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Investigation of Bio-hydrogen Production Optimization from Synthetic and Real Wastes using Pure and Mixed Cultures

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Abstract

Dark fermentative H_2 production is an important route to renewable energy as it is based on a known technology and can utilize a wide range of available waste streams as substrate. However, more research is required to overcome the technical barriers to practical application. The aim of this study is to investigate different scenarios towards the optimization of fermentative H_2 production from synthetic and real wastes using pure and mixed cultures. Lignocellulosic biomass, i.e. pretreated corn cobs and poplar wood hydrolysate were evaluated for H_2 production using mixed anaerobic cultures and yields of 141 and 169 mL $H_2/gCOD_{added}$ were determined, respectively. Also, substrate utilization kinetic parameters for selected mesophilic and thermophilic H_2-producing pure cultures utilizing hexose and pentose sugars were determined. Furthermore, the effect of co-fermentation and co-cultures on H_2 production was studied. This work proved that headspace CO_2 sequestration in a continuous-flow system producing H_2 from glucose increased H_2 yield from 2.4 to 3.0 mol/mol glucose, i.e. approximately 90% of the theoretical yield. An extensive comparative study of mesophilic and thermophilic anaerobic digester sludges confirmed the superiority of thermophilic cultures which produced 23.8 L H_2/L poplar wood hydrolysate. The Monod kinetic parameters of mono- and co-culture of *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum* on glucose, starch, and cellulose were derived.

Keywords

Biohydrogen, Lignocellulosic waste, CO_2 sequestration, Microbial community analysis, *Clostridium*, co-culture, co-substrate.

Co-Authorship Statement

Chapter 3: Biohydrogen Production from Pretreated Corn Cobs

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“O Lord, to You is Praise as befits the Glory of Your Face

and the Greatness of Your Might”

To my Dear Father, Mother and Family for their Love & Prayers

To my Beloved Husband Ahmed for his Love, Care & Great Support

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Chapter 1

Introduction

1.1 Background

Dark fermentative hydrogen production is now being widely investigated for its promising advantages for the future of H_2 energy. It is a light-independent anaerobic process that utilizes a wide variety of feedstocks, and that can produce valuable metabolites such as acetic and butyric acids as by products [Azbar and Levin, 2012]. One of the main factors affecting H_2 production pathways, end products and yields is the inoculum type. The most widely used inoculum for bio-H_2 production is either mixed cultures as anaerobic digester sludge (ADS) or pure cultures of a known H_2-producing species. The use of mixed cultures in fermentative H_2 production has many advantages in terms of practicality, where it is easier to control, does not require sterile environment, and can utilize a wide range of substrate from simple pure sugars to complex real wastes [Li and Fang, 2007; Ntaikou et al., 2010]. However, H_2 produced by H_2-producing bacteria may be consumed by H_2 consuming bacteria and the end products will depend on the type of species in the culture.

Another factor that plays an important role in H_2 production is the substrate (i.e. carbon source). Pure substrates as monosaccharides (e.g. glucose, xylose, arabinose), disaccharides (e.g. sucrose, cellobiose, maltose), and polysaccharides (e.g. starch, cellulose) have been used in many studies for better understanding of cultures kinetics and optimal operational conditions [Wang and Wan, 2008; Fernendez et al., 2011; Mullai et al., 2013; Holwerda and Lynd, 2013]. Biohydrogen production from real waste streams depends on the substrate composition and its biodegradability, for example, lignocellulosic feedstocks may need a pre-hydrolysis step in order to break down its complex structure and facilitate the fermentation process [Monlau et al., 2013]. Many pretreatment methods have been investigated for lignocellulosic feedstocks hydrolysis

such as grinding, milling, pyrolysis, steam explosion, acid, alkaline, and enzymatic hydrolysis [Galbe and Zacchi, 2002; Sun and Cheng, 2002].

Temperature has been considered as one of the main physiological parameters that affect biohydrogen production, where fermentation process can be operated at mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C), or hyperthermophilic (>80°C) temperatures [Azbar and Levin, 2012]. Many studies have investigated the effect of temperature on biohydrogen production with mixed cultures utilizing pure sugars [Karadag and Puhakka, 2010; Gadow et al., 2012], or real waste [Zhang et al., 2015], and even pure strains [Munro et al., 2009]. Generally, the specific rate of H_2 production increases with temperature increase which was due to lower biomass production at elevated temperatures in many studies [Azbar and Levin, 2012].

1.2 Problem Statement

Many studies have investigated co-fermentation for anaerobic digestion, however, more research should be directed towards co-fermenting different lignocellulosic waste streams for H_2 production. In addition, while studies compare the performance of either mesophilic mixed cultures under mesophilic versus thermophilic temperatures, or mesophilic versus thermophilic mixed cultures, it is important to assess the performance of the three conditions (i.e. mesophilic culture at mesophilic temperature, mesophilic culture at thermophilic temperature, and thermophilic culture at thermophilic temperature) to have more consistent data.

The production of H_2 in dark fermentation results in a mixture of H_2 and CO_2 gases, which creates challenges for the useful application of H_2 as a fuel [Azbar and Levin, 2012]. With the emerging technology of microbial fuel cells, high purity H_2 is required while CO_2 is considered the main contaminant in this technology [Larminie and Dicks, 2003]. Although CO_2 sequestration from biohydrogen reactors headspace is a promising method for enhancing H_2 production, however, previous studies neither investigated its impact in continuous-flow systems nor on the metabolic pathways and microbial community structure.

Pure H_2-producing cultures have been investigated a lot by many researchers, utilizing different substrates and operating at different optimal operational conditions [Elsharnouby et al., 2013]. However, H_2 production experimental results have been contradictory even when utilizing the same substrate. For example, the ability of *Clostridium beijerinckii* to utilize starch has been confirmed by Taguchi et al. [1992] while George et al. [1983] reported the opposite. Also, more research should be directed towards *Clostridium saccharoperbutylacetonicum*, a well-known alcohol-producing bacteria that has been recently used for H_2 production, where its potential for utilizing different substrates should be investigated. In addition, *Clostridium thermocellum* experiments have been focusing on cellulosic substrates neglecting other important ones such as glucose.

Finally, In addition, H_2 production kinetics are important for system design, analysis, and process control [Azbar and Levin, 2012; Huang and Wang, 2010]. The modified Gompertz and the Monod-based kinetic models are widely used for modeling H_2 production and substrate utilization [Wang and Wan, 2009; Gnanapragasam et al., 2011]. However, studies reporting H_2 production parameters as yields and rates usually use Gompertz model which ignores the substrate utilization kinetics [Pan et al., 2008] and hence is of limited utility in bioreactor design. On the other hand, studies reporting the metabolic and growth kinetics ignore the H_2 production parameters [Hernandez, 1982; Ng and Zeikus, 1982].

1.3 Research Objectives

In the present research, four main approaches have been investigated to optimize fermentative H_2 production: pretreatment of biomass (i.e. corn cobs and poplar wood hydrolysates), system operational parameters (i.e. headspace CO_2 sequestration), physiological parameters (i.e. mesophilic Vs. thermophilic), and designal pure cultures (i.e. co-substrate, co-culture, kinetics data). The specific objectives of this study are:

1. Assessment of impact of furfural and HMF on H_2 production from co-fermentation of four different pretreated corn cobs streams

2. Comparative evaluation of mesophilic anaerobic digester sludge at mesophilic (MADS) and thermophilic (TADS) temperatures, and thermophilic anaerobic digester sludge (TADS) for H_2 production using hydrolyzed poplar wood
3. Evaluating the impact of headspace CO_2 removal on H_2 production, metabolic pathways, and microbial community structure in a continuous-flow system
4. Providing Monod kinetic parameters for *Clostridium thermocellum* on glucose and cellobiose
5. Providing Monod kinetic parameters for *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii* on glucose
6. Assessing the effect of co-substrate and co-culture on H_2 production and substrate utilization kinetics using *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii*

1.4 Research Contributions

The effect of important operational and physiological parameters on H_2 production has been investigated in this study, which lead to these main contributions:

1. Determining the inhibition threshold for furfural and HMF on H_2 production from corn cobs hydrolysate in a co-fermentation batch study
2. Determining the correlation between monomeric-to-polymeric sugars composition and H_2 production yields and rates
3. Providing Monod kinetic parameters for MADS, TMADS, and TADS on poplar wood hydrolysate for H_2 production
4. Evaluating for the first time, the impact of headspace CO_2 sequestration on H_2 production and microbial community structure in a continuous-flow system
5. Providing Monod kinetic parameters for *Clostridium thermocellum* on glucose and cellobiose
6. Providing Monod kinetic parameters for *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii* as mono-and co-cultures on glucose, starch, and cellulose as mono- and co-substrate
7. Confirming the inability of *Clostridium beijerinckii* for hydrolyzing insoluble starch

8. Assessing H_2 production for the first time using a co-culture of *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii* from mono- and co-substrate of glucose, starch, and cellulose

1.5 Thesis Organization

This thesis includes nine 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 Western University.

Chapter 1 presents a general introduction on fermentative H_2 production including research objectives and contributions. A literature review including background on dark fermentative H_2 production and different approaches for enhancing H_2 production yields and rates is presented in Chapter 2.

Chapter 3 presents a batch co-fermentation H_2 production experiment from different streams of corn cobs hydrolysate. Chapter 4 presents a comparative assessment of using MADS, TMADS, and TADS for H_2 production from poplar wood hydrolysate with a kinetic study. Chapter 5 assesses the impact of headspace CO_2 sequestration on H_2 production and microbial community structure in a continuous-flow system. Chapter 6 presents a comparative assessment of glucose utilization kinetics using *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii*. Chapter 7 introduces mono- and co-substrate utilization kinetics of glucose, starch, and cellulose using mono- and co-culture of *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii*.

Finally, Chapter 8 summarizes the major contributions and conclusions of this research and provides future work recommendations based on the findings of this study.

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Chapter 2

Literature Review

2.1 Introduction

High production costs, technical storage requirements, and distribution system are problems that limit the use of hydrogen gas as an energy source [Dunn, 2002]. However, it is widely used as a chemical reactant in fertilizers production, for diesel refinement, and in ammonia synthesis [Guo et al., 2010].

Dark fermentative biohydrogen production is a promising technology that has the potential for use in H_2 production from renewable resources such as lignocellulosic waste streams [Lin et al., 2007]. It allows faster production rates than the photosynthetic route, and eliminates light requirements [Azbar and Levin, 2012; Urbaniec and Bakker, 2015]. Lignocellulosics are carbohydrate-based feedstocks containing oligosaccharides and/or polymers (e.g. cellulose, hemicellulose, and starch) which are considered good organic carbon sources for dark fermentative H_2 production [Hawkes et al., 2002]. Different groups of microorganisms have been investigated over decades for biological H_2 production such as algae and cyanobacteria (biophotolysis), photosynthetic bacteria (photofermentation), and fermentative bacteria (dark fermentation) [Hallenbeck and Benemann, 2002]. On the other hand, the complexity of these organic wastes makes them difficult for H_2 producing bacteria to utilize directly without pretreatment [Masset et al., 2012].

2.2 Dark Fermentative H_2 Production

Biohydrogen production through anaerobic dark fermentation involves a wide variety of bacterial species that can be strictly anaerobic (*Clostridia*, *methylotrophs*, rumen bacteria, archaea), or facultative anaerobic (*Escherichia coli*, *Enterobacter*, *Citrobacter*) [Li and Fang, 2007; Lin et al., 2007; Yokoi et al., 2001]. Each culture has its optimal operating temperature that can be mesophilic (25-40°C), thermophilic (40-65°C),

extreme thermophilic (65-80°C), or hyperthermophilic (>80°C) [Levin et al., 2004]. Cultures used for fermentative H_2 production include: mixed anaerobic bacteria obtained from anaerobic sludge digesters [Morimoto et al., 2004; Zhu and Beland, 2006], natural microflora [Ling et al., 2009; Li et al., 2008], composts [Ginkel and Sung, 2001; Fan et al., 2004] or pure cultures that operates at mesophilic [Lin et al., 2007] or thermophilic conditions [Masset et al., 2012]. Fermentative H_2 production is also affected by the carbon source used, preferring carbohydrate-rich substrates that can be simple as like glucose [Zhang et al., 2015a] or complex as starch [Gupta et al., 2014], cellulose [Gomez-Flores et al., 2015], food waste [Hu et al., 2014], or lignocellulosic waste [Nissila et al., 2014].

H_2 production yields depend on the fermentation pathways and the produced end-products [Levin et al., 2004]. The most common dark fermentation pathways for H_2 production from glucose are the acetate, butyrate, and propionate pathways (Equations 2.1, 2.2, 2.3) [Nath and Das, 2004; Guo et al., 2010]. The three reactions are thermodynamically favourable (i.e. negative ΔG values) with acetate and butyrate pathways associated with H_2 production while propionate pathway associated with H_2 consumption [Hussy et al., 2003]. This limits the theoretical H_2 yield to between 2 and 4 moles of H_2 per mole of glucose, and the greater the acetate-to-butyrate ratio, the higher is the H_2 yield. Therefore, directing the metabolism of the culture towards acetate formation by providing its optimum operational conditions is key to achieving higher H_2 yields [O-Thong et al., 2009] as well as avoiding propionate production [Hussy et al., 2003].

$$C_6H_{12}O_6\ 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \Delta G_R^\circ = -196\ KJ \quad (2.1)$$

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2 \qquad \Delta G_R^\circ = -224\ KJ \quad (2.2)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad \Delta G_R^\circ = -279\ KJ \quad (2.3)$$

Two H_2-producing pathways from butyrate and propionate that are thermodynamically unfavourable (reactions 2.4 and 2.5) [Stams and Plugge, 2009] can occur if H_2 as a product is decreased to its minimum concentration, converting Gibbs free

energy from positive to negative values [Stams and Plugge, 2009]. Similarly, the propionate to acetate pathway (reaction 2.4), which is thermodynamically unfavourable, could be shifted forward if CO_2 was removed from the headspace [Stams and Plugge, 2009; Nasr et al., 2015].

$$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2 \qquad \Delta G_R^\circ = +72 \text{ KJ} \quad (2.4)$$

$$CH_3(CH_2)_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad \Delta G_R^\circ = +48 \text{ KJ} \quad (2.5)$$

2.2.1 System operation

Nath and Das [2004] stated that removing CO_2 efficiently from the culture medium will shift H_2-synthesizing reactions in the forward direction, increasing H_2 production, and decreasing the consumption of reducing equivalents carried by electron carriers like nicotinamide adenine dinucleotide (NADH) by competing reactions [Nath and Das, 2004]. Kraemer and Bagley [2007] discussed several methods for improving the H_2 yield, one of which was removing dissolved H_2 and CO_2 from the liquid phase of the fermentation process.

In addition, H_2 and CO_2 are the main substrates for both hydrogenotrophic methanogenic bacteria and homoacetogenic bacteria to produce methane (reaction 2.6) and acetate (reaction 2.7), respectively [Kotsyurbenko et al., 2004; Saady, 2013]. Mayumi et al. [2013] observed that increasing CO_2 concentrations accelerated the rate of hydrogenotrphic methanogenesis in oil reservoirs. Also, Saady [2013] indicated that controlling CO_2 concentrations during dark fermentative H_2 production needs further investigation as a potential approach of controlling homoacetogenesis. Therefore, dissolved CO_2 removal from the liquid phase may prevent the consumption of H_2 for methane (CH_4) or acetate production.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G_R^\circ = -131 \text{ KJ} \qquad (2.6)$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G_R^\circ = -104 \text{ KJ} \qquad (2.7)$$

2.2.1.1 Gas sparging techniques

Gas sparging is one of the common techniques used for dissolved gas removal. Table 2.1 shows that nitrogen (N_2) gas has been used in many studies while few studies investigated the effect of gas sparging on H_2 production using other gases such as argon (Ar) [Tanisho et al., 1998; Oh et al., 2002], CO_2 [Kim et al., 2006a], or biogas [Kim et al., 2006a]. Crabbendam et al. [1985] observed an increase in the glucose utilization efficiency from 65% to 73% when continuously purging N_2 gas in a 0.5 L chemostat operating with a dilution rate of 0.2 h^{-1}. The aforementioned authors did not report H_2 production data as they focused on substrate utilization and adenosine triphosphate (ATP) generation, however, the increase in glucose utilization efficiency implies better H_2 production performance [Crabbendam et al., 1985]. Hussy et al. [2005] observed an increase in the H_2 yield from 1.0 to 1.9 mol/mol hexose using sucrose as the substrate in a continuous stirred-tank reactor (CSTR) operated at a hydraulic retention time (HRT) of 15 hours and achieved 95% sucrose conversion after sparging N_2 gas continuously in the reactor. In another study, the aforementioned authors tested the effect of N_2 gas sparging and reported an increase in the H_2 yield from 1.26 to 1.87 mol/mol hexose utilizing wheat starch as the carbon source [Hussy et al., 2003]. Kim et al. [2006a] compared the utilization of N_2, CO_2, and biogas as sparging gases in H_2 production from sucrose in a CSTR operated at an HRT of 12 hours and loading of 40 gCOD/L.d and observed 24%, 118%, and 12% increase in the H_2 yield to 0.93, 1.68, and 0.86 mol/mol hexose, respectively. Gas sparging was also tested in pure cultures experiments. Tanisho et al. [1998] observed a 110% increase in the H_2 yield to 1.09 mol/mol hexose by continuous sparging of argon gas in a H_2 producing batch experiment by *Enterobacter aerogenes* using molasses as the carbon source. Also, Oh et al. [2002] tested Ar gas sparging in a H_2 production from glucose batch experiment using *Rhodopseudomonas Palustris* achieving a 47% increase in the H_2 yield to 1.06 mol/mol hexose. It can be depicted from the previous studies (Table 2.1) that N_2 gas was the most common sparging gas used, however, a wide range of H_2 yields increase was observed (i.e. 24%-90%). In addition, the highest increase in H_2 yield observed of 118% was associated with CO_2 gas sparging

which contradicts the idea of shifting the H_2 production reaction forward by removing CO_2 gas from the head space [Nath and Das, 2004].

Table 2.1 - Effect of gas sparging on H_2 production yields

Gas Sparged	H_2 Yield (mol/mol_{hexose})		Yield Increase (%)	Carbon Source	Inoculum	Reference
	No sparging	With sparging				
N_2	1.00	1.90	90	Sucrose	ADS*	Hussy et al., 2005
	0.85	1.43	68	Glucose	Anaerobic microflora	Mizuno et al., 2000
	1.26	1.87	48	Wheat Starch	ADS	Hussy et al., 2003
	1.30	1.80	38	Glucose	ADS	Kraemer & Bagley, 2006
	0.85	1.15	35	Sucrose	ADS	Kyazze et al., 2006
	0.77	0.95	23	Sucrose	ADS	Kim et al., 2006a
CO_2	0.77	1.68	118	Sucrose	ADS	
Biogas**	0.77	0.86	12	Sucrose	ADS	
Ar	0.52	1.09	110	Molasses	*Enterobacter aerogenes*	Tanisho et al., 1998
	0.72	1.06	47	Glucose	*Rhodopseudomonas Palustris*	Oh et al., 2002

* ADS: Anaerobic digester sludge

** Biogas produced (i.e. $H_2 + CO_2$) was recycled back to the reactor

2.2.1.2 Non-gas sparging techniques

Non-sparging techniques that decrease the dissolved gas concentrations include increasing stirring speed, applying vacuum in the headspace (i.e. decreasing the reactor headspace pressure), using in-reactor ultrasonication, and using an immersed membrane to remove the dissolved gases [Kraemer and Bagley, 2007; Elbeshbishy et al., 2011a; Elbeshbishy et al., 2011b]. Lamed et al. [1988] observed that vigorous stirring of *Clostridium thermocellum* batches utilizing cellobiose decreased the ethanol-to-acetate ratio producing more H_2 through the acetate pathway and increasing the H_2 yield by 129% to 0.78 mol/mol hexose. Mandal et al. [2006] observed an increase of 105% in the H_2 yield to 3.9 mol/mol hexose of a batch H_2 producing experiment from glucose by *Enterobacter cloacae* by decreasing the headspace total pressure. The increase in H_2 yield was attributed to inhibition of H_2 consumption due to the decrease in total pressure that lead to the production of reduced by-products such as ethanol and organic acids [Mandal et al., 2006]. The aforementioned authors also used a potassium hydroxide (KOH) trap outside the batch reactor headspace to absorb CO_2. Liang et al. [2002] used a silicone rubber membrane to separate biogas from the liquid phase in a H_2 fermentation batch reactor using glucose as the substrate, and observed 15% and 10% increases in H_2 yield and H_2 production rate, respectively.

Park et al. [2005] were the first to apply headspace CO_2 sequestration using KOH in batch H_2 glucose fermentation, and achieved a H_2 content of 87.4% in the headspace. They recommended assessing CO_2 removal from the headspace of continuous-flow systems instead of batches to measure how effectively CO_2 would be removed, specially under different organic loading rates (OLRs) [Park et al., 2005].

2.2.2 Operating temperature

Temperature is another important physical factor that influences the activity of H_2 producing bacteria [Wang and Wan, 2009]. As reported in the literature, H_2 production can be enhanced under thermophilic conditions [Elsharnouby et al., 2013]. However, maintaining mesophilic (25-40°C) conditions is less expensive than maintaining

thermophilic conditions (40-65°C). H_2 production from high value cellulosic feedstocks requires full fermentation of pentose and hexose sugars (i.e. xylose, glucose, sucrose, and cellobiose) [Ngo et al., 2011]. One of the advantages of operating at thermophilic conditions over the mesophilic one is the elimination of hydrolytic enzymes used for H_2 production from complex carbohydrates such as cellulase [Liu et al., 2008]. Another advantage is the reduction of contamination by mesophilic microorganisms as well as the reduction of molecular H_2 uptake by hydrogenases [Munro et al., 2009]. Higher hydrolysis rates and H_2 yields were reported using thermophilic cultures [Ngo et al., 2011; van Groenestijn et al., 2002]. In a fermentative reaction for H_2 production, an increase in temperature will increase the equilibrium kinetic constant keeping the reactants concentration constant, which enhances H_2 production [Sinha and Pandey, 2011]. Valdez-Vazquez et al. [2005] observed an increase in H_2 yield from 1.5 (at mesophilic temperature, 37°C) to 3.2 mol/mol hexose (at thermophilic temperature, 55°C) using a real waste containing 26% (by weight) cellulose. Gupta et al. [2015] achieved H_2 yield of 0.42 mol/mol hexose at thermophilic temperature (60°C) compared to yield of 0.13 mol H_2/mol hexose at mesophilic temperature (37°C) using cellulose as the carbon source in batch experiments. Gadow et al. [2012] observed an increase in H_2 yields from 0.1 to 2.5 to 2.9 mol/mol hexose using 5 g/L of cellulose at mesophilic (37°C), thermophilic (55°C), and hyper-thermophilic (80°C) temperatures, respectively in a continuous-flow system with a hydraulic retention time of 10 days.

2.2.2.1 Pure cultures

Many studies have been conducted using pure cultures for H_2 production from various substrates. *Clostridium* species, strict anaerobes, gram-positive, rod-shaped, and endospore formers are the most widely used species for H_2 production [Wang and Wan, 2009]. One of the main differences between H_2 production using pure and mixed cultures is the end products, where in mixed cultures it depends on the type of species within the used culture, while it can be predicted in pure cultures experiments since it depends on the species type. For instance, some *Clostridium* species are non-butyrate producers such as *Clostridium cellulolyticum* that produces acetate and ethanol [Ren et al., 2007], and

Clostridium stercorarium that produces acetate, lactate, and ethanol [Fardeau et al., 2001]. Although most of the studies using pure cultures were conducted in batch experiments using simple sugars as substrate, however, it is more beneficial to produce H_2 from organic wastes in continuous-flow systems.

H_2 yields achieved from soluble substrates such as glucose are comparable using pure and mixed cultures. However, pure H_2 producing bacteria achieved higher yields from complex substrates such as cellulose. Table 2.2 shows H_2 production yields achieved by mesophilic and thermophilic pure cultures from different pure carbohydrates. Simple sugars such as glucose, xylose, and cellobiose can be found in real wastes and their hydrolysates and are easily biodegradable due to their simple structures. A wide range of H_2 yields by mesophilic and thermophilic strictly anaerobic pure cultures have been reported in the literature using glucose as the carbon source (Table 2.2). The highest yield of 2.8 mol/mol hexose has been reported by the mesophilic bacteria *Clostridium beijerinckii* in an 80 mL batch test utilizing 3 g/L glucose [Lin et al., 2007]. Masset et al. [2012] reported a low H_2 yield of 0.7 mol/mol hexose using the mesophilic culture *Clostridium pasteurianum* and utilizing 5 g/L glucose in a 3 L batch experiment. *Thermoanaerobacterium thermosaccharolyticum* achieved a high H_2 yield of 2.4 mol/mol hexose utilizing 10 g/L glucose under thermophilic temperature [O-Thong et al., 2008]. Higher H_2 yields from xylose were reported at thermophilic temperatures than mesophilic ones. At a xylose concentration of 10 g/L in batch studies, *Thermoanaerobacterium thermosaccharolyticum* achieved 2.6 mol/mol hexose [Ren et al., 2008], while *Clostridium butyricum* achieved 0.6 mol/mol hexose [Junghare et al., 2012]. Similarly, cellobiose was degraded at a yield of 1.7 mol/mol hexose using *Clostridium thermocellum* at thermophilic temperature [Levin et al., 2006], while lower yields of 1.1 and 0.9 mol/mol hexose were reported at mesophilic temperatures using *Clostridium termitidis* [Gomez-Flores et al., 2015] and *Clostridium butyricum* [Junghare et al., 2012], respectively. Although it is easier to degrade simple sugars, real wastes contain complex substrates such as starch and cellulose which require an additional hydrolysis step. Specific pure cultures were found to have the ability of hydrolyzing and utilizing complex substrates. For example, *Clostridium termitidis* is a mesophilic cellulolytic bacteria that can produce H_2 by hydrolyzing and consuming cellulose [Ramachandran et

al., 2008]. *Clostridium thermocellum* and *Clostridium cellulolyticum* were also able to utilize cellulose with H_2 yields of 1.9 and 1.6 mol/mol hexose at thermophilic and mesophilic temperatures, respectively [Lin et al., 2007; Ren et al., 2007]. Other *Clostridium* species achieved very low H_2 yields of 0.1 and 0.6 mol/mol hexose from cellulose like *Clostridium butyricum* and *Clostridium acetobutylicum*, respectively (Table 2.2). Masset et al. [2012] reported a H_2 yield of 2.9 mol/mol hexose utilizing 5 g/L starch as the carbon source and using the mesophilic anaerobic bacteria *Clostridium butyricum*. The aforementioned authors achieved a lower yield of 1.8 mol/mol hexose using *Clostridium pasteurianum* [Masset et al., 2012]. As depicted from Table 2.2, a very wide range of H_2 yields can be produced using different types of H_2 producing pure cultures from the same carbon source, which is due to the variation of the growth kinetics as well as the optimum operational conditions for each culture.

Table 2.2 - H_2 production yields from sugars by pure cultures

Substrate	S* (g/L)	Culture	T** (°C)	H_2 Yield (mol/mol_{hex})	Reference
Glucose	3	*Clostridium beijerinckii*	35	2.8	Lin et al., 2007
	10	*Thermoanaerobacterium thermosaccharolyticum*	60	2.4	O-Thong et al., 2008
	3	*Clostridium butyricum*	36	2.3	Lin et al., 2007
	3	*Clostridium acetobutylicum*	37	1.8	
	3	*Clostridium tyrobutyricum*	35	1.5	
	20	*Clostridium saccharoperbutylacetonicum*	30	1.3	Ferchichi et al., 2005
	5	*Clostridium pasteurianum*	35	0.7	Masset et al., 2012
Xylose	10	*Thermoanaerobacterium thermosaccharolyticum*	60	2.62	Ren et al., 2008
	10	*Clostridium butyricum*	37	0.59	Junghare et al., 2012
Cellobiose	1	*Clostridium thermocellum*	60	1.7	Levin et al., 2006
	2	*Clostridium termitidis*	37	1.1	Gomez-Flores et al., 2015
	10	*Clostridium butyricum*	37	0.9	Junghare et al., 2012
Starch	5	*Clostridium butyricum*	35	2.9	Masset et al., 2012
	5	*Clostridium pasteurianum*	35	1.8	
	10	*Clostridium beijerinckii*	35	1.8	Taguchi et al., 1992
	10	*Clostridium butyricum*	37	0.6	Junghare et al., 2012
Cellulose	1	*Clostridium thermocellum*	60	1.9	Lin et al., 2007
	5	*Clostridium cellulolyticum*	35	1.6	Ren et al., 2007
	2	*Clostridium termitidis*	37	1.5	Gomez-Flores, 2015
	10	*Clostridium acetobutylicum*	37	0.6	Wang et al., 2008
	10	*Clostridium butyricum*	37	0.1	Junghare et al., 2012

* S: Initial substrate concentration ** T: Temperature

Although H_2 can be produced from a wide spectrum of carbohydrates, most of the pure cultures studies reported in the literature have investigated H_2 production from pure sugars which is easier in terms of providing a sterile environment [Elsharnouby et al., 2013]. However, renewable feedstocks should be more investigated as they are more beneficial to the environment. Table 2.3 shows H_2 production yields achieved by mesophilic and thermophilic pure cultures from real wastes. Starch containing wastes like corn, rice, and potato produced high H_2 yields using mesophilic and thermophilic pure cultures. Dada et al. [2013] achieved a H_2 yield of 2.9 mol/mol hexose from rice bran hydrolysate using the mesophilic bacteria *Clostridium saccharoperbutylacetonicum* in a 100 mL batch experiment at sugar concentration of 29 g/L. The anaerobic thermophilic bacteria *Thermoanaerobacterium thermosaccharolyticum* utilized hydrolyzed corn stover producing H_2 at a yield of 2.2 mol/mol hexose in a 50 mL batch experiment at a temperature of 60°C [Cao et al., 2009]. Cheng and Liu [2011] reported a lower H_2 yield of 1.5 mol/mol hexose from untreated corn stalk powder at a concentration of 30 g/L using the thermophilic bacteria *Clostridium thermocellum* in a 10 L CSTR. Hydrolyzed potato steam peels were used at a concentration of 10 g/L in 1 L batch experiments using the thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and produced a H_2 yield of 2.4 mol/mol hexose at a temperature of 72°C [Mars et al., 2010]. Sugar containing crops like sugarcane bagasse and sugar beet were also used in pure cultures experiments. Pattra et al. [2008] produced 1.7 mol H_2/mol hexose from 20 gCOD/L sugarcane bagasse hydrolysate using the anaerobic mesophilic bacteria *Clostridium butyricum* in a 70 mL batch experiment. Also, Plangklang et al. [2012] utilized sugarcane juice by *Clostridium butyricum* and produced H_2 at a yield of 1.3 mol/mol hexose. Lignocellulosic wastes such as agricultural residues, paper waste, and wood are cheap renewable feedstocks that have a high potential for fermentative biohydrogen production. The cellulolytic thermophilic bacteria *Clostridium thermocellum* utilized delignified wood fiber at a concentration of 0.1 g/L in a 26 mL batch experiment achieving a H_2 yield of 2.3 mol/mol hexose at 60°C [Levin et al., 2006]. The same bacteria produced H_2 with a lower yield of 0.7 mol/mol hexose using pulp and paper sludge as the carbon source in a 50 mL batch test at an initial substrate concentration of 5 g/L [Moreau et al., 2015]. At a higher temperature of

72 and 70°C, de Vrije et al. [2009; 2010] used *Caldicellulosiruptor saccharolyticus* and achieved high H_2 yields of 3.3 and 2.8 mol/mol hexose from 10 g/L miscanthus and carrot pulp hydrolysates, respectively. The high H_2 yield (83% of theoretical yield) obtained from the miscanthus hydrolysate is due to the high percentage of sugars obtained from alkaline pretreatment, where 61% of the hydrolysate COD was sugars [de Vrije et al., 2009].

Table 2.3 - H_2 production yields from real waste by pure cultures

Substrate	S[a] (g/L)	Culture	Temp. (°C)	H_2 Yield (mol/mol_{hex})	Reference
Corn stalk powder	30	*Clostridium thermocellum*	55	1.46	Cheng and Liu 2011
Sugarcane bagasse hydrolysate	20[b]	*Clostridium butyricum*	37	1.73	Pattra et al., 2008
Sugarcane juice	22.3	*Clostridium butyricum*	37	1.33	Plangklang et al., 2012
Rice bran hydrolysate	29[c]	*Clostridium saccharoperbutylacetonicum*		2.87	Dada et al., 2013
Hydrolyzed corn stover		*Thermoanaerobacterium thermosaccharolyticum*	60	2.24	Cao et al., 2009
Delignified wood fiber	0.1	*Clostridium thermocellum*	60	2.32	Levin et al., 2006
Pulp and paper sludge	5	*Clostridium thermocellum*	60	0.67	Moreau et al., 2015
Miscanthus hydrolysate	10	*Caldicellulosiruptor saccharolyticus*	72	3.3	de Vrije et al., 2009
Carrot pulp hydrolysate	10	*Caldicellulosiruptor saccharolyticus*	70	2.80	de Vrije et al., 2010
Potato steam peels	10	*Caldicellulosiruptor saccharolyticus*	72	2.4	Mars et al., 2010

[a] S: Initial substrate concentration [b] gCOD/L [c] g sugars/L

2.2.2.2 Mixed cultures

Mixed cultures of bacteria from anaerobic sludge, composts, and municipal sewage sludge have been used in many studies as the inoculum in fermentative H_2 production. The main advantages of mixed cultures are operation in non-sterile environments, which are critical to maintain for pure cultures, as well as the wide range of feedstocks that can be utilized by mixed cultures [Fang and Li, 2007]. In mixed cultures, H_2 produced by H_2-producing bacteria may be consumed by H_2 consuming bacteria, which requires pretreatment to suppress bacteria that consume H_2 [Fang and Li, 2007]. Operational temperature is one of the main factors that affect fermentative H_2 production using mixed cultures, since it contains a variety of H_2 producing species with different optimum operating temperatures. Many studies have reported enhancement in H_2 production parameters using mesophilic cultures operated at thermophilic conditions relative to mesophilic conditions (Table 2.4). Zhang et al. [2015b] studied biohydrogen production from corn stover acid hydrolysate at a concentration of 5 g/L and a pH of 7 in batches using anaerobic granular sludge obtained from a bench-scale expanded granular sludge bed reactor treating starch wastewater. The aforementioned culture was tested at mesophilic temperature (37°C) and thermophilic temperature (55°C) at a substrate-to-biomass (S°/X°) ratio of 5.6 gCOD/gVSS. The authors reported that the H_2 production yield at thermophilic condition (55°C) was 802 mL H_2/L (0.95 mol H_2/mol hexose) with acetate and butyrate as the predominant soluble by-products while at mesophilic condition (37°C), H_2 production yield was 223 mL H_2/L (0.32 mol H_2/mol hexose) with predominantly acetate, ethanol, and propionate as the soluble by-products. In the aforementioned study, the authors attributed better H_2-producing performance at thermophilic conditions to the selective enrichment of some efficient H_2-producing thermophiles, which are capable of producing more H_2 by utilizing complex substrate components. Luo et al. [2010] studied biohydrogen production from cassava stillage at a concentration of 26.9 g sugar/L (48 gCOD/L), a pH range from 5.4 to 5.8, and an S°/X° ratio of 2.4 gCOD/gVSS using mesophilic anaerobic digester sludge obtained from an up-flow anaerobic sludge blanket (UASB) reactor operating at mesophilic temperature

(37°C) in a thermophilic (60°C) continuously stirred tank reactor (CSTR) and obtained hydrogen production yields of 12 and 58 mL H_2/gCOD at mesophilic and thermophilic conditions, respectively. The aforementioned authors attributed the better performance of mesophilic anaerobic digested sludge at thermophilic conditions to the lower propionate production and lower activity of homoacetogens. In the aforementioned study, the distribution of VFAs was quite different as butyrate was the main soluble by-product at the thermophilic temperature, while butyrate, propionate, and acetate were predominant at the mesophilic temperature, with propionate concentration 5 times higher than that observed at the thermophilic one [Luo et al., 2010]. Gavala et al. [2006] observed a 31% increase in the H_2 yield to 2.1 mol/mol hexose utilizing glucose at thermophilic conditions, while the H_2 production rates were comparable for both temperature ranges. The inocula used in the abovementioned study was anaerobic digester sludge (ADS) in a 0.5 L CSTR operating at an HRT of 2 hours. Zhang et al. [2003] observed an increase of 66% in the H_2 yield from starch to 66 mL/gCOD under thermophilic conditions as compared to mesophilic conditions. Microbial culture analysis conducted on the culture operating at thermophilic conditions showed 86% of the developed clones closely affiliated with the genus *Thermoanaerobacterium*. However, a 24% decrease in the production rate (1.9 mL/h) was observed at thermophilic conditions, which can be attributed to the fact that the used seed originally was obtained from a mesophilic digester [Zhang et al., 2003].

Other studies reported H_2 production enhancement using thermophilic anaerobic cultures or acclimatized thermophilic cultures compared to mesophilic cultures (Table 2.4). Cheng and Liu [2012] studied biohydrogen production from raw cornstalk and a mixture of raw and fungal pretreated cornstalk using mesophilic and thermophilic cultures. The thermophilic seed was obtained from a 4 L anaerobic digester operating at 55°C for more than 6 months utilizing glucose as the carbon source with total and suspended solids (TSS and VSS) of 24 and 12 g/L, respectively. Cheng and Liu [2012] observed the highest H_2 production yield of 54 mL/gVS for the experiment utilizing thermophilic seed with raw and pretreated cornstalk mixture as the substrate, producing acetate, butyrate, propionate, and ethanol as the main by-products. Also, Kargi et al.

[2012] achieved a 72% increase in the H_2 production yield at thermophilic conditions utilizing cheese whey powder as the carbon source. The aforementioned authors observed yields of 0.47 and 0.81 mol/mol hexose using mesophilic ADS and acclimatized mesophilic ADS at 55°C on 60 g/L glucose [Kargi et al., 2012]. Cakır et al. [2010] investigated biohydrogen production from acid-hydrolyzed wheat starch at a concentration of 18.5 g/L and a neutral pH using mesophilic anaerobic digester sludge at mesophilic temperature (37°C) and thermophilic temperature (55°C). The mesophilic anaerobic digester sludge was acclimatized at 55°C using glucose at a concentration of 60 g/L for 3 days prior switching to acid-hydrolyzed wheat starch at a concentration of 18.5 g/L at thermophilic temperature. The aforementioned authors reported that dark fermentative H_2 production of acid-hydrolyzed ground wheat was more beneficial under thermophilic condition (55°C) than mesophilic condition (37°C). A yield of 2.4 mol H_2/mol hexose consumed was obtained at thermophilic temperature compared to 1.6 mol H_2/ mol hexose consumed at mesophilic condition. Interestingly, the lag phase for thermophilic fermentation (31.6 hr) was much lower than for mesophilic one (44.3 hr). Total final VFAs concentration were much higher at thermophilic fermentation (10.1 g/L) compared to at mesophilic one (6.9 g/L) suggesting that VFAs and biohydrogen production were directly related as high final VFAs concentrations yielded high hydrogen production [Cakır et al ., 2010].

Table 2.4 - Effect of temperature on H_2 production yields using mixed cultures

Inoculum	Carbon source	Reactor	Temp. (°C)	H_2 Yield (mol/mol_{hexose})	Reference
ADS	Glucose	CSTR	35	1.60	Gavala et al., 2006
		(HRT 2 hrs)	55	2.10	
ADS	Starch	Batch	37	1.00	Gupta et al., 2015
	(13.5 gCOD/L)	(200 mL)	60	1.13	
Mesophilic sucrose fed WW	Starch WW	Batch	37	0.30	Zhang et al., 2003
	(4.6 g/L)	(280 mL)	55	0.47	
AS* from a bench-scale reactor	Corn stover hydrolysate	Batch	37	0.32	Zhang et al., 2015b
treating starch WW**	(6.2 g/L)	(50 mL)	55	0.95	
AS from UASB reactor	Cassava stillage	Batch	37	14***	Luo et al., 2010
	(34 gCOD/L)	(200 mL)	60	70	
ADS	Cellulose	CSTR	37	0.10	Gadow et al., 2012
	(5 g/L)	(HRT 10 d)	55	2.46	
ADS	Cellulose	Batch	37	0.13	Gupta et al., 2015
	(13.5 gCOD/L)	(200 mL)	60	0.42	
ADS	Wheat starch hydrolysate	Batch	37	1.6	Cakır et al ., 2010
ADS acclimatized with glucose at 55°C	(18 g/L)	(500 mL)	55	2.4	
ADS	Cheese whey powder	Batch	35	0.47	Kargi et al., 2012
ADS acclimatized with glucose at 55°C	(10.8 g/L)	(150 mL)	55	0.81	
ADS	Raw cornstalk (8 g/L)	Batch	35	25.7***	Cheng & Liu, 2012
	Raw + pretreated^{+} (8 g/L)	(500 mL)	35	35.9	
AS from lab-scale thermophilic	Raw cornstalk (8 g/L)	Batch	55	29.8	
treating glucose at 55°C	Raw + pretreated (8 g/L)	(500 mL)	55	54.1	

* AS: Anaerobic sludge ** WW: Wastewater *** H_2 yields in mL/gVS

$^{+}$ Substrate used was a mixture of raw and fungal pretreated cornstalk

2.2.3 H_2-producing cultures optimization

Recently, there has been a renewed interest in benefiting from the synergistic effect of designed co-cultures as opposed to undefined consortia as well as co-substrates or co-fermentation of different types of substrates. Designed co-cultures are used in fermentative H_2 production in order to improve yields, production rates, and extend the range of substrate utilization [Azbar and Levin, 2012]. Based on the synergetic effects between selected cultures, designed co-cultures may offer a better performance than mixed cultures for H_2 production and can overcome some of the shortcomings of pure cultures. Masset et al. [2012] observed an average increase of 80% in H_2 yields by testing co-cultures of clostridia species using glucose as the substrate at mesophilic temperature. In co-substrate experiments, the presence of different types of carbon sources stimulated the utilization of substrates that were poorly degraded as single substrate, leading to an overall substrate utilization enhancement and consequently increasing the H_2 production yield. In a H_2-producing system, the enhancement of H_2 production kinetics and/or substrate utilization kinetics reduces the reaction times, leading to a reduction in the system size, equipment maintenance cost, and process control equipment which leads to capital and operational costs reduction.

2.2.3.1 Substrate concentration

Initial substrate concentration is an important factor that affects fermentative H_2 production using pure and mixed cultures. Studies in the literature have shown that in mixed cultures the microbial community structures as well as the metabolic pathways are affected by the initial substrate concentration [Kyazze et al., 2006; Kim et al., 2006b]. Wang and Wan [2008] investigated the effect of glucose concentration on H_2 production in 100 mL batch experiments using anaerobic digester sludge at 35°C. The aforementioned authors observed a constant substrate degradation efficiency of 96±2% with initial substrate concentrations in the range of 1-25 g/L, however, a drastic decline occurred for higher substrate concentrations till it reached only 30% at 300 g/L glucose. H_2 yield increased from 2.0 to 3.0 mol/mol hexose for initial glucose concentrations of 1

and 2 g/L, respectively, then remained at 2.2±0.1 mol/mol hexose for glucose concentrations in the range of 5-15 g/L, after which it declined to zero H_2 yield at 300 g/L glucose [Wang and Wan, 2008]. A metabolic shift was observed with the increase in initial glucose concentration, where acetate and butyrate were the main end-products (94±3% of soluble metabolites) and propionate contributed only with 4±3% at 1-50 g/L glucose, then propionate production increased to 23±9% of the soluble metabolites at 100-300 g/L with same acetate production while butyrate production decreased [Wang and Wan, 2008]. Kim et al. [2006b] studied the effect of initial sucrose concentration on H_2 production in a 5 L CSTR (HRT 12 hrs) at 35°C. The aforementioned authors observed a maximum sucrose consumption (99%) at an initial concentration of 10 gCOD/L after which it decreased to 88% at 30 gCOD/L. At the higher substrate concentrations of 35 to 60 gCOD/L, sucrose consumption decreased drastically from 75% to 39%, respectively [Kim et al., 2006b]. At an initial sucrose concentration of 30 gCOD/L the DGGE analysis revealed all bands for H_2-producing *Clostridium* species such as *Clostridium butyricum* and *Clostridium tyrobutyricum*, which is consistent with the high butyrate-to-acetate ratio observed (>1), where acetate and butyrate were 50% of the soluble end-products. At lower glucose concentration of 10 gCOD/L, acetate increased leading to butyrate-to-acetate ratio less than 1 along with the detection of the spore-forming acetogen *Clostridium scatologenes*. At 60 gCOD/L sucrose an increase in the lactate production was detected associated with the presence of the spore-forming lactic acid bacterium *Bacillus racemilacticus* [Kim et al., 2006b].

In pure culture experiments, initial substrate concentration affects the end-products and H_2 production yields and rates. The effect of initial glucose concentration on H_2 production using the mesophilic *Clostridium beijerinckii* was tested in 50 mL batch experiments by Skonieczny and Yargeau [2009]. Increasing the initial glucose concentration in the range of 1-3 gCOD/L was accompanied by an increase in the butyrate and a decrease in the formate and propionate concentrations along with an increase in H_2 production yields and rates [Skonieczny and Yargeau, 2009]. Chen et al. [2005] investigated the effect of initial sucrose concentration on H_2 production using the mesophilic bacteria *Clostridium butyricum* in batch experiments varying the sucrose

concentration from 5-30 gCOD/L. The achieved H_2 yield achieved was 2.0 mol/mol hexose at sucrose initial concentrations 5 and 10 gCOD/L, which peaked to 3.1 mol/mol hexose at a concentration of 20 gCOD/L after which it declined to 1.8 mol/mol hexose at 30 gCOD/L sucrose [Chen et al., 2005]. Butyrate and acetate were the main end-products accounting for 70±5% of the soluble metabolites, with the butyrate-to-acetate molar ratio greater than one. Ethanol production was constant (12% of soluble metabolites) at initial sucrose concentrations of 5-20 gCOD/L with almost no ethanol produced at the 30 gCOD/L. However, propionate production increased with the increase in initial sucrose concentration which is consistent with the low H_2 production yield at the 30 gCOD/L experiment [Chen et al., 2005].

2.2.3.2 Co-fermentation

To date, the majority of the research on biohydrogen production using dark fermentation has mainly focused on single substrates and very few studies have explored co-fermentation of different substrates. Prakasham et al. [2009] investigated the role of glucose to xylose ratio on fermentative mesophilic biohydrogen production using a mixed culture as inoculum in 1 L batch experiments. The authors observed a 23% increase in H_2 production from the glucose-xylose co-fermentation when compared to glucose-only fermentation, and a 9% increase in H_2 production from the glucose-xylose co-fermentation when compared to the xylose-only experiment. In another study, co-substrates including glucose, xylose, and starch, were investigated for thermophilic anaerobic conversion of microcrystalline cellulose using ADS in batch tests [Xia et al., 2012]. Xylose increased the cellulose degradation efficiency by three times compared to the cellulose mono-substrate, where nearly no cellulose was degraded. Gupta et al. [2014] assessed the synergistic effect of using glucose, starch, and cellulose as co-substrates in batch experiments using ADS as the inoculum. H_2 yields of the glucose, starch, and cellulose mono-substrate experiments were 1.2, 1.0, and 0.2 mol/mol hexose, respectively. However, the yields increased by an average of 27±4% in all different co-substrate mixtures (i.e. glucose+starch, glucose+cellulose, starch+cellulose, glucose+starch+cellulose) with a maximum H_2 yield of 1.4 mol/mol hexose from glucose and starch co-substrate experiment. Microbial community analysis confirmed the

synergistic effect in the co-substrate experiments with a 51, 10, and 9-fold increase in *Clostridium* species compared to the seed control in glucose-starch, glucose-cellulose, and starch-cellulose experiments, respectively [Gupta et al., 2014]. The thermophilic bacteria *Thermoanaerobacterium thermosaccharolyticum* was tested for H_2 production from mono- and co-substrate of glucose and xylose in batch tests [Ren et al., 2008]. H_2 production yields of 2.4 and 2.6 mol/mol hexose were achieved using mono-substrate of glucose and xylose, respectively. The aforementioned authors reported no significant difference in H_2 yields of the co-substrate experiments with different mixing ratios (2.5 mol/mol hexose), however, H_2 production rates increased by 12±1% compared to the xylose only experiment [Ren et al., 2008]. Fangkum and Reungsang [2011a] studied the thermophilic co-digestion of xylose and arabinose at 2.5 g/L each concentrations using anaerobic mixed cultures, and obtained a maximum hydrogen yield of 2.9 mol H_2/mol hexose with 95% substrate degradation. Substrate degradation was observed to decrease with the increase in xylose/arabinose concentrations.

Co-fermentation of different organic residues has demonstrated H_2 production enhancement in a number of studies in the literature suggesting synergistic and complementary effects [Wang et al., 2013]. Some of the reported advantages of co-digestion of organic wastes are toxic compounds dilution, nutrients balance enhancement, buffering capacity improvement, and synergistic microbial effects [Wang et al., 2013]. Nasr et al. [2014] investigated the co-fermentability of four different corn cobs hydrolysates and its effect on H_2 production as well as its impact on the inhibitory compounds present in the hydrolysates (i.e. furfural and hydroxymethylfurfural "HMF"). Co-fermentation of two acid hydrolysates enhanced H_2 production yield achieving 174 mL/gCOD, while co-fermentation of an acid hydrolysate and a high pressure hydrolysate resulted in enhancing H_2 production potential achieving 145 mL/gCOD. It has been reported that furfural at concentration of 0.2-1.1 g/L and HMF of lower than 0.14 g/L had no impact on H_2 production yields and rates [Nasr et al., 2014].

2.2.3.3 Co-cultures

The motivation for using co-cultures rather than mono-cultures is either economic or technical [Elsharnouby et al., 2013]. From the economical viewpoint, co-cultures can help ensure strictly anaerobic conditions and replace the use of expensive reducing agents. Yokoi et al. [1998] used strictly anaerobic bacteria (*Clostridium butyricum*) and facultative bacteria (*Enterobacter aerogenes*) for H_2 production from starch in batch experiments under mesophilic (37°C) conditions and observed a reduction in the lag phase from 12 and 5 hours without and with a reducing agent, to only 2 hours using the co-culture. The aforementioned authors also observed a 25% increase in the H_2 yield in batches using the reducing agent and batches using the co-culture. Beckers et al. [2010] reported a 49% increase in the H_2 production yield from starch using co-culture of *Cirobacter freundii* and *Clostridium butyricum* in batch experiments compared to the 0.5 mol/mol hexose achieved by *Clostridium butyricum* mono-culture. From a technical perspective, co-cultures can enhance H_2 production from complex sugars such as cellulose by using one culture that is capable of cellulose degradation with another culture that can utilize the cellulose degradation end-products for H_2 production. Liu et al. [2008] enhanced H_2 production from cellulose in batch experiments under thermophilic conditions, where *Clostridium thermocellum* produced 0.8 mol H_2/mol hexose with lactate as the main by-product, while co-culture of *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* produced 1.8 mol H_2/mol hexose with butyrate as the main by-product. Masset et al. [2012] studied the synergistic effects between three different clostridia cultures; *Clostridium butyricum, Clostridium pasteurianum, and Clostridium felsineum*. The authors observed enhancement in H_2 yields when co-culturing *Clostridium butyricum* and *Clostridium pasteurianum* (1.33 mol/mol hexose), *Clostridium butyricum* and *Clostridium felsineum* (1.02 mol/mol hexose), and *Clostridium pasteurianum* and *Clostridium felsineum* (1.61 mol/mol hexose). Geng et al. [2010] reported 8-fold H_2 yield (1.4 mol/mol hexose) in the co-culture of *Clostridium thermocellum* and *Clostridium thermopalmarium* utilizing cellulose as the carbon source over the yield achieved by *Clostridium thermocellum* mono-culture. In addition, most of the co-cultures studies used single substrate such as

cellulose [Liu et al., 2008; Wang et al., 2008], starch [Yokoi et al., 1998; Beckers et al., 2010], or glucose [Seppälä et al., 2011; Masset et al., 2012]. Table 2.5 compares H_2 yields using mono- and co-culture batch experiments in the literature.

Table 2.5 - H_2 Yields for Mono- and Co-culture Studies

Mono-/Co-Culture	Substrate	H_2 Yield (mol H_2/mol$_{hexose}$)	Reference
C. butyricum	Starch	0.49	Beckers et al., 2010
Cirobacter freundii	Starch	0.00	
C. butyricum + Cirobacter freundii	Starch	0.73	
C. butyricum	Glucose	0.97	Masset et al., 2012
C. pasteurianum	Glucose	0.66	
C. felsineum	Glucose	0.62	
C. butyricum + C. pasteurianum	Glucose	1.33	
C. butyricum + C. felsineum	Glucose	1.02	
C. pasteurianum + C. felsineum	Glucose	1.61	
C. thermocellum	Cellulose	0.80	Liu et al., 2008
C. thermocellum + Th. thermosaccharolyticum	Cellulose	1.80	
C. thermocellum	Cellulose	0.17	Geng et al., 2010
C. thermocellum + C. thermopalmarium	Cellulose	1.36	
C. acetobutylicum	Microcrystalline cellulose	0.58	Wang et al., 2008
C. acetobutylicum + Ethanoigenens harbinese	Microcrystalline cellulose	1.40	

2.2.4 Lignocellulosic feedstocks

Lignocellulosic biomass, of which two thirds are carbohydrate polymers of cellulose and hemicellulose is the most abundant raw material [Ren et al., 2009]. Cellulose is the most abundant component of lignocellulosic wastes representing 30-70% which depends on the nature of the feedstock [Monlau et al., 2013a]. Corn cobs contain 32.3%-45.6% cellulose, 39.8% hemicelluloses-mostly pentosan, and 6.7%-13.9% lignin [Zych, 2008]. Monlau et al. [2013a] reported the composition of different lignocellulosic compounds like wheat straw and bran, rice straw, barley straw, maize bran and stover, and poplar wood with cellulose, hemicellulose, and lignin compositions ranging between 32%-45%, 18%-37%, and 3%-26%, respectively. Cellulose is a linear polymer of cellobiose (glucose-glucose dimer) and upon hydrolysis yields free glucose molecules. Hemicellulose, on the other hand, consists mainly of xylose, arabinose, galactose, glucose, and mannose which are easily fermentable [Hamelinck et al., 2005]. The difficulty of producing H_2 from raw lignocellulosic wastes comes from the complex structure that does not facilitate the hydrolysis step during fermentation, existing pentose sugars are not readily fermented, and the formation of many compounds and by-products such as furans (furfural and HMF), organic acids (e.g. acetate), and phenolic monomers (e.g. vanillin and syringaldehyde) that negatively affect fermentation [Galbe and Zacchi, 2012; Quéméneur et al., 2012]. Thus, prehydrolysis to convert carbohydrate polymers in to fermentable monomeric sugars is needed.

2.2.4.1 Pretreatment methods

Several pretreatment methods have been investigated in the literature on different lignocellulosic wastes for their effect on dark fermentative processes. Mechanical methods such as grinding, milling, and chipping convert the biomass into a fine powder, which increase the surface area of cellulose facilitating its consumption [Monlau et al., 2013a]. However, this process is not cost effective as it requires too much energy especially for lignocellulosic wastes with high moisture contents [Yu et al., 2006]. Thermal pretreatment like steam explosion is conducted by rapidly heating the biomass to high temperature (160-260°C) with pressure (7-50 bar) enabling water molecules to enter

the biomass structure, after which pressure is released causing water to explode. This procedure opens the plant cells and increases the biomass surface area leading to biomass digestibility enhancement [Ballesteros et al., 2000; Monlau et al., 2013b]. The problem with steam explosion is the incomplete disruption of the lignin-carbohydrate matrix [Kumar et al., 2009]. Chemical methods such as acid and alkaline pretreatments are used efficiently for breaking ether and ester bonds in lignin/phenolics-carbohydrates complexes. Acid pretreatment is used to convert glucan in the biomass into glucose with a conversion efficiency that can reach 90% [Monlau et al., 2013a]. Acid pretreatment is the most commonly used method for treating substrates of fermentation processes and is considered the most efficient and easiest method for releasing simple sugars [Mosier et al., 2005]. However, acid pretreatment can produce inhibitory compounds and fermentation can be inhibited by acid residues [Nissila et al., 2014]. In addition, acid recovery and hydrolysates neutralization are sometimes required after pretreatment [Akobi, 2016]. Pan et al. [2008] investigated the effect of acid pretreatment of wheat bran on H_2 production. Soluble saccharides contents in the acid pretreated biomass increased from 0.1 to 0.4 g/gTS compared to raw wheat bran, leading to a 60% increase in the H_2 production yield [Pan et al., 2008]. Also, Zhang et al. [2007] reported a H_2 yield of 106 mL/gCOD from acid hydrolyzed cornstalk which was 46-fold the value obtained from the raw substrate. The aforementioned authors compared acid, alkaline, and steam explosion pretreatment methods on H_2 production and reported values of 2.6 and 2.3-fold for H_2 yield using acid hydrolysate compared to alkaline and steam explosion hydrolysates, respectively [Zhang et al., 2007].

2.2.4.2 Hydrolysates composition

The pretreatment of lignocellulosic biomass is required to hydrolyze and breakdown the biomass structure into monomer sugars such as glucose and xylose [Sun and Cheng, 2002]. The composition of the hydrolysate depends on the biomass type as well as the pretreatment method itself. Generally, pretreatment breaks the lignin seal of biomass and modifies its size, structure, and chemical composition, moreover, it hydrolyses part of the hemicellulose, decreases the crystallinity of cellulose, and increases cellulose surface area [Nissila et al., 2014]. During pretreatment processes,

different degradation products of cellulose and lignin are formed, which contain some undesired inhibitory compounds that negatively affect both hydrolysis and fermentation processes [Zha et al., 2012]. Inhibitory compounds can be organic acids (e.g. acetic acid), furan derivatives (e.g. furfural and HMF), and phenolic compounds (e.g. vanillin, syringaldehyde, 4-hydoxylbenzoic acid). Phenolic compounds, furfural and HMF are considered the strongest inhibitors to fermentative H_2 production [Haroun et al., 2016].

Furfural is the main degradation product of pentoses and it affects microbial growth by interfering with glycotic and/or fermentative enzymes and also disturb the membrane integrity of diverse microorganisms, with concentrations as low as 1g/L considered inhibitory [Quéméneur et al., 2012]. HMF compromises the cell membrane integrity, and intracellular sites are the primary inhibition targets [Mills et al., 2009]. In order to release the inhibitory effects of these furan compounds, microorganisms perform metabolic pathway switching and convert HMF and furfural to less toxic compounds, provided the initial concentrations are not beyond threshold levels [Boyer et al., 1992]. Furfural is known to be converted to furfuryl alcohol and furoic acid and HMF is converted to 5-hydroxymethyl furfuryl alcohol or 2,5-bis-hydroxymethylfuran [Boopathy et al., 1993; Liu et al., 2004; Liu et al., 2005]. Quéméneur et al. [2012] assessed the impact of 1 g/L furfural and HMF concentrations on fermentative H_2 production from xylose at an initial concentration of 5 g/L using ADS in a batch experiment. H_2 production inhibition in terms of lag phase duration, H_2 yield, and maximum H_2 production was observed. In the aforementioned study, H_2 yields decreased from 2.0 mol H_2/mol hexose in the control (xylose-only) batch bottles to 0.5 (±0.10) mol H_2/mol hexose, and with no gas production from furfural or HMF when added as the sole carbon source at 1g/L [Quéméneur et al., 2012]. In another batch experiment, Nasr et al. [2014] observed no inhibition of H_2 production with furfural and HMF concentrations of 0.21-1.09 g/L and below 0.14 g/L, respectively. Haroun et al. [2016] reported the inhibition threshold for furfural in the range 2-4 g/L using glucose (10 g/L) as the carbon source and acclimatized ADS as the seed in a continuous-flow system. The aforementioned authors observed an increase of 17% and 6% in the H_2 production yields with furfural concentrations of 0.25 and 0.5 g/L, respectively, compared to the 2.3 mol/mol hexose produced with no furfural. Then, H_2 yield decreased by 21%, 29%, and 62% at furfural

concentrations of 1, 2, and 4 g/L, respectively [Haroun et al., 2016]. The revivability of inhibited sludge was also tested by removing furfural from the feed and H_2 yield of 1.6 mol/mol hexose was achieved compared to the 2.3 mol/mol hexose achieved before furfural addition [Haroun et al., 2016].

2.2.4.3 H_2 production potential of hydrolysates

Various types of hydrolysates have been tested for their fermentative H_2 production potential. Table 2.6 shows some potential biomass hydrolysates for fermentative H_2 production that have been investigated in the literature. High H_2 yields have been reported from hydrothermal, steam explosion, and dilute acid pretreated hydrolysates (Table 2.6). Datar et al. [2007] reported a high H_2 yield of 270 mL/gCOD from corn stover hydrolyzed using steam explosion using ADS. Kongjan et al. [2010] reported H_2 yield of 298 mL/gCOD initial from hydrothermal pretreated wheat straw using anaerobic sludge, however, this was associated with very low H_2 production rate of 0.8 mL/hr. Dilute acid hydrolysis has been reported as an effective pretreatment method associated with high yields such as the 234 and 174 mL/gCOD produced from sunflower stalks and corn cobs using ADS, respectively [Monlau et al., 2013b; Nasr et al., 2014]. The increase in H_2 production yields from hydrothermal and steam explosion hydrolysates over the dilute acid one may not be feasible economically considering how energy intensive these methods are [Nissila et al., 2014].

Table 2.6 - H_2 production potential from hydrolysates

Lignocellulosic biomass	**Pretreatment method**	**Inoculum**	**H_2 Yield ($mL/gCOD_i$)**	**HPR* (mL/hr)**	**Reference**
Wheat straw	Hydrothermal	AS	298	0.8	Kongjan et al., 2010
Marine algae	Hydrothermal	ADS	110	3.1	Jung et al., 2011
Sunflower stalks	Dilute acid	ADS	234	-	Monlau et al., 2013b
Corn cobs	Dilute acid	ADS	174	8.7	Nasr et al., 2014
Sugarcane bagasse	Dilute acid	Elephant dung	94	0.2	Fangkum & Reungsang, 2011b
Sugarcane bagasse	Acid	*C. butyricum*	129	4.7	Pattra et al., 2008
Corn stover	Steam explosion	ADS	270	-	Datar et al., 2007
Corn stalks	Dilute Acid	Cow dung compost	106	7.6	Zhang et al., 2007
Beet pulp	Alkaline	AS	116	-	Ozkan et al., 2011

* HPR: H_2 Production Rate

2.3 Biological H_2 Production Modeling

Modeling fermentative H_2 production is one of the most critical requirements for improving our ability to predict biohydrogen processes and parameters that are essential for systems design, control, optimization, and scale-up [Prakasham et al., 2011]. Improving H_2 production kinetics would decrease reaction times, which is reflected in system size as well as capital and operational costs reduction.

2.3.1 Gompertz kinetics

The modified Gompertz equation (Equation 2.8) was commonly used in the literature to model biohydrogen production, where P is the cumulative H_2 production, P_{max} is the maximum cumulative H_2 production, R_{max} is the maximum H_2 production rate, λ is the lag time, and t is the fermentation time [Lay et al., 1999]. Although Gompertz kinetic parameters are important for better understanding H_2 production systems, however, it does not reflect a whole picture of the process lacking substrate utilization and microbial growth parameters. Most of the studies in the literature that reported Gompertz kinetics ignored other kinetic parameters like Monod kinetic parameters [Hu et al., 2013; Zhao et al., 2011; Liu et al., 2011]. A correlation between Gompertz and other kinetic models would be very useful and a lot of kinetic parameters would be estimated from Gompertz kinetic parameters available in the literature.

$$P = P_{max}\, exp\left\{-exp\left[\frac{R_{max}\, e}{P_{max}}(\lambda - t) + 1\right]\right\} \tag{2.8}$$

2.3.2 Monod kinetics

The classical Monod kinetic model and its various modified forms have been successfully used to describe the cell growth kinetics as a function of substrate for biological H_2 production [Gnanapragasam et al., 2011]. Equation 2.9 describes the basic Monod model [Lobry et al., 1992]:

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{2.9}$$

where μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (g/L), K_s is the saturation concentration (g/L) or half-velocity constant and is equal to the concentration of the rate-limiting substrate when the specific growth rate is equal to one half of the maximum. The Monod kinetic model has also been used to describe substrate utilization as well as the effect of substrate concentration on substrate degradation rates, H_2-producing bacterial growth, and H_2 production [Wang and Wan, 2009]. Most of the studies in the literature that have reported Monod kinetics for H_2 production systems have focused on substrate utilization and microbial growth parameters ignoring H_2 production parameters such as yields and rates and sometimes even not reporting H_2 production potential data [Hernandez, 1982; Ng et al., 1977; Linville et al., 2013]. Table 2.7 shows the Monod kinetic parameters; maximum specific growth rate (μ_{max}) and the half velocity constant (K_s) reported by many studies in the literature. As depicted in Table 2.7, the Monod kinetic parameters reported in the literature vary widely depending on the culture type, substrate type, as well as other operational conditions like pH and temperature. For instance, glucose consumption was associated with the maximum specific growth rates in the range of 0.03-0.17 h^{-1} in studies using mixed cultures [Sharma and Li, 2009; Mullai et al., 2013], while a higher value of 0.4 h^{-1} was obtained using a pure culture [Nath et al., 2008].

Table 2.7 - Monod Kinetic Parameters for mixed and pure cultures

Inoculum	**Reactor**	**T (°C)**	**Substrate**	**μ_{max} (h^{-1})**	**K_s (g/L)**	**Reference**
Soil from organic farm	Batch	30	Glucose	0.03	-	Sharma and Li, 2009
Activated Sludge	Fed-Batch	35	Glucose	0.13	0.01	Fernandez et al., 2010
Activated Sludge	Sequential Batch	35	Glucose	0.16	0.01	Fernandez et al., 2011
Sediments	Batch	35	Glucose	0.17	0.11	Mullai et al., 2013
AS	CSTR	35	Sucrose	0.17	0.06	Chen et al., 2001
ADS	Batch	37	Starch	0.05	0.20	Gupta et al., 2015
ADS	Batch	37	Cellulose	0.05	2.10	Gupta et al., 2015
Enterobacter cloacae	Batch	34	Glucose	0.40	5.51	Nath et al., 2008
C. termitidis	Batch	37	Glucose	0.30	0.87	Gomez-Flores et al., 2015
Ca. saccharolyticus	Batch	70	Sucrose	0.13	0.75	van Niel et al., 2003
Th. Thermosaccharolyticum	Batch	60	Sucrose	0.31	1.47	O-Thong et al., 2008
C. termitidis	Batch	37	Cellobiose	0.34	0.37	Gomez-Flores et al., 2015
C. thermocellum	Batch	58	Cellobiose	0.57	0.92	Linville et al., 2013

2.4 Biological H_2 Production Modeling

H_2 production has shown high potential to replace fossil fuels with a great advantage of using lignocellulosic wastes. However, it hasn't reached the commercial stage yet because of the low production yields, rates, and efficiencies reported. Therefore, more research should be directed to enhance H_2 production by overcoming the obstacles towards commercialization which include, low H_2 production yields and rates, as well as lack of substrate utilization and microbial kinetics data. An extensive literature search revealed the following knowledge gaps:

- The impact of furfural and HMF on co-fermentative H_2 production
- Comparing the use of MADS, TMADS, and TADS for H_2 production from poplar wood hydrolysate, with no studies that have been conducted using ADS obtained from a full-scale thermophilic anaerobic digester
- Biogas cleanup methods for pure H_2 utilization
- Impact of CO_2 sequestration on microbial community structures and metabolic pathways from a thermodynamic perspective in continuous-flow systems
- Contradictory data for kinetic parameters on glucose and cellobiose utilization using *Clostridium thermocellum*
- Limited data on Monod and Gompertz kinetics for *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum* utilizing glucose
- Limited data on H_2 production kinetics on cellulose and starch using *Clostridium saccharoperbutylacetonicum*
- Contradictory data on the ability of *Clostridium beijerinckii* for degrading starch
- No available data on co-culturing the cellulose degrading *Clostridium saccharoperbutylacetonicum* and the glucose utilizing *Clostridium beijerinckii*

This study investigated the potential of real lignocellulosic wastes for H_2 production in batch studies using mixed cultures at mesophilic and thermophilic conditions. Then the effect of headspace CO_2 removal was tested in a continuous system to study the effect on H_2 production parameters as well as the microbial community structure. The aforementioned studies promoted the work on pure cultures in order to

fully understand the substrate utilization, microbial growth, and H_2 production kinetics with implementing various techniques for enhancing H_2 production properties such as co-culturing and co-fermentation processes. In light of the highlighted paucity of information on fermentative H_2 production, the novelty of this research stems primarily in:

- Assessing the potential inhibitory impact of furfural and HMF in a co-fermentation study using pretreated corn cobs
- Evaluating the impact of monomeric-to-polymeric sugars composition in a co-fermentation study on H_2 production yields and rates
- Providing Monod kinetic parameters for MADS, TMADS, and TADS on poplar wood hydrolysate
- Evaluating the impact of CO_2 sequestration on H_2 production yields and rates, chemical buffering requirements, metabolic pathways, and microbial community structure in a continuous-flow system
- Providing Monod kinetic parameters of *Clostridium thermocellum* on cellobiose and glucose
- Providing Monod and Gompertz kinetic parameters for *Clostridium beijerinckii* and the new H_2 producer *Clostridium saccharoperbutylacetonicum* as mono- and co-culture on glucose, starch, and cellulose as mono-and co-substrate
- Confirming the inability of *Clostridium beijerinckii* to utilize insoluble starch
- Investigating the potential of cellulose degradation by the new H_2 producer *Clostridium saccharoperbutylacetonicum*
- Assessing the effect of co-substrate and co-culture on H_2 production and substrate utilization kinetics using *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum*

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Chapter 3

Biohydrogen Production from Pretreated Corn Cobs

3.1 Introduction

A wide variety of feedstocks and wastes that are rich in carbohydrate content have the potential to produce hydrogen using dark fermentation [Azbar and Levin, 2012]. A number of studies have utilized real waste streams for biohydrogen production like sweet potato-starch residue [Yokoi et al., 2002], insoluble co-products of wheat starch food industry [Hussy et al., 2003], sugarcane bagasse [Pattra et al., 2008], thin stillage from bioethanol processing [Nasr et al., 2012], and cassava stillage from ethanol processing [Luo et al., 2010].

Lignocellulosic biomass, of which two thirds are carbohydrate polymers of cellulose and hemicellulose [Ren et al., 2009a] is the most abundant raw material. Corn cobs contain 32.3%-45.6% cellulose, 39.8% hemicelluloses-mostly pentosan, and 6.7%-13.9% lignin [Zych, 2008]. Cellulose is a linear polymer of cellobiose (glucose-glucose dimer) and upon hydrolysis yields free glucose molecules. Hemicellulose, on the other hand, consists mainly of xylose, arabinose, galactose, glucose, and mannose which are easily fermentable [Hamelinck et al., 2005]. Prehydrolysis is required to convert carbohydrate polymers to fermentable monomeric sugars [Ren et al., 2009a].

Xylose is the second most common product of saccharification of organics after glucose [Lin and Chen, 2006]. Lin and Chen [2006] investigated mesophilic hydrogen production from xylose using a mixed anaerobic culture in both chemostat and batch bioreactors, and achieved hydrogen yields of 0.7 and 2.25 mol H_2/mol-xylose, respectively, with the major observed VFAs being acetate, propionate, and butyrate, with butyrate as the major component. Danko et al. [2008] observed a hydrogen yield of 1.98 mol H_2/mol substrate consumed for arabinose at a concentration 10 g/L using a mixed-culture anaerobic sludge and the soluble products released in addition to n-butyrate were formate, propionate, valerate, and ethanol. Cheng et al. [Cheng et al., 2012] obtained a hydrogen yield of 1.12 mol H_2/mol xylose while de Sa et al. [de Sá et al., 2013] achieved

1.88 mol H_2/ mol xylose, both using mesophilic anaerobic sludge. Yokoi et al. [1995] studied hydrogen production using a mesophilic facultative anaerobe, *Enterobacter aerogenes* strain HO-39 and, obtained hydrogen yields of 0.95, 0.98, and 2.16 mol H_2/mol-substrate for the monosaccharides galactose, and mannose as well as the disaccharide, maltose, respectively. In a more recent study, *Enterobacter aerogenes* IAM 1183 utilized xylose, galactose, and mannose mesophilically yielding 2.2, 2.35, and 2.62 mol H_2/ mol substrate, respectively [Ren et al., 2009b]. Ghosh and Hallenbeck [2009] studied *Escherichia coli* strain DJT135 for mesophilic biohydrogen production from arabinose, galactose, maltose, and xylose, and achieved hydrogen yields of 1.02, 0.69, 0.72 and 0.57 mol H_2/ mol-substrate, respectively.

Apart from carbohydrates and depending on the raw material and the pre-treatment applied, the resulting hydrosylates may contain substances such as furfural and HMF that could be potentially inhibitory to fermentation [Klinke et al., 2004]. Furfural derivatives affect microbial growth by interfering with glycotic and/or fermentative enzymes and also disturb the membrane integrity of diverse microorganisms, with concentrations as low as 1g/L considered inhibitory [Quéméneur et al., 2012]. Quéméneur et al. [2012] assessed the impact of 1 g/L furfural and HMF concentrations on H_2 production from xylose at 5 g/L concentration by anaerobic digester sludge, and observed inhibition of H_2 production in terms of the duration of the lag phase, H_2 yield, and maximum H_2 production. In the aforementioned study, H_2 yields decreased from 1.67 mol H_2/ mol xylose in the control (xylose-only) batch bottles to 0.45 (±0.10) mol H_2/ mol xylose, and with no gas production from furfural or HMF when added as the sole carbon source at 1g/L.

HMF compromises the cell membrane integrity, and intracellular sites are the primary inhibition targets [Mills et al., 2009]. Microorganisms are known to relieve the inhibitory effects of these furan compounds by metabolic pathway switching, thereby converting HMF and furfural to less toxic compounds, provided the initial concentrations are not beyond threshold levels [Boyer et al., 1992]. Furfural is converted to furfuryl alcohol and furoic acid while HMF is converted to 5-hydroxymethyl furfuryl alcohol or 2,5-bis-hydroxymethylfuran [Liu et al., 2005; Boopathy et al., 1993; Liu et al., 2004]. Chemical potential fluctuations in the microenvironment, differences in the type and

quantity of microorganisms, pH variations, and concentrations affect the metabolic pathways.

Co-fermentation of different organic residues has demonstrated enhanced hydrogen production in a number of studies suggesting synergistic and complementary effects [Wang et al., 2013]. Some of the reported advantages of co-digestion are dilution of toxic compounds, improved nutrients balance, improved buffering capacity, and synergistic microbial effects [Wang et al., 2013]. Fangkum and Reungsang [2011a] studied the thermophilic co-digestion of xylose and arabinose at 2.5 g/L each concentrations using anaerobic mixed cultures, and obtained a maximum hydrogen yield of 2.59 mol H_2/mol-sugar consumed with 95% substrate degradation. Substrate degradation was observed to decrease with the increase in xylose/arabinose concentrations.

In light of the reported advantages of co-fermentation as well as limited literature on the impact of HMF and furfural on biohydrogen production, the main objectives of this study were to: a- evaluate the co-fermentability of four different pretreated corn cob streams at different mixing ratios; b- assess the potential inhibitory impact of furfural and HMF; and c- examine the impact of monomeric-to-polymeric sugars composition on H_2 yields and rates. This study examined the biodegradation of specific polymeric carbohydrates, that is, arabinose, xylose, mannose, galactose, and glucose.

3.2 Materials and Methods

3.2.1 Seed sludge and substrate

Anaerobic digester sludge (ADS) was collected from St. Mary's wastewater treatment plant (St. Mary's, Ontario, Canada) and preheated at 70°C for 30 min prior to use. Four different pretreated corn cob streams, for potential use in the bioethanol industry, were obtained from an industrial facility (Ontario, Canada) and used as substrates. Table 3.1 shows the characteristics of the four streams where sugars including xylose, mannose, galactose, and glucose were measured in both their polymeric and monomeric forms as explained in the analytical methods section. Dilute Acid

Pretreatment (DAP) using sulphuric acid and High Pressure Autohydrolysis (HPA) at a temperature of 235°C were used as a first stage pretreatment to facilitate the second stage pretreatment for hemicellulose solubilization. Purge and Squeeze streams differ in their location in the cellulosic pretreatment process; where “Purge” is taken from a steam percolation reactor during cooling while “Squeeze” is recovered from the cooked biomass via pressing. The four streams are denoted henceforth as DP (dilute acid pretreatment - purge stream), DS (dilute acid pretreatment - squeeze stream), HP (high pressure autohydrolysis pretreatment - purge stream), and HS (high pressure autohydrolysis pretreatment - squeeze stream).

Table 3.1 - Substrates characteristics

	HPA-Purge (HP)	**HPA-Squeeze (HS)**	**DAP-Purge (DP)**	**DAP-Squeeze (DS)**
Solids (%)	6.69	14.14	4.14	8.21
pH	3.40	3.82	2.31	2.37
Sugars (polymers)				
Arabinose (g/L)	5.63	7.94	3.60	6.24
Xylose (g/L)	31.85	89.19	23.22	55.00
Mannose (g/L)	0.28	0.48	0.22	0.22
Galactose (g/L)	2.13	3.60	1.48	2.71
Glucose (g/L)	5.21	9.01	4.43	7.96
Total poly-sugars (g/L)	**45**	**110**	**33**	**72**
Sugars (monomers)				
Arabinose (g/L)	2.45	6.36	2.37	4.46
XMG* (g/L)	3.97	25.18	14.39	44.83
Glucose (g/L)	1.95	4.01	1.91	4.52
Total mono-sugars (g/L)	**8**	**36**	**19**	**54**
(M/P)%**	19	32	57	75
VFAs				
Succinate (g/L)	0.00	0.00	0.00	0.00
Formate (g/L)	0.18	2.49	0	0
Acetate (g/L)	1.35	5.70	3.10	2.61
HMF (g/L)	0	0.64	0	0.73
Furfural (g/L)	0	2.27	3.79	0

*XMG: Total xylose, mannose, and galactose concentrations

**(M/P)%: monomeric-sugars to polymeric-sugars percentage

3.2.2 Batch setup

Batch anaerobic experiments were conducted in serum bottles with a liquid volume of 200 mL. Volumes of substrates and seed were calculated based on a substrate to-biomass (S°/X°) ratio of 2 gCOD/gVSS using the following equation:

$$S°/X° = \frac{V_{sub}(L) * TCOD_{eq}(\frac{g}{L})}{V_{seed}(L) * VSS_{seed}(\frac{g}{L})} \quad (3.1)$$

Where V_{sub} is the volume of substrate, V_{seed} is the volume of seed, and $TCOD_{eq}$ is the equivalent total chemical oxygen demand (TCOD) for different volumetric mixing ratios of the four streams (HP, HS, DP, and DS) as shown in Table 3.2. A total of 18 different mixing ratios for the four streams were tested with no replication. A control batch was prepared using ADS without any substrate. The initial pH for the mixed solution in each bottle was adjusted to 5.50±0.04 using HCl and NaOH. A 5 g/L buffer solution ($NaHCO_3$) was also added for pH control.

Table 3.2 - Experimental substrates mixing ratios

Batch #	% Volume				$TCOD_{eq}$	M/P	Initial HMF	Initial Furfural
	HP	HS	DP	DS	g/L	%	g/L	g/L
1	100	0	0	0	92	19	0.00	0.00
2	0	100	0	0	121	32	0.11	0.39
3	0	0	100	0	63	57	0.00	1.09
4	0	0	0	100	107	75	0.14	0.00
5	50	50	0	0	107	28	0.06	0.22
6	50	0	50	0	77	35	0.00	0.47
7	50	0	0	50	99	53	0.07	0.00
8	0	50	50	0	92	38	0.07	0.66
9	0	50	0	50	114	97	0.12	0.21
10	0	0	50	50	85	69	0.08	0.44
11	25	25	25	25	96	45	0.07	0.32
12	35	15	15	35	97	48	0.07	0.19
13	15	35	35	15	94	42	0.07	0.45
14	15	55	15	15	106	38	0.09	0.35
15	15	15	55	15	82	48	0.05	0.57
16	15	15	15	55	105	57	0.10	0.18
17	33.3	0	33.3	33.3	87	54	0.05	0.29
18	33.3	33.3	0	33.3	107	43	0.09	0.15

3.2.3 Analytical methods

The biogas production was measured using appropriately sized glass syringes in the range of 5-100 mL. The gas in the headspace of the serum bottles was released to equilibrate with the ambient pressure [Nasr et al., 2011]. The biogas composition including hydrogen, methane, and nitrogen was determined by a gas chromatograph (Model 310, SRI Instruments, Torrance, CA) equipped with thermal conductivity detector (TCD) and a molecular sieve column (Mole sieve 5A, mesh 80/100, 6 ft x 1/8 in). Argon was used as the carrier gas at a flow rate of 30 mL/min and the temperature of the column and the TCD detector were 90°C and 105°C, respectively. Total and soluble chemical oxygen demand (TCOD/ SCOD) were measured using HACH methods and test kits (HACH Odyssey DR/2500 spectrophotometer manual) [Nasr et al., 2011]. TSS and VSS were analyzed using standard methods [Clesceri et al., 1998]. Polymeric sugars were measured based on the NREL method [Sluiter et al., 2012] and an additional analytical step was added where the sugar monomers were acetylated into alditols and quantified by gas chromatography (Tappi method 249 cm-85). Monomeric sugars were measured using an HPLC, consisting of an Agilent 1200 isocratic pump, autosampler, column compartment, and a refractive index detector (RID). The method parameters were: pump run time was 50 minutes; pump flow rate was 0.6 ml/min; mobile phase of 5.0 mM H_2SO_4; a column temperature of 65ºC, a detector temperature of 35ºC, and an injection volume of 10 µL. Components were separated using PL Hi-Plex guard column (50x7.7) and Hi-Plex H column (300x7.7) from Agilent (Palo Alto, CA, USA). Sulphuric acid (0.005M) was used as the mobile phase at a flow rate of 0.7mL min^{-1}. The column temperature was maintained at 60°C. Data was acquired and processed using Agilent ChemStation for LC systems software version B.04.01 (Agilent, Palo Alto, CA, USA).

3.3 Results and Discussion

3.3.1 Biohydrogen production

Figure 3.1 shows the H_2 production profiles after deducting the blank (inoculum only) for the various individual and mixtures of HP, HS, DP, and DS mixtures. The

maximum H_2 production potential of 141 mL/$gCOD_{initial}$ was achieved in batch 9 with an HS:DS mixing ratio of 50/50 % by volume, and the highest monomeric-to-polymeric sugars (M/P)% of 97%. It is noteworthy that the four highest batches (9, 10, 7, and 16) consisted of 50% or more DS, which has the highest individual H_2 production potential of the four streams. Also, two lag phases which can be attributed to the similar furfural and HMF concentrations in both batches with DS as the main stream were observed in batches 9 and 16. Batch 2 with HS as the substrate had the lowest hydrogen production potential of 5 mL/$gCOD_{initial}$. Although HP has a lower (M/P)% of 19% compared to the 32% of HS, batch 1 had a higher H_2 production of 23 mL/$gCOD_{initial}$ than batch 2. As depicted in Figure 3.1, the lag phases were mostly less than 4 hours for both individual and co-substrate fermentations. A mildly negative correlation ($R^2 = 0.61$) between the lag phase and the (M/P)% was observed, i.e., the higher the (M/P)%, the lower was the lag phase since more monomeric sugars were available for degradation and less polymeric sugars needed to be hydrolyzed prior to utilization.

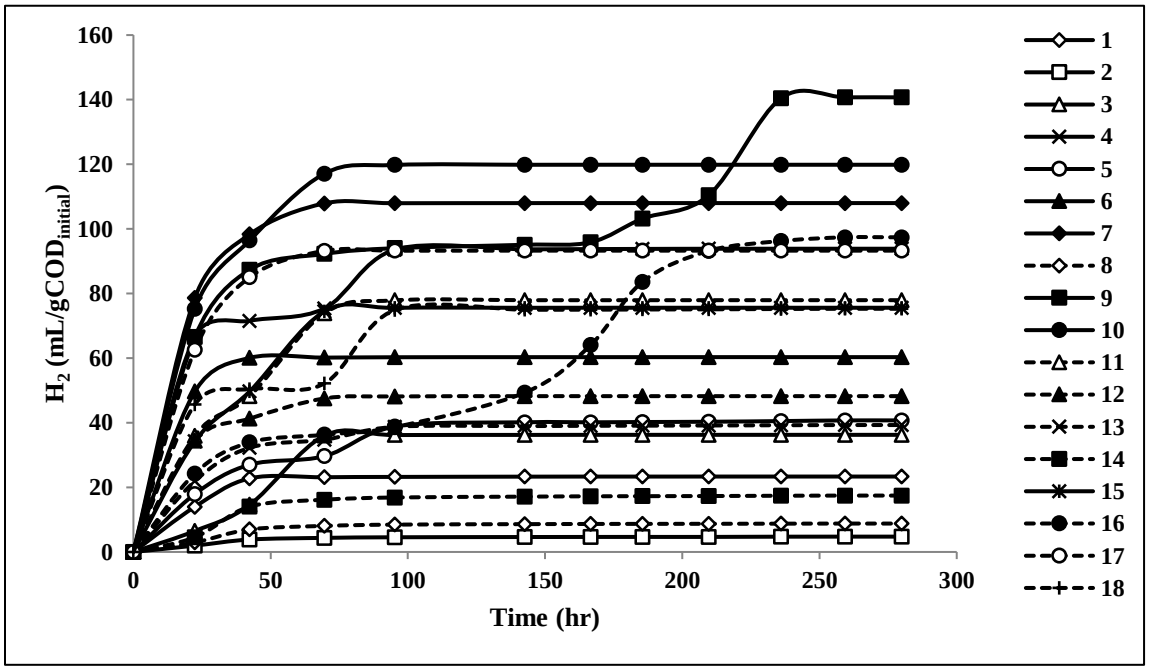

Figure 3.1 - Hydrogen Production Profile

3.3.2 Hydrogen yields and production rates

Figure 3.2 shows the H_2 yields based on the sugars consumed (as COD). As depicted in Figure 3.2, amongst the individual substrates (batches 1-4), the highest hydrogen yield of 94 mL H_2/gCOD sugars consumed was achieved for DS and the lowest hydrogen yield of 5 mL H_2/gCOD sugars consumed was observed for HS. It seems that the presence of the HMF, furfural, and acetate in the substrate had a negative impact on the hydrogen production when the M/P ratio is low. As shown in Table 3.1, the M/P ratio in the HS was only 32%, compared to 75% in the DS. Although the M/P of the DP was higher than that of the HP (57% vs 19%), the hydrogen yields of the two substrates were very close (36 and 31 mL H_2/gCOD sugars consumed) for the DP and HP, respectively. This may be attributed to the absence of the furfural and the relatively low acetate concentration. Figure 3.2 also shows that for runs 5 to 10, mixing the two substrates improved the hydrogen yields for all mixtures except batch 8 (mixture of HS and DP at 50/50 by volume). The highest hydrogen yield of 265 mL H_2/gCOD sugars consumed was achieved when DP was mixed with DS, followed by 148 mL H_2/gCOD sugars consumed for HS and DS mixture. The lowest hydrogen yield of 9 mL H_2/gCOD sugars consumed was observed for HS and DP mixture. When the four substrates were mixed at different ratios (batches 11-16), there was a slight enhancement in the hydrogen yield compared with the individual substrate. The highest hydrogen yield in the range of 101 mL H_2/gCOD sugars consumed was observed when DS was predominant in the mixture (55%). When DP was predominant in the mixture (55%), the hydrogen yield of 82 mL H_2/gCOD sugars consumed was observed. This is consistent with the hydrogen yields of mixing the two aforementioned substrates (50:50)% (batches 5-10) as the maximum hydrogen yield was achieved when DP and DS were mixed together (batch 10). Furthermore, the maximum individual hydrogen yields for the single streams were achieved for DS and DP, respectively. In batches 17 and 18, where three substrates were mixed, a hydrogen yield of 97 mL H_2/gCOD sugars consumed was observed for HP, DP, and DS mixture and 78 mL H_2/gCOD sugars consumed for HP, HS, and DS mixture. This also confirms that the presence of both DP and DS increased the H_2 yield.

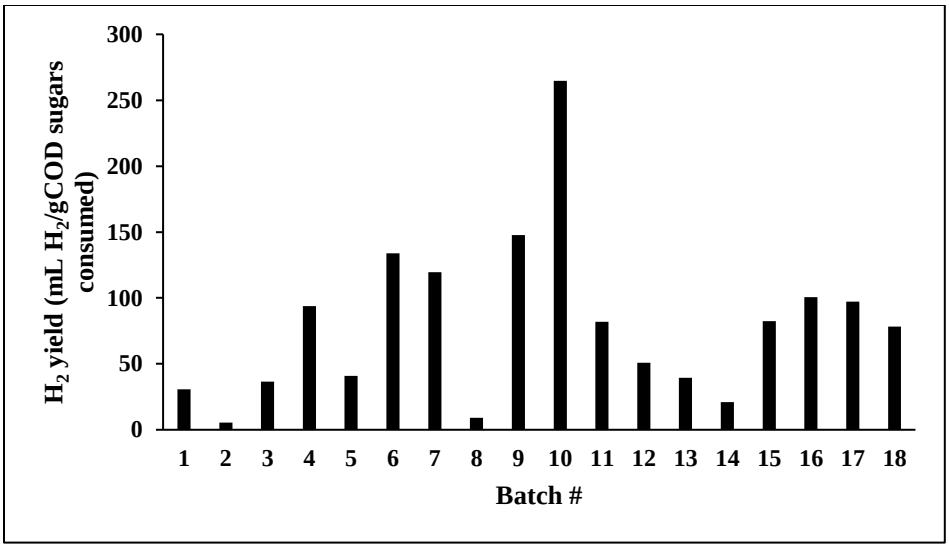

Figure 3.2 - Hydrogen Yields

The maximum yield of 265 (mL H_2/gCOD sugars consumed) obtained for the DP:DS mix was only 50% of the theoretical yield of 527 (mL H_2/gCOD sugars consumed) based on 4 mol H_2/mol hexose. However, this maximum yield was 50% higher than the maximum yield achieved in batch experiments using thin stillage from a conventional ethanol plant as the substrate, at the same (S°/X°) ratio using ADS as the seed [Nasr et al., 2011].

Figure 3.3 shows the H_2 yields (mol/mol $_{\text{T-sugars initial}}$) and the maximum H_2 production rates (MHPR) (mL/hr). The highest MHPR of about 8.8 mL/hr was achieved in batches 4, 7, and 10 and the lowest MHPR of 0.4 mL/hr was observed in batch 2. A positive correlation was observed between the MHPR and the H_2 yield, which is consistent with Nasr et al. [2011] who observed the same behaviour in batch experiments using thin stillage as the substrate. Fangkum and Reungsang [2011b] reported a H_2 yield of 0.34 mol/mol$_{\text{T-sugars initial}}$ in a batch experiment using preheated elephant dung as inoculum and sugarcane bagasse hydrolysate as the substrate at similar operating conditions of pH 5.5 and mesophilic temperature, which is only 20% of the average H_2 yield of 1.72 mol/mol$_{\text{T-sugars initial}}$ observed in this study. H_2 yields and MHPR correlated positively with the (M/P)% with R^2 values of 0.70 and 0.69, respectively, i.e., higher H_2 production yields and rates at higher (M/P)% are attributed to the availability of more readily-fermentable monomeric sugars.

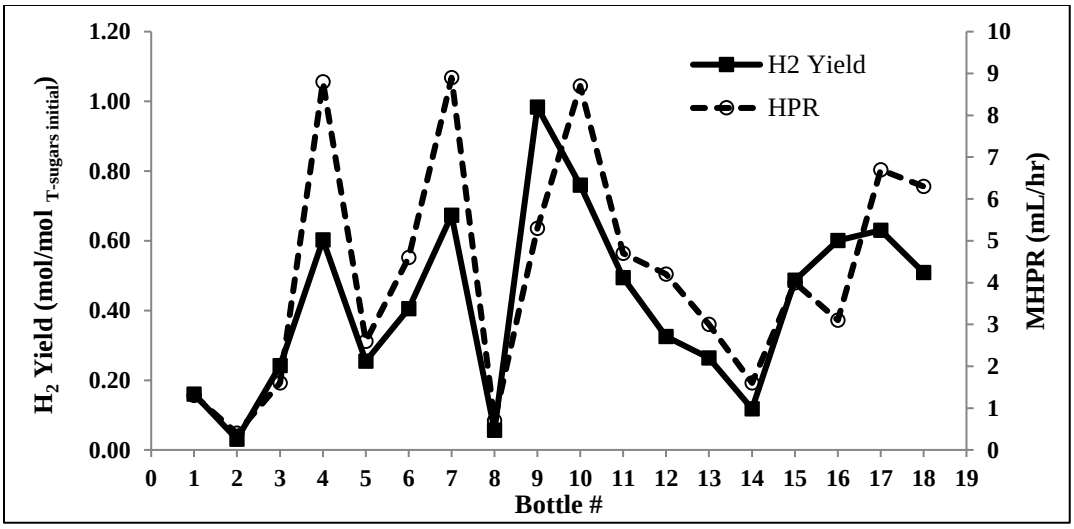

Figure 3.3 - Maximum H_2 Production Rates and Yields

3.3.3 Conversion of sugars

Figure 3.4 shows the initial and final concentrations of polymeric sugars in all batch experiments. Degradation efficiency of monomeric sugars for all batches was 100%. An average conversion efficiency of 94% for polymeric sugars was observed in all batches except batches 6 and 10. These were the two anomalies that could not be explained, with both batches exhibiting only 45% degradation efficiency.

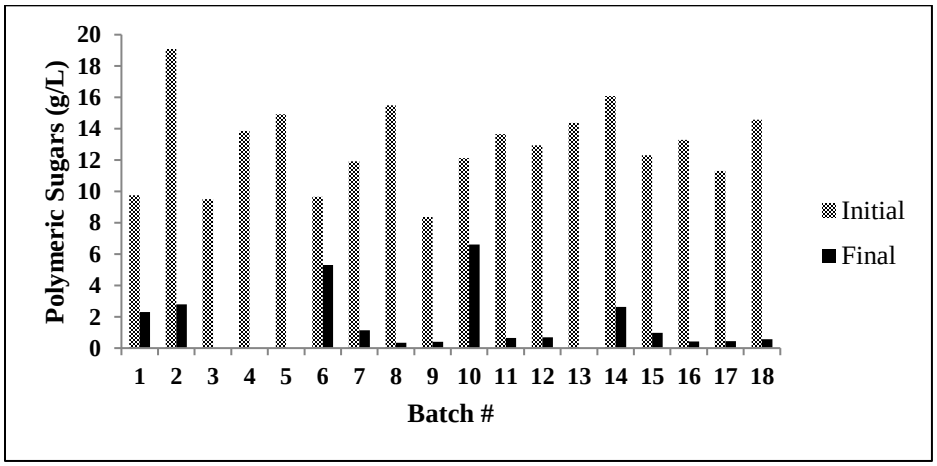

Figure 3.4 - Initial and Final Polymeric Sugars Concentrations

3.3.4 Impact of HMF and furfural

No correlation was observed between initial HMF and furfural with the MHPR and the H_2 production yield. The HMF concentrations in the different batches ranged from 0.05-0.14 g/L. On addition of furfural and HMF at 1 g/L each, Quéméneur et al. [2012] observed a decrease in hydrogen yield from 1.67 to 0.45 mol H_2/mol xylose. de Vrije et al. [2009] studied the effects of 0-4 g/L of furfural and HMF on H_2 production and growth of the pure thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. *C. saccharolyticus* was observed to be more sensitive than *T. neapolitana* with 1-2 g/L of furfural and HMF identified as the concentrations at which 50% inhibition of growth and H_2 production was observed (IC_{50}). The observed discrepancy in the impact of furfural on biohydrogen production between this study and the two aforementioned studies [Quéméneur et al., 2012; de Vrije et al., 2009] is attributed to the widely different ratios of initial substrate concentration to furfural and/or HMF. On average, the ratio of initial substrate concentration to initial furfural and HMF was 30:1, which nullified the effect of these inhibitors.

3.4 Conclusions

The outcome of this study revealed the high impact of monomeric-to-polymeric sugars ratio on the co-fermentability of four different partially hydrolyzed corn cob streams. It appears that the fermentability of the dilute acid streams was better than the high pressure streams. The following conclusions can be drawn:

- The maximum H_2 production potential of 141 mL/$gCOD_{initial}$ was achieved from the co-fermentation of HS and DS
- The maximum H_2 yield of 265 (mL/gCOD sugars consumed) was achieved using DP and DS co-substrate
- A positive correlation between H_2 production rates and yields was observed
- The ratio of monomeric-to-polymeric sugars correlated positively with H_2 production rates and yields, and negatively with the lag times

- HMF in the range of 0.05-0.14 g/L did not impact H_2 production and hydrogen yield
- Furfural concentration of 0.21-1.09 g/L had no discernible impact on H_2 production and yield

3.5 References

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Chapter 4

Comparative Assessment of Mesophilic and Thermophilic Biohydrogen Production from Poplar Wood Hydrolysate

4.1 Introduction

Hydrogen production from lignocellulosic materials through anaerobic dark fermentation is recognized as a potential and environmental friendly process and can be an effective way to utilize lignocellulosic waste biomass [Qiu et al., 2016]. Lignocellulosic materials from agriculture and forest management are the largest sources of carbohydrates, mainly hexose and pentose, and possess the potential for biofuels production [Singh et al., 2014; Nissilä et al., 2014]. Hexose and pentose sugars from lignocellulose can be effectively converted to various biofuels with relatively high yields and productivity, including bioethanol [Sommer et al., 2004] and biohydrogen [Zhang et al., 2015; Haroun et al., 2016] through dark anaerobic fermentation.

Dark fermentative H_2 production can be operated at mesophilic (25-40°C), thermophilic (40-65°C), and extreme thermophilic (65-80°C) conditions [Cavinato et al., 2011]. Mesophilic digestion has been commonly adopted for fermentative H_2 production in many studies [Temudo et al., 2009; Nasr et al., 2011; Makinen et al., 2012; Haroun et al., 2016]. Recently, thermophilic digestion has attracted much attention for H_2 production [Kim and Kim, 2012; Gokfiliz and Karapinar, 2016; Zheng et al., 2016] due to the many advantages such as efficient utilization of complex substrates, better thermodynamic conditions, and suppression of methanogens [Shanmugam et al., 2014; Zhang et al., 2015]. Moreover, the predominance of some efficient H_2-producing thermophiles, such as *Thermoanaerobacterium* spp., is considered as a key microbial factor responsible for better performances in these cases [Zhang et al., 2015].

Many studies have reported enhancement in H_2 production parameters using mesophilic culture operated at thermophilic temperature. Zhang et al. [2015] studied biohydrogen production from corn stover acid hydrolysate at a concentration of 5 g /L and a pH of 7 in batches using anaerobic granular sludge obtained from a bench-scale expanded granular sludge bed reactor treating starch wastewater. The abovementioned culture was tested at mesophilic temperature (37°C) and thermophilic temperature (55°C) at an S°/X° ratio of 5.6 g COD/gVSS. The authors reported that the H_2 production yield at thermophilic temperature (55°C) was 802 mL H_2/L (0.95 mol H_2/ mol hexose) with acetate and butyrate as the predominant soluble by-products while at mesophilic temperature (37°C), H_2 production yield was 223 mL H_2/L (0.32 mol H_2/ mol hexose) with predominantly acetate, ethanol, and propionate as the soluble by-products. In the aforementioned study, the authors attributed better H_2 production at thermophilic conditions to the selective enrichment of some efficient H_2-producing thermophiles, which are capable of producing more H_2 by utilizing complex substrate components. Luo et al. [2010] studied biohydrogen production from cassava stillage at a concentration of 26.9 g sugar/L (40 gVS/L), a pH range from 5.4 to 5.8, and an S°/X° ratio of 2.4 gCOD/gVSS using mesophilic anaerobic digester sludge obtained from an up-flow anaerobic sludge blanket (UASB) reactor operating at mesophilic temperature (37°C) and a thermophilic temperature (60°C) in a continuously stirred tank reactor (CSTR) and obtained hydrogen production yields of 14 and 70 mL H_2/gVS at mesophilic and thermophilic conditions, respectively. The aforementioned authors attributed the better performance of mesophilic sludge at thermophilic conditions to the lower propionate production and lower activity of homoacetogens. In the abovementioned study, although the acetate-to-butyrate ratio was higher at mesophilic temperature, however, the decrease in propionate production at the thermophilic temperature resulted in the higher H_2 yield. The distribution of VFAs was quite different as butyrate was the main soluble by-product at thermophilic temperature with an acetate-to-butyrate ratio of 0.3, while butyrate, propionate, and acetate were predominant at mesophilic temperature with an acetate-to-butyrate ratio of 0.1, with propionate concentration 5.4 times higher than that observed at the thermophilic one [Luo et al., 2010].

Other studies reported H_2 production enhancement using thermophilic anaerobic cultures or acclimatized thermophilic cultures compared to mesophilic cultures. Cheng and Liu [2012] studied biohydrogen production from raw cornstalk and a mixture of raw and fungal pretreated cornstalk using mesophilic and thermophilic cultures, obtained from a 4 L anaerobic digester treating glucose for more than 6 months and reported the highest H_2 production yield of 54 mL/gVS for the experiment utilizing thermophilic seed with raw and pretreated cornstalk mixture as the substrate, producing acetate, butyrate, propionate, and ethanol as the main by-products. Cakır et al. [2010] investigated biohydrogen production from acid-hydrolyzed wheat starch at an initial total sugars concentration of 18.5 g/L and a neutral pH using mesophilic anaerobic digester sludge at mesophilic temperature (37°C) and thermophilic temperature (55°C). The mesophilic anaerobic digester sludge was acclimatized at 55°C using glucose at a concentration of 60 g/L for 3 days prior switching to acid-hydrolyzed wheat starch at a concentration of 18.5 g/L at thermophilic temperature. The aforementioned authors reported that dark fermentative H_2 production of acid-hydrolyzed ground wheat was more beneficial at thermophilic conditions (55°C) than mesophilic conditions (37°C). A yield of 2.4 mol H_2/mol hexose consumed was obtained at thermophilic temperature compared to 1.6 mol H_2/ mol hexose consumed at mesophilic condition. Interestingly, the lag phase for thermophilic fermentation (31.6 hr) was much lower than for mesophilic one (44.3 hr). Total final VFAs were much higher at thermophilic fermentation (10.1 g/L) compared to at mesophilic one (6.9 g/L) suggesting that VFAs and biohydrogen production were directly related as high final VFAs concentrations yielded high hydrogen production [Cakır et al., 2010]. Lab-scale studies that acclimatized biomass to thermophilic temperature utilize synthetic carbon source (usually glucose) for the acclimatization process [Cheng and Liu, 2012; Cakır et al., 2010]. This limits the diversity of the developed culture to H_2-producers from simple sugars rather than from complex sugars, and complicates scale-up to full-scale thermophilic cultures which utilize real wastes with both simple and complex sugars. In addition, the period of acclimatization to thermophilic temperature in lab-scale experiments varies significantly which affects the degree of acclimatization from one study to another. For example, Cheng and Liu [2012]

acclimatized the seed for 6 months, while Cakır et al. [2010] acclimatized for only 3 days.

The complex structure of lignocellulosic materials makes it difficult to access cellulose and hemicellulose polymers to yield sugars for H_2 production [Galbe and Zacchi, 2012]. Therefore, lignocellulosic biomass needs to be pretreated to break down the complex compounds into simpler ones to facilitate H_2 production. In addition to the desired simple compounds produced during the pretreatment of lignocellulosic biomass, harmful by-products are produced in the form of organic acids, furan derivatives, and phenolic compounds [Palmqvist and Hagerdal, 2000; Quéméneur et al., 2012]. Among the aforementioned group of compounds, furan derivatives (i.e. hydroxymethyl furfural (HMF) and furfural) are reported to strongly inhibit H_2 production [Fangkum and Reungsang, 2011; Haroun et al., 2016]. Most studies in the literature focused on the effect of furan derivatives on H_2 production under mesophilic temperature [Liu et al., 2015; Monlau et al., 2013; Quéméneur et al., 2012] while few studies investigated its effect at thermophilic temperatures [Cao et al., 2010; Akobi, 2016]. Liu et al. [2015] tested pretreated cornstalk at a concentration of 73% VS using mesophilic anaerobic digester sludge (MADS) for H_2 production and observed that the H_2 yield decreased by 50% at 0.5 g/L furfural but increased by 50% at 0.5 g/L HMF. Monlau et al. [2013] studied H_2 production using a mixture of glucose and sunflower stalks hydrolysate and observed a 78% reduction in the H_2 yield to 0.45 mol/mol hexose at furfural concentration of 0.09 g/L. Quéméneur et al. [2012] conducted H_2 production batches using MADS and 5 g/L xylose and reported a 70% decrease in the H_2 yield to 0.51 mol/mol xylose at a furfural concentration of 1 g/L. Cao et al. [2010] investigated H_2 production from corn stover hydrolysate using *Thermoanaerobacterium thermosaccharolyticum* and observed no significant change in H_2 yield at furfural and HMF concentrations of 0.5 g/L each, while a 30% decrease in the yield occurred at 0.8 g/L furfural and HMF. Akobi [2016] used a xylose-based synthetic hydrolysate for H_2 production and reported an enhancement in the H_2 yield from 1.1 to 1.6 mol/mol hexose using MADS while a reduction in the yield was observed from 1.4 to 0.7 mol/mol hexose using thermophilic anaerobic digester sludge (TADS) at a furfural concentration of 1 g/L.

From the literature survey, some studies have been conducted to compare either mesophilic culture with thermophilic one or mesophilic culture with mesophilic acclimatized to thermophilic temperature for biohydrogen production from lignocellulosic hydrolysates. In addition, the thermophilic cultures used in H_2 production experiments were either obtained from hot springs or from lab-scale mesophilic cultures that have been acclimatized to thermophilic temperature, with no studies that have been conducted using seed obtained from a full-scale thermophilic anaerobic digester. Furthermore, the impact of potential inhibitors such as furfural and HMF present in real hydrolysates simultaneously with complex sugars, on mesophilic and thermophilic cultures has been sparsely addressed in the literature. Thus, the novelty of this paper stems primarily from the very limited data available in the literature on the comparison of fermentative H_2 production from poplar wood hydrolysate using MADS, MADS at thermophilic temperature (TMADS), and TADS from a full-scale thermophilic digester as well as comparing Monod kinetic parameters for the aforementioned three different seed sludges.

4.2 Materials and Methods

4.2.1 Seed sludge

Mesophilic anaerobic digester sludge (MADS) collected from the St. Marys wastewater treatment plant (St. Marys, Ontario, Canada) and thermophilic anaerobic digester sludge (TADS) collected from the Ravensview wastewater treatment facility (Kingston, Ontario, Canada) and were used as seed for the experiment. The total and volatile suspended solids (TSS and VSS) concentrations were 19.8 and 12.2 g/L for the MADS and 19.0 and 11.2 g/L for the TADS, respectively. Both seeds were heat pretreated at 70°C for 30 min to inhibit methanogens.

4.2.2 Poplar wood hydrolysate (substrate)

Poplar wood biomass was treated using the twin screw extrusion (TSE) technology. The hydrolysate was collected from the extruder part operating at 170°C and

100 psig. Table 4.1 lists the different characteristics of poplar wood hydrolysate measured in triplicates.

Table 4.1 - Poplar wood hydrolysate characteristics

Parameter (g/L)	**Poplar wood hydrolysate quality (Average ± STDEV)**
TCOD	140.7 ± 0.9
SCOD	137.0 ± 0.6
TS	120.9 ± 0.4
VS	110.1 ± 0.1
TSS	2.6 ± 0.3
VSS	2.5 ± 0.2
T-carbohydrates	103.2 ± 1.3
S-carbohydrates	100.4 ±0.6
Glucose	0.31 ± 0.03
Xylose	9.11 ± 0.7
Arabinose	0.23 ± 0.0
Acetate	3.72 ± 0.2
Furfural	1.36 ± 0.1
HMF	0.31 ± 0.0

4.2.3 Experimental design

Batch anaerobic fermentations were conducted in 310 mL serum bottles with a working volume of 250 mL. Experiments were conducted in triplicates for initial substrate-to-biomass (S°/X°) ratios of 0.5 and 1 $gCOD_{substrate}/gVSS_{seed}$. Volumes of seed sludge and poplar wood hydrolysate were calculated using the following equation:

$$\frac{S^o}{X^o}\left(\frac{gCOD}{gVSS}\right) = \frac{V_f(L) * TCOD_{feed}\left(\frac{g}{L}\right)}{V_s(L) * VSS_{seed}\left(\frac{g}{L}\right)} \quad (4.1)$$

where V_f is the volume of feed (poplar wood hydrolysate) and V_s is the volume of seed sludge. The initial pH value for each batch bottle was adjusted to 5.64±0.14 using HCl. $NaHCO_3$ buffer was added at 5 g/L for pH control. An initial sample of 20 mL was collected from each bottle. Batch bottles headspace were flushed with oxygen-free nitrogen gas for two minutes and capped tightly with rubber stoppers, after which the bottles were placed in swirling-action shakers (Max Q 4000, Fisher Scientific, ON, CA) operating at 180 rpm. The temperatures were set 37°C and 55°C for mesophilic and thermophilic experiments, respectively. Two control bottles were prepared using seed without any substrate for each set of experiment (MADS, TMADS, and TADS). Final samples were analyzed at the end of the batch experiment and the final pH was measured to be 5.08±0.29 for all batches.

4.2.4 Analytical methods

Glass syringes of appropriate sizes in the range of 5-100 mL were used to measure the volume of gas produced by releasing the gas to equilibrate with the ambient pressure [Owen et al., 1979]. A gas chromatograph (Model 310, SRI instruments, ON, CA) was used to determine the gas composition. The GC is equipped with a thermal conductivity detector (TCD) of temperature 90°C and a molecular sieve column of temperature 105°C. Argon was used as the carrier gas at a flow rate of 30 mL/min. H_2 gas production was calculated using Equation (4.2):

$$V_{H_2,i} = V_{H_2,i-1} + C_{H_2,i} * V_{G,i} + V_{h,i}(C_{H_2,i} - C_{H_2,i-1}) \quad (4.2)$$

where $V_{H_2,i}$ and $V_{H_2,i-1}$ are cumulative H_2 gas volumes at the current (i) and previous (i - 1) time intervals. $V_{G,i}$ is the total gas volume accumulated between the previous and current time intervals. $C_{H_2,i}$ and $C_{H_2,i-1}$ are the fractions of H_2 gas in the headspace of the reactor in the current and previous intervals, and $V_{h,i}$ is the total volume of the headspace of the reactor in the current interval [López et al., 2007]. HACH methods and testing kits (HACH Odyssey DR/2500) were used to measure the total and soluble chemical oxygen demand (TCOD and SCOD). Glucose was analyzed by BioPacific Diagnostic glucose kit (BC, Canada). The volatile fatty acids (VFAs) concentrations were analyzed using Varian 8500 has chromatography (Varian Inc., ON, CA) with a flame ionization detector (FID) of temperature 250°C and equipped with a fused silica column (30 m * 0.32 mm) of temperature 110°C. Helium was used as the carrier gas at a flow rate of 5 mL/min. Ethanol, xylose, arabinose, glucose, furfural, and HMF were measured using an HPLC consisting of a Dionex GP50 Gradient pump and a Dionex LC25 Chromatography oven equipped with an Aminex HPX-87H column (Bio-Rad) at 30°C and 9mM H_2SO_4 at 0.6 mL/min as mobile phase, connected to a Perkin Elmer 200 series refractive index detector (RID).

4.2.5 Biohydrogen production modeling

The modified Gompertz equation (Equation 4.3) was used to model biohydrogen production, where P is the cumulative H_2 production, P_{max} is the maximum cumulative H_2 production, R_{max} is the maximum H_2 production rate, λ is the lag time, and t is the fermentation time [Lay et al., 1999].

$$P = P_{max}\, exp\left\{-exp\left[\frac{R_{max}\, e}{P_{max}}(\lambda - t) + 1\right]\right\} \tag{4.3}$$

Monod kinetic parameters were determined using MATLAB R2014a with a modified non-linear least square fit model established by Gomez-Flores et al. [2015]. Equation (4.4) shows Monod kinetics [Mu et al., 2006]:

$$\frac{1}{X}\frac{dS}{dt} = \frac{-KS}{K_s + S} \tag{4.4}$$

where X is the biomass concentration (g/L), S is the substrate concentration (g/L), K is the maximum specific substrate utilization rate (g substrate/gVSS.hr), K_s is the saturation concentration (g/L) or half-velocity constant and is equal to the concentration of the rate-limiting substrate when the substrate degradation rate is equal to one half of the maximum [Mu et al., 2006]. Average percentage errors (APE), root mean square errors (RMSE), and coefficient of determination (R^2) were used to assess the model fit.

4.3 Results and Discussion

4.3.1 COD balance

Table 4.2 presents the COD mass balance for all experiments using MADS, TMADS, and TADS at S°/X° ratios of 0.5 and 1.0 gCOD/gVSS. The closure of the COD balance at an average of 92±4% verifies the reliability of the data.

Table 4.2 - Summary of COD balance

S°/X° (gCOD/gVSS)	**0.5**			**1.0**		
Seed	**MADS**	**TMADS**	**TADS**	**MADS**	**TMADS**	**TADS**
$COD_{initial}$ (gCOD)	6.01	6.10	6.00	7.21	7.25	7.55
COD_{final} (gCOD)	5.43	5.57	5.05	6.46	6.13	7.14
Cumulative H_2 (mL)	181	129	228	385	369	391
H_2 (gCOD)	0.11	0.08	0.14	0.24	0.22	0.24
COD balance[a] (%)	92	93	87	93	88	98

[a] COD balance (%) = [H_2 (gCOD) + COD_{final} (gCOD)]*100/[$COD_{initial}$ (gCOD)]

4.3.2 Biohydrogen production

Figure 4.1 shows the cumulative H_2 production profiles as mL H_2/gCOD added for batches using MADS, TMADS, and TADS at the two tested S°/X° ratios 0.5 and 1 gCOD/gVSS. Coefficients of variation (calculated as standard deviation divided by the

average) in all experiments were less than 10% confirming data reproducibility. It is evident from Figure 4.1 that at both S°/X° ratios, H_2 potentials using TADS were the highest, followed by MADS then TMADS. However, the percentage increase in H_2 production per gCOD added (91%) at S°/X° of 0.5 gCOD/gVSS was much higher than the percentage increase (14%) at S°/X° of 1 gCOD/gVSS. The increase in H_2 production potential is similar to Cheng and Liu [2012] who observed a 15% increase in the volumetric H_2 production potential from 81 to 93 mL in batches using MADS and TADS, respectively, treating raw cornstalk. The aforementioned authors observed a higher increase in the H_2 production potential of 50% using a mixture of raw and fungal treated cornstalk. The TADS used in their experiment was obtained from a 4 L anaerobic digester operating at 55°C for 6 months utilizing glucose as the carbon source. The decrease in the H_2 potential in batches using TMADS compared to MADS is consistent with Gupta et al. [2015] who observed a volumetric H_2 production potential decrease from 269 to 218 mL utilizing insoluble starch and cellulose as substrate and using MADS and TMADS, respectively. However, this decrease contradicts Zhang et al. [2015] who observed an increase in the H_2 potential from 224 to 822 mL/L_{media} using anaerobic granular sludge from a bench-scale expanded granular sludge bed reactor treating starch wastewater at 37°C and 55°C, respectively.

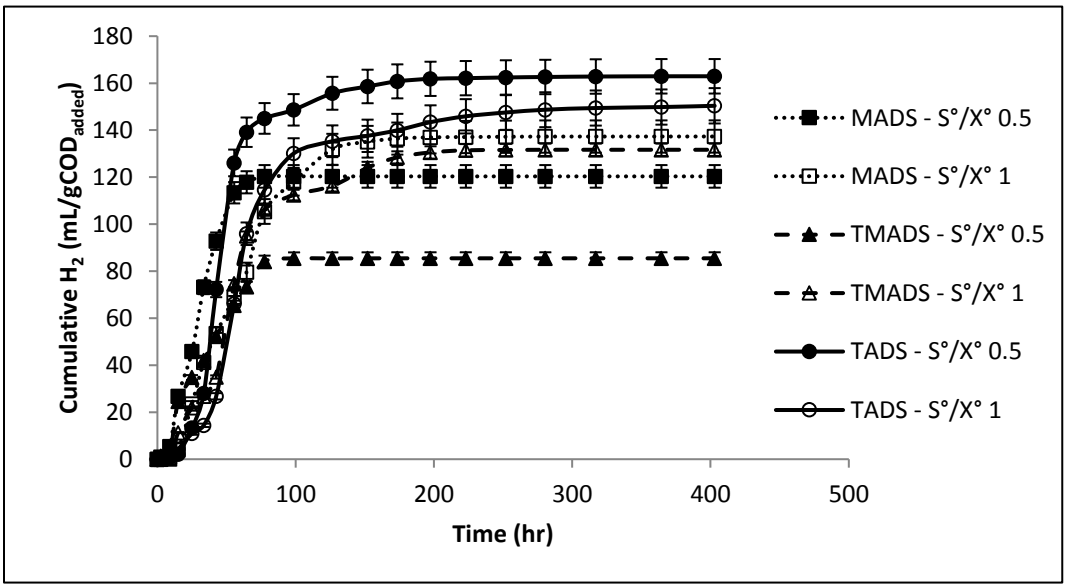

Figure 4.1 - Cumulative H_2 production per gCOD added

Table 4.3 shows the Gompertz kinetics and H_2 production yields for experiments using MADS, TMADS, and TADS at S°/X° ratios of 0.5 and 1.0 gCOD/gVSS. The coefficient of determination R^2 was 0.999 for all Gompertz data. It is apparent from Table 4.3 that the lag phase for the mesophilic culture (MADS) was not highly affected by the thermophilic conditions (TMADS) increasing from 7.2 to 9.0 hours and from 14.1 to 19.0 hours at S°/X° ratios of 0.5 and 1.0 gCOD/gVSS, respectively. However, the thermophilic culture took triple and double the time (26.5 and 30.5 hours) to produce H_2 at S°/X° ratios of 0.5 and 1.0 gCOD/gVSS, respectively. Zhang et al. [2015] observed the same slight increase in the lag phase from 15.4 to 16.6 hours using mesophilic anaerobic granular sludge at mesophilic and thermophilic conditions, respectively. The longer lag phase in batches using TADS is consistent with Shin et al. [2004] who observed a 12 hours lag phase in batches using thermophilic sludge obtained from an acidogenic CSTR treating food waste operating at 55°C (HRT of 5 days) compared to only 1 hour when using mesophilic sludge obtained from a similar CSTR operating at 35°C. The longer lag phase using the TADS is due to the low microbial diversity known for thermophilic anaerobic mixed cultures [Mäkinen et al., 2012]. While the high diversity of H_2 producing bacteria in mesophilic cultures allows faster production of H_2 with shorter lag phases. On the other hand, since MADS and TMADS are the same culture operating at different temperatures, the slight increase in lag phase is due to the adaptation of the culture to a different temperature or the enrichment of thermophilic H_2 producing bacteria that exist in the mesophilic culture [Qui et al., 2016]. The aforementioned authors observed a lag phase of 4 and 8 hours in H_2 batches utilizing xylose and using a mixture of thickened anaerobic sludge and cow manure at mesophilic and thermophilic conditions, respectively. Qui et al. [2016] reported a decrease in the microbial diversity at thermophilic temperature, although *Clostridium* species were dominant at both mesophilic and thermophilic conditions. However, fewer H_2-producing species were identified in the thermophilic microflora which is due to the enrichment of specific microbial species associated with thermophilic H_2 production increasing the H_2 yield at thermophilic temperature.

Higher maximum H_2 production rates of 6.4 and 7.0 mL/hr were obtained using the TADS compared to 4.7 and 4.9 mL/hr using MADS and 2.3 and 5.1 mL/hr using TMADS at S°/X° ratios of 0.5 and 1.0 gCOD/gVSS, respectively. Cakır et al. [2010] showed Gompertz kinetics for batches using MADS and TADS utilizing acid hydrolyzed wheat starch, where the maximum H_2 production rate increased from 4.3 to 7.4 mL/hr, respectively. Also, Pan et al. [2008] observed an increase in the H_2 production rate from 2 mL/hr using MADS to 10 mL/hr using TADS obtained from a thermophilic pilot-scale digester.

Table 4.3 - Gompertz kinetics data and H_2 yields

Seed	**MADS**		**TMADS**		**TADS**	
S°/X° (gCOD/gVSS)	**0.5**	**1.0**	**0.5**	**1.0**	**0.5**	**1.0**
P_{max} (mL)	181.4	385.0	128.8	363.2	224.1	390.7
R_{max} (mL/hr)	4.7	4.9	2.3	5.1	6.4	7.0
λ (hr)	7.2	14.1	9.0	18.8	26.5	30.5
H_2 yield						
($mL/gCOD_{added}$)	123	137	88	132	169	151
(mL/gVS_{added})	158	175	112	168	216	193
($L/L_{substrate\ added}$)	17.4	19.3	12.3	18.5	23.8	21.3

At an S°/X° ratio of 0.5 gCOD/gVSS, the H_2 yield decreased from 169 to 123 to 88 $mL/gCOD_{added}$ in the TADS, MADS, and TMADS, respectively. The decrease in H_2 yield by 29% in the TMADS compared to the MADS, is comparable with Gupta et al. [2015] who reported a 19% decrease in the H_2 yield using MADS and TMADS, utilizing a mixture of starch and cellulose at an S°/X° ratio of 4 gCOD/gVSS. However, this contradicts the findings of Luo et al. [2010] who reported an increase in the H_2 yield from 14 to 70 mL/gVS_{added} utilizing cassava stillage at an S°/X° ratio of 2.4 gCOD/gVSS using

MADS and TMADS, respectively. The increase in the H_2 yields using TADS is consistent with Cakır et al. [2010] who reported 206 and 312 mL/gCOD using MADS and TADS, respectively. The TADS used in the aforementioned study was acidogenic anaerobic sludge acclimated at 55°C with 60 g/L glucose for three days prior its use in the H_2 production batches [Cakır et al., 2010].

The effect of furfural concentration in the hydrolysate on the H_2 production yield ($L/L_{substrate}$) was observed at mesophilic and thermophilic conditions. It has been reported in the literature that 1 g/L furfural is considered inhibitory [Quéméneur et al., 2012], while other studies reported the inhibition threshold to be as high as 2-4 g/L [Haroun et al., 2016] at mesophilic conditions. For thermophilic conditions, Cao et al. [2010] reported the inhibition threshold to be 1.5-2.0 g/L. At low furfural concentration below 0.12 g/L, H_2 yields increased with furfural concentration increase in experiments using the mesophilic culture (i.e. MADS and TMADS) by 11% and 50%, respectively. This agrees with Akobi [2016] who observed a 45% increase in the H_2 yield using a xylose-based synthetic hydrolysate and MADS at furfural concentration of 1 g/L, but contradicts Liu et al. [2015] who observed a 50% decrease in the H_2 yield at 0.5 g/L furfural using pretreated cornstalk as the substrate. The increase in H_2 yield can be attributed to furfural degradation to acetic acid with H_2 production at a theoretical yield of 6 mol H_2/mol furfural through a thermodynamically favorable reaction [Haroun et al., 2016]. In contrast, H_2 yields decreased by 11% with the increase in furfural concentration during thermophilic culture experiment (i.e. TADS), which agrees with the findings of Cao et al. [2010] who observed 30% decrease in the H_2 yield using corn stover hydrolysate and *Thermoanaerobacterium thermosaccharolyticum* at 0.8 g/L furfural, as well as Akobi [2016] who observed a 50% decrease in the yield using xylose based synthetic hydrolysate and TADS. The aforementioned results confirms the high diversity of mesophilic H_2-producing cultures compared to thermophilic cultures, where thermophilic cultures are more inhibited by furfural even below the inhibition concentration (1 g/L) reported in the literature.

4.3.3 Monod growth kinetics

The Monod kinetic equation (4.4) was used to estimate the kinetic coefficients by modeling the substrate (i.e. sugars) degradation for MADS, TMADS, and TADS while neglecting the temporal change in biomass concentration. Figure 4.2 shows the experimental and modeled substrate degradation (i.e. sugars degradation) for experiments using MADS, TMADS, and TADS at the tested S°/X° ratios of 0.5 (Figure 4.2a) and 1.0 gCOD/gVSS (Figure 4.2b). Table 4.4 presents the estimated kinetic parameters derived from only the growth phase as well as the APE, RMSE, and R^2 that indicates the goodness of fit for substrate concentrations. Values of APE (2.3-8.0%), RMSE (0.014-0.045 g/L), and R^2 (0.97-1.00) confirm the MATLAB model reliability. Figure 4.3 shows the correlation between the modeled and experimental sugars concentration, with absolute fraction of variance (R^2), calculated with respect to the equity line, of 0.79 and 0.83 for S°/X° ratios of 0.5 and 1.0 gCOD/gVSS, respectively. At S°/X° ratio of 0.5 gCOD/gVSS, the decrease in the K value from 0.020 g substrate/gVSS.hr using MADS to 0.012 g substrate/gVSS.hr using TMADS is consistent with Gupta et al. [2015] who observed a decrease from 0.023 to 0.014 g substrate/gVSS.hr utilizing starch as the carbon source, and using MADS and TMADS, respectively. However, the aforementioned authors operated their batch experiment at an S°/X° ratio of 4 gCOD/gVSS. Also, at S°/X° ratio of 0.5 gCOD/gVSS, the K value of 0.02 g substrate/gVSS.hr was not affected by the change of culture from mesophilic (i.e. MADS) to thermophilic (i.e. TADS) which is consistent with Akobi [2016] who observed no change in the K value (0.14 g substrate/gVSS.hr) utilizing synthetic lignocellulosic hydrolysate with no furfural and comprised of 96% sugars. The aforementioned authors used MADS and TADS and operated their batches at an S°/X° ratio of 4 gCOD/gVSS. On the contrary, at S°/X° ratio of 1.0 gCOD/gVSS, the value of K increased from 0.022 at 37°C to 0.03 at 55°C g substrate/gVSS.hr reflecting better microbial kinetics for the thermophilic mixed culture.

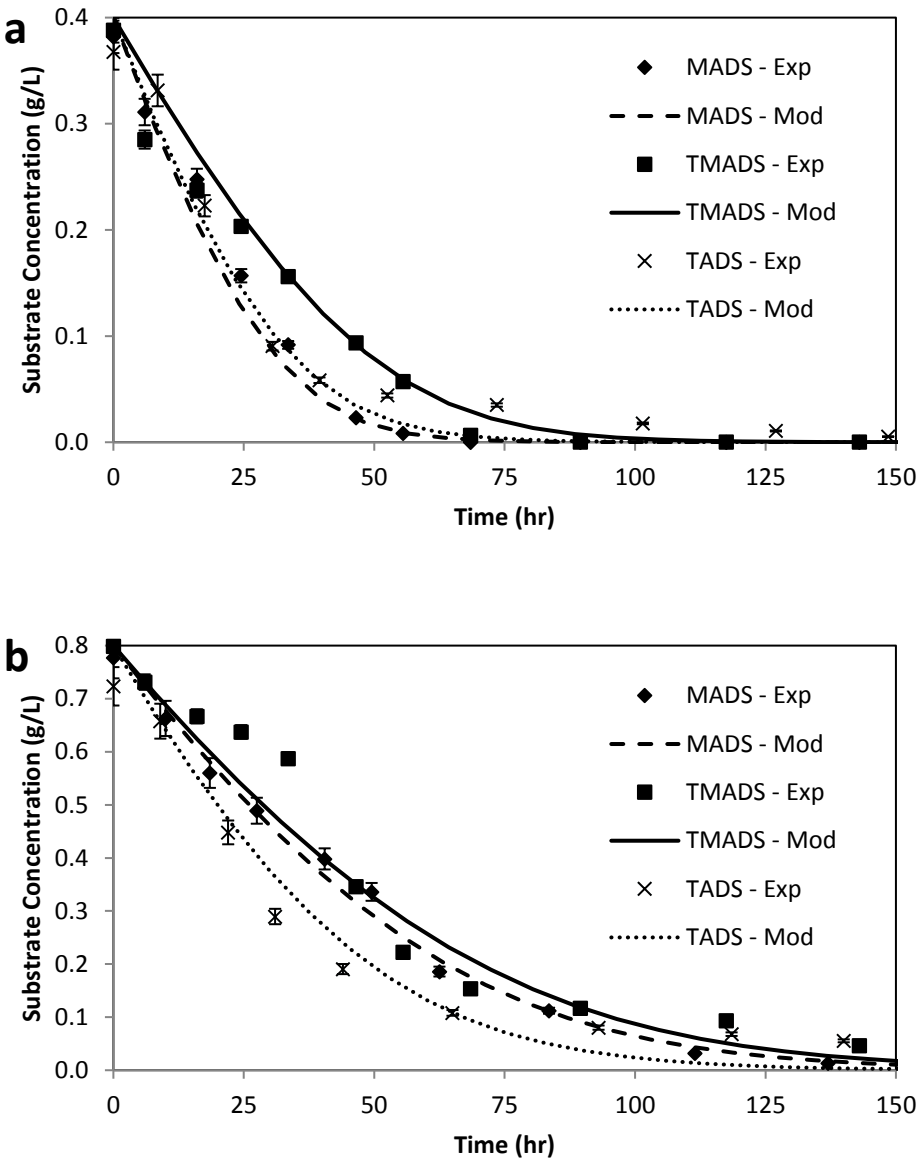

Figure 4.2 - Experimental and modeled substrate utilization profiles for MADS, TMADS, and TADS at S°/X° ratio of a) 0.5 and b) 1.0 gCOD/gVSS

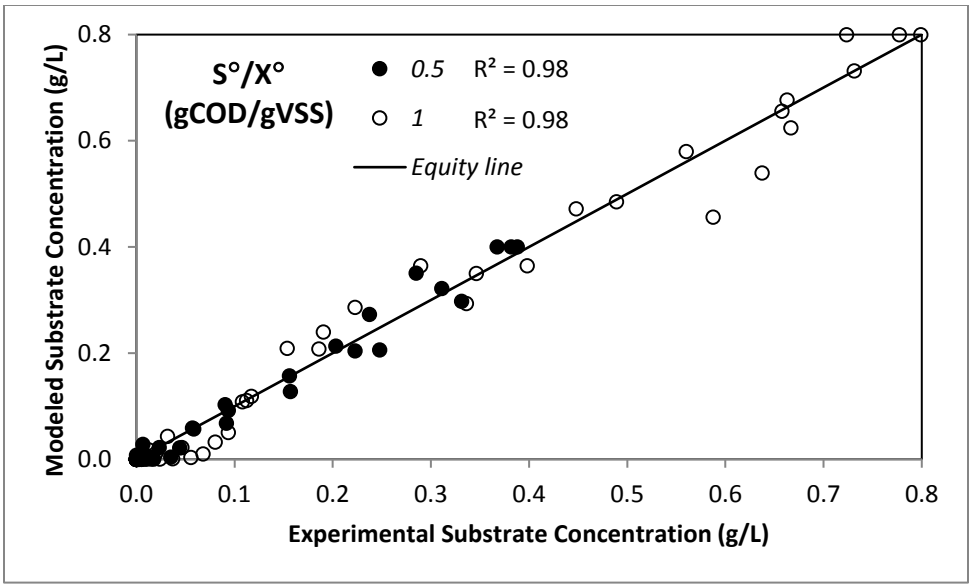

Figure 4.3 - Linear regression of experimental against modeled substrate concentrations for MADS, TMADS, and TADS at S°/X° ratio of 0.5 and 1.0 gCOD/gVSS

Table 4.4 - Monod kinetic parameters of MADS, TMADS, and TADS

Seed	**MADS**		**TMADS**		**TADS**	
S°/X° (gCOD/gVSS)	**0.5**	**1.0**	**0.5**	**1.0**	**0.5**	**1.0**
K (g substrate/gVSS.hr)	0.020	0.022	0.012	0.020	0.020	0.030
K_s (g/L)	0.19	0.58	0.17	0.58	0.23	0.63
APE (%)	4.4	2.3	2.7	5.8	5.5	8.0
RMSE (g/L)	0.014	0.016	0.018	0.045	0.017	0.040
R^2	0.99	1.00	0.98	0.98	0.98	0.97

4.3.4 Volatile fatty acids

VFAs are the desirable products as opposed to ethanol, formate, and lactate in fermentative H_2 production. Table 4.5 shows that acetate, butyrate, and propionate were the main end products in all experiments, however, ethanol was detected only in experiments using MADS at both S°/X° ratios. Theoretical H_2 production from VFAs produced was calculated based on 0.84 L H_2/g acetate, 0.58 L H_2/g butyrate, and 0.34 L H_2/g propionate [Nasr et al., 2015]. The stoichiometric H_2 produced was estimated from the measured VFAs showing an average measured-to-theoretical H_2 of 94±6% which confirms the consistency of experimental and stoichiometric data. The average acetate-to-butyrate ratio was 0.9±0.1 which is similar to Cheng and Liu [2012] who observed the same ratio of 0.9±0.2 in batches using MADS and TADS utilizing raw cornstalk and a mixture of raw and fungal treated cornstalk. Although the aforementioned authors observed a similar decrease in the propionate concentrations at thermophilic conditions, however, ethanol was detected in both experiments with even higher concentrations of 0.2 g/L in thermophilic experiments [Cheng and Liu, 2012]. The decrease in the propionate concentration in experiments using TADS is consistent with the increase in H_2 production, since propionate production pathway consumes H_2 [Batstone et al., 2002]. Shin et al. [2004] also reported acetate, butyrate, and propionate as the main end-products for H_2 production from food waste using MADS. However, no propionate was detected in experiments using TADS and ethanol was not detected in any of the experiments [Shin et al., 2004].

Table 4.5 - Stoichiometric H_2 production

S°/X° (gCOD/gVSS)	0.5			1.0		
Seed	MADS	TMADS	TADS	MADS	TMADS	TADS
HAc (g/L)	0.70	0.60	0.67	1.29	1.17	1.11
HBu (g/L)	0.48	0.36	0.45	0.81	0.93	0.93
HPr (g/L)	0.18	0.23	0.05	0.09	0.12	0.04
EtOH (g/L)	0.11	ND	ND	0.18	ND	ND
HAc/HBu (mol/mol)	0.86	0.98	0.87	0.93	0.74	0.70
Theoretical H_2[a] (mL)	203	147	217	408	400	406
Measured/Theoretical H_2 (%)	89	88	105	94	92	96

[a] Theoretical H_2 = [HAc (g/L) * 0.84 (L H_2/g HAc) + HBu (g/L) * 0.58 (L H_2/g HBu) – HPr (g/L) * 0.34 (L H_2/g HPr)] * batch working volume (mL)

4.4 Summary and Conclusions

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

- Poplar wood hydrolysate has the maximum H_2 production potential with a yield of 23.8 $L/L_{substrate}$ corresponding to 169 mL/gCOD added using TADS at an S°/X° ratio of 0.5 gCOD/gVSS.
- The use of TADS compared to MADS and TMADS increased H_2 production yields by 37% and 92% at an S°/X° ratio of 0.5 gCOD/gVSS, and by 10% and 14% at an S°/X° ratio of 1.0 gCOD/gVSS, respectively.
- The use of TADS compared to MADS and TMADS increased the lag phase by 19.3 and 17.5 hours at an S°/X° ratio of 0.5 gCOD/gVSS, and by 16.4 and 11.7 hours at an S°/X° ratio of 1.0 gCOD/gVSS, respectively.

- At low furfural concentration below 0.12 g/L and with the increase in furfural concentration, H_2 yields increased using MADS and TMADS by 11% and 50%, respectively, but decreased by 11% using TADS.
- Highest K of 0.03 g substrate/gVSS.hr was achieved by the TADS at an S°/X° ratio of 1.0 gCOD/gVSS with K_s of 0.63 g/L.
- Acetate, butyrate, and propionate were the main end-products in all experiments at both S°/X° ratios, while ethanol was detected only in experiments using MADS.
- Propionate concentrations decreased in experiments using TADS which was reflected in higher H_2 yields at both S°/X° ratios.

4.5 References

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Chapter 5

Effect of Headspace Carbon Dioxide Sequestration on Microbial Biohydrogen Communities

5.1 Introduction

Hydrogen (H_2) production by dark fermentation is characterized by relatively low yields, with higher yields only possible through thermodynamically unfavourable pathways. In addition, the product gas is a mixture of H_2 and carbon dioxide (CO_2), which creates challenges for the useful application of the H_2 as a fuel [Sabaratnam and Hassan, 2012]. Specifically, CO_2 is a major contaminant in fuel cell technologies that generate electricity from H_2 gas [Dayton, 2001], as proton exchange membrane fuel cells (PEMFCs) require high-purity H_2 (greater than 99%) [Larminie and Dicks, 2003].

The two most common dark fermentation pathways for H_2 production from glucose are the acetate and butyrate pathways (reactions 5.1 and 5.2) [Nath and Das, 2004], which limit the theoretical H_2 yield to between 2 and 4 moles of H_2 per mole of glucose. Both reactions are thermodynamically favourable (i.e. negative ΔG values) and the greater the acetate to butyrate ratio, the higher is the H_2 yield. Therefore, directing the metabolism of the culture towards acetate formation is key to achieving higher H_2 yields [O-Thong et al., 2009]. Also, in order to maximize the H_2 yield, metabolism should be directed away from alcohols (ethanol, butanol) and reduced acids (lactate) towards volatile fatty acids (VFAs) production [Levin et al., 2004]. However, propionate production decreases the H_2 yield, since it is a H_2-consuming pathway (reaction 5.3) [Hussy et al., 2003].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \Delta G_R^\circ = -196 \text{ KJ} \quad (5.1)$$

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2 \qquad \Delta G_R^\circ = -224 \text{ KJ} \quad (5.2)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad \Delta G_R^\circ = -279 \text{ KJ} \quad (5.3)$$

Nath and Das [2004] stated that removing CO_2 efficiently from the culture medium will shift H_2-synthesizing reactions in the forward direction, increasing H_2 production, and decreasing the consumption of reducing equivalents carried by electron carrier's molecules like Nicotinamide Adenine Dinucleotide (NADH) by competing reactions [Nath and Das, 2004]. Kraemer and Bagley [Kraemer and Baley, 2007] discussed several methods for improving the H_2 yield, one of which was removing dissolved H_2 and CO_2 from the liquid phase of the fermentation process.

In addition, H_2 and CO_2 are the main substrates for both hydrogenotrophic methanogenic bacteria and homoacetogenic bacteria to produce methane (reaction 5.4) and acetate (reaction 5.5), respectively [Kotsyurbenko et al., 2004; Saady, 2013]. Mayumi et al. [2013] observed that increasing CO_2 concentrations accelerated the rate of hydrogenotrphic methanogenesis in oil reservoirs. Also, Saady [2013] indicated that controlling CO_2 concentrations during dark fermentative H_2 production needs further investigation as a potential approach towards controlling homoacetogenesis. Therefore, dissolved CO_2 removal from the liquid phase may prevent the consumption of H_2 for methane (CH_4) or acetate production.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G_R^\circ = -131\ KJ \qquad (5.4)$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G_R^\circ = -104\ KJ \qquad (5.5)$$

One of the common techniques used for dissolved gas removal is gas sparging. Hussy et al. [2005] observed an increase in the H_2 yield from 1.0 to 1.9 mol/mol $hexose_{converted}$ using sucrose as the substrate in a continuous stirred-tank reactor (CSTR) operated at a hydraulic retention time (HRT) of 15 hours and achieving 95% sucrose conversion after sparging nitrogen (N_2) gas continuously in the reactor. Kim et al. [2006] tested the utilization of N_2 as a sparging gas in H_2 production from sucrose in a CSTR operated at an HRT of 12 hours and loading of 40 gCOD/L.d and observed a 24% increase in the H_2 yield to 0.93 mol H_2/mol hexose. Tanisho et al. [1998] observed a 110% increase in the H_2 yield to 1.09 mol H_2/mol hexose by continuous purging of argon gas in a H_2 producing batch experiment by *Enterobacter aerogenes* using molasses as the carbon source.

Non-sparging techniques to decrease the dissolved gas concentrations include increasing of stirring speed, applying vacuum in the headspace (i.e. decreasing the reactor headspace pressure), using in-reactor ultrasonication, and using an immersed membrane to remove the dissolved gases [Kraemer and Bagley, 2007; Elbeshbishy et al., 2011a; Elbeshbishy et al., 2011b]. Mandal et al. [2006] observed an increase of 105% in the H_2 yield to 3.9 mol H_2/mol hexose of a batch H_2 producing experiment from glucose by *Enterobacter cloacae* by decreasing the headspace total pressure. The increase in H_2 yield was attributed to inhibition of H_2 consumption due to the decrease in total pressure that lead to the production of reduced by-products such as ethanol and organic acids [Mandal et al., 2006]. The aforementioned authors also used a potassium hydroxide (KOH) trap outside the batch reactor headspace to absorb CO_2. Liang et al. [Liang et al., 2002] used a silicone rubber membrane to separate biogas from the liquid phase in a H_2 fermentation batch reactor using glucose as the substrate, and observed 15% and 10% increases in H_2 yield and H_2 production rate, respectively.

Park et al. [2005] were the first to apply headspace CO_2 sequestration using KOH in batch H_2 glucose fermentation, and achieved a H_2 content of 87.4% in the headspace. They recommended assessing CO_2 removal from the headspace of a continuous system instead of batches to measure how effectively CO_2 would be removed, specially under different OLRs [Park et al., 2005].

Two H_2-producing pathways from butyrate and propionate that are thermodynamically unfavourable (reactions 5.6 and 5.7) [Stams and Plugge, 2009] can occur if H_2 as a product is decreased to its minimum concentration, converting Gibbs free energy from positive to negative values [Stams and Plugge, 2009]. Similarly, the propionate to acetate pathway (reaction 5.6), which is thermodynamically unfavourable, could be shifted forward if CO_2 was removed from the headspace.

$$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2 \qquad \Delta G_R^\circ = +72 \text{ KJ} \quad (5.6)$$

$$CH_3(CH_2)_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad \Delta G_R^\circ = +48 \text{ KJ} \quad (5.7)$$

Microbial community composition in a H_2 reactor directly affects the fermentation efficiency [Song et al., 2012]. Therefore, it is important to explore the changes in species diversity and population distribution of the predominant H_2 producers due to the removal of CO_2 from the reactor headspace. 16S rDNA-based techniques have been widely used for the qualitative and quantitative analysis of microbial communities [Fang et al., 2002].

As depicted in this brief introduction, CO_2 presents several challenges to the application of biohydrogen systems, not the least of which is reduced H_2 yield due to hydrogenotrophic methanogens and homoacetogens, and the necessity for biogas cleanup prior to utilization. In addition, the literature is devoid of information on the impact of CO_2 sequestration from continuous flow systems, as most of the few published studies that attempted to sequester CO_2 were done in batch reactors. Moreover, previous studies did not investigate the impact of sequestration on metabolic pathways and microbial community structure, and have only focused on H_2 yield. Therefore, the objective of this study is to evaluate the impact of CO_2 sequestration on H_2 yield, H_2 production rate, chemical buffering requirements, metabolic pathways, and microbial community structure in a novel continuous flow biohydrogen production system.

5.2 Materials and Methods

5.2.1 IBRCS setup

The patented integrated biohydrogen reactor clarifier system (IBRCS) consisting of a CSTR (7 L working volume), followed by a gravity settler (8 L volume), shown in Figure 5.1, was operated at an HRT of 8 hours and an OLR of 25.7 gCOD/L-d. For further details on the system design, refer to Hafez et al. [2014]. A cylindrical CO_2 trap (0.25 L volume, which represents about 10% of the reactor's headspace volume) with KOH pellets and a porous base was introduced to the system and fixed in the reactor cover [Hafez, 2013]. The CO_2 trap was fixed in the headspace of the reactor to allow maximum and continuous exposure of the KOH pellets to the produced biogas prior its exit from the reactor. The CO_2 trap had a porous base facing the headspace of the reactor

and an outlet extending outside the reactor's top and connected with a tube to a wet-tip gas meter. The IBRCS was operated in two conditions in series: 18 days without CO_2 sequestration followed by 17 days with CO_2 sequestration by adding KOH pellets (60 g) in the CO_2 trap fixed in the headspace. Samples were taken at the end of the steady state period for the two experimental phases; phase A: before adding KOH and phase B: after adding KOH in the reactor's headspace.

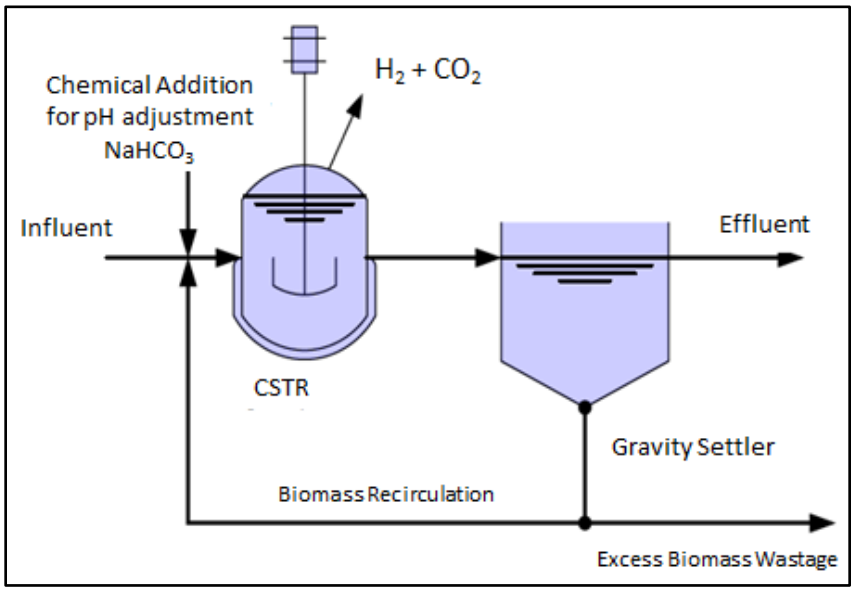

Figure 5.1 - Schematic diagram for the Integrated Biohydrogen Reactor Clarifier System (IBRCS)

5.2.2 Seed sludge and substrate

Anaerobic digester sludge (ADS) was collected from St. Mary's wastewater treatment plant (St. Mary's, Ontario, Canada) and preheated at 70°C for 30 min to be used as the seed. Total and volatile suspended solids (TSS, VSS) of the seed sludge were 16.4 and 11.4 g/L, respectively. Glucose was used as the substrate with a concentration of 8 g/L, i.e. 25.7 gCOD/L-d. The feed contained sufficient inorganics and trace minerals [Hafez et al., 2009]. Buffer used in the feed was sodium bicarbonate ($NaHCO_3$) at a concentration of 3 g/L. A pH of 5.2±0.2 in the bioreactor was maintained during the experiment using $NaHCO_3$ solution at a concentration of 168 g/L.

5.2.3 Analytical methods

The volume of biogas was measured using a wet-tip gas meter (Rebel Wet-tip Gas Meter Company, Nashville, TN, USA), while the biogas composition (N_2, H_2, and CH_4) was determined using a gas chromatograph (Model 310, SRI instruments, Torrance, CA) with a thermal conductivity detector (TCD) temperature of 90°C and a molecular sieve column (Molesieve 5A, mesh 80/100, 6 ft * 1/8 in) at a temperature 105°C. Argon was used as the carrier gas at a flow rate of 30 mL/min. The VFAs concentrations were analyzed using a gas chromatograph (Varian 8500, Varian Inc., Toronto, Canada) with a flame ionization detector (FID) of temperature 250°C equipped with a fused silica column (30 m * 0.32 mm) of temperature 110°C. Helium was used as the carrier gas at a flow rate of 5 mL/min. TSS and VSS were measured according to the standard methods [Clasceri et al., 1998]. Glucose was analyzed by Genzyme Diagnostics P.E.I. Inc. glucose kit, PE, Canada. HACH methods and testing kits (HACH Odyssey DR/2500) were used to measure the total and soluble chemical oxygen demands (TCOD, SCOD).

5.2.4 Microbial analysis

5.2.4.1 DNA extraction

Approximately 200 mg of each sample were used for DNA extraction using E.Z.N.A. DNA isolation kit (manufacturer information), which included a bead-beating step for the mechanical lysis of the microbial cells. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). DNA samples were normalized to 20 ng/µl, and quality checked by polymerase chain reaction (PCR) amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. [2009]. Amplicons were verified by agarose gel electrophoresis.

5.2.4.2 Library construction and Illumina sequencing

Library construction and Illumina sequencing were performed as described by Derakhshani et al. [2014]. In brief, the V4 region of 16S rRNA gene was targeted for PCR amplification using modified F515/R806 primers [Caporaso et al., 2012]. The reverse PCR primer was indexed with 12-base Golay barcodes allowing for multiplexing of samples. PCR reaction for each sample was performed in duplicate and contained 1.0 µL of pre-normalized DNA, 1.0 µL of each forward and reverse primers (10 µM), 12 µL HPLC grade water (Fisher Scientific, ON, Canada) and 10 µL 5 Prime Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) to remove primers, dNTPs and reaction components. The V4 library was then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (Invitrogen, NY, USA). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, measured by Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers for read1 (5´-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3´), read2 (5´-AGTCAGTCAGCCGGACT ACHVGGGTWTCTAAT-3´) and index read (5´-ATTAGAWACCCBDGTAGTCCGGCTGAC TGACT-3´) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

5.2.4.3 Bioinformatics analysis

Bioinformatics analyses were performed as described by Derakhshani et al. [2014]. In brief, the PANDAseq assembler [Masella et al., 2012] was used to merge overlapping paired-end Illumina fastq files. All the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME [Caporaso et al., 2010a]. Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with more than 3 consecutive bases with quality scores below 1e-5 were truncated, and those with a read length shorter than 75 bases were removed from the downstream analysis. Chimeric reads were filtered using UCHIME [Edgar et al., 2011] and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST [Edgar, 2010] at 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier [Wang et al., 2007] and aligned with the Greengenes Core reference database [DeSantis et al., 2006] using PyNAST algorithms [Caporaso et al., 2010b]. Phylogenetic tree was built with FastTree 2.1.3 [Prince et al., 2010] for further comparisons between microbial communities.

Within community diversity (α-diversity) was calculated using QIIME. Alpha rarefaction curve was generated using Chao 1 estimator of species richness [Chao, 1984] with ten sampling repetitions at each sampling depth. An even depth of approximately 15,700 sequences per sample was used for calculation of richness and diversity indices. To compare microbial composition between samples, β-diversity was measured by calculating the weighted and unweighted Unifrac distances [Lozupone and Knight, 2005] using QIIME default scripts. Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using PRIMER software (version 6; Warwick R, Clarke K. 2006. PRIMER-E Ltd, Plymouth). Permutational multivariate analysis of variance PERMANOVA software (Anderson M. 2005. A FORTRAN computer program for permutational multivariate analysis of variance, Department of Statistics, University of Auckland, New Zealand) was used to calculate *P*-values and test for significant differences of beta-diversity among treatment groups.

5.2.5 Statistical analysis

Statistical analyses were performed as described by Derakhshani et al. [2014]. In brief, partial least square discriminant analysis (PLS-DA; SIMCA P, SIMCASIMCA software, version 13.0, 2008, Umetrics, Umea, Sweden) was performed on genus data to identify the effects of treatments. The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the categories of a categorical variable on X. In this case, X variables were bacterial genera and Y variables were observations of different days post- or pre-parturition versus each other. For this analysis, data were scaled using Unit Variance in SIMCA. Cross-validation then was performed to determine the number of significant PLS components and a permutation testing was conducted to validate the model. To avoid over parameterization of the model, variable influence on projection value (VIP) was estimated for each genus and genera with VIP < 0.50 were removed from the final model [Verhulst et al., 2011; Pérez-Enciso and Tenenhaus, 2003]. R^2 estimate then was used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to identify genera that were most characteristics of each treatment group and the results were visualized by PLS-DA loading scatter plots.

The UNIVARIATE procedure of SAS/STAT (version 9.3, 2012, SAS Institute Inc, Cary, NC, US) was used to test the normality of residuals for Alpha biodiversity data. Non-normally distributed data were log transformed and then used to assess the effect of sampling date (pre-/post-calving) using MIXED procedure of SAS. Phylum percentage data was also used to evaluate statistical differences among different days. The MIXED procedure of SAS was utilized, as described above, to test for significant changes in the proportions of different phyla among the groups of interest. All the phyla were divided into two groups of abundant, above 1% of the population, and low-abundance, below 1% of the population. The differences between groups were considered significant at $P < 0.05$ while trends were observed at $P < 0.1$.

5.3 Results and Discussion

5.3.1 Hydrogen production

Figure 5.2 shows the change in H_2 content due to the addition of KOH in the headspace. No CH_4 was detected in the headspace before or after KOH application. H_2 content reached 57.3 ± 4% without KOH, increasing rapidly to 100% after application of KOH in the headspace. Park et al. [2005] achieved only 87.4% H_2 after adding KOH in the headspace of H_2 batches, due to incomplete sequestration of headspace CO_2. Since in batches, after the maximum production rates are established, biogas production rates usually decline with time due to lower substrate utilization rates, the extrapolation of batch biogas composition data to continuous-flow systems depends on numerous factors related to operational conditions i.e. OLR, HRT, biomass concentration, etc.

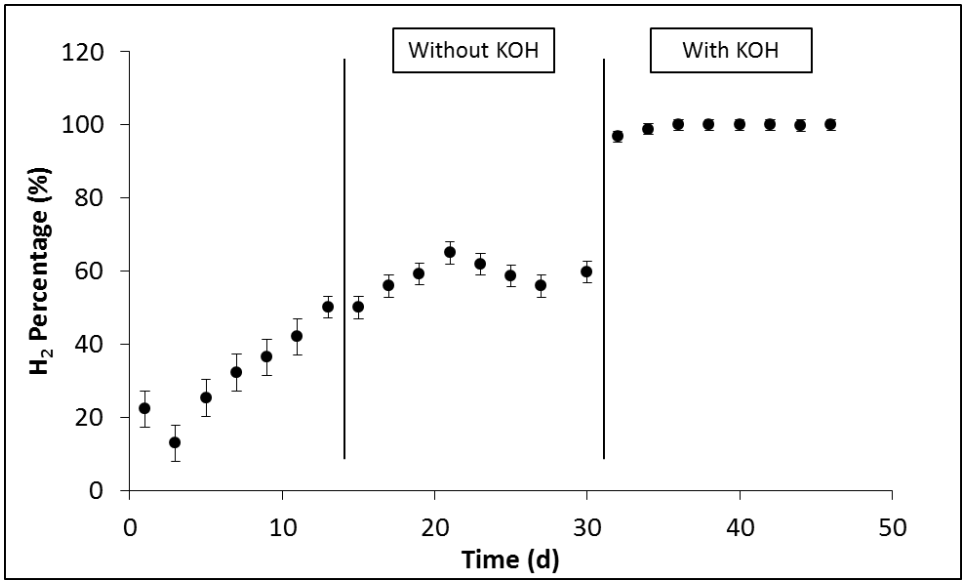

Figure 5.2 - Hydrogen content in the IBRCS reactor headspace with and without CO_2 sequestration

As depicted in Figure 5.3, H_2 production rates increased from 57 to 70 L H_2/d after applying the CO_2 sequestration with an increase of 23%. H_2 production rate before CO_2 sequestration was consistent with Hafez et al. [2010b] who achieved 48 L H_2/d at the same OLR and HRT. Before adding KOH to the headspace, steady state H_2 production was reached after 12 days with an average fluctuation in production rates of 3.4% was observed.

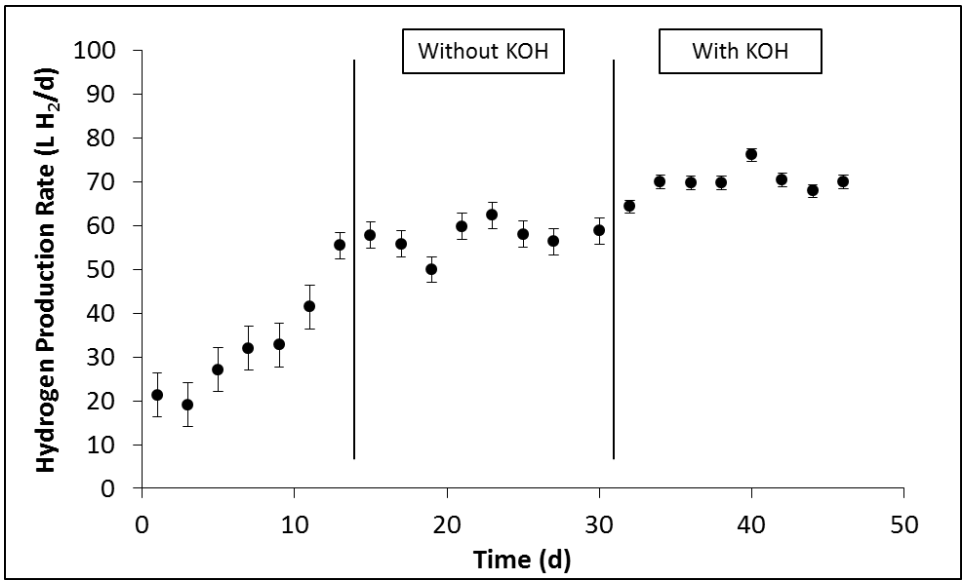

Figure 5.3 - Hydrogen production rate in the IBRCS with and without CO_2 sequestration

H_2 production rates per unit reactor volume before applying KOH was 8.2 ± 0.5 L/L-d, which is consistent with Hafez et al. [2010b] who achieved 9.6 L/L-d at the same OLR and HRT in the IBRCS. After applying KOH, the rate increased to 10 ± 0.4 L/L-d. It is postulated that removing CO_2 from the headspace favoured the forward direction for reactions 5.1, 5.2, and 5.3, which lead to an increase in the H_2 production rate in order to compensate for the decrease in the CO_2 concentration.

5.3.2 Hydrogen yields

The H_2 yield achieved before sequestering CO_2 was 2.42 ± 0.15 mol/mol$_{glucose}$, which is 13% lower than Hafez et al. [2010b] who achieved a H_2 yield of 2.8 mol/mol$_{glucose}$ at the same OLR and HRT in the IBRCS. The decrease in yield can be attributed to differences in the microbial culture. This result is 27% higher than the maximum H_2 yield of 1.93 mol/mol$_{glucose}$ observed by Zhang et al. [2006] at an OLR of 32.1 gCOD/L-d and an HRT of 8 hours in a CSTR using glucose and mixed anaerobic culture.

A H_2 yield of 2.96 ± 0.14 mol/mol$_{glucose}$ was achieved after CO_2 sequestration; with an increase of 22%. The increase in the H_2 yield is attributed to favouring the shift of reactions 5.1, 5.2, and 5.6 to the forward direction due to CO_2 sequestration. With a maximum theoretical H_2 yield of 4 mol/mol$_{glucose}$, a maximum practical yield of 3.4 mol/mol$_{glucose}$ taking the biomass yield of 0.15 gCOD/gCOD$_{converted}$ into consideration [Chen et al., 2001], the 22% increase in the yield due to sequestering CO_2 achieved 87% of the practical yield. The impact of headspace CO_2 sequestration on the H_2 yield would be more drastic for systems achieving low H_2 yields, such as 1.8 mol/mol$_{glucose}$ in a CSTR [Zhang et al., 2007; Show et al., 2007], 1.57 mol/mol$_{glucose}$ in an agitated granular sludge bed reactor [Wu et al., 2008], and 1.83 mol/mol$_{glucose}$ in an AFBR [Zhang et al., 2008; Show et al., 2010].

5.3.3 Volatile fatty acids

Table 5.1 shows the effluent VFAs concentrations before and after applying KOH in the headspace together with the estimated glucose consumption rates and H_2 production rates. It is noteworthy that there were three major changes in the effluent VFAs concentrations after sequestering CO_2; 1) an increase in the acetate concentration by 44%, 2) a decrease in the butyrate concentration to 53% of its original concentration, and 3) the complete elimination of the propionate. In contrast, Park et al. [2005] observed a decrease in the acetate concentration after applying KOH in the headspace of their batch experiments, in addition to an increase in the ethanol production, with acetate and

ethanol as the two main by-products. The aforementioned authors attributed the decrease in acetate concentration to the inhibition of homoacetogenesis [Park et al., 2005]. Also, Kim et al. [2006] observed a decrease in the acetate concentration to only 35% of its original value, and an increase in both butyrate and propionate concentrations by 101% and 28%, respectively, after applying continuous N_2 and CO_2 gas sparging in a CSTR producing H_2 from sucrose at an OLR of 40 gCOD/L.d and an HRT of 12 hours. However, the aforementioned authors observed low H_2 yields of 0.75, 0.93, and 1.20 mol/mol $hexose_{added}$ without gas sparging, with N_2 sparging, and with CO_2 sparging, respectively, indicative of H_2 production mainly through the butyrate pathway. Also, it should be noted that since the aforementioned systems were operated at low biomass concentrations of ~1 gVSS/L, specific H_2 production rates are lower than in the current study. Interestingly, with N_2 sparging only, Kim et al. [2006] observed a 24% increase in H_2 yield in agreement with the 22% observed in the current study, without any changes in microbial community structure i.e. the predominance of the butyrate pathway without gas sparging continued after N_2 sparging. However, Kim et al. [2006] reported that with CO_2 sparging, the improved yield is due to inhibition of acetogens and lactic acid bacteria, which compete with H_2 producers.

High H_2 yields have been associated with acetate and butyrate as fermentation products [Show et al., 2007]. Acetate and butyrate pathways limit the H_2 yield to the range of 2 to 4 moles of H_2 per 1 mole of glucose (reactions 5.1 and 5.2), but even lower H_2 yields are associated with propionate coexistence [Hawkes et al., 2002]. The propionate pathway is a H_2 consuming reaction which negatively affects H_2 yields (reaction 5.3), so production of propionate should be avoided [Vavilin et al., 1995]. In addition, from a thermodynamic point of view, reaction (5.6) shows that the propionate consuming reaction that produces H_2 and acetate is thermodynamically unfavourable (positive ΔG). Consequently, removing CO_2 from the headspace will shift reaction (5.6) forward, making this reaction more thermodynamically favourable. Stams and Plugge [2009] showed that the ΔG for the propionate to acetate pathway (reaction 5.6) and the butyrate to acetate pathway (reaction 5.7) can shift from +72 to -21 kJ/mol and from +48 to -22 kJ/mol, respectively, under low H_2 concentrations, due to syntrophic microorganism interactions at 25°C. Similarly, since CO_2 is an end-product in the

propionate to acetate pathway (reaction 5.6), based on the observed concentrations in this study, ΔG changed from +72 kJ/mol before CO_2 sequestration to -29 kJ/mol after CO_2 sequestration, respectively at 37°C. Accordingly, both H_2 and acetate production would increase, and propionate would be consumed, which explains the increase in acetate concentration and the sharp reduction in propionate concentration below its detection limit of 0.1 mg/L.

Table 5.1 - Stoichiometric glucose consumption, VFAs and H_2 production

CO_2 Sequestration	VFAs	VFAs Measured		VFAs Estimated	Estimated Glucose[1] Consumption	Actual Glucose Consumption	H_2 Theoretical	H_2 Measured	Reaction
		g/L	mol/d	mol/d	mol/d	mol/d	mol/d	mol/d	
Before (Reactions 5.1-5.3)	**HAc**	2.72	0.95	NA	0.47		+1.90		5.1
	HBu	0.90	0.21	NA	0.21		+0.42		5.2
	HPr	1.00	0.28	NA	0.14		-0.28		5.3
	Total	-	1.44	-	0.82	0.93	2.04	2.09	
After "Scenario I" (Reactions 5.1-5.3)	**HAc**	3.92	1.37	1.37	0.68		+2.74		5.1
	HBu	0.48	0.11	0.11	0.11		+0.22		5.2
	HPr	ND	ND	0.00	0.00		0.00		5.3
	Total	-	1.48	1.48	0.79	0.93	2.96	2.52	
After "Scenario II" (Reactions 5.1-5.3 & 5.6)	**HAc**	3.92	1.37	0.95	0.47		+1.90		5.1
				0.42			+1.26		5.6
	HBu	0.48	0.11	0.11	0.11		+0.22		5.2
	HPr	ND	ND	0.70	0.35		-0.70		5.3
	Total	-	1.48	2.18	0.93	0.93	2.68	2.52	

[1] Glucose consumed calculated based on VFAs produced

Theoretical H_2 production from VFAs produced was calculated based on 0.84 L H_2/g acetate, 0.58 L H_2/g butyrate, 0.34 L H_2/g propionate, and 1.27 L H_2/g acetate (reactions 5.1, 5.2, 5.3, and 5.6). Table 5.1 shows the detailed stoichiometric estimates for glucose consumption and H_2 production, based on the measured VFAs concentrations as compared to the experimental measurements. Since experimentally the contribution of each pathway to the consumption of glucose is not measured, but only the total glucose consumed, the estimated glucose consumption was based on the measured VFAs. As apparent from Table 5.1, in phase A, before CO_2 sequestration, glucose consumption by the thermodynamically favourable reactions 5.1 to 5.3 was 0.82 mol/d, which is 88% of the actual glucose consumption of 0.93 mol/d. The remaining glucose consumed of 0.11 mol/d can be attributed to glucose fermentation through other non-H_2 producing pathways such as lactate and ethanol, which were not quantified in the study. It should be noted that the ratio of VFAs (as COD)-to-SCOD in the effluent in phase A was 0.97:1 i.e. the other intermediates are present at very low concentrations. The theoretical H_2 production rates, shown in Table 5.1, were consistent with the H_2 measured during the experiment with a measured:theoretical ratio of 0.98. In phase B, after CO_2 sequestration, as a result of the observed increase in acetate and disappearance of propionate, two scenarios are analyzed denoted here as scenario 1 and 2. In scenario 1, it was assumed that only reactions 5.1 to 5.3 involving glucose occurred i.e. glucose was fermented directly to acetate and butyrate, with no propionate formation (reaction 5.3 did not occur). In scenario 2, it was assumed that reactions 5.1 to 5.3 proceeded exactly like before the CO_2 sequestration, but the propionate formed in reaction 5.3 was completely converted to acetate according to reaction 5.6, which became thermodynamically favourable with CO_2 sequestration i.e. the observed increase in acetate production in phase B relative to phase A is due to reaction 5.6. It is obvious that scenario 1 does not close the mass balance for both glucose and H_2. The estimated glucose consumption of 0.79 mol/d for scenario 1 accounts only for 85% of the actual glucose consumed. Also, the theoretical H_2 production rate of 2.96 mol/d is 17% higher than the actual H_2 production (2.52 mol/d). On the other hand, scenario 2 which is based on the assumption that the unaccounted 0.35 mol/d of glucose (the measured 0.93 mol/d of glucose consumed minus the 0.47 mol/d glucose consumed by reaction 5.1 prior to CO_2 sequestration minus the 0.11 mol/d for

butyrate production according to reaction 5.2) was consumed for propionate production with H_2 consumption (reaction 5.3), after which the propionate produced was converted to acetate and H_2 (reaction 5.6), which is supported by the calculated negative ΔG due to CO_2 sequestration. It is interesting to note that theoretical H_2 production in scenario 2 differed by only 6% from the actual H_2 production. Furthermore, even if we assume that 3% of the influent glucose was fermented through the lactate and ethanol non-H_2 producing pathways since the effluent VFAs in phase B were 97% of the SCOD, the estimated H_2 production rate is 2.7 mol/d, in close agreement with the observed 2.52 mol/d. The increase in H_2 production may be due to a microbial shift to lactate production, which is supported by the microbial community analysis results discussed later. OTUs in the genus *Streptococcus* were present in both phases and are known as lactate producing bacteria [Hino et al., 1994]. In addition, the microbial community analysis showed that bacteria from the genus *Megasphaera* were enriched 3-fold, from 9.7% in phase A to 27.4% in phase B. The two aforementioned bacteria utilize lactate in preference to glucose and produce propionate [Hino et al., 1994], which may explain the 22% increase in H_2 production after CO_2 sequestration.

5.3.4 pH, buffer, and KOH requirements

Reactor pH was maintained at 5.2 ± 0.2 during the experiment using a buffer solution of 168 g/L $NaHCO_3$. The buffer concentration of 3 g $NaHCO_3$/L in the feed was kept constant before and after CO_2 sequestration from the headspace. It is noteworthy that using KOH in the headspace for CO_2 sequestration decreased the $NaHCO_3$ buffer consumption by the pH controller to only 17% of its consumption before adding the KOH, while overall $NaHCO_3$ buffer consumption i.e. feed and reactor pH control system decreased by 57%. Table 5.2 shows buffer concentrations used in the feed and consumed by the pH controller to maintain a constant pH of 5.2 ± 0.2 during H_2 production.

Table 5.2 - Buffer requirements

	$NaHCO_3$ added						
	Feed		**pH controller**			**Total**	
	Solution concentration						
CO_2 Sequestration	**g/L**	**g/d**	**mL/d**	**g/L**	**g/d**	**g/d**	**g $NaHCO_3$/g glucose feed**
Before	3	63	825	168	139	202	1.2
After	3	63	140	168	24	87	0.52

Theoretical KOH consumption of 117 g/d was calculated based on reaction 5.8, where 1 mole of CO_2 would consume 1 mole of KOH (i.e. 1.27 g KOH/g CO_2). CO_2 production rates were 43 and 53 L/d before and after applying KOH in the headspace, respectively. However, the experimental KOH consumption rate was observed to be 136 g/d which is 14% higher than the theoretical value, KOH was deemed to be exhausted when the H_2 percentage in the biogas started dropping, at which point KOH was replaced.

$$KOH + CO_2 \rightarrow KHCO_3 \quad (5.8)$$

Overall alkalinity consumption including both feed $NaHCO_3$ and headspace KOH consumption was calculated to be 120 mg$CaCO_3$/d before KOH application and 173 mg$CaCO_3$/d after KOH application. However, although the overall alkalinity consumption increased after KOH application by 44%, both H_2 production yields and rates increased, and gas composition shifted to 100% H_2, indicating that the increase in alkalinity was greatly beneficial. In addition, the $KHCO_3$ produced can be recycled and used as a buffer, which could reduce the overall buffer consumption.

Chemical CO_2 produced (H_2CO_3*) during KOH application from buffer addition was calculated based on a pH of 5.2 ($[H^+] = 10^{-5.2}$) and carbonic acid dissociation constant ($K_{a1}=4.9*10^{-6}$ at 37°C) to be 0.27 mol/d, whereas the biological CO_2 produced was measured to be 2.07 mol/d. The low contribution of chemical CO_2 to the total CO_2 produced (12%) supports the idea that CO_2 produced from microbial metabolism is the main CO_2 that is being sequestered.

5.3.5 Microbial community analysis

The composition of the bacterial communities present in the IBRCS was assessed and compared before and after CO_2 removal from the reactor headspace. Samples were taken from the IBRCS in triplicates from phase A and phase B, and total DNA extracted from samples was amplified using primers specific to the V4 hyper-variable region of 16S rDNA. The PCR amplicons were sequenced by high-throughput Illumina sequencing, and the nucleotide sequence data was subjected to bioinformatics analyses to determine species identity, diversity, and richness in the samples. IBRCS samples were complex due to the presence of multiple organic compounds, diverse degradation products, and mixed microbial cultures. Figure 5.4 shows amplification of the 16S rDNA V4 region using the 515 F and 806 R primers for phase A and phase B samples, as demonstrated by the presence of the PCR products of the expected size (300-350 bp).

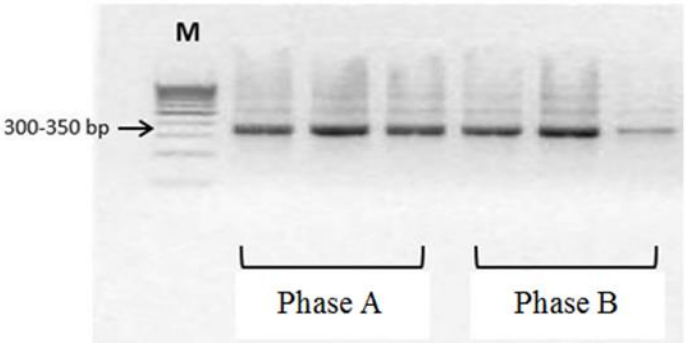

Figure 5.4 - 16S rDNA PCR products amplified from DNA extracted from anaerobic digester samples collected from different experimental phases

Rarefaction analysis was used to estimate the species richness of the samples by QIIME software [Caporaso et al., 2010a]. The average numbers of sequence per phase were plotted vs. rarefaction measures [Tracy et al., 2012]. The microbial richness was measured based on number of operational taxonomic units (OTUs) between phases. Figure 5.5 depicts species richness between the two different phases applied in the IBRCS. This study revealed a greater number of OTUs in phase B samples (after addition of KOH pellets in the IBRCS headspace) than in samples from phase A (before addition of KOH pellets in the IBRCS headspace) which contained fewer OTUs than phase B, and thus had lower species diversity. These data indicate that the microbial community structure was impacted by CO_2 sequestration at the tested OLR. It can be inferred from the microbial community richness results that the richer microbial community, affected lower buffer consumption, since as the richness increased after applying the KOH in the IBRCS headspace, the total buffer consumption decreased to 43% of its original value before applying the KOH. Normalizing the total buffer consumption to the VFAs produced also showed a decrease in the total buffer consumption from 1.6 to 0.8 $gNaHCO_3/gCOD_{TVFAs}$ at the tested OLR.

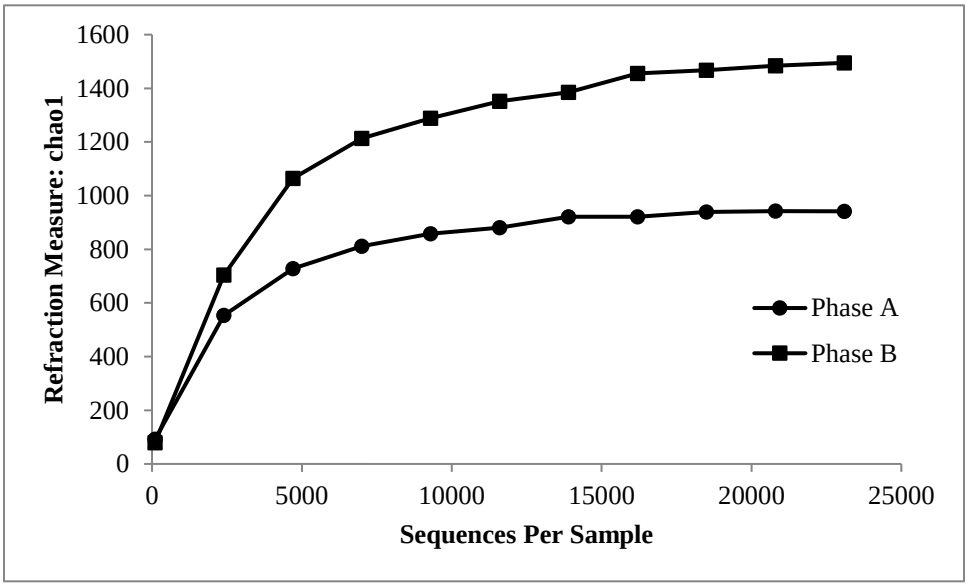

Figure 5.5 - Alpha diversity analysis

The bacterial richness and diversity for each phase were calculated using the Mixed Procedure (Table 5.3). Percentage of coverage of phase A and phase B was significant ($p < 0.05$), which indicates that samples of both phases had different number of species. The Simpson and Shannon species diversity indices for phase A and phase B were also significantly correlated, which indicates that species diversity within both samples was different.

Table 5.3 - Effect of bacterial richness and diversity indices calculated from illumine sequences in sludge samples collected from IBRCS

Item	Phase A	Phase B	SED	p-Value
Average no. of sequences per sample	11600	11600	3253	-
Richness chao1	781	1181	152	0.0161
Coverage (%)	0.045	0.035	0.003	0.0299
Observed species	500	648	117	0.2237
Shannon	3.846	4.397	0.117	0.0001
Simpson	0.735	0.849	0.002	0.0001

5.3.5.1 Principal coordinate analysis (PCoA)

The species diversity relationships among the samples can be viewed based on weighted or unweighted unifrac distances measured between the microbial communities, and visualized by phylogenetic trees illustrated using the PCoA plot (Figure 5.6). The

taxa in each sample were clustered in the phylogenetic tree and the UniFrac distance values were created separately [Lozupone et al., 2007]. These UniFrac values of each phase were used to construct 2D plots by Principal Coordinate Analysis (PCoA), presented in Figure 5.6. Samples of each phase clustered based unweighted UniFrac distances with clear separation with PC1 of 28.36% and PC2 of 10.6% between the different phases. In this graph, samples are clustered by similar OTUs, implying that overall phylogenetic diversity changed due to the addition of KOH to the headspace. QIIME pipeline demonstrates beta diversity by different cluster affinities of V4 hyper variable region of 16S rDNA sequenced by illumina sequencer. The samples of each phase are separated and grouped together in the plot with variation in the UniFrac distance values. It is evident from Figure 5.6 that Phase A and Phase B stand unique from each other due to total variation in species diversity among the samples.

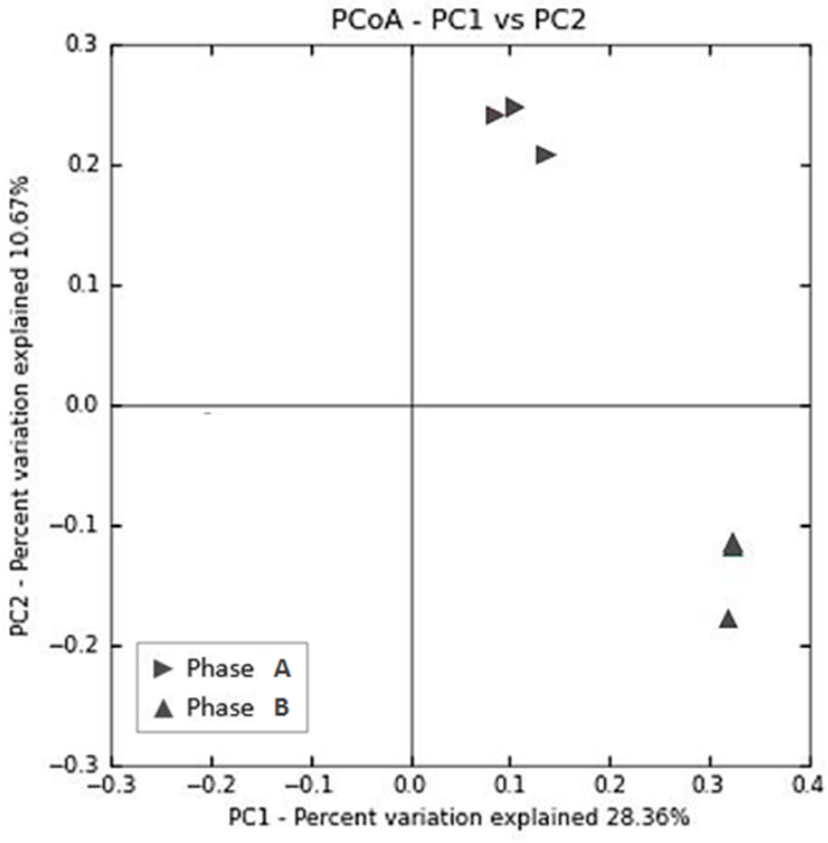

Figure 5.6 - Principal coordinates analysis of unweighted UniFrac distances between samples

5.3.5.2 Partial least square analysis

The influential contribution distinguishing between phases based on the abundance of each OTU has been analyzed using the partial least square analysis. OTUs in the Family *Streptococcaceae* were the major dominant species. However, OTUs in the Family *Clostridiaceae* and in the genus *Blautia* were the next most prevalent species in phase A. The other species, i.e. OTUs in the Phyla *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* were comparatively low in quantity before adding KOH to the reactor headspace. On the other hand, phase B was significantly influenced by an abundance of OTUs in the genus *Megasphaera*. The Phylum *Firmicutes* contains OTUs in the Order *Clostridiales*, the Class *Clostridia*, the Family *Coriobacteriaceae*, and the genera *Ruminococcus*, *Streptococcus*, and *Atopobium*, which were, on average, present at > 2% of the total population after addition of KOH in the IBRCS headspace. The OTUs of Phylum *Firmicutes*, in the Order *Bacteroidales*, the Family *Paraprevotellacea*, and in the genera *Desulfovibrio*, *Ethanoligenens*, and *Ruminococcus* were present in equal amounts both before and after addition of KOH to the IBRCS headspace.

5.3.6 Statistical analysis

Normality of residuals of OTUs was statistically analyzed using the Mixed Procedure. This analysis revealed the importance of genera in each phase. The p-values ($p < 0.05$) indicate that a particular OTU was unique to a particular phase sample. According to the statistical analysis, OTUs in the Phylum *Firmicutes*, in the Order *Bacteroidales*, and in the genus *Streptococcus* were present in both phase A and phase B and these OTUs were significantly correlated among these phases.

Certain taxa were significantly enriched in the IBRCS at the tested OLR, in the presence of KOH (Table 5.4). OTUs in the genus *Blautia* were enriched 17.9-fold in phase B compared to phase A, and the populations of other H_2 producing OTUs in the genera *Ruminococcus*, *Ethanoligenens*, *Megasphaera*, and *Clostridium* were enriched 4.1-fold, 3.9-fold, 3.7-fold, and 3.2-fold, respectively, in phase B compared with phase A. Bacteria in the genus *Blautia* are gram-positive, obligate anaerobes that produce acetate,

CO_2, H_2, and other end-products when fermenting glucose [Park et al., 2013]. Bacteria in the genus *Ruminococcus* also produce acetate, CO_2, H_2, and other end-products during glucose fermentation, with an observed H_2 yield of 2 mol/$mol_{glucose}$ [Ntaikou et al., 2009]. Bacteria in the genus *Ethanoligenens* produce H_2 through the acetate pathway with a H_2 yield of 1.83 mol/$mol_{glucose}$ [Tsygankov and Tekucheva, 2012]. The enrichment of these cultures with the ability to produce H_2 through the acetate pathway supports the fact that acetate concentrations increased after CO_2 sequestration. *Megasphaera* is an important taxon in H_2 fermentation systems from glucose, fructose, and lactate as the main carbon source and acetate, butyrate, CO_2, and H_2 as the end products [Ohnishi et al., 2010]. It is noteworthy, that bacteria in the genus *Megasphaera* are known as propionate producers from lactate, but are not capable of producing propionate from glucose [Hino et al., 1994]. Also, bacteria in the genus *Clostridium* are well-known H_2 producers through acetate and/or butyrate pathways [Tsygankov and Tekucheva, 2012]. The total percentages of H_2 producers were 13% and 37% of the total sequences in phases A and B, respectively.

CO_2 sequestration affected the population of non-H_2 producers as well as the H_2 producers, where certain taxa were significantly reduced in the presence of KOH (Table 5.4). OTUs in the genus *Veillonella* are obligate anaerobes that are capable of producing H_2 from a variety of carbon sources as lactate, malate, and fumarate, while glucose is not its favourite substrate [Ohnishi et al., 2010]. OTUs in the genus *Veillonella* were observed to decrease from phase A to phase B by 85%. OTUs in the genus *Faecalibacterium*, decreased by 80% from phase A to phase B. These bacteria cannot produce H_2 as fermentation product, and consume acetate during fermentation process [Duncan et al., 2002]. OTUs in the genus *Dialister*, which have been reported as non-H_2 producers [Lin et al., 2008], also decreased by 51%. Wexler et al. [1996] stated that OTUs in the genus *Sutterella* are H_2 consumers that require formate and fumarate, or H_2 for growth, and this OTU decreased by 40%. OTUs in the genus *Desulfovibrio* are sulfate-reducing bacteria that obtain energy by oxidizing organic compounds or H_2 while reducing sulfate to hydrogen sulfide [Martins and Pereira, 2013]. *Desulfovibrio* was observed to decrease by 30% at the tested OLR.

Table 5.4 - Enrichment of selected bacterial species

Taxa	Phase A		Phase B		Fold enrichment: Phase B/Phase A
	%	Observed species	%	Observed species	
Taxa at genus level known as H_2 producers					
Blautia	0.04	0	0.49	3	17.9
Ruminococcus	1.28	6	4.07	26	4.1
Ethanoligenens	0.04	0	0.12	1	3.9
Megasphaera	9.66	48	27.40	178	3.7
Clostridium	2.05	10	5.07	33	3.2
Taxa at genus level known as non-H_2 producers					
Veillonella	0.52	3	0.08	1	0.2
Faecalibacterium	1.36	7	0.25	2	0.2
Dialister	2.24	11	1.10	7	0.6
Sutterella	0.15	1	0.09	1	0.8
Desulfovibrio	5.38	27	3.77	24	0.9

In summary, the microbial population of IBRCS before the addition of KOH, was dominated by OTUs in the Families *Streptococcaceae* and *Clostridiaceae,* and in the genera *Blautia* and *Ethanoligenens*. On the other hand, after the addition of KOH pellets in the headspace the microbial population was found to be dominated by OTUs in the Class *Clostridia*, the Order *Clostridiales*, the Family *Coriobacteriaceae*, and the genera *Megasphaera*, *Ruminococcus*, *Streptococcus*, and *Atopobium*. A survey of the literature revealed that bacteria in the genera *Megasphaera* and *Ruminococcus* are H_2 producers [Castelló et al., 2009; Ntaikou et al., 2008]. Species of bacteria in the Phylum *Firmicutes,*

the Class *Clostridia*, the Family *Clostridiaceae*, and the genus *Clostridium* were present in all phases indicating that these species are not affected by the addition KOH pellets in the headspace reactor.

5.4 Conclusions

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

- Removal of CO_2 from the headspace shifted the H_2 producing pathways forward, increasing H_2 yield by 22% to 2.96 mol/mol and H_2 production rate by 23%
- CO_2 sequestration changed the propionate consumption pathway to be thermodynamically favourable producing more acetate and H_2
- Microbial analysis based on OTUs revealed higher bacterial richness and diversity due to CO_2 sequestration
- Percentage of identified H_2 producers of the total sequences increased from 13% before CO_2 sequestration to 37% after CO_2 sequestration
- Percentage of identified non-H_2 producers of the total sequences decreased from 10% before CO_2 sequestration to 5% after CO_2 sequestration

5.5 Acknowledgment

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5.6 References

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Chapter 6

Comparative Assessment of Glucose Utilization Kinetics using *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii*

6.1 Introduction

Dark fermentative biohydrogen production is a promising area of technology development that shows a potential for H_2 production from lignocellulosic waste streams [Lin et al., 2007]. Different groups of microorganisms have been investigated over decades for biological H_2 production such as algae and cyanobacteria (biophotolysis), photosynthetic bacteria (photofermentation), and fermentative bacteria (dark fermentation) [Hallenbeck and Benemann, 2002]. Dark fermentative H_2 production has the advantages of potentially using waste and biomass residues as feedstocks, faster production rates than the photosynthetic route, and no light requirements [Urbaniec and Bakker, 2015; Azbar and Levin, 2012].

Fermentative H_2-producing bacteria are classified into: strict anaerobes (*Clostridia*, methylotrophs, rumen bacteria, archaea), facultative anaerobes (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and aerobes (*Alcaligenes*, *Bacillus*) [Li and Fang, 2007]. Many studies have shown that *Clostridium* species were dominant in anaerobic fermentative H_2 production processes [Lin et al., 2007; Pan et al., 2008; Hafez et al., 2010]. However, H_2 production experiments reported in the literature using *Clostridium* species have shown a wide range of observed H_2 production parameters for the same species i.e. H_2 yields, production rates, lag phases, and end-products [Elsharnouby et al., 2013]. In addition, most of the studies on H_2 production focused mainly on H_2 yields, production rates, and end-products, without reporting kinetic parameters [Liu et al., 2011; Zhao et al., 2011; Hu et al., 2013]. On the other hand, studies which focused on kinetic parameters and metabolic pathways ignored H_2 production parameters [Linville et al., 2013].

Clostridium species are strict anaerobic bacteria that can ferment a wide range of different substrates to many important end-products. Some species such as *C. termitidis* have the ability to hydrolyze lignocellulosic substrates to simple monosaccharides (mainly glucose) under mesophilic conditions [Ramachandran et al., 2008; Gomez-Flores et al., 2015]. However, a low H_2 yield of 0.62 mol H_2/mol hexose equivalent was obtained when using cellulose as the carbon source [Ramachandran et al., 2008], necessitating the use of other species that can efficiently utilize monosaccharides enhancing H_2 production yields. *C. beijerinckii* is a mesophilic H_2 producer that cannot utilize cellulose but is adept at utilizing glucose [Masset et al., 2012]. Different H_2 production rates and yields that have been reported for different strains of *C. beijerinckii* are shown in Table 6.1. At an initial glucose concentration of 10 g/L, the H_2 yield of 2.52 mol/mol glucose reported by Pan et al. [2008] was 26% higher than the 2.00 mol/mol glucose reported by Taguchi et al. [1992]. However, the higher H_2 production rate of 36.5 mL/hr [Taguchi et al., 1992] was associated with the lower yield, while Pan et al. [2008] reported only a rate of 15.2 mL/hr for the 2.52 mol/mol glucose. Also, at an initial glucose concentration of 6 g/L, Liu et al. [2011] achieved a H_2 yield of 1.72 mol/mol glucose using L9 strain, which was 72% higher than the yield achieved by Zhao et al. [2011] using RZF-1108 strain.

C. saccharoperbutylacetonicum has been known as an alcohol-producing bacteria in fermentative acetone-butanol-ethanol (ABE) production which is optimum at pH of 4.5 to 5.5 [Biebl, 1999; Kalil et al., 2003; Al-Shorgani et al., 2012] and its H_2 production potential has not been well studied [Alalayah et al., 2008]. Ferchichi et al. [2005a] reported a H_2 yield of 1.30 mol/mol glucose at an initial glucose concentration of 10 g/L using *C. saccharoperbutylacetonicum* ATCC 27021, while at the same glucose concentration Alalayah et al. [2008] reported only 0.57 mol/mol glucose using strain ATCC 13564. However, higher yields of 2.70 mol/mol lactose and 2.87 mol/mol sugars were reported for *C. saccharoperbutylacetonicum* using carbohydrate-rich substrates of cheese whey and rice bran, respectively [Ferchichi et al., 2005b; Dada et al., 2013]. *C. saccharoperbutylacetonicum* has not been thoroughly investigated for H_2 production except by Alalayah et al. [2008] and Ferchichi et al. [2005a] for utilizing glucose, and by Dada et al. [2013] and Ferchichi et al. [2005b] for utilizing carbohydrate-rich wastes.

Table 6.1 - H_2 production rates and yields reported for *C. beijerinckii* strains

Strain	Glucose (g/L)	Production rate (mL H_2/hr)	Yield (mol H_2/mol glucose)	Reference
RZF-1108	6	2.0	1.00	Zhao et al., 2011
	8	3.2	1.20	
	9	6.00	1.97	
DSM 791	5-20	0.3-0.7	0.60-1.60	Hu et al., 2013
ATCC 8260	0.9	1.0	1.05	Skonieczny and Yargeau, 2009
	1.4	2.0	1.31	
	1.9	2.4	1.44	
	2.3	3.6	1.30	
	2.8	3.3	1.57	
DSM 1820	5	-	1.45	Masset et al., 2012
L9	6	-	1.72	Liu et al., 2011
	3	20.0	2.81	Lin et al., 2007
AM21B	10	36.5	2.00	Taguchi et al., 1992
Fanp 3	10	15.2	2.52	Pan et al., 2008

The study of substrate utilization kinetics is important for the analysis, design, operation, and scale-up of H_2 production processes [Huang and Wang, 2010]. The Monod-based kinetic model is widely used to define substrate utilization [Gnanapragasam et al., 2011] and particularly to describe the influence of initial substrate concentration on the substrate utilization rates [Wang and Wan, 2009]. Lin et al. [2007] reported the maximum specific glucose consumption rate and Monod half-saturation constant for *C. beijerinckii* L9 to be 1.03 h^{-1} and 0.47 g/L, respectively using initial

glucose concentration of 3 g/L. For ABE production, the specific growth rate of *C. saccharoperbutylacetonicum* has been reported to be 0.2 h^{-1} using 10 g/L glucose as the substrate [Soni et al., 1987]. For H_2 production, only Alalayah et al. [2008] reported the maximum specific growth rate and saturation constant for *C. saccharoperbutylacetonicum* to be 0.4 h^{-1} and 5.51 g/L, respectively using initial glucose concentration of 10 g/L. An extensive literature search revealed that while few studies reported Gompertz kinetics for *C. beijerinckii* on sugars [Pan et al., 2008; Skonieczny and Yargeau, 2009], only one study reported Monod kinetics on glucose [Lin et al., 2007]. For *C. saccharoperbutylacetonicum*, only Alalayah et al. [2008; 2010] reported Monod kinetics on glucose, and no Gompertz data were reported. Thus, the aim of this study is to provide and compare H_2 production, Monod kinetics, and Gompertz model parameters for the known H_2 producer, *C. beijerinckii* and the new H_2 producer, *C. saccharoperbutylacetonicum*.

6.2 Materials and Methods

6.2.1 Microbial strain and media

Clostridium beijerinckii strain DSM 1820 and *Clostridium saccharoperbutylacetonicum* strain DSM 14923 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany). Cultures inoculations of 10% (v/v) were conducted in ATCC 1191 medium at 37°C and pH 7.2 using filter-sterilized glucose at different concentrations. The medium contained (per liter of double-distilled water): KH_2PO_4, 1.5 g; Na_2HPO_4, 3.35 g; NH_4Cl, 0.5 g; $MgCl_2.6H_2O$, 0.18 g; yeast extract, 2 g; resazurin, $2.5*10^{-4}$ g; mineral solution, 1 mL; vitamin solution, 0.5 mL, and L-cysteine (reducing agent), 1 g. The mineral solution contained (g per liter): trisodium nitrilotriacetate 20.2; $FeCl_3.6H_2O$, 2.1; $CoCl_2.6.\ H_2O$, 2; $MnCl_2.4H_2O$, 1; $ZnCl_2$, 1; $NiCl_2.6H_2O$, 1; $CaCl_2.2H_2O$, 0.5; $CuSO_4.5H_2O$, 0.64; and $Na_2MoO_4.2H_2O$, 0.5. The vitamin solution contained (mg per liter): pyridoxine-HCl, 100; riboflavin, 50; thiamine, 50; nicotinic acid, 50; *p*-aminobenzoic acid, 50; lipoic acid (thioctic acid), 50; biotin, 20; folic acid, 20; and cyanocobalamin, 10.

6.2.2 Experimental setup

Batch anaerobic fermentations were conducted in 180 mL serum bottles with a working volume of 100 mL. All bottles containing 1191 media were initially degassed by applying vacuum then sparged with N_2 gas, and autoclaved. Filter-sterilized glucose was added to media bottles at concentrations of 4, 6, and 8 g/L in triplicates. Fresh cultures of *C. beijerinckii* and *C. saccharoperbutylacetonicum* at 10% (v/v) were inoculated in each bottle then incubated at 37°C in a swirling-action shaker (MaxQ 4000, Fisher Scientific, ON, CA) operating at 100 rpm. Control bottles using media and glucose without cultures were prepared and incubated in duplicates at the same experimental conditions.

6.2.3 Analytical methods

Glucose was analyzed by BioPacific Diagnostic glucose kit (BC, Canada). HACH methods and testing kits (HACH Odyssey DR/2500) were used to measure the chemical oxygen demand (COD). The volatile fatty acids (VFAs) concentrations were analyzed using Varian 8500 has chromatography (Varian Inc., ON, CA) with a flame ionization detector (FID) of temperature 250°C and equipped with a fused silica column (30 m * 0.32 mm) of temperature 110°C. Helium was used as the carrier gas at a flow rate of 5 mL/min.

6.2.4 Gas measurements

Glass syringes of appropriate sizes in the range of 5-100 mL were used to measure the volume of gas produced by releasing the gas to equilibrate with the ambient pressure [Owen et al., 1979]. A gas chromatograph (Model 310, SRI instruments, ON, CA) was used to determine the gas composition. The GC is equipped with a thermal conductivity detector (TCD) of temperature 90°C and a molecular sieve column of temperature 105°C. Argon was used as the carrier gas at a flow rate of 30 mL/min. H_2 gas production was calculated using Equation 6.1:

$$V_{H_2,i} = V_{H_2,i-1} + C_{H_2,i} * V_{G,i} + V_{h,i}(C_{H_2,i} - C_{H_2,i-1}) \qquad (6.1)$$

where $V_{H_2,i}$ and $V_{H_2,i-1}$ are cumulative H_2 gas volumes at the current (i) and previous (i - 1) time intervals. $V_{G,i}$ is the total gas volume accumulated between the previous and current time intervals. $C_{H_2,i}$ and $C_{H_2,i-1}$ are the fractions of H_2 gas in the headspace of the reactor in the current and previous intervals, and $V_{h,i}$ is the total volume of the headspace of the reactor in the current interval [López et al., 2007].

6.2.5 Modeling

A modified non-linear least square fit model established by Gomez-Flores et al. [2015] using MATLAB R2014a was used to determine Monod kinetic parameters (Equation 6.2) [Mu et al., 2006]:

$$\frac{1}{X}\frac{dS}{dt} = \frac{-KS}{K_S+S} \tag{6.2}$$

where X is the biomass concentration (g/L), S is the substrate concentration (g/L), K is the maximum specific substrate utilization rate (g substrate/gVSS.hr), K_s is the saturation concentration (g/L) or half-velocity constant and is equal to the concentration of the rate-limiting substrate (glucose) when the substrate degradation rate is equal to one half of the maximum [Mu et al., 2006]. Average percentage errors (APE), root mean square errors (RMSE), and coefficient of determination (R^2) were used to assess the model fit.

The modified Gompertz equation (Equation 6.3) was used to model biohydrogen production, where P is the cumulative H_2 production, P_{max} is the maximum cumulative H_2 production, R_{max} is the maximum H_2 production rate, λ is the lag time, and t is the fermentation time [Lay et al., 1999].

$$P = P_{max}\, exp\left\{-exp\left[\frac{R_{max}\, e}{P_{max}}(\lambda - t) + 1\right]\right\} \tag{6.3}$$

6.3 Results and Discussion

6.3.1 H_2 production

Figure 6.1 shows the experimental and stoichiometric cumulative H_2 production profiles for *C. beijerinckii* and *C. saccharoperbutylacetonicum* using initial glucose concentrations of 4, 6, and 8 g/L. Coefficients of variation (calculated as standard deviation divided by the average) in all experiments were less than 8% confirming data reproducibility. Stoichiometric H_2 production was calculated from the VFAs produced and will be further discussed later. The maximum H_2 content reached was 57±2% in all experiments using both cultures. The initial pH (7.2) dropped to an average of 5.5±0.2 and 5.6±0.1 in experiments using *C. beijerinckii* and *C. saccharoperbutylacetonicum*, respectively.

Table 6.2 shows the Gompertz kinetics for both experiments at each initial glucose concentration. The coefficient of determination R^2 was 0.999 for all Gompertz data. The maximum H_2 production rate of *C. beijerinckii* achieved was 34.2 mL/hr at initial glucose concentration of 8 g/L, which is consistent with Pan et al. [2008] who reported a rate of 30.3 mL/hr at a glucose concentration of 10 g/L using Gompertz model. For *C. saccharoperbutylacetonicum*, there are no available Gompertz kinetic parameters in the literature. Average lag phase and maximum H_2 production rate of 11.0±0.4 hours and 21.8±2.3 mL/hr for *C. saccharoperbutylacetonicum* were determined using Gompertz kinetics compared to 16.6±2.7 hours and 29.2±6.9 mL/hr for *C. beijerinckii*. It is apparent from Figure 6.1 and Table 6.2 that for *C. saccharoperbutylacetonicum*, the lag phase and H_2 production rates were not drastically affected by the change in initial glucose concentration with a 7% change in the lag phase from 10.6 to 11.3 hours and an 11% change in the maximum H_2 production rate from 19.2 to 23.7 mL/hr. On the other hand, for *C. beijerinckii* experiments, the lag phase increased by 37% from 14.2 hours at an initial glucose concentration of 4 g/L to 19.5 hours at 8 g/L. Also, the H_2 production rate increased by 61% from 21.3 at 4 g/L glucose to 34.2 mL/hr at 8 g/L glucose. The H_2 production rate for *C. beijerinckii* achieved in this study (21.3 mL/hr) at an initial glucose concentration of 4 g/L was six times the production rate achieved by Skonieczny and

Yargeau [2009] at an initial glucose concentration of 2.8 g/L. The aforementioned authors used *C. beijerinckii* ATCC 8260, i.e. the different strain used may explain the big difference in H_2 production rates.

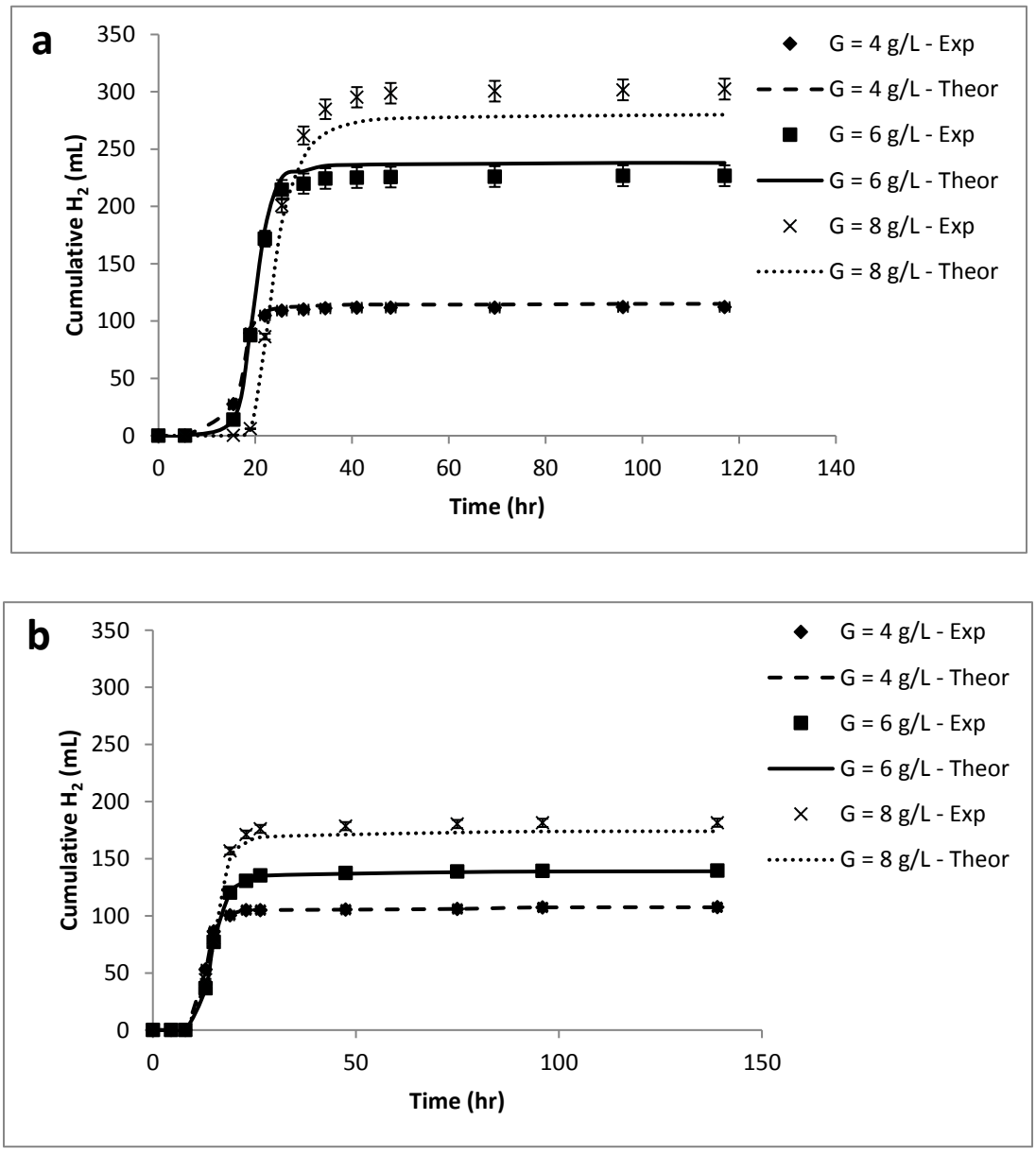

Figure 6.1 - Experimental and Theoretical Cumulative H_2 production from different glucose concentrations using a) *C. beijerinckii* and b) *C. saccharoperbutylacetonicum*

Table 6.2 - Gompertz data and H_2 production yields

	C. beijerinckii			*C. saccharoperbutylacetonicum*		
G (g/L)	**4**	**6**	**8**	**4**	**6**	**8**
P^a (mL)	111	226	298	106	138	180
$R_m{}^b$ (mL/hr)	21.3	32.1	34.2	22.4	19.2	23.7
λ^c (hr)	14.2	16.1	19.5	10.6	11.0	11.3
H_2 Yield (mol H_2/$mol_{glucose}$)	2.00	1.72	1.58	1.91	1.65	1.61

[a] Ultimate H_2 production, [b] Maximum H_2 production rate, [c] Lag phase

The maximum H_2 production yields achieved for *C. beijerinckii* and *C. saccharoperbutylacetonicum* were 2.00±0.07 and 1.91±0.08 mol/mol glucose, respectively, and were both in experiments using initial glucose concentration of 4 g/L (Table 6.2). At an initial glucose concentration of 6 g/L, *C. beijerinckii* L9 strain achieved the same H_2 yield as this study of 1.72 mol/mol glucose [Liu et al., 2011]. However, at the same initial glucose concentration of 6 g/L using *C. beijerinckii* RZF-1108 strain, this study achieved 72% higher yield than Zhao et al. [2011] who achieved only 1.00 mol/mol glucose. Also, at 8 g/L glucose, the 2.00 mol/mol glucose achieved was 67% higher than the yield achieved by the aforementioned authors [Zhao et al., 2011]. At a lower glucose concentration of 3 g/L, *C. beijerinckii* L9 was able to achieve higher H_2 yield of 2.81 mol/mol glucose [Lin et al., 2007]. Alalayah et al. [2008; 2010] investigated the effect of inoculum size, initial glucose concentration, initial pH, and operational temperature on H_2 production from *C. saccharoperbutylacetonicum* ATCC 13564. The aforementioned authors observed maximum H_2 yields of 0.73 and 0.55 mol/mol glucose at 5 and 10 g/L, respectively, and found the optimum inoculation size, glucose concentration, initial pH, and temperature to be 10% (v/v), 10 g/L, 6-7, and 37°C, respectively. The maximum H_2 production yield achieved in this study of 1.91

mol/mol glucose using *C. saccharoperbutylacetonicum* DSM 14923 is 2.6 fold the yield achieved by Alalayah et al. [2008; 2010].

6.3.2 COD balance

Although COD balances are essential to assess the quality of data reported, reporting COD mass balances in H_2 production experiments using pure cultures is limited in the literature compared to studies using mixed cultures. Table 6.3 presents the COD mass balance for all experiments using both cultures. The closure of COD balance at an average of 99±4% verifies the reliability of the data.

Table 6.3 - Summary of COD balance

	C. beijerinckii			***C. saccharoperbutylacetonicum***		
G (g/L)	**4**	**6**	**8**	**4**	**6**	**8**
$COD_{initial}$ (gCOD)	0.76	0.93	1.12	0.75	0.94	1.12
COD_{final} (gCOD)	0.68	0.84	0.95	0.66	0.80	0.97
Cumulative H_2 (mL)	112.3	226.8	302.4	107.5	139.6	181.5
H_2 (gCOD)	0.07	0.14	0.19	0.07	0.09	0.11
COD balance[a] (%)	98	106	102	97	95	97

[a] COD balance (%) = [H_2 (gCOD) + COD_{final} (gCOD)]*100/[$COD_{initial}$ (gCOD)]

6.3.3 Monod growth kinetics

The Monod kinetic equation (Equation 6.2) was used to estimate the kinetic coefficients by modeling the glucose degradation for *C. beijerinckii* and *C. saccharoperbutylacetonicum* and neglecting the change in biomass concentration. Figure 6.2 shows the experimental and modeled substrate degradation for experiments using the

three tested initial substrate concentrations for *C. beijerinckii* (Figure 6.2a) and *C. saccharoperbutylacetonicum* (Figure 6.2b). The APE, RMSE, and R^2 were calculated to assess the goodness of fit for substrate concentrations and are presented in Table 6.4. APE values ranged from 7.2% to 19.2%, RMSE values ranged from 0.14 to 1.00 g/L, and R^2 ranged from 0.80 to 0.99. In addition, Figure 6.3 shows the correlation between the modeled and experimental glucose concentrations, with absolute fraction of variance (R^2), calculated with respect to the equity line, of 0.93 and 0.99 for *C. beijerinckii* and *C. saccharoperbutylacetonicum*, respectively. In conclusion, the calculated statistical parameters and correlations prove the good fitness of the MATLAB model. Table 6.4 presents the estimated kinetic parameters derived from only the growth phase as shown in Figure 6.2. For *C. beijerinckii*, K and K_s increased with the increase in the initial glucose concentration as shown in Table 6.4 with an average of 0.50±0.18 g substrate/gVSS.hr and 1.43±0.56 g/L, respectively. Lin et al. [2007] reported the maximum specific glucose consumption rate to be 1.03 mmol/mmol.hr (1.58 g/g.hr) using *C. beijerinckii* L9, which is double the value reported in this study, and the Monod half-saturation constant to be 0.47 g/L which is only one-third the value reported in this study. The aforementioned authors used *C. beijerinckii* strain L9 and an initial glucose concentration of 3 g/L [Lin et al., 2007], which is less than the range tested in this study (4-8 g/L). Average K and K_s for *C. saccharoperbutylacetonicum* were determined to be 0.57±0.05 g substrate/gVSS.hr and 0.78±0.04 g/L for the initial glucose concentrations tested. For *C. saccharoperbutylacetonicum*, the value of K (0.57 g substrate/gVSS.hr, i.e. μ_{max} of 0.11 h^{-1} assuming a biomass yield of 0.2 gVSS/g glucose) is 30% of the maximum specific growth rate (μ_{max}) reported by Alalayah et al. [2008; 2010] who used *C. saccharoperbutylacetonicum* ATCC 13564 at an initial glucose concentration of 10 g/L. Also, the K_s of 5.51 g/L reported by the aforementioned authors is much higher than the value reported in this study (0.78 g/L). The lower K_s reported in this study indicates better growth kinetics for the DSM 14923 strain used compared to the ATCC 13564 strain used by Alalayah et al. [2008; 2010], however, the initial glucose concentration is different in both studies.

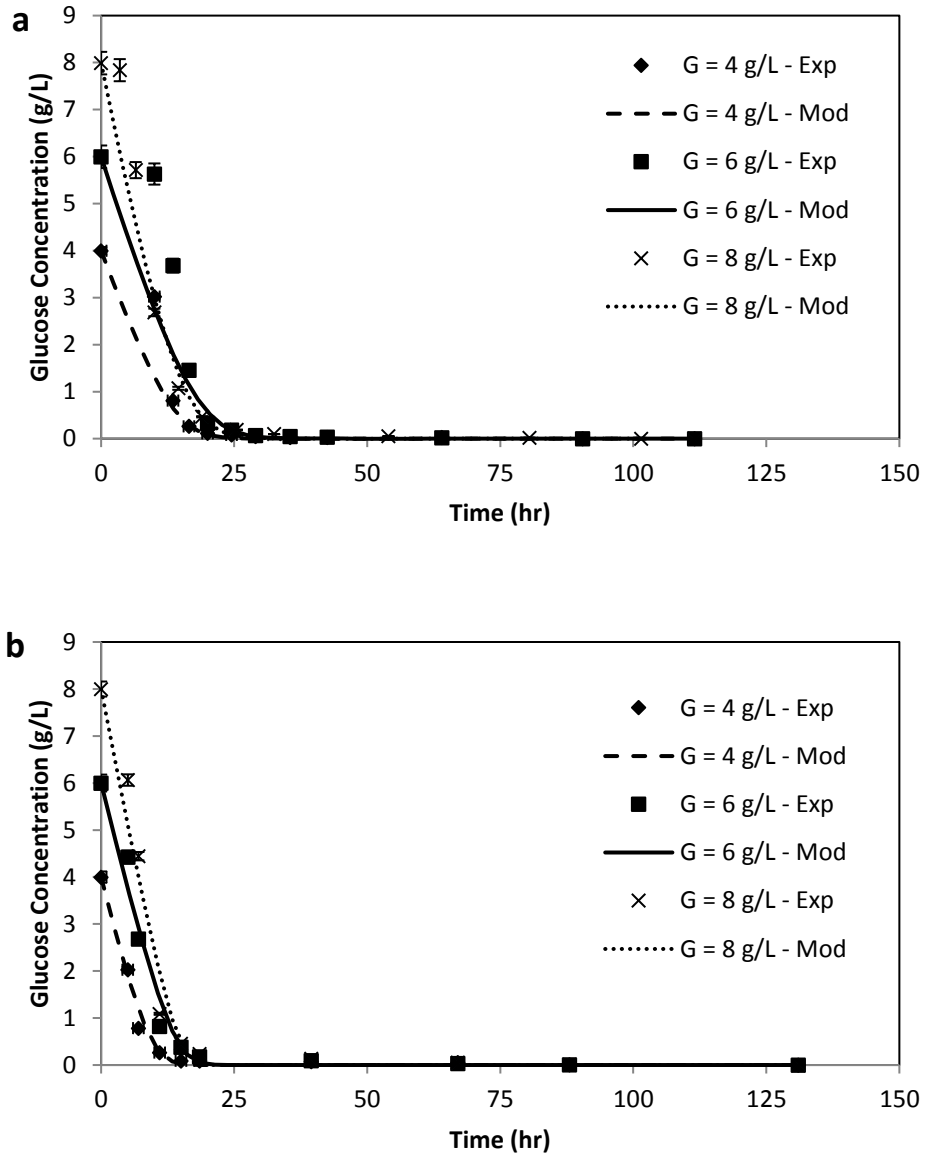

Figure 6.2 - Experimental and modeled substrate utilization profiles for a) *C. beijerinckii* and b) *C. saccharoperbutylacetonicum*

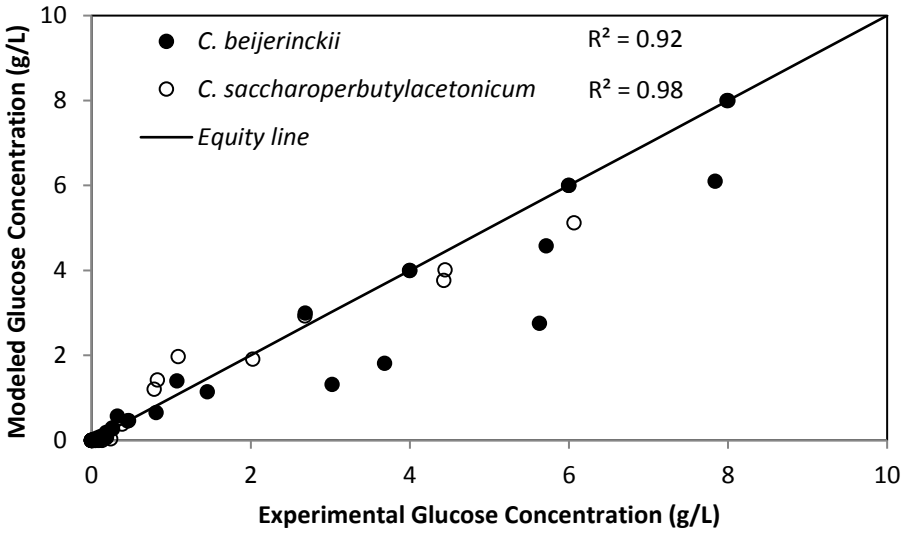

Figure 6.3 - Linear regression of experimental against modeled glucose concentrations for *C. beijerinckii* and *C. saccharoperbutylacetonicum*

Table 6.4 - Monod kinetic parameters of *C. beijerinckii* and *C. saccharoperbutylacetonicum*, APE, RMSE, and R^2

	C. beijerinckii			***C. saccharoperbutylacetonicum***		
G (g/L)	**4**	**6**	**8**	**4**	**6**	**8**
K (g substrate/gVSS.hr)	0.37	0.43	0.70	0.54	0.52	0.64
K_s (g/L)	0.91	1.35	2.03	0.82	0.79	0.72
APE (%)	7.2	16.6	13.8	13.2	16.5	19.2
RMSE (g/L)	0.50	1.00	0.64	0.14	0.30	0.44
R^2	0.85	0.80	0.96	0.99	0.98	0.98

6.3.4 End products

The different concentrations of glucose were completely utilized during the batch experiments using *C. beijerinckii* and *C. saccharoperbutylacetonicum*. This is consistent with Zhao et al. [2011] who observed 100% glucose utilization from 5 to 8 g/L initial glucose concentration, and observed a decline in glucose utilization at greater glucose concentrations using *C. beijerinckii* RZF-1108. *Clostridium* species can produce different soluble products based on the strain used as well as the operational conditions, where the metabolic breakdown of glucose yields acetate, butyrate, propionate, lactate, ethanol, butanol, and acetone [Azbar and Levin, 2012]. However, from a H_2 production perspective, VFAs are the desirable products as opposed to ethanol, formate, and lactate. Acetate, butyrate, and propionate were the main end products in all experiments. This is similar to Zhao et al. [2011] who used *C. beijerinckii* RZF-1108 and observed butyrate and acetate as the main end-products, but with a molar acetate-to-butyrate ratio of 1.1 and 1.0 at glucose initial concentrations of 6 and 8 g/L, respectively. On the contrary, Skonieczny and Yargeau [2009] observed butyrate, formate, and ethanol in 50 mL H_2 batches using *C. beijerinckii* ATCC 8260 and 2.8 g/L of glucose. The aforementioned authors observed a H_2 yield of 1.57 mol H_2/mol glucose which is 22% less than the yield observed in this study at a glucose concentration of 4 g/L. H_2 producing bacteria utilize glucose to produce acetate, butyrate, and propionate through the following pathways [Batstone et el., 2002]:

$$C_6H_{12}O_6 + H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (6.3)$$

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2H_2 + 2CO_2 \quad (6.4)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (6.5)$$

Table 6.5 shows the stoichiometric H_2 produced estimated from measured VFAs, where the average measured-to-theoretical H_2 of 102±5% shows the consistency of experimental and stoichiometric data. Figure 6.1 also shows the measured and theoretical H_2 calculated from VFAs produced with APE and RMSE ranging from 0.4% to 9% and 0.5 to 16.5 mL, respectively. Theoretical H_2 production and consumption from VFAs

produced was calculated based on 0.85 L H_2/g acetate, 0.58 L H_2/g butyrate, and 0.34 L H_2/g propionate (Equations 6.3-6.5). It is obvious from equations 6.3 and 6.4 that the H_2 yield would increase as the acetate production increase. Previous studies observed a positive correlation between the molar H_2 production and the molar acetate-to-butyrate ratio [Hafez et al., 2010]. However, as propionate production pathway is H_2 consuming, it directly affects the H_2 production yield. As depicted in Table 6.5, the molar acetate-to-butyrate ratio decreased with increasing the initial glucose concentration for both experiments, however, H_2 yields were not significantly affected with an average of 1.91±0.08 and 2.00±0.07 mol H_2/mol glucose for *C. saccharoperbutylacetonicum* and *C. beijerinckii* experiments, respectively. This is due to the decrease in propionate produced along with the decrease in acetate-to-butyrate ratio, which results in less H_2 consumption through the propionate production pathway (Equation 6.5).

Table 6.5 - Stoichiometric H_2 production

	C. beijerinckii			***C. saccharoperbutylacetonicum***		
G (g/L)	**4**	**6**	**8**	**4**	**6**	**8**
HAc (g/L)	3.0	3.0	2.8	2.9	2.6	2.5
HBu (g/L)	0.7	1.3	1.7	0.6	1.2	1.8
HPr (g/L)	4.9	4.5	3.4	5.0	4.0	3.6
HAc/HBu (mol/mol)	6.2	3.5	2.4	5.6	3.2	2.0
Theoretical H_2[a] (mL)	115	238	280	98	139	174
Measured/Theoretical H_2 (%)	97	96	108	109	101	104

[a] Theoretical H_2 = [HAc (g/L) * 0.84 (L H_2/g HAc) + HBu (g/L) * 0.0.58 (L H_2/g HBu) – HPr (g/L) * 0.34 (L H_2/g HPr)] * batch working volume (mL)

6.4 Conclusions

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

- Maximum H_2 yields obtained were 2.00 and 1.91 mol H_2/mol glucose for C. *beijerinckii* and *C. saccharoperbutylacetonicum*, respectively.
- Maximum H_2 production rates of 34.2 and 23.7 mL/hr obtained from Gompertz kinetics model were obtained at initial glucose concentration of 8 g/L for C. *beijerinckii* and *C. saccharoperbutylacetonicum*, respectively.
- K and K_s were 0.50 g substrate/gVSS.hr and 1.43 g/L for C. *beijerinckii* DSM 1820 and 0.57 g substrate/gVSS.hr and 0.78 g/L for *C. saccharoperbutylacetonicum* DSM 14923.
- Acetate, butyrate, and propionate were the main end products in both cultures experiments, with the measured and theoretical H_2 production from VFAs comparable with APE of less than 10% for both cultures.

6.5 References

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Chapter 7

Mono- and Co-Substrate Utilization Kinetics using Mono- and Co-Culture of *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii*

7.1 Introduction

Dark fermentation provides a promising alternative to light dependent processes, particularly with the utilization of waste biomass for H_2 production [Azbar and Levin, 2012]. Carbohydrate-based feedstocks containing oligosaccharides and/or polymers (e.g. cellulose, hemicellulose, and starch) are considered good organic carbon sources for fermentative H_2 production [Hawkes et al., 2002]. However, the complexity of these organic wastes makes them difficult for H_2 producing bacteria to utilize directly without pretreatment [Masset et al., 2012].

Dark fermentative H_2 production by pure cultures has achieved higher H_2 yields than mixed cultures [Masset et al., 2012]. In addition, the idea of using microbial co-cultures has the advantage of performing complex functions, to overcome economic or technical barriers [Elsharnouby et al., 2013]. From an economical point of view, a facultative anaerobe can maintain anaerobic conditions for strict H_2-producing anaerobes, eliminating the need for expensive reducing agents [Seppälä et al., 2011; Yokoi et al., 2001]. From the technical perspective, co-cultures can enhance the utilization of complex sugars using a culture with hydrolysis capabilities and a high H_2 producer that consumes simple sugars [Liu et al., 2008; Gomez-Flores, 2015].

Co-cultures for H_2 production from cellulose have been considered in many literature studies. Liu et al. [2008] reported an enhancement in the H_2 production yield of cellulose from 0.8 mol/mol glucose by *C. thermocellum* alone to 1.8 mol/mol glucose when co-cultured with *Thermoanaerobacterium thermosaccharolyticum*. Li and Liu [2012] used the aforementioned co-culture with cornstalk as the carbon source and

achieved a H_2 yield of 68.2 mL/g-cornstalk, which was 94% higher than the yield achieved by *C. thermocellum* as a mono-culture. At mesophilic temperature, Gomez-Flores [2015] achieved a yield of 2.1 mol/mol hexose using a co-culture of the cellulolytic bacterium *C. termitidis* and the high H_2 producer *C. beijerinckii*, 45% higher than the yield achieved by the *C. termitidis* mono-culture.

Although the concept of co-culturing was successfully implemented for cellulose and lignocellulosic wastes utilization, fewer studies applied co-culturing for H_2 production from starch-based wastes. Masset et al. [2012] tested mono- and co-cultures of *C. butyricum* and *C. pasteurianum* for H_2 production using starch as the carbon sources. The aforementioned authors observed an enhancement in the H_2 production rate of the co-culture experiment over the mono-culture experiments, while the H_2 yield of the co-culture (2.32 mol/mol hexose) was higher than for *C. pasteurianum* alone (1.79 mol/mol hexose) but lower than for *C. beijerinckii* alone (2.91 mol/mol hexose) [Masset et al., 2012].

C. saccharoperbutylacetonicum, a mesophilic alcohol-producing bacteria in acetone-butanol-ethanol (ABE) fermentation, has been recently found to produce H_2 efficiently utilizing glucose and starch with no evidence of cellulose utilization [Al-Shorgani et al., 2014; Alalayah et al., 2008]. Alalayah et al. [2008] reported a H_2 yield of 0.57 mol/mol glucose by *C. saccharoperbutylacetonicum* ATCC 27021 strain from glucose (10 g/L), while Ferchichi et al. [2005] reported a higher yield of 1.3 mol/mol glucose from glucose (20 g/L). On the other hand, a high H_2 yield of 2.87 mol/mol sugars was reported by *C. saccharoperbutylacetonicum* utilizing rice bran hydrolysate [Dada et al., 2013]. *C. beijerinckii* is a strict anaerobe that utilizes glucose efficiently for H_2 production, but cannot utilize cellulose, and has contradictory results on starch utilization depending on its strain [Masset et al., 2012]. George et al. [1983] were not able to degrade starch using ATCC 25752, ATCC 11914, and ATCC 14949 strains of *C. beijerinckii*. On the contrary, Taguchi et al. [1992; 1994] reported that *C. beijerinckii* AM21B and RZF-1108 strains can utilize starch as the carbon source producing 1.8 mol H_2/mol hexose, however, the starch used in their experiments was soluble, which does not prove the ability of *C. beijerinckii* to degrade insoluble starch. However, *C.*

beijerinckii is a good candidate for co-culturing with *C. saccharoperbutylacetonicum* for starch utilization, due to its high H_2 yields of up to 2.81 mol/mol glucose reported for *C. beijerinckii* L9 strain at an initial glucose concentration of 3 g/L [Lin et al., 2007].

In addition, H_2 production kinetics are important for system design, analysis, and process control [Azbar and Levin, 2012; Huang and Wang, 2010]. Improving the kinetics of H_2 production systems would decrease the reaction time, which consequently will reduce the system size as well as capital and operational costs. The modified Gompertz and the Monod-based kinetic models are widely used for modeling H_2 production and substrate utilization [Wang and Wan, 2009; Gnanapragasam et al., 2011]. However, studies reporting H_2 production parameters as yields and rates usually use Gompertz model which ignores the substrate utilization kinetics [Pan et al., 2008] and hence is of limited utility in bioreactor design. On the other hand, studies reporting the metabolic and growth kinetics ignore the H_2 production parameters.

In light of the highlighted paucity of information on H_2 production kinetics from cellulose and starch by *C. saccharoperbutylacetonicum* coupled with no specific data on the co-culture of the two important aforementioned species, the specific objectives of this study are:

- To confirm the inability of *C. beijerinckii* to utilize insoluble starch
- Test the potential of H_2 production from cellulose by *C. saccharoperbutylacetonicum*
- Assess the effect of co-substrate and co-culture on H_2 production and substrate utilization kinetics

7.2 Materials and Methods

7.2.1 Microbial strain and media

Clostridium beijerinckii strain DSM 1820 and *Clostridium saccharoperbutylacetonicum* strain DSM 14923 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany). Cultures inoculations of 10% (v/v) were conducted in ATCC 1191 medium at pH 7.2 and mesophilic temperature of 37°C.

The medium contained (per liter of double-distilled water): KH_2PO_4, 1.5 g; Na_2HPO_4, 3.35 g; NH_4Cl, 0.5 g; $MgCl_2.6H_2O$, 0.18 g; yeast extract, 2 g; resazurin, $2.5*10^{-4}$ g; mineral solution, 1 mL; vitamin solution, 0.5 mL, and L-cysteine (reducing agent), 1 g. The mineral solution contained (g per liter): trisodium nitrilotriacetate 20.2; $FeCl_3.6H_2O$, 2.1; $CoCl_2.6.\ H_2O$, 2; $MnCl_2.4H_2O$, 1; $ZnCl_2$, 1; $NiCl_2.6H_2O$, 1; $CaCl_2.2H_2O$, 0.5; $CuSO_4.5H_2O$, 0.64; and $Na_2MoO_4.2H_2O$, 0.5. The vitamin solution contained (mg per liter): pyridoxine-HCl, 100; riboflavin, 50; thiamine, 50; nicotinic acid, 50; *p*-aminobenzoic acid, 50; lipoic acid (thioctic acid), 50; biotin, 20; folic acid, 20; and cyanocobalamin, 10.

7.2.2 Experimental setup

Batch anaerobic fermentations were conducted in 180 mL serum bottles with a working volume of 100 mL. All bottles containing 1191 media were initially degassed by applying vacuum then sparged with N_2 gas, and autoclaved. An initial substrate concentration of 2 g/L was set using different mixing ratios of glucose, starch, and cellulose as shown in Table 7.1. Fresh cultures of *C. beijerinckii* and *C. saccharoperbutylacetonicum* were inoculated in each bottle at 10% (v/v) for mono-culture experiments and 5% (v/v) of each culture for the co-culture experiment, which is equivalent to 0.11 g biomass of each culture. Bottles were incubated at 37°C in a swirling-action shaker (MaxQ 4000, Fisher Scientific, ON, CA) operating at 100 rpm. Control bottles using media and substrate without cultures were prepared and incubated in duplicates at the same experimental conditions.

Table 7.1 - Initial substrate weights in experimental bottles

Substrate	G: Glucose (g)	S: Starch (g)	C: Cellulose (g)
G	0.2	-	-
S	-	0.2	-
C	-	-	0.2
GS	0.1	0.1	-
GC	0.1	-	0.1
SC	-	0.1	0.1
GSC	0.067	0.067	0.067

7.2.3 Analytical methods

HACH methods and testing kits (HACH Odyssey DR/2500) were used to measure the chemical oxygen demand (COD). The volatile fatty acids (VFAs) concentrations were analyzed using Varian 8500 has chromatography (Varian Inc., ON, CA) with a flame ionization detector (FID) of temperature 250°C and equipped with a fused silica column (30 m * 0.32 mm) of temperature 110°C. Helium was used as the carrier gas at a flow rate of 5 mL/min. Glucose was analyzed by BioPacific Diagnostic glucose kit (BC, Canada).

7.2.4 Gas measurements

Glass syringes of appropriate sizes in the range of 5-100 mL were used to measure the volume of gas produced by releasing the gas to equilibrate with the ambient pressure [Owen et al., 1979]. A gas chromatograph (Model 310, SRI instruments, ON, CA) was used to determine the gas composition. The GC is equipped with a thermal conductivity detector (TCD) of temperature 90°C and a molecular sieve column of

temperature 105°C. Argon was used as the carrier gas at a flow rate of 30 mL/min. H_2 gas production was calculated using Equation 7.1:

$$V_{H_2,i} = V_{H_2,i-1} + C_{H_2,i} * V_{G,i} + V_{h,i}(C_{H_2,i} - C_{H_2,i-1}) \quad (7.1)$$

where $V_{H_2,i}$ and $V_{H_2,i-1}$ are cumulative H_2 gas volumes at the current (i) and previous (i - 1) time intervals. $V_{G,i}$ is the total gas volume accumulated between the previous and current time intervals. $C_{H_2,i}$ and $C_{H_2,i-1}$ are the fractions of H_2 gas in the headspace of the reactor in the current and previous intervals, and $V_{h,i}$ is the total volume of the headspace of the reactor in the current interval [López et al., 2007].

7.2.5 Modeling

A modified non-linear least square fit model established by Gomez-Flores et al. [2015] using MATLAB R2014a was used to determine Monod kinetic parameters (Equation 7.2) [Mu et al., 2006]:

$$\frac{1}{X}\frac{dS}{dt} = \frac{-KS}{K_S+S} \quad (7.2)$$

where X is the biomass concentration (g/L), S is the substrate concentration (g/L), K is the maximum specific substrate utilization rate (h^{-1}), K_s is the saturation concentration (g/L) or half-velocity constant and is equal to the concentration of the rate-limiting substrate (glucose) when the substrate degradation rate is equal to one half of the maximum [Mu et al., 2006]. Average percentage errors (APE), root mean square errors (RMSE), and coefficient of determination (R^2) were used to assess the model fit.

7.3 Results and Discussion

7.3.1 H_2 production potential

Figures 7.1-7.3 show the experimental and stoichiometric cumulative H_2 production profiles for *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and their co-culture, respectively. Coefficients of variation (calculated as standard deviation divided

by the average) in all experiments were less than 10% confirming data reproducibility. The maximum H_2 content reached was 46±2% in the glucose utilizing experiments for mono- and co-culture experiments, while H_2 content in starch and co-substrate batches reached 32±5%. The initial pH (7.2) dropped to an average of 6.3±0.1 in experiments utilizing glucose and 6.6±0.2 in experiments utilizing starch and co-substrate of glucose, starch, and cellulose. Stoichiometric H_2 production was calculated from the VFAs produced and will be further discussed later. Both *C. beijerinckii* and *C. saccharoperbutylacetonicum* utilized glucose efficiently consistent with many studies in the literature [Hu et al., 2013; Alalayah et al., 2008]. Starch was only fermented by *C. saccharoperbutylacetonicum* and both cultures were not able to utilize cellulose as a mono-substrate, which confirms the limited ability of butanol-producing bacteria to utilize cellulose [Nakayama et al., 2011]. H_2 production profiles for *C. beijerinckii* were consistent at the various substrate mixing ratios, showing the same lag phase of 5.5 hours in the G, GS, GC, and GSC experiments (Figure 7.1). This is due to the production of H_2 by *C. beijerinckii* from glucose without being affected by the presence of starch and/or cellulose. The inability of *C. beijerinckii* to degrade starch or cellulose as mono- or co-substrate indicates the absence of hydrolytic enzymes [Al-Shorgani et al., 2014]. For *C. saccharoperbutylacetonicum*, glucose was degraded after a lag phase of 5.5 hours, while starch took 87 hours to hydrolyze before H_2 was produced (Figure 7.2). It is evident from Figure 7.2 that, two lag phases were observed in the GS experiment and H_2 was produced in two stages. *C. saccharoperbutylacetonicum* produced H_2 first from the readily biodegradable glucose after 5.5 hours, then after 37 hours it produced H_2 after starch hydrolysis.

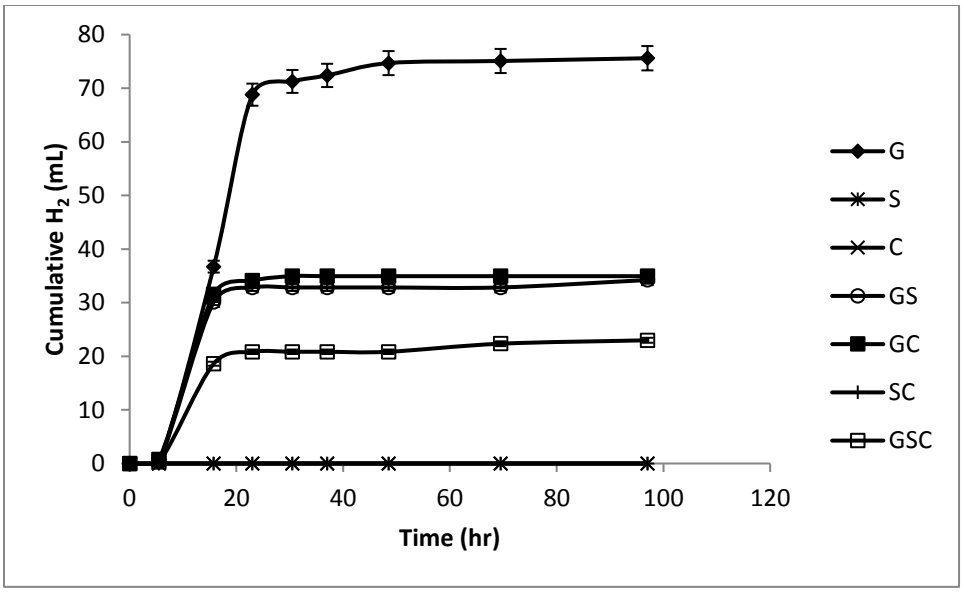

Figure 7.1 - Experimental Cumulative H_2 production from mono- and co-substrate using mono-culture of *C. beijerinckii*

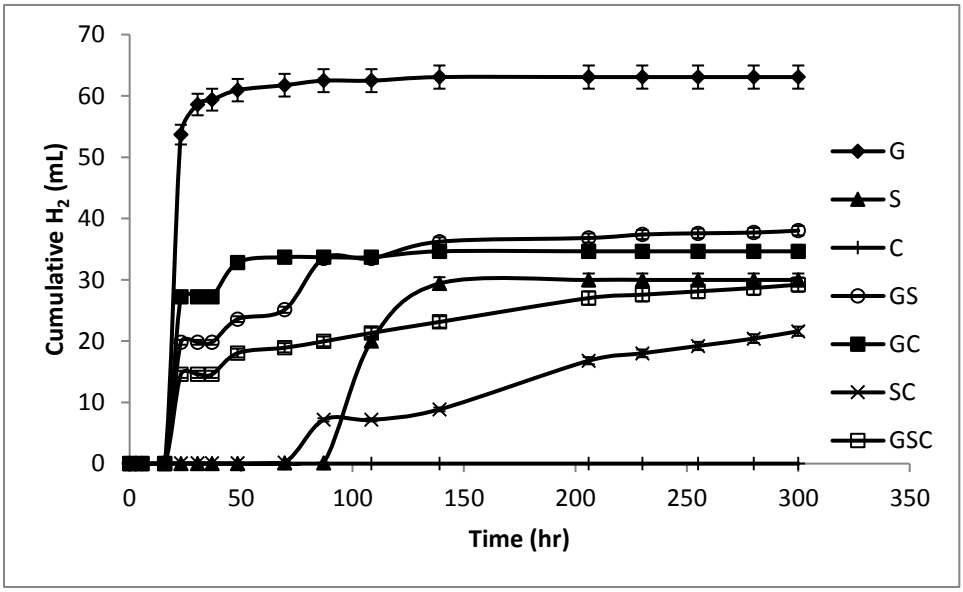

Figure 7.2 - Experimental Cumulative H_2 production from mono- and co-substrate using mono-culture of *C. saccharoperbutylacetonicum*

Clostridium species utilize glucose to produce acetate, butyrate, and propionate through the following pathways [Batstone et al., 2002; Azbar and Levin, 2012]:

$$C_6H_{12}O_6 + H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (7.4)$$

$$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2H_2 + 2CO_2 \quad (7.5)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (7.6)$$

In the *C. saccharoperbutylacetonicum* co-substrate experiments with glucose (i.e. GS, GC, and GSC), the percentage of H_2 produced during the first stage was 35%, 49%, and 39% from the theoretical H_2 based on glucose only, respectively, where the theoretical H_2 was calculated assuming an average H_2 yield through acetate and butyrate pathways (Equations 7.4 and 7.5). It is evident from Figure 7.2 that when glucose was present, the lag phase for the second stage ended at 37 hours (i.e. in GS, GC, and GSC experiments). In the SC experiment the second lag phase ended at 70 hours, which coupled with no H_2 production in cellulose experiment, and the starch alone experiment had a lag phase of 87 hours suggesting that starch hydrolysis takes about 37-87 hours. Interestingly, in the GC and SC experiments, although *C. saccharoperbutylacetonicum* did not utilize cellulose alone, H_2 was produced in two stages with the second stage starting after 37 and 109 hours, respectively, suggesting cellulose consumption after the culture has developed more biomass, which facilitated cellulose hydrolysis.

On the other hand, Figure 7.3 shows H_2 production profiles for the co-culture experiments with only one initial lag phase, which indicates the synergism between the two cultures in utilizing soluble substrate, particulate substrate, and particulate substrate hydrolysates. It is also evident from Figure 7.3 that the lag phase for S and SC experiments was 73 hours which lies within the 37-87 hours that requires *C. saccharoperbutylacetonicum* to hydrolyze starch confirming that *C. beijerinck*i could not utilize starch, since the lag phase with and without *C. beijerinckii* were the same. Also, comparing the starch only experiment (Figures 7.2 and 7.3) shows the synergetic effect between *C. saccharoperbutylacetonicum* and *C. beijerinckii*, where it took the co-culture

only 22 hours to produce 27 mL of H_2 while the mono-culture *C. saccharoperbutylacetonicum* took 52 hours to produce almost the same amount of H_2.

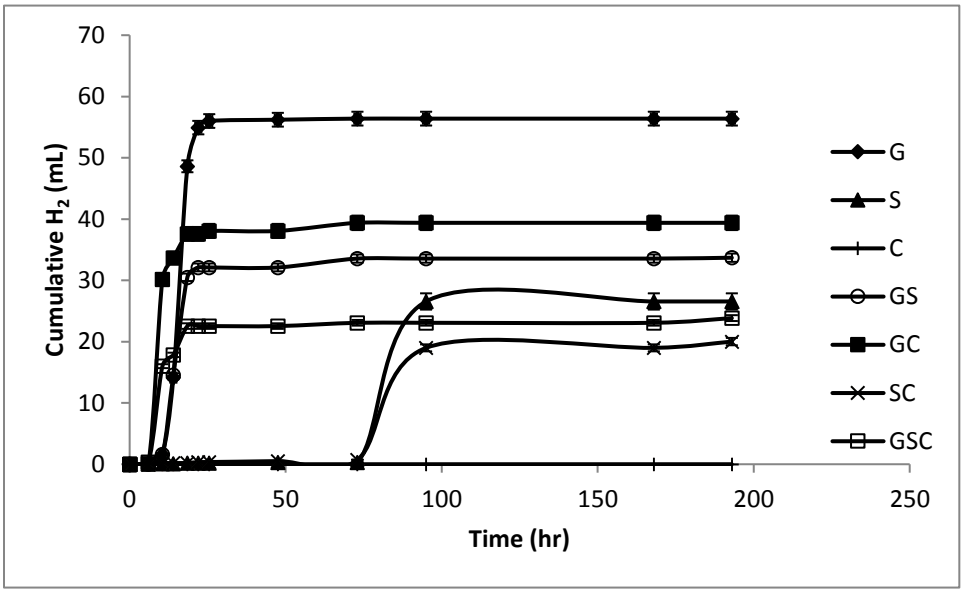

Figure 7.3 - Experimental Cumulative H_2 production from mono- and co-substrate using co-culture of *C. beijerinckii* and *C. saccharoperbutylacetonicum*

7.3.2 COD balance

Table 7.2 presents the COD mass balance for *C. beijerinckii* and *C. saccharoperbutylacetonicum* mono- and co-culture experiments. The closure of COD balance at an average of 99.8±3.0% verifies the reliability of the data.

Table 7.2 - Summary of COD mass balance

Experiment		Substrate					
		G	**S**	**GS**	**GC**	**SC**	**GSC**
C. beijerinckii	$COD_{initial}$ (gCOD)	0.53	-	0.57	0.58	-	0.58
	COD_{final} (gCOD)	0.52	-	0.55	0.56	-	0.57
	Cumulative H_2 (mL)	75.6	0	34.2	35.0	0	23.0
	H_2 (gCOD)	0.05	0	0.02	0.02	0	0.01
	COD balance[a] (%)	107.1	-	100.1	100.5	-	101.3
C. saccharoperbutylacetonicum	$COD_{initial}$ (gCOD)	0.58	0.57	0.57	0.58	0.59	0.58
	COD_{final} (gCOD)	0.50	0.56	0.54	0.56	0.56	0.56
	Cumulative H_2 (mL)	63.1	30.0	38.0	34.7	22.1	30.3
	H_2 (gCOD)	0.04	0.02	0.02	0.02	0.01	0.02
	COD balance[a] (%)	92.7	101.8	98.9	100.3	97.6	99.8
C. beijerinckii + *C. saccharoperbutylacetonicum*	$COD_{initial}$ (gCOD)	0.56	0.60	0.63	0.60	0.60	0.62
	COD_{final} (gCOD)	0.54	0.58	0.56	0.59	0.59	0.60
	Cumulative H_2 (mL)	56.4	26.6	33.7	39.4	20.0	23.9
	H_2 (gCOD)	0.04	0.02	0.02	0.02	0.01	0.02
	COD balance[a] (%)	102.3	98.2	93.6	103.5	101.3	97.8

[a] COD balance (%) = [H_2 (gCOD) + COD_{final} (gCOD)]*100/[$COD_{initial}$ (gCOD)]

7.3.3 *C. beijerinckii* bioH_2 production

Table 7.3 shows the H_2 production yields as mol/mol hexose initial for the mono-culture of *C. beijerinckii* experiments, which is based on the hexose equivalent for the total initial substrate (i.e. biodegradable and non-biodegradable substrate). H_2 yields based on biodegradable substrate were calculated using the initial glucose concentration and excluding starch and cellulose which are non-degradable by the bacteria. *C. beijerinckii* consumed glucose with a H_2 yield of 2.7±0.2 mol/mol glucose, which is consistent with Lin et al. [2007] who used initial glucose concentration of 3 g/L and reported a yield of 2.8 mol/mol glucose by *C. beijerinckii* L9 strain, and higher than the 1.4 mol/mol glucose reported by Skonieczny and Yargeau [2009] who used 1.9 g/L glucose by *C. beijerinckii* ATCC 8260 strain. The decrease in H_2 yields from 2.7 mol/mol hexose in the glucose experiment (G) to 1.2 mol/mol hexose in the glucose co-substrate with starch and cellulose experiments (GS and GC) to 0.8 mol/mol hexose in the co-substrate of glucose, starch, and cellulose experiment (GSC) is due to considering both degradable and bio-degradable initial substrate concentration in the yields calculation. However, when the biodegradable substrate is only taken into account (i.e. glucose), the calculated yields were consistent as illustrated in Table 7.3. Since glucose was the only biodegradable substrate for *C. beijerinckii*, H_2 yields for all experiments ranged from 2.4 to 2.7 mol/mol hexose with a percent difference of 12%. The observed-to-expected ratio reported in Table 7.3 reflects the effect of co-substrate utilization on H_2 production. For *C. beijerinckii*, the presence of co-substrates did not enhance H_2 production with an observed-to-expected ratio ranging from 90% to 93%. This is attributed to the fact that the culture only utilizes glucose and is not affected by the presence of insoluble starch or cellulose that it cannot degrade.

Table 7.3 - H_2 production potentials and yields for *C. beijerinckii*

Substrate	PS/B* (g/g)	H_2 Yield (mol/mol)	H_2 Yield (mol/mol$_{biodeg.}$)**	Observed H_2 Potential (mL)	Expected H_2 Potential*** (mL)	Observed/Expected (%)
G	ND	2.7±0.2	2.7±0.2	75.6	-	-
GS	0.9:1	1.2±0.0	2.4±0.0	34.2	37.8	90
GC	0.9:1	1.2±0.1	2.5±0.1	35.0	37.8	93
GSC	1.2:1	0.8±0.1	2.5±0.1	23.0	25.2	91

* PS:B is the initial particulate substrate-to-biomass ratio (g particulate substrate/g biomass)

** H_2 yield calculated based on the biodegradable substrate (i.e. glucose)

*** Expected H_2 is calculated based on H_2 produced in the mono-substrate experiments [e.g. expected H_2 in GS experiment (37.8 mL) = 75.6/2 (from G)]

7.3.4 *C. saccharoperbutylacetonicum* bioH_2 production

Table 7.4 shows the H_2 production yields as mol/mol hexose initial for the mono-culture of *C. saccharoperbutylacetonicum* experiments, which is based on the hexose equivalent for the total initial substrate. H_2 yields based on biodegradable substrate were calculated using initial concentrations of glucose and starch, excluding the cellulose concentration. *C. saccharoperbutylacetonicum* consumed glucose with a lower H_2 yield of 2.2±0.2 mol/mol glucose than the 2.7±0.2 mol/mol glucose achieved by *C. beijerinckii*. For glucose experiments (i.e. G and GC) H_2 yields based on the biodegradable substrate were 2.2 and 2.5 mol/mol hexose with a percent difference of 13% and in the glucose and starch experiments (i.e. GS and GSC) H_2 yields were 1.4 and 1.6 mol/mol hexose with a percent difference of 13%. On the other hand, for the starch only experiments (i.e. S and SC) H_2 yields were 1.1 and 1.6 mol/mol hexose with a high

percent difference of 37%, which supports the idea of cellulose degradation in the SC experiment. It can be deduced from the H_2 yields values presented in Table 7.4 that when glucose is a co-substrate with starch and/or cellulose, H_2 production is not greatly affected since utilizing glucose is not associated with producing any hydrolytic enzymes; however, H_2 production was enhanced in the SC experiment due to the presence of hydrolytic enzymes associated with starch utilization. Although the utilization of pure starch by *C. saccharoperbutylacetonicum* has never been reported in the literature, Ferchichi et al. [2005] reported a high H_2 yield of 2.77 mol/mol maltose at an initial maltose concentration of 20 g/L. Since maltose is the main hydrolysis product of starch [Antranikian, 1992], *C. saccharoperbutylacetonicum* may potentially be able to degrade insoluble starch. Also, Thang and Kobayashi [2014] used *C. saccharoperbutylacetonicum* to convert cassava, corn, and wheat starch for ABE production. On the other hand, the inability of *C. beijerinckii* to utilize starch is consistent with the findings of George et al. [1983] who used ATCC 25752, ATCC 11914, and ATCC 14949 strains, but contradictory to the observations of Taguchi et al. [1992; 1994] who reported a H_2 yield of 1.8 mol/mol hexose from 10 g/L starch using AM21B and RZF-1108 strains. Taguchi et al. [1992; 1994] used soluble starch in their experiments, however, the starch used in this experiment was insoluble which confirms the ability of *C. saccharoperbutylacetonicum* to hydrolyze and utilize insoluble starch producing H_2. The 1.07 mol/mol hexose achieved in the starch experiment is the first reported yield on insoluble starch by *C. saccharoperbutylacetonicum*. Al-Shorgani et al. [2014] reported a H_2 production yield of 92.6 mL/g starch from enzymatically hydrolyzed sago starch by *C. saccharoperbutylacetonicum* which is 62% of the 150 mL H_2/g starch (1.07 mol/mol hexose) achieved in this study.

Although *C. saccharoperbutylacetonicum* did not utilize cellulose alone, cellulose hydrolysis is rationalized by the 47% increase in the expected H_2 produced for the SC experiment as presented in Table 7.4, since hydrolytic enzymes would have been already produced by the culture after degradation of starch. Another factor that supports the premise of cellulose degradation is the particulate substrate-to-biomass ratio (PS:B) (g/g) shown in Table 7.4. The initial PS:B in the GC and GSC experiments were 0.9:1 and 1.2:1, respectively. After glucose consumption and biomass growth in the first stage (23

hours), the PS:B decreased to 0.5:1 and 0.7:1 in the GC and GSC experiments, respectively. However, in the cellulose only experiment, since *C. saccharoperbutylacetonicum* could not utilize cellulose and develop more biomass, the PS:B remained the same (1.8:1). The 82% observed-to-expected ratio reported in Table 7.4 for GS reflecting the effect of co-substrate utilization on H_2 production may be attributed to the 2-phase H_2 production observed, where the bacteria were acclimatized on the readily biodegradable glucose in the first phase which affected its utilization for starch in the second phase. This behaviour is similar to Masset et al. [2012] who observed a decrease in the H_2 production rates for 4 different *Clostridium* species after changing the carbon source from glucose to starch. Although *C. saccharoperbutylacetonicum* could not degrade cellulose, co-substrate of cellulose with glucose and starch individually showed increases of 10% and 47%, respectively. This indicates that the co-substrate enhanced cellulose degradation producing more H_2, especially with starch due to its similar chemical composition to cellulose, which activated the production of hydrolytic enzymes that helped degrade the cellulose. Xia et al. [2012] reported the enhancement of cellulose degradation when glucose, starch and xylose were used as a co-substrate individually with a cellulose-to-sugar mixing ratio of 10:1 using anaerobic digester sludge as the inoculum at thermophilic temperature. The aforementioned authors achieved a cellulose conversion of 8% in cellulose batches which doubled to 16% when using glucose and starch individually as a co-substrate and tripled when using xylose as a co-substrate [Xia et al., 2012]. For the co-substrate of glucose, starch, and cellulose there was almost no change in H_2 production with an observed-to-expected ratio of 98%. This may be due to the competition between glucose and starch for utilization by *C. saccharoperbutylacetonicum* that was compensated by an enhancement in H_2 production from cellulose degradation.

Table 7.4 - H_2 production potentials and yields for *C. saccharoperbutylacetonicum*

Substrate	PS/B* (g/g)	H_2 Yield (mol/mol)	H_2 Yield ($mol/mol_{biodeg.}$)**	Observed H_2 Potential (mL)	Expected H_2 Potential*** (mL)	Observed/Expected (%)
G	ND	2.2±0.2	2.2±0.2	63.1	-	-
S	1.8:1	1.1±0.0	1.1±0.0	30.0	-	-
GS	0.9:1	1.4±0.2	1.4±0.2	38.0	46.6	82
GC	0.9:1	1.2±0.0	2.5±0.0	34.7	31.6	110
SC	1.8:1	0.7±0.0	1.6±0.0	22.1	15.0	**147**
GSC	1.2:1	1.0±0.1	1.6±0.1	30.3	31.0	98

* PS:B is the initial particulate substrate-to-biomass ratio (g particulate substrate/g biomass)

** H_2 yield calculated based on the biodegradable substrate (i.e. glucose and starch)

*** Expected H_2 is calculated based on H_2 produced in the mono-substrate experiments [e.g. expected H_2 in GS experiment (46.6 mL) = 63.1/2 (from G) + 30.0/2 (from S)]

7.3.5 Co-culture bioH_2 production

Table 7.5 shows the H_2 production yields for the co-culture experiments. H_2 yield for glucose decreased from 2.7 and 2.2 mol/mol glucose in mono-culture experiments of *C. beijerinckii* and *C. saccharoperbutylacetonicum*, respectively, to 2.0±0.1 mol/mol glucose in the co-culture experiment. Also, the observed-to-expected ratio for utilizing glucose based on the mono-culture experiments was 81%, which indicates that both cultures competed for utilizing glucose as they both have the ability to consume glucose.

On the other hand, the high observed-to-expected ratio of 177% for starch utilization shows clearly the positive impact of the co-culture where *C. saccharoperbutylacetonicum* degraded complex starch and *C. beijerinckii* consumed the simple sugars produced from hydrolysis. The same concept has been implemented in many studies by using a cellulose degrading culture and a H_2 producing culture to enhance H_2 production from cellulose [Liu et al., 2008; Geng et al., 2010]. Liu et al. [2008] reported a H_2 yield of 0.8 mol/mol hexose utilizing 5 g/L cellulose by *C. thermocellum*, which increased to 1.8 mol/mol hexose when co-cultured with *Thermoanaerobacterium thermosaccharolyticum*. Masset et al. [2012] applied co-culturing on starch (5 g/L) using *C. butyricum* and *C. pasteurianum*. The aforementioned authors observed H_2 yields of 2.91 and 1.79 mol/mol hexose for *C. butyricum* and *C. pasteurianum*, respectively, while the co-culture achieved a yield of 2.32 mol/mol hexose [Masset et al., 2012]. In the GC and SC experiments, the 40% and 50% increase in the observed-to-expected ratio from a mono-substrate perspective, confirms the enhancement of H_2 production due to cellulose degradation after both cultures have developed more biomass. On the other hand, the 77% and 80% increases in the observed-to-expected ratio from a mono-culture perspective, confirms the synergism between *C. beijerinckii* and C. *saccharoperbutylacetonicum* in hydrolyzing and consuming starch in the absence of glucose. In the GS and GSC experiments, H_2 yields based on the biodegradable substrate (i.e. glucose and starch) were 1.2 and 1.3 mol/mol hexose with a low percent difference of 8%, however, for the particulate substrate (i.e. S and SC experiments), the high difference of 33% confirms the degradation of cellulose in the SC experiment.

Table 7.5 - H_2 production potentials and yields for co-culture experiments

Substrate	H_2 Yield (mol/mol)	H_2 Yield ($mol/mol_{biodeg.}$)*	Observed H_2 Potential (mL)	Expected H_2 Potential** (mL)	Observed/ Expected (%)	Expected H_2 Potential*** (mL)	Observed/ Expected (%)
G	2.0±0.1	2.0±0.1	56.4	-	-	69.4	81
S	1.0±0.0	1.0±0.0	26.6	-	-	15.0	177
GS	1.2±0.1	1.2±0.1	33.7	41.5	81	36.1	93
GC	1.3±0.1	2.8±0.1	39.4	28.2	140	34.9	113
SC	0.7±0.0	1.4±0.0	20.0	13.3	150	11.1	180
GSC	0.8±0.0	1.3±0.0	23.9	27.7	86	26.7	90

* H_2 yield calculated based on the biodegradable substrate (i.e. G and S for the co-culture)

** Expected H_2 is calculated based on H_2 produced in the mono-substrate experiments [e.g. expected H_2 in GS experiment (41.5 mL) = 56.4/2 (from G) + 26.6/2 (from S)]

*** Expected H_2 is calculated based on H_2 produced in the mono-culture experiments [e.g. expected H_2 in GS experiment (36.1 mL) = 34.2/2 (from *C. beijerinckii* GS) + 38.0/2 (from *C. saccharoperbutylacetonicum* GS)]

7.3.6 H_2 production rates

Linear regression for each growth phase of the mono- and co-culture experiments was estimated and presented in Table 7.6. *C. beijerinckii* produced H_2 from glucose at a specific H_2 production rate (SHPR) of 9.90 mL/gVSS.hr (3.9 mL/hr) which is almost double the production rate of 2.4 mL/hr reported by Skonieczny and Yargeau [2009] at an initial glucose concentration of 1.9 g/L for ATCC 8260 strain. SHPR decreased in the co-substrate experiments to 4.87 and 5.42 mL/gVSS.hr (1.9 and 2.1 mL/hr) in GS and

GC experiments, respectively, with an initial glucose concentration of 1 g/L and further to 3.20 mL/gVSS.hr (1.3 mL/hr) in the GSC experiment with initial glucose concentration of 0.67 g/L. These values were also higher than those of Skonieczny and Yargeau [2009] who reported a production rate of 1.0 mL/hr at 0.9 g/L initial glucose concentration.

The SHPR of *C. saccharoperbutylacetonicum* utilizing starch (1.37 mL/gVSS.hr) was much slower than the rate for glucose utilization, which is due to the additional hydrolysis step needed to release the fermentable sugars. In the 2-stage H_2 production utilizing glucose experiments by *C. saccharoperbutylacetonicum*, SHPR decreased from 9.90 mL/gVSS.hr in the G experiment to 2.90 and 3.99 mL/gVSS.hr in the GS and GC experiments, respectively, and further to 2.46 mL/gVSS.hr in the GSC experiment. Although *C. saccharoperbutylacetonicum* could not utilize cellulose as a mono-substrate, however, the different rates in the 2-phase H_2 production supports the idea of cellulose degradation with relatively high SHPR in the second stage of GC and SC experiments of 0.46 and 0.24 mL/gVSS.hr, respectively. The very low SHPR in the second phase of the GSC experiment (0.05 mL/gVSS.hr) agrees with the overall no enhancement in expected H_2 production.

For glucose utilization, SHPR was the same for mono- and co-culture experiments with a value of 9.9 mL H_2/gVSS.hr, while the synergistic effects of the co-culture are obvious for the remaining mono- and co-substrate experiments, as presented in Table 7.6. For example, in the starch only experiment, the SHPR increased from 1.37 to 3.01 mL H_2/gVSS.hr in the *C. saccharoperbutylacetonicum* and the co-culture experiments, respectively.

Table 7.6 - H_2 production rates of *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and their co-cultures

Experiment	Phase	Parameters	Substrate					
			G	**S**	**GS**	**GC**	**SC**	**GSC**
C. beijerinckii		SHPR*	9.90	-	4.87	5.42	-	3.20
		R^2	0.99	-	0.88	0.89	-	0.89
C. saccharoperbutylacetonicum	1	SHPR	9.90	1.37	2.90	3.99	1.00	2.46
		R^2	0.80	0.91	0.81	0.81	0.99	0.80
	2	SHPR	-	-	0.62	0.46	0.24	0.05
		R^2	-	-	0.90	0.81	0.98	0.98
C. beijerinckii +		SHPR	9.90	3.01	5.86	5.12	1.67	5.68
C. saccharoperbutylacetonicum		R^2	0.90	0.99	0.93	0.82	0.94	0.88

* SHPR: Maximum specific hydrogen production rate in mL/gVSS.hr

7.3.7 Monod growth kinetics

The Monod kinetic equation (Equation 7.2) was used to estimate the kinetic coefficients by modeling the substrate degradation for the mono- and co-culture while neglecting the change in biomass concentration. Figure 7.4 shows the experimental and modeled substrate degradation for *C. beijerinckii* (Figure 7.4a), *C. saccharoperbutylacetonicum* (Figure 7.4b), and co-culture of *C. beijerinckii* and *C. saccharoperbutylacetonicum* (Figure 7.4c). Table 7.7 presents the estimated kinetic parameters, derived from only the overall growth phase as shown in Figure 7.4. For *C. saccharoperbutylacetonicum*, it can be depicted from Figure 7.4b that the overall kinetics neglected the 2-phase substrate utilization observed in GS, GC, SC, and GSC experiments. However, Figure 7.5 shows the experimental and modeled substrate

degradation for *C. saccharoperbutylacetonicum* for the 2-phase substrate utilization observed. Table 7.8 presents the estimated kinetic parameters taking into consideration the 2-phase substrate utilization, which reflects the different kinetics for utilizing each sugar separately in the co-substrates experiments. The goodness of fit for substrate concentrations are assessed by calculating the APE, RMSE, and R^2. APE values ranged from 0.3% to 19.8%, RMSE values ranged from 0.01 to 0.25 g/L, and R^2 ranged from 0.85 to 1.00 as shown in Table 7.7. Also, Figure 7.6 shows the correlation between the modeled and experimental substrate concentrations with absolute fraction of variance (R^2), calculated with respect to the equity line, of 0.98 for *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and the co-culture experiments. In summary, the calculated statistical parameters and correlations verify the good fitness of the MATLAB model.

For *C. beijerinckii*, the value of K increased from 0.30 g substrate/gVSS.h in the G experiment to 0.37 g substrate/gVSS.h in the GS and GC experiments, and then decreased to 0.23 g substrate/gVSS.h in the GSC experiment. This is due to the different initial glucose concentration in the G (2 g/L), GS and GC (1 g/L), and GSC (0.67 g/L) experiments. Lin et al. [2007] reported the value of K to be 1.03 mmol/mmol.h (1.58 g/g.h) for *C. beijerinckii* L9 strain utilizing 3 g/L glucose. The value of K_s remained at an average of 0.92±0.02 g/L for G, GS, GC, and GSC experiments, which is double the value of 0.47 g/L (2.6 mmol/L) reported by Lin et al. [2007].

For *C. saccharoperbutylacetonicum*, the value of K (0.48 g substrate/gVSS.h) achieved in this study is similar to Alalayah et al. [2008] who achieved a value of 0.4 g substrate/gVSS.h utilizing glucose at an initial concentration of 10 g/L. The higher K achieved by *C. saccharoperbutylacetonicum* (0.48 g substrate/gVSS.h) than that achieved by *C. beijerinckii* (0.30 g substrate/gVSS.h) reflects better glucose utilization kinetics for *C. saccharoperbutylacetonicum*. For the starch experiment, a lower K of 0.11 g substrate/gVSS.h was achieved, which is the first to be reported for *C. saccharoperbutylacetonicum* utilizing insoluble starch. The average value of K_s for all mono- and co-substrate experiments was 0.91±0.01 g/L which is very low compared to the value of 5.5 g/L reported by Alalayah et al. [2010]. However, the aforementioned authors estimated the Monod kinetic parameters using the Lineweaver-Burk linearization

method and used *C. saccharoperbutylacetonicum* ATCC 13564 strain. The inconsistency of the overall K values for the GS, SC, and GSC experiments (Table 7.7) reflects the low accuracy inherent in neglecting the 2-phase substrate utilization. In contrast, the K values for each phase separately (Table 7.8) are more representative for the H_2 production profiles and are more consistent with the SHPR data.

The co-culture experiment showed an obvious enhancement in the maximum specific substrate utilization rate except for the glucose experiment. The co-culture of *C. beijerinckii* and *C. saccharoperbutylacetonicum* utilizing glucose, achieved a K value of 0.44 g substrate/gVSS.h which is 8% less than that for the *C. saccharoperbutylacetonicum* but almost twice the K achieved by *C. beijerinckii* (Table 7.7). This confirms the co-culture competition for glucose utilization as both cultures have the ability to utilize glucose. In contrast, the K value increased by 14%, 13%, and 65% compared to mono-culture of *C. beijerinckii* in the GS, GC, and GSC experiments, respectively, and increased by 255%, 71%, 95%, 270%, and 111% compared to K values of the first stage (Table 7.8) in the mono-culture of *C. saccharoperbutylacetonicum* in the S, GS, GC, SC, and GSC experiments, respectively. This confirms the advantage of using a starch-degrading bacterium co-cultured with a H_2 producing bacteria that utilizes the starch hydrolysis products, increasing the H_2 production potential from starch. The average value of K_s achieved in the co-culture experiment was 0.95±0.04 g/L, which is almost the same as the values achieved in the mono-culture experiments.

Figure 7.7 shows the correlation between the Monod kinetics and SHPR for all mono- and co-culture experiments. The initial degradable substrate concentration was used to calculate the Monod term along with the modeled K and K_s values obtained. The 2-phase coefficients were considered for the *C. saccharoperbutylacetonicum* data points taking into account the degradable substrate for each phase separately (e.g. in the GS experiments, initial substrate concentrations for the first and second phases were 1 g/L glucose and 1 g/L starch, respectively). This correlation can be utilized to obtain the SHPR from the Monod kinetics.

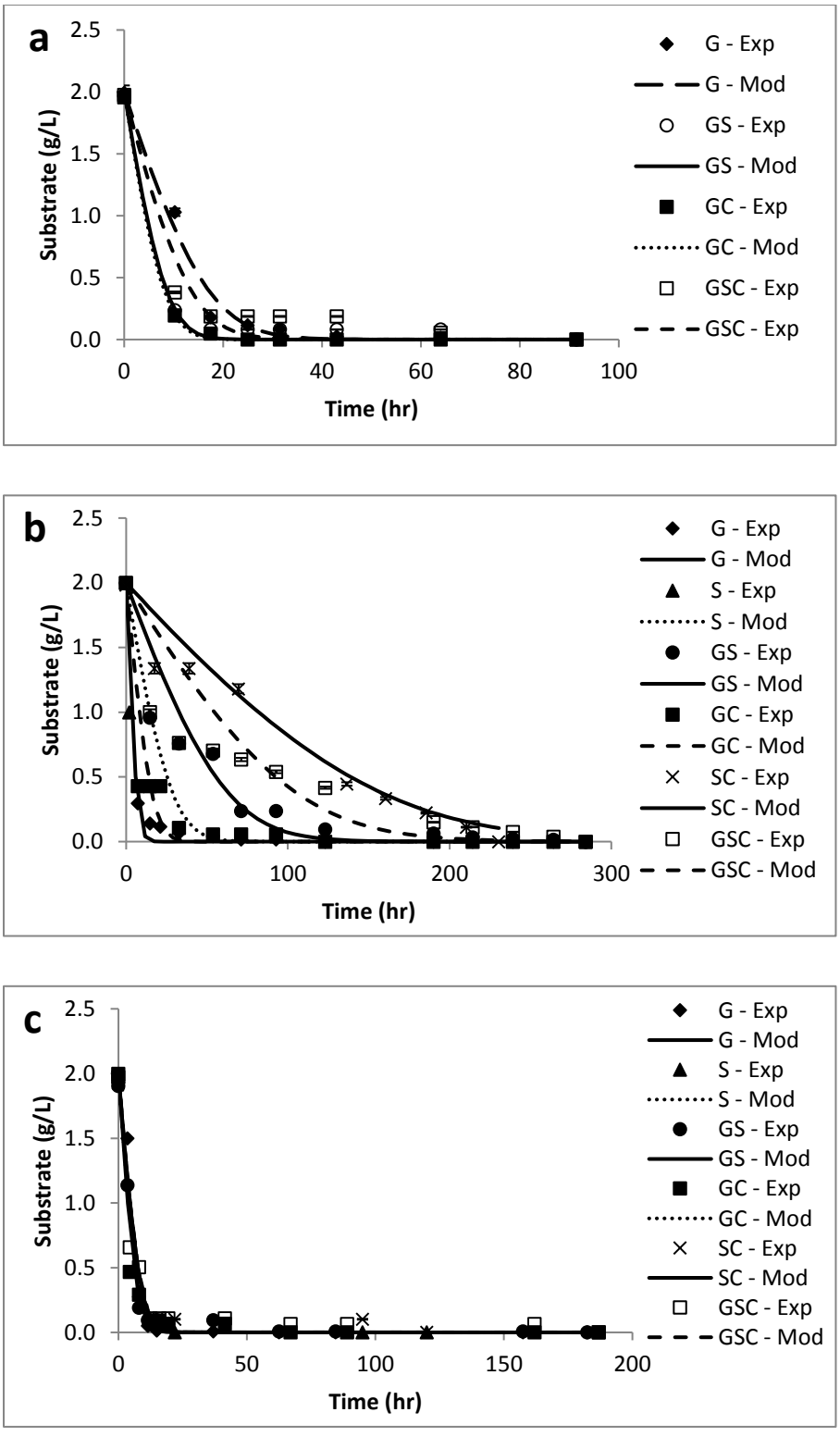

Figure 7.4 - Experimental and modeled substrate utilization profiles for a) *C. beijerinckii*, b) *C. saccharoperbutylacetonicum*, and c) co-culture of *C. beijerinckii* and *C. saccharoperbutylacetonicum*

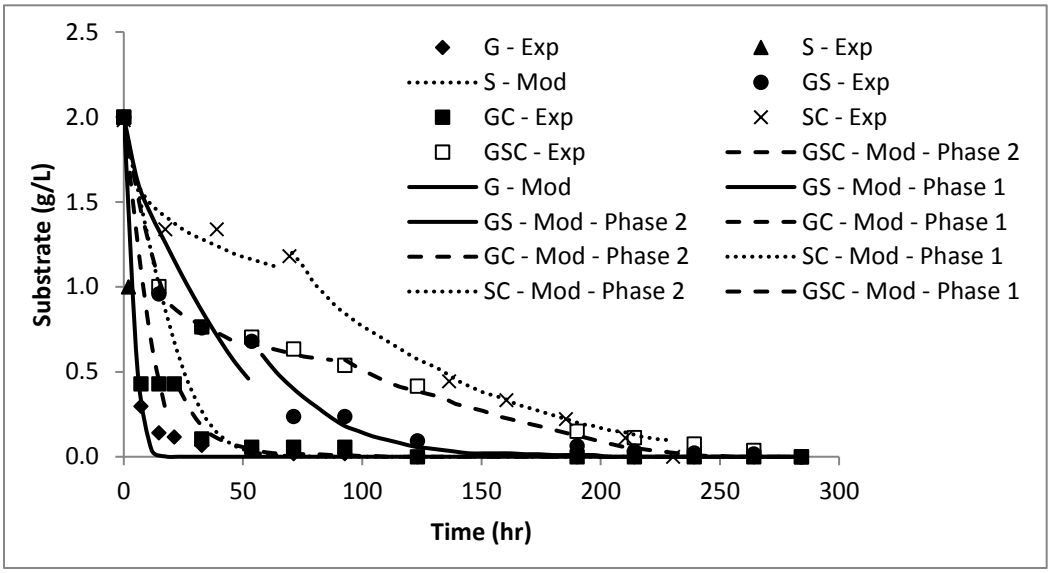

Figure 7.5 - Experimental and modeled 2-phase substrate utilization profiles for *C. saccharoperbutylacetonicum*

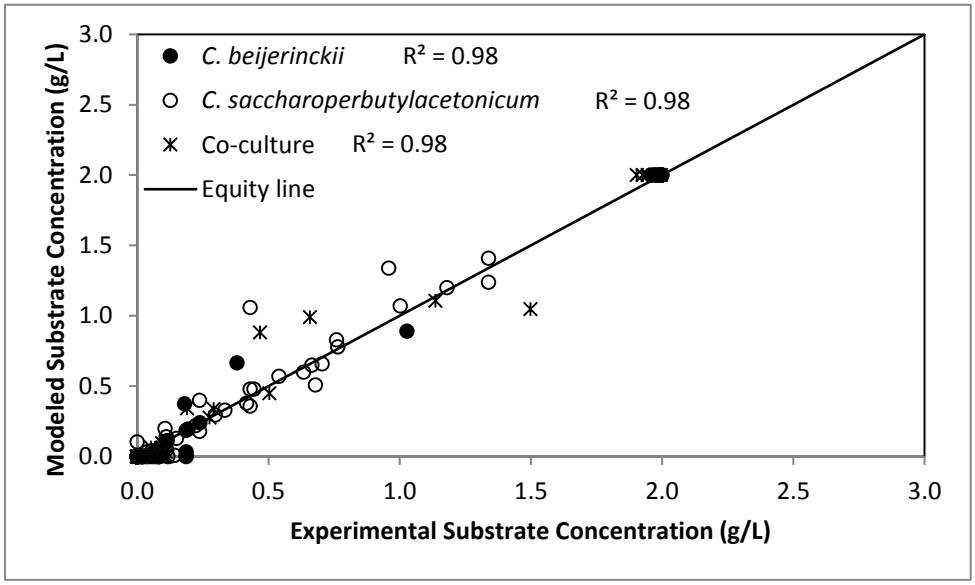

Figure 7.6 - Linear regression of experimental against modeled substrate concentrations for a) *C. beijerinckii*, b) *C. saccharoperbutylacetonicum*, and c) co-culture

Table 7.7 - Monod kinetic parameters of *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and their co-culture

Experiment	Parameters	Substrate					
		G	**S**	**GS**	**GC**	**SC**	**GSC**
C. beijerinckii	K (g substrate/gVSS.h)	0.30	-	0.36	0.38	-	0.23
	K_s (g/L)	0.93	-	0.91	0.90	-	0.93
	APE (%)	16.4	-	10.1	10.0	-	19.8
	RMSE (g/L)	0.09	-	0.06	0.02	-	0.15
	R^2	0.98	-	0.99	1.00	-	0.94
C. saccharoperbutylacetonicum	K (g substrate/gVSS.h)	0.48	0.11	0.05	0.20	0.02	0.05
	K_s (g/L)	0.90	0.92	0.92	0.90	0.92	0.92
	APE (%)	13.9	12.5	15.3	15.9	13.1	12.0
	RMSE (g/L)	0.05	0.01	0.19	0.20	0.16	0.25
	R^2	0.99	1.00	0.89	0.85	0.94	0.87
C. beijerinckii + *C. saccharoperbutylacetonicum*	K (g substrate/gVSS.h)	0.44	0.39	0.41	0.43	0.37	0.38
	K_s (g/L)	0.91	0.93	0.92	1.00	0.93	1.00
	APE (%)	16.6	0.3	16.5	12.2	0.9	12.5
	RMSE (g/L)	0.14	0.01	0.07	0.13	0.08	0.12
	R^2	0.96	1.00	0.99	0.95	0.99	0.95

Table 7.8 - Monod kinetic parameters of *C. saccharoperbutylacetonicum* for the 2-phase substrate utilization

Phase	Parameters	Substrate					
		G	**S**	**GS**	**GC**	**SC**	**GSC**
1	K (g substrate/gVSS.h)	0.48	0.11	0.24	0.22	0.10	0.18
	K_s (g/L)	0.90	0.92	0.90	0.90	0.92	0.92
2	K (g substrate/gVSS.h)	-	-	0.03	0.07	0.02	0.02
	K_s (g/L)	-	-	0.90	0.92	0.90	0.90
	APE (%)	13.9	10.9	14.9	16.9	14.1	16.0
	RMSE (g/L)	0.05	0.01	0.20	0.04	0.16	0.17
	R^2	0.99	1.00	0.92	0.89	0.94	0.87

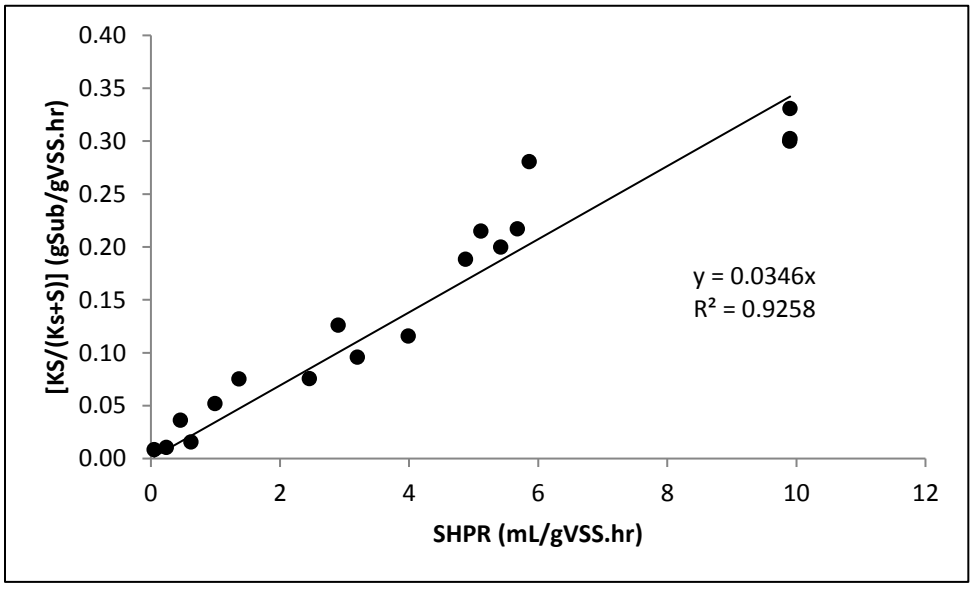

Figure 7.7 - Monod kinetics and maximum SHPR correlation

7.3.8 End products

Clostridium species have a diversity of end products depending on the bacterial strain, operational conditions, and type of substrate [Azbar and Levin, 2012]. Since, H_2 production pathways are associated with VFAs production except for propionate, acetate and butyrate production is more favourable than ethanol, formate, and lactate. In the mono- and co-culture experiments, acetate, butyrate, and propionate were the main end products. Table 7.9 shows the stoichiometric H_2 produced estimated from measured VFAs and the acetate-to-butyrate ratio for mono- and co-culture experiments. The average measured-to-theoretical H_2 was calculated to be 106±10%, which reflects the consistency of experimental and stoichiometric data. Also, Figures 7.1-7.3 show the measured and theoretical temporal H_2 profiles with an average calculated APE and RMSE of 0.1±0.0% and 2.9±1.8 mL, respectively, for mono- and co-culture experiments. For glucose utilization by *C. beijerinckii* at 6 g/L, Liu et al. [2011] reported acetate and butyrate as the main end products, however, the acetate-to-butyrate ratio of 0.69 was much lower than the 5.2 reported in this study. This indicates that for the *C. beijerinckii* L9 strain used in the aforementioned study, butyrate was the preferred pathway for H_2 production [Liu et al., 2011]. Lin et al. [2007] reported a higher acetate-to-butyrate ratio of 1.82 utilizing 3 g/L glucose and using *C. beijerinckii* L9 strain as well.

The end products from glucose and insoluble starch utilization by *C. saccharoperbutylacetonicum* have not been reported in the literature. Dada et al. [2013] reported similar acetate-to-butyrate ratio of 5.1 using rice bran hydrolysate (10 g/L) as the substrate and *C. saccharoperbutylacetonicum* N1-4 strain. Also, Al-Shorgani et al. [2014] observed acetate and butyrate as the main end products by *C. saccharoperbutylacetonicum* ATCC 13564 strain utilizing hydrolyzed rice bran. As depicted in Table 7.9, the molar acetate-to-butyrate ratio increased from 3.8 in the *C. saccharoperbutylacetonicum* experiment to 4.7 in the co-culture experiment utilizing starch only, confirming the synergism between the two cultures in hydrolyzing the insoluble starch and utilizing the simple sugars resulting from hydrolysis. Although, the molar acetate-to-butyrate ratio decreased in the co-culture experiments utilizing GS, GC,

and SC, the acetate pathway was still more favourable than the butyrate pathway with higher acetate-to-butyrate ratios than one.

Table 7.9 - VFAs and Stoichiometric H_2 production

Experiment	Parameters	Substrate					
		G	**S**	**GS**	**GC**	**SC**	**GSC**
C. beijerinckii	HAc (g/L)	1.0	-	0.5	0.6	-	0.5
	HBu (g/L)	0.3	-	0.1	0.1	-	0.1
	HPr (g/L)	0.6	-	0.3	0.5	-	0.2
	HAc/HBu (mol/mol)	5.2	-	7.5	7.8	-	4.6
	Theoretical H_2[a] (mL)	67	-	32	40	-	22
	Measured/Theoretical H_2 (%)	112	-	106	87	-	106
C. saccharoperbutylacetonicum	HAc (g/L)	1.3	0.4	0.8	0.5	0.4	0.5
	HBu (g/L)	0.3	0.2	0.2	0.1	0.2	0.2
	HPr (g/L)	0.2	0.4	0.8	0.5	0.4	0.5
	HAc/HBu (mol/mol)	7.4	3.8	6.4	6.1	4.0	4.2
	Theoretical H_2[a] (mL)	70	27	33	31	24	26
	Measured/Theoretical H_2 (%)	90	112	114	113	91	117
C. beijerinckii + *C. saccharoperbutylacetonicum*	HAc (g/L)	1.5	0.4	0.6	0.6	0.3	0.4
	HBu (g/L)	0.4	0.1	0.2	0.2	0.1	0.1
	HPr (g/L)	2.1	0.3	0.8	0.8	0.4	0.4
	HAc/HBu (mol/mol)	5.5	4.7	5.0	5.0	3.4	4.9
	Theoretical H_2[a] (mL)	61	24	30	34	18	22
	Measured/Theoretical H_2 (%)	93	111	113	116	110	110

[a] Theoretical H_2 = [HAc (g/L) * 0.84 (L H_2/g HAc) + HBu (g/L) * 0.0.58 (L H_2/g HBu) – HPr (g/L) * 0.34 (L H_2/g HPr)] * batch working volume (mL)

7.4 Conclusions

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

- Maximum H_2 yields achieved on glucose and starch were 2.69 and 1.07 mol/mol hexose by *C. beijerinckii* and *C. saccharoperbutylacetonicum*, respectively.
- K of 0.48 g substrate/gVSS.h was the highest for utilizing glucose and was achieved by *C. saccharoperbutylacetonicum*.
- K of 0.39 g substrate/gVSS.h was the highest for utilizing starch and was achieved by *C. beijerinckii* and *C. saccharoperbutylacetonicum* co-culture.
- An average K_s of 0.93±0.03 g/L was achieved in all mono- and co-cultures experiment.
- Acetate, butyrate, and propionate were the main end products in both cultures experiments with the measured and theoretical H_2 production from VFAs comparable with APE less than 1%.
- Co-substrate did not affect H_2 production by *C. beijerinckii* as it utilized only glucose and had no ability of starch or cellulose degradation.
- Co-substrate had a negative effect on *C. saccharoperbutylacetonicum* mono-culture as glucose and starch competed for substrate utilization.
- Co-culture had a negative effect on glucose degradation as both cultures competed for glucose utilization.
- Co-culture had a positive effect on starch degradation as *C. beijerinckii* utilized the starch-hydrolysis products degraded by *C. saccharoperbutylacetonicum*.

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Chapter 8

Conclusions and Recommendations

8.1 Contributions and Conclusions

The major scientific contributions of this research are reflected in the fact that this is the first study to:

1. Investigate CO_2 sequestration in a continuous-flow system resulting in the awarded patent US20150111273 A1
2. Prove microbial shift happening due to CO_2 sequestration
3. Prove that co-culture of *C. beijerinckii* and *C. saccharoperbutylacetonicum* worked in synergy improving starch utilization and develop their microbial kinetics

The following findings summarize the overall conclusions of this research:

- *Effect of feedstock quality on H_2 production:*
    1. *through biomass selection* Furfural up to 1100 mg/L and HMF up to 140 mg/L had no impact on H_2 production
    2. Monomeric-to-polymeric sugars ratio correlated positively with H_2 production yields and rates, and negatively with lag times
- *Process stability enhancement through biomass selection:*
    1. Microbial community diversity of MADS is higher than in TADS, which is reflected in TADS sensitivity to furfural below 120 mg/L
    2. The use of TADS compared to MADS enhanced H_2 yields but increased the lag phase
    3. Co-culture had a positive effect on degradation as *C. beijerinckii* utilized the starch-hydrolysis products degraded by *C. saccharoperbutylacetonicum*
- *H_2 production optimization through end products manipulation:*
    1. Removal of CO_2 from the headspace of a continuous-flow system shifted the H_2 production pathways forward increasing H_2 yields and rates

2. CO_2 sequestration changed the propionate consumption pathway to be thermodynamically favourable producing more acetate and H_2

8.2 Recommendations

Based on the findings of this research, the recommended future research should include:

1. Studying furfural and HMF inhibitory effect in continuous-flow systems taking into consideration the effect of initial sugars concentration in lignocellulosic biomass
2. Conducting pure cultures batch experiments with temporal substrate analysis to enhance kinetic studies
3. Developing inhibition models for furfural, HMF, substrate, and volatile fatty acids in mixed and pure cultures experiments
4. Studying the impact of CO_2 sequestration using real feedstocks in presence of other biogas pollutants as H_2S

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