	4.64	0	1.58	0.03	0.86	1.39	0
306	0.23	7.24	0	4.63	0	2.00	0.06	1.18	1.39	0
310	0.21	6.55	0	4.65	0	2.04	0.06	1.21	1.40	0
320	0.21	5.23	0	4.66	0	2.51	0.06	1.26	1.40	0
324	0.20	4.79	0	4.68	0	2.61	0.06	1.70	1.42	0

Thermophilic experiment at 1 g/L furfural cont'd

Time (h)	Sugars			VFAs						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
332	0.17	4.32	0	4.70	0	2.67	0.03	1.79	1.44	0
342	0.15	3.53	0	4.74	0	2.79	0.04	1.83	1.45	0
350	0.10	2.75	0	4.78	0	2.87	0.02	1.92	1.45	0
359	0.11	2.31	0	4.84	0	3.02	0.03	1.93	2.04	0
370	0.10	1.83	0	4.85	0	3.20	0.04	2.01	2.28	0
379	0.10	1.57	0	4.94	0	3.28	0.04	2.02	2.32	0
394	0.10	1.30	0	4.77	0	3.30	0.04	2.12	2.31	0
403	0.09	1.21	0	4.72	0	3.35	0.03	2.13	2.26	0
451	0.09	1.07	0	4.77	0	3.48	0.03	2.14	2.35	0
475	0.09	0.74	0	4.67	0	3.52	0.04	2.32	2.35	0
499	0.09	0.73	0	4.68	0	3.42	0.02	2.33	2.39	0
514	0.08	0.68	0	4.64	0	3.32	0.03	2.71	2.39	0
539	0.08	0.40	0	4.59	0	3.29	0.05	2.75	2.27	0
562	0.08	0.17	0	4.39	0	3.25	0.06	2.88	2.20	0
589	0.07	0.01	0	4.39	0	3.39	0.07	3.38	2.12	0
615	0.07	0	0	4.37	0	3.49	0.06	3.39	2.14	0
625	0.06	0	0	4.39	0	3.43	0.05	3.39	2.15	0
642	0.05	0	0	4.24	0	3.24	0.07	3.39	2.16	0
671	0.04	0	0	4.30	0	3.05	0.06	3.38	2.19	0
690	0.03	0	0	4.13	0	2.97	0.06	3.38	2.23	0
713	0.03	0	0	4.13	0	2.90	0.08	3.38	2.19	0
765	0.02	0	0	4.14	0	2.87	0.05	3.38	2.08	0
810	0.01	0	0	4.15	0	2.82	0.06	3.37	2.06	0
872	0	0	0	4.15	0	2.85	0.05	3.37	2.05	0

Appendix B6: Sugars degradation and metabolite formation data with time at 4 g/L furfural under thermophilic conditions for Chapter 4

Thermophilic experiment at 4 g/L furfural

Time (h)	Sugars			VFAs						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
0	3.10	24.00	2.70	0	0	0.82	0.01	0.01	0.03	3.98
36	3.01	23.05	2.58	0	0	0.86	0.01	0.01	0.06	3.91
61	2.98	22.94	2.52	0	0	0.90	0.02	0.01	0.01	3.65
84	2.95	22.83	2.50	0	0	1.05	0.01	0.02	0.02	3.59
108	2.89	22.72	2.48	0	0	1.04	0.01	0.02	0.03	3.45
135	2.77	22.48	2.47	0	0	0.98	0.01	0.02	0.02	3.43
157	2.75	22.33	2.45	0	0	1.02	0.01	0.01	0.02	3.42
179	2.68	22.30	2.19	0	0	1.02	0.02	0.00	0.01	3.38
202	2.68	21.37	2.12	0	0	1.02	0.02	0.00	0.02	3.10
226	2.09	19.42	2.09	0	0	1.01	0.02	0.01	0.02	2.92
250	2.06	18.39	1.92	0	0	1.05	0.01	0.02	0.01	2.62
273	2.05	18.36	1.87	0	0	1.11	0.01	0.01	0.03	2.58
295	2.05	18.36	1.74	0	0	1.14	0.02	0.01	0.01	2.57
301	1.76	18.36	1.63	0	0	1.17	0.01	0.01	0.01	2.55
320	1.74	18.23	1.55	0	0	1.09	0.01	0.01	0.01	2.55
350	1.72	18.11	1.52	0	0	1.05	0.01	0.02	0.01	2.53
370	1.72	18.04	1.52	0	0	1.04	0.01	0.00	0.01	2.52
394	1.70	18.00	1.46	0	0	1.12	0.01	0.00	0.02	2.49
403	1.72	17.98	1.40	0	0	1.14	0.02	0.01	0.01	2.46

Thermophilic experiment at 4 g/L furfural cont'd

Time (h)	Sugars			VFAs						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
475	1.66	17.76	1.37	0	0	1.19	0.01	0	0.02	2.42
499	1.53	17.75	1.37	0	0	1.01	0.02	0	0.02	2.40
514	1.51	17.74	1.36	0	0	1.23	0.02	0	0.02	2.38
539	1.50	17.72	1.31	0	0	1.16	0.02	0	0.02	2.29
562	1.49	17.68	1.30	0	0	1.15	0.02	0	0.01	2.27
589	1.46	17.61	1.30	0	0	1.12	0.02	0	0.02	2.16
615	1.45	17.57	1.24	0	0	1.14	0.02	0	0.02	2.08
625	1.43	17.57	1.24	0	0	1.18	0.02	0	0.02	2.06
642	1.36	17.56	1.20	0	0	1.06	0.02	0	0.02	2.03
671	0.79	17.53	1.16	0.5	0	1.13	0.02	0	0.05	2.00
690	0.35	17.18	1.04	1.5	0	1.11	0.00	0	0.01	1.98
713	0	17.00	1.03	2.0	0	1.10	0.02	0	0.06	1.97
765	0	16.36	0.83	3.6	0	0.93	0.01	0	0.03	1.88
810	0	16.04	0.76	3.8	0	0.97	0.03	0	0.04	1.86
872	0	15.47	0.76	4.3	0	1.01	0.02	0	0.09	1.84
1011	0	14.01	0.64	5.1	0	0.90	0.14	0	0.08	1.80
1139	0	12.62	0.63	5.3	0	0.86	0.14	0	0.02	1.79
1253	0	11.78	0.62	5.4	0	0.86	0.14	0	0.06	1.45
1354	0	11.40	0.47	5.9	0	0.86	0.06	0	0.06	1.19
1515	0	11.15	0.40	6.9	0	0.86	0.12	0	0.06	1.00
1588	0	11.04	0	7.8	0	0.85	0.06	0	0.09	0.98
1667	0	10.94	0	7.8	0	1.17	0.01	0	0.13	0.93

Thermophilic experiment at 4 g/L furfural cont'd

Time (h)	Sugars			VFAs						
	Glucose	Xyl, Man & Galac	Arabinose	Lactate	Formate	Acetate	Propionate	Butyrate	Valerate	Furfural
1757	0	10.50	0	8.2	0	1.19	0.17	0	0.10	0.8
1875	0	9.8	0	10.6	0	0.84	0.03	0	0.08	0.71
1948	0	9.4	0	11.1	0	0.68	0.02	0	0.06	0.66
2068	0	5.5	0	13.2	0	0.46	0.02	0	0	0.48
2193	0	3.6	0	16.6	0	0.91	0.09	0	0.15	0.48
2428	0	2.4	0	18.8	0	0.53	0.02	0	0.04	0.32
2596	0	2.2	0	19.1	0	0.86	0.03	0	0.11	0.15
2700	0	1.9	0	20.0	0	0.84	0	0	0.1	0
2900	0	1.8	0	20.6	0	0.82	0	0	0.11	0

Appendix C

Determination of structural carbohydrates (cellulose and hemicellulose), lignin, and ash

C1. Determination of acid-insoluble lignin

1.1 Ignite glass filters at 575 ± 25ºC to achieve a constant weight and then store the ignited filters in a desiccator until needed.

1.2 Weigh 0.3 ± 0.01 g prepared sample to the nearest 0.1 mg and place in a test tube. Record as W_1, the initial sample weight.

1.3 Add 3.00 ± 0.01 mL of 72% (w/w) H_2SO_4 and use a glass stirring rod to mix until the sample is thoroughly wetted.

1.4 Place the test tube in the water bath controlled to 30 ºC and hydrolyze for 2 hours

1.5 Stir the sample every 15 minutes to assure complete mixing and wetting.

1.6 Transfer the hydrolysate to a glass bottle and dilute to a 4% (w/w) acid concentration by adding 84.00 ± 0.04 mL of water. Stopper each of the bottles and crimp aluminum seals into place.

1.7 Autoclave the samples in their sealed bottles for 1 hour at 121ºC. After completion of the autoclave cycle, allow the samples to cool for about 20 minutes at room temperature.

1.8 Vacuum filter the hydrolysis solution through one of the previously ignited filters.

1.9 If a structural carbohydrate analysis or an acid-soluble lignin analysis is desired, decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis,

store in refrigerator at 4ºC. Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

1.10 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible.

1.11 Dry the filter and contents at 105ºC for 2 hours or until constant weight is achieved.

1.12 Cool in desiccator and record the weight, W_2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash to the nearest 0.1 mg.

1.13 Place the filter and contents in the muffle furnace and ignite at 575 ± 25ºC for a minimum of 3 hours, or until all the carbon is eliminated.

1.14 Cool in desiccator and record the weight, W_3, the weight of the filter and acid- insoluble ash, to the nearest 0.1 mg.

1.15 Calculation

$$\%\ (\text{wt})\ \text{acid} - \text{insoluble lignin} = \frac{W_2 - W_3}{W_1} \times 100$$

W_1 = initial sample weight.

W_2 = weight of crucible, acid-insoluble lignin, and acid-insoluble ash.

W_3 = weight of crucible and acid-insoluble ash.

C2. Determination of acid-soluble lignin

2.1 Set up and calibrate the spectrophotometer following the protocols recommended in the instrument manual.

2.2 Measure the absorbance of the hydrolysate at 205 nm, using the 1-cm light path cuvette. A 4% solution of H_2SO_4 should be used as a reference blank.

2.3 If the absorbance reading exceeds 0.7, the sample must be diluted. Dilute the sample so the resulting absorbance reading falls between 0.2 and 0.7. The 4% H_2SO_4 must be diluted in the same ratio as the sample and used as the reference blank for this repeat analysis.

2.4 Calculation

$$\%\ \text{acid} - \text{soluble lignin} = \frac{\frac{\mathrm{A}}{\mathrm{b} \times \mathrm{a}} \times d_f \times \mathrm{V} \times \frac{\mathrm{L}}{1000\ \mathrm{mL}}}{\mathrm{W}_1} \times 100$$

A = absorbance (λ=320 nm for corn stover; λ= 240 nm for poplar)

d_f = dilution factor.

b = cell path length, 1 cm.

a = absorptivity (30 L/g-cm for corn stover; 25 L/g-cm for poplar wood)

V = filtrate volume, this volume will be 87 mL.

W_1 = initial biomass sample weight in grams.

C3. Total lignin determination

Total lignin (% wt) = acid-insoluble lignin (% wt) + acid-soluble lignin (% wt)

C4. Determination of structural carbohydrates in biomass

4.1 Using the hydrolysis liquor obtained in the determination of lignin in biomass, transfer an approximately 20 mL aliquot of each liquor to a 50 mL Erlenmeyer flask.

4.2 Use calcium carbonate to neutralize each sample to pH 5 – 6. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5 – 6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant. The pH of the liquid after settling will be approximately 7. (Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.)

4.3 Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 μm filter into a vial. Seal and label the vial. If necessary, neutralized samples may be stored in the refrigerator for three or four days.

4.4 Analyze the calibration standards and samples by HPLC-RID using a Biorad Aminex HPX-87P column equipped with the appropriate guard column.

Note: Standard curves for cellobiose, glucose, xylose, arabinose, galactose, and mannose (0.1 – 4 g/L for each)

4.5 Calculate the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or162/180) for C-6 sugars (glucose, galactose, and mannose)

$C_{anhydro} = C_{sugar} \times$ Anhydro correction

4.6 Calculate the percentage of each sugar

$$\% \text{ Sugar} = \frac{C_{anhyhdro} \times V_f \times \frac{1\text{ g}}{1000\text{ mg}}}{W_1} \times 100$$

where;

V_f = volume of filtrate, 87 mL

W_1 = initial weight of sample

Note: Cellulose = Glucan, Hemicellulose = Xylan + Arabian + Galactan + Mannose

C5. Determination of ash in biomass

5.1 Mark a pan or crucible with a unique identification using a porcelain marker, place it in the muffle furnace, and bring to constant weight by igniting at 575 ± 25 °C. Remove the pan or crucible from the furnace, cool to room temperature in a desiccator, and weigh to the nearest 0.1 mg. Record this weight as the tare weight. Keep the pan or crucible in a desiccator until used.

5.2 Weigh approximately 0.5 to 1.0 g, to the nearest 0.1 mg, of a test specimen into the tared pan or crucible. If the sample being analyzed is a 105 °C dried test specimen, the sample should be stored in a desiccator until use. Record the weight (container plus sample minus tare weight of container) as the initial weight of the test specimen, W_2.

5.3 Place the container and contents in the muffle furnace and ignite at 575 ± 25oC for a minimum of four hours, or until all the carbon is eliminated. Heat slowly at the start to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of test specimen.

5.4 Remove the pan or crucible with its contents to a desiccator, cool to room temperature, weigh to the nearest 0.1 mg, and record this weight. Repeat the heating for one hour periods until the

weight after cooling is constant to within0.3 mg. Record the final weight of the ash, W_1, as the container plus ash weight minus container tare weight.

5.5 Calculation

Ash, % = (W_1/W_2) x 100

where:

W_1 = weight of ash

W_2 = initial weight of dried sample.

Appendix D1: Batch design for BHP experiment for Chapter 5

Samples		S°/X° (gCOD/gVSS)	Volume of substrate (mL)	Mass of substrate (g)	Volume of seed (mL)
S1	Solid	0.5	-	3.4	220
S2			-	3.9	220
S3			-	3.2	220
S4			-	5.2	220
S5			-	4.2	220
L1	Liquid		25	-	195
L2			33	-	187
L3			8	-	212
S1	Solid	1	-	6.8	220
S2			-	7.7	220
S3			-	6.5	220
S4			-	10.4	220
S5			-	8.5	220
L1	Liquid		46	-	174
L2			57	-	163
L3			16	-	204
Blank			-	-	220

Appendix D2: Batch design for single-stage BMP experiment for Chapter 5

Samples		S°/X° (gCOD/gVSS)	Volume of substrate (mL)	Mass of substrate (g)	Volume of seed (mL)
S1	Solid	0.5	-	3.0	220
S2			-	3.4	220
S3			-	2.9	220
S4			-	4.6	220
S5			-	3.8	220
L1	Liquid		23	-	197
L2			30	-	190
L3			8	-	212
S1	Solid	1	-	6.0	220
S2			-	6.9	220
S3			-	5.7	220
S4			-	9.3	220
S5			-	7.6	220
L1	Liquid		41	-	179
L2			52	-	168
L3			15	-	205
Blank			-	-	220

Appendix D3: Batch design for second-stage BMP experiment for Chapter 5

Samples		S°/X° (gCOD/gVSS)	Volume of substrate (mL)	Volume of seed (mL)
S1	Solid	0.5	112	108
S2			119	101
S3			117	103
S4			113	107
S5			113	107
L1	Liquid		90	130
L2			91	129
L3			87	133
S1	Solid	1	128	92
S2			136	84
S3			136	84
S4			139	81
S5			132	88
L1	Liquid		116	104
L2			120	100
L3			105	115
Blank			-	220

Curriculum Vitae

Name: Chinaza Okeoghene Akobi

Post-secondary Education and Degrees:

MESc Chemical and Biochemical and Engineering
The University of Western Ontario, London, Canada
2014 - 2016

MSc (Eng) Environmental and Energy Engineering
The University of Sheffield, Sheffield, UK
2009 - 2010

BSc Biochemistry
The University of Benin, Benin City, Nigeria
2003 - 2007

Honours and Awards:

Western Graduate Research Scholarship (WGRS)
Chemical and Biochemical Engineering
The University of Western Ontario
London, Canada
2014 – 2016

1st place prize in the Bioenergy series
51st Central Canadian Symposium on Water Quality Research
Canadian Association on Water Quality Conference
Ryerson University, Toronto, Canada
2016

Mitacs grant for Industrial Research
2015

2nd place prize for poster presentation
Energy Institute, Yorkshire, UK
2010

Related Work Experience:

Lead Research Intern
GreenField Specialty Alcohols Inc.
Chatham, Canada
2015

Research Assistant
The University of Western Ontario
London, Canada
2014 – 2016

Publications, Conferences and Workshops:

Akobi, C., Hafez, H., Nakhla, G. (2016). Biological hydrogen production from synthetic lignocellulosic hydrolysate using mesophilic anaerobic digester sludge: Impact of furfural. Submitted to the *Bioresource Technology* journal. Submission number: BITE-D-16-04188

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