ANAEROBIC FERMENTATION FOR BIOLOGICAL HYDROGEN PRODUCTION IN A SEQUENCING BATCH REACTOR

by

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Abstract

Biological hydrogen production via anaerobic fermentation of organic waste can be potentially a greener and sustainable technology. Thus far, most research has been conducted using continuous stirred tank reactors (CSTR). Anaerobic sequencing batch reactors (ASBR) have advantages over CSTR, but there are disadvantages in terms of their operation. The overall goal of the thesis research is to enhance hydrogen production by optimizing the operational conditions in an ASBR using agri-food wastewater as substrate.

An ASBR with 6-L working volume was inoculated with sewage sludge from the anaerobic zone of a sewage treatment facility and was not pretreated to select the hydrogen-producing bacteria. Hydrogen productivity was estimated by hydrogen content (%), hydrogen production rate (HPR) and hydrogen yield as the performance indicators in response to changes in pH, hydraulic retention time (HRT), organic loading rate (OLR), and cyclic duration (CD) as the key operational parameters.

Using dairy wastewater as substrate, the suppression of methanogenesis was feasible without pretreatment of inoculum under the conditions of higher OLR and shorter HRT, which favoured hydrogen production. With carbohydrate-rich synthetic wastewater as substrate, the combination of relatively low pH 4.5 and HRT 30 hr was found to be the optimal condition for hydrogen production. For higher hydrogen production, ethanol-to-acetic acid ratio of 1.25 and food-to-microorganism ratio of 0.84 were revealed as threshold values. Higher hydrogen productivity at longer CD was not necessarily accompanied with higher microbial growth that occurred at shorter CD.

Subsequently, real sugar refinery wastewater was used in the tests for biohydrogen production. Based on statistical analysis and curve fitting by the modified Gompertz model

of the data as well as microbial identification, the operational setting of (pH 5.5, HRT 10 hr, OLR 15 kg/m^3.d) was concluded to be optimal with the performance indicators of (71.8±10.5% H_2, HPR 2.11±0.31 L H_2/L reactor.d and yield 0.95±0.13 mol H_2/mol sucrose). Taxonomic analysis confirmed the presence of dominant hydrogen-producing bacteria among the diverse microbial genera, and in particular, the *Clostridia* spp. without the pretreatment of inocula. Further studies with the optimization of operational conditions would contribute towards making the best possible decision for ASBR.

Preface

The literature review, experimental design, all experiments and data analysis throughout the whole thesis were conducted by the Ph.D. candidate, Seung Gun Won under supervision of Dr. Anthony K. Lau in the Department of Chemical and Biological Engineering at the University of British Columbia. Dr. Lau also provided direct supervision in the preparation of the dissertation, and the preparation of manuscripts for publication of the research work. The supervisory committee members, Dr. Sheldon Duff, Dr. Madjid Mohseni, and Dr. Keng Chou have given me meaningful and important comments for revisions. The assistance with the analysis of dominant microorganisms in section 5.3.7 was provided by Dr. Susan Baldwin.

The manuscripts included in this dissertation are listed below.

1. Won, S.G. and Lau, A.K. (2011). Effects of key operational parameters on biohydrogen production via anaerobic fermentation in a sequencing batch reactor. *Bioresource Technology, 102*, 6876-6883. A version of this manuscript is included in Chapter 3, except for section 3.3.3.

2. Won, S.G. and Lau, A.K. Effects of manipulating cyclic duration and pH for fermentative hydrogen production in a sequencing batch reactor. To be submitted for publication. Sections of this manuscript are included in Chapter 4.

3. Won, S.G., Baldwin, S., and Lau, A.K. Optimizing the operational parameters in the anaerobic fermentation of sugar refinery wastewater for biohydrogen. To be submitted for publication. Sections of this manuscript are included in Chapter 5.

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List of Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
ASBR	anaerobic sequencing batch reactor
ATP	adenosine triphosphate
BNR	biological nutrients removal
BOD	biochemical oxygen demand, mg/L
CD	cyclic duration, hr
COD	chemical oxygen demand, mg/L
CSTR	continuous stirred tank reactor
DNA	deoxyribonucleic acid
EtOH	ethanol
Fd	ferredoxine
FID	flame ionization detector
FISH	fluorescence in-situ hybridization
F/M	food to microorganism ratio, g COD or sucrose/g MLVSS.d
GC	gas chromatography
HAc	acetic acid
HBu	butyric acid
HPr	propionic acid
HPR	hydrogen production rate, L H_2/L reactor.d
HRT	hydraulic retention time, hr or d
MLVSS	mixed liquor volatile suspended solids, g MLVSS/L

NAD^+	nicotinamide adenine dinucleotide
OLR	organic loading rate, kg COD or sucrose/m^3 reactor.d
OTU	operational taxonomic unit
PCR-DGGE	polymerase chain reaction – denaturing gradient gel electrophoresis
PEMFC	proton exchange membrane fuel cell
PFL	pyruvate formate lyase
SMP	soluble metabolite products
SOFC	solid oxide fuel cell
SRT	solids retention time, d
TCD	thermal conductivity detector
TS	total solids, mg/L
TDS	total dissolved solids, mg/L
TVS	total volatile solids, mg/L
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acids
VS	volatile solids, mg/L

List of Symbols

A	asymptote on cumulative hydrogen production curve, mL
b_0	offset term
$b_{1,2,3}$	linear coefficients
$b_{11,22,33}$	squared coefficients
$b_{12,13,23}$	interaction coefficients
C_{H2}	hydrogen content, %
C_{in}	initial carbohydrate concentration (per cycle) in the reactor, mg/L
E_f	theoretical maximum output electricity
E_0	redox potential at standard conditions
e	exp(1) = 2.71828
F	Faraday constant, 96485 C/mol
$\Delta\bar{h}_f$	enthalpy of combustion, kJ/mol
μ_m	hydrogen production rate, mL H_2/hr
N_c	the number of cycles, #cycles/d
n	amount of required hydrogen in fuel cell, mol/s
η	fuel cell efficiency
η_{cu}	carbohydrate degradation efficiency
P_{H2}	partial pressure of hydrogen, atm
R	universal gas constant, 8.314 J/mol.K
r_{tv}	the ratio of influent volume to working volume of reactor per cycle
S	substrate concentration, mg/L
S_{ef}	carbohydrate concentration in the effluent, mg/L

S_{ef-1}	residual carbohydrate concentration from a previous cycle in the reactor
S_{in}	initial carbohydrate concentration of the substrate, mg/L
S_{mol}	molar mass of substrate loaded, mol
T	temperature, K
Q	flow rate of influent, L/d
μ_f	fuel utilization efficiency in fuel cell
V_c	fuel cell output voltage
v_{in}	the influent or effluent volume in each cycle, L/cycle
V_g	volume of total biogas produced, L
V_r	working volume of the reactor, L
$x_{1,2,3}$	variable
X_i	uncoded value of the independent variable
X_0	centre point value of X_i
x_i	coded value
y	predicted response
z	number of electrons
λ	lag time
ΔX	step change value

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It is really a pleasure to thank all people in Department of Chemical and Biological Engineering who made this thesis possible. Especially, I would like to show my sincere gratitude to my supervisor, Dr. Anthony K. Lau for the continuous supports of my Ph.D. study, for his patience, guidance, encouragement, and motivation. His encouragement and guidance from the initial to the final level enabled me to develop an understanding of the research and finally break an egg. This thesis would not have been possible without his devotional supports.

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Certainly, I shall not forget my former supervisor for my Master's degree and a mentor of my life, Dr. Changsix Ra, who has been cheering me up to finish this long journey far from Korea. And I owe my special thanks to Dr. Sung Chan Nam who was once a visiting scientist in our department from the Korea Institute of Energy Research.

Last but not least, I would like to give deep appreciation to my parents-in-law who have waited for me with cheers. I heartily thank my parents for their love and supporting me during all my studies. Also, I was able to finish my thesis with support from my elder brother and his wife who have given huge love to my family.

Dedication

This thesis is lovingly dedicated to

My wife, Sooyoung Hwang,

My son, Joseph,

My daughter, Anne,

And my parents, Yong-Jin Won and Ok Soon Choi.

Chapter 1: Introduction

Global climate change and energy security are the major driving forces for the gradual shift towards renewable and sustainable energy sources. There is a wide range of renewable and sustainable energy technologies such as solar, wind, biomass, hydroelectric, geothermal, ocean, and arguably nuclear energy (Evans, 2007). Biomass conversion processes include thermochemical and biochemical methods. For instance, woody biomass in densified form (such as wood pellets) or non-densified form (such as wood chips) may be used as solid biofuel in an industrial combustion or gasification plant. On the other hand, liquid fuels (such as bioethanol and biodiesel) may be more readily integrated into the present infrastructure, and they are primarily used in the transportation sector. According to the U.S. Energy Information Administration (USEIA, 2011), the demands of liquid fuels will keep increasing by 2035 and the growth of the transportation sector needs will occupy 85%, despite rising fuel prices.

For more than two decades, anaerobic digestion technology for biogas production has also been successfully commercialized for the treatment of wastewater and solid wastes (Mata-Alvarez et al., 2000). Anaerobic digestion is the decomposition of organic matter in the absence of oxygen. In the process, a series of chain reaction takes place which involve distinct groups of anaerobic microorganisms. Complex organics are first hydrolyzed and fermented into fatty acids; while, significant reduction in BOD (biochemical oxygen demand) or COD (chemical oxygen demand) with respect to wastewater treatment is not expected, since complex molecules are converted to smaller molecules such as short chain fatty acids (propionate, butyrate), alcohols, and new biomass. Then, they are further converted into acetate, carbon dioxide and hydrogen. The final

gaseous mixture contains methane, carbon dioxide and trace amounts of hydrogen sulfide (**Figure 1.1**). Hence, anaerobic digestion systems are often referred to as "biogas systems". It is a process found in many naturally occurring anoxic environments including watercourses, sediments, waterlogged soils and the mammalian gut.

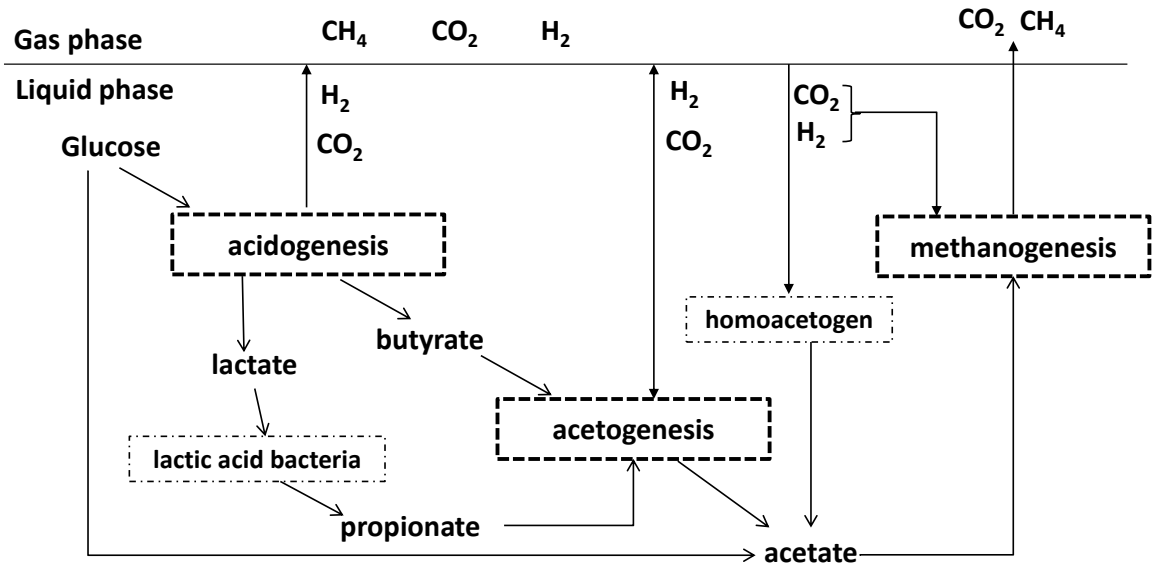

Figure 1.1. A schematic of anaerobic digestion from glucose (Costello et al., 1991; Minton and Clarke, 1989)

It can also be applied to a wide range of feedstock including industrial and municipal wastewater, agricultural, municipal, food industry wastes, and plant residues. Methane produced as an end product via anaerobic digestion has received great attention over a century and various technologies have been developed and conventionally used. The bioenergy derived from anaerobic digestion can take different forms. For instance, biogas can be purified to different extents and fed to engines, microturbines or fuel cells to produce combined heat and electricity. Purified biogas can also produce pipeline grade methane. In addition, this methane production as an established technology had been

spotlighted as the way to decrease the use of fossil fuel, reduce greenhouse gas and odour emissions, give the cost benefit to farmers, recover residues as useful products such as bedding materials and eco-friendly organic fertilizer, generate revenue for rural communities and so on.

The release of CO_2 from the burning of biomass-derived biogas is carbon neutral. However, for long-term sustainability, it is preferable to burn hydrogen, provided that hydrogen can be produced using clean technologies in a cost-effective way. Hydrogen is the simplest form of elements and plentiful in the universe but it is found only in combined form on earth. Hydrogen as an energy carrier can be produced from several resources including fossil fuels, nuclear, biomass, and other renewable energy technologies. At present, supply of hydrogen is achieved through energy intensive processes such as steam reforming of methane, partial oxidation of hydrogen-rich feedstock, and electrolysis of water. Hydrogen production via anaerobic processes could be less energy intensive, though again, it ought to be economically viable if commercialization of the technology is to be realized. Hydrogen has been deemed the future energy carrier, due to its high energy content and non-polluting nature upon combustion to release water vapour. When hydrogen is used in a fuel cell, it is converted to electricity through a chemical reaction, releasing water vapour as exhaust. The energy content of hydrogen is greater than hydrocarbon fuels (Kapdan and Kargi 2006). For instance, the higher heating value HHV and lower heating value LHV of hydrogen are 142 MJ/kg and 120 MJ/kg, respectively. By comparison, the HHV and LHV of methane and propane are (55.5, 50) MJ/kg and (50.5, 46.5) MJ/kg, respectively. Besides, the conversion of hydrogen to energy is more efficient than methane. Hydrogen has a wide range of industrial applications. It can be used for the

syntheses of ammonia, alcohols, and aldehydes, as well as for the hydrogenation of edible oil, petroleum, coal, and shale oil (Hart, 1997), whereas methane is mostly used as fuel.

From the life cycle analysis point of view, production of hydrogen from the recycling of organic waste is potentially a greener technology compared to conventional method of hydrogen production from methane, a non-renewable fossil fuel source. Aside from thermal processes such as gasification of solid waste, researchers have investigated biological hydrogen production via anaerobic fermentation under dark conditions (dark fermentation) since the 1980's using a variety of pure or mixed organic substrates, as well as photo-fermentation of organic materials (Benemann, 1996).

Light-dependent processes to produce hydrogen may be achieved by biophotolysis of water and photofermentation. Photofermentation is conducted by photosynthetic bacteria which are not required to split water to obtain electrons, since organic acids (such as acetic, lactic, succinic and butyric acids, or alcohols) play the role as electron donor. However, oxygen gas highly inhibits hydrogenase activity and light conversion efficiency was very low at 1–5 % (Nath and Das, 2004). In addition, many other studies have described that the light-independent processes have fewer barriers than the light-dependent process, although both light dependent and independent processes have their own problems in order to be commercialized (Kapdan and Kargi, 2006).

Anaerobic fermentation without using light energy is called dark fermentation. Dark fermentation has proven to be more feasible for practical applications, including integration with fuel cell technologies, due to its much higher hydrogen synthesis rate and no requirements of additional light energy (Cicha, 2009; Levin and Chahine, 2010).

Many fermentative bacteria produce hydrogen, which provides a specific mechanism to dispose of excess electrons through the activity of hydrogen producing enzymes in bacteria. As distinguished from methane production, hydrogen is one of the intermediates formed during anaerobic fermentation, which means, hydrogen is not always released to the outer surface during the reaction. It can be available for other reactions where necessary. Hydrogen-producing enzymes catalyze the chemical reaction: $2H^+ + 2e^- \rightarrow H_2$. At present, three enzymes that carry out this reaction are known: nitrogenase, Fe-hydrogenase, and NiFe-hydrogenase; however, nitrogenase is not a very metabolically effective way to produce H_2, compared to Fe-hydrogenases (Hallenbeck and Benemann, 2002). Bacteria that possess such capability include strict anaerobes such as *Clostridium, Bacillus, Ruminococcus (e.g. Ethanoligenens) and Escherichia coli,* and facultative anaerobes such as *E. coli*, *Enterobacter* and *Citrobacter* (Nandi and Sengupta, 1998). Among the hydrogen-producing bacteria, genera *Clostridium* and *Enterobacter* are the most widely studied. Species of genus *Clostridium* are gram-positive, rod-shaped, strict anaerobes; they produce hydrogen gas during the exponential growth phase and form spores in response to unfavourable environmental conditions (Levin et al., 2004), whereas *Enterobacter* are gram-negative, rod-shaped, and facultative anaerobes (Holt et al., 1994). Thermophiles that include *Thermotoga* spp. and *Caldicellulosiruptor* spp. (de Vrije et al., 2002) are also capable of producing hydrogen. Studies were mostly conducted at 36-38 ℃ for *Clostridium* and *Enterobacter*, and 65-80 ℃ for thermophiles (de Vrije and Claassen, 2003).

In addition to the type of microbial species, there are diverse factors related to hydrogen production in anaerobic fermentation, such as the source of feedstocks, and

strategies of bioprocesses. Carbohydrate-rich wastewater as feedstock has been preferred and various pretreatment methods of inoculum have been studied in order to eliminate hydrogen-consuming bacteria such as methanogens and homoacetogens. The technologies of biological wastewater treatment via anaerobic fermentation have also developed very well, along with proven energy production (CH_4). In order to overcome the barriers of improved hydrogen production, both biotechnological and engineering strategies are required. As an engineering strategy, the type of reactors could also exert influence on biological hydrogen productivity. Current studies for fermentative hydrogen production have been achieved mostly through batch, continuous stirred tank reactor (CSTR) or upflow anaerobic sludge blanket (UASB). However, only a few studies via anaerobic sequencing batch reactor (ASBR) have been reported. Since each type of reactors has different intrinsic attributes, ASBR has also been expected to achieve the improved hydrogen productivity. During the last two decades, many studies to enhance hydrogen production using the tools of process engineering have been conducted; they may be generally categorized by improvement of reactor design and optimization of the operational parameters.

1.1 Metabolic Pathways for Biohydrogen Production

All living organisms have a functioning reaction in order to conserve energy, which is achieved by reduction/oxidation reaction. Aerobic, anoxic, and anaerobic conditions could be separated by means of electron acceptors. Aerobic heterotrophic bacteria obtain electrons from organic compounds and oxygen is the final electron acceptor. Energy for growth is conserved through substrate level phosphorylation and ATP synthesis is coupled

to the electron transport chain reaction. In the absence of oxygen, anoxic condition could be formed when inorganic compounds play the role of electron acceptors. However, anaerobic bacteria are able to live without suitable inorganic electron acceptors; energy conservation may be achieved only with substrate level phosphorylation during which ATP is generated. Whereas, electron transport to other molecules is not usually coupled to energy conservation.

The bacterial groups participating in each step are roughly categorized as follows: 1) fermentative bacteria, 2) hydrogen-producing acetogenic bacteria, 3) hydrogen-consuming acetogenic bacteria, 4) carbon dioxide-reducing methanogens, 5) aceticlastic methanogens (Pavlostathis and Giraldo-Gomez, 1991). Hydrogen is presumably consumed by the hydrogen-consuming acetogenic bacteria and the carbon dioxide reducing methanogens. *Methanobacterium* are known to consume hydrogen and carbon dioxide. However, methane-producing bacteria are known to be slow growers compared to acid-producing bacteria so that methane producing step is the rate-limiting step in anaerobic processes. Besides, methane producing bacteria are very sensitive to low pH and the methanogenic activity is inhibited at a pH below 6.8 (Metcalf & Eddy Inc., 2003).

Various metabolic pathways of dark fermentation have been proposed for hydrogen production (Yan et al., 1988; Tanisho, 2001; Liu, 2002; Ren et al., 2006). With glucose as the model substrate, it is first converted to pyruvate, producing adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and the reduced form of nicotinamide adenine dinucleotide (NADH) via the glycolytic pathway. Pyruvate is then converted to acetylcoenzyme A (acetyl-CoA), carbon dioxide, and hydrogen by the enzymes pyruvate-ferredoxin oxidoreductase and hydrogenase. Pyruvate may also be converted to acetyl-

CoA and formate, which may be readily converted to hydrogen and carbon dioxide by bacteria such as *E. coli*. Acetyl-CoA is finally converted into acetate, butyrate, and ethanol, depending on the microorganisms and the environmental conditions. NADH is used in the formation of butyrate and ethanol and the residual NADH may be oxidized, producing hydrogen and NAD^+. ATP is generated in the formation of butyrate and acetate from acetyl-CoA (**Figure 1.2**).

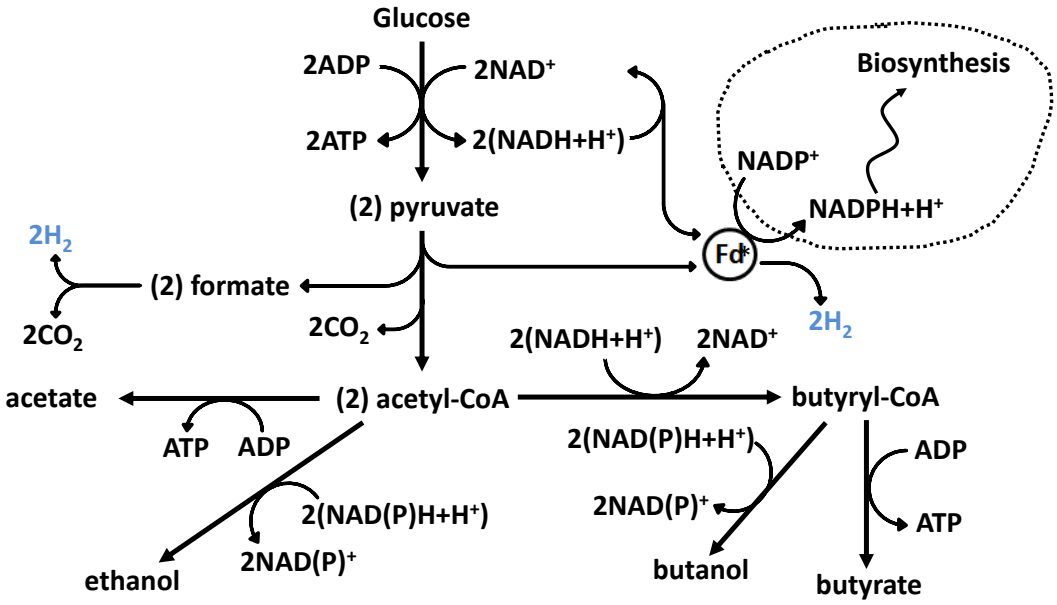

Figure 1.2. Metabolic pathway of hydrogen production from anaerobic fermentation of glucose to selected by-products (Minton and Clarke, 1989; Tanisho et al., 1998; Jungermann et al., 1973) *Fd, ferredoxine

About 40 hydrogenase genes have been sequenced and all of them have been reported to contain Fe and some contain Ni and Se as well (Voordouw, 1992). Those hydrogenases containing Ni and Se facilitate the uptake of hydrogen, whereas those containing Fe alone (Fe hydrogenases) catalyze the production of hydrogen (Cammack, 1999). They catalyze the conversion between hydrogen and proton depending on the

oxidation state (Fontecilla-Camps et al., 2007). In addition, they are classified according to their location in the cytoplasm, periplasm and cellular membrane. Calusinska et al. (2010) proposed the following three pathways to produce hydrogen; 1. the oxidation of reduced ferredoxin by pyruvate:ferredoxin oxidoreductase; 2. the re-oxidation of ferredoxin-mediated NADH by NADH:ferredoxin oxidoreductase; and 3. an alternative pathway with trimeric bifurcating hydrogenase.

Soluble metabolites during dark fermentation indicate metabolic pathways in microbial activity since hydrogen is an intermediate rather than an end-product, as distinguished from methane formation. Ren et al. (2008) showed that mixed-acid type fermentation was achieved when no pretreatment was applied to the inocula. Based on the volatile fatty acids profiles obtained, Arooj et al. (2008) suggested that the HBu:HPr (butyric acid/propionic acid) ratio was the most important parameter to justify hydrogen yield at various HRTs. Wu et al. (2010) reported butyric acid-type fermentation occurring in most tests involved in their study; at pH 5.5, 5.0 and 4.0, the effluent contained mostly butyric acid (43–57%), followed by acetic acid (25–30%). However, from the study by Wu et al. (2009), ethanol and organic acids were the major aqueous metabolites produced during fermentation, with acetic acid accounting for 56–58%. Hydrogen yield was found to be proportional to the HAc:HBu (acetic acid/butyric acid) ratio, though they cautioned that other researchers have observed the opposite trends thus rendering the HAc:HBu ratio an insufficient indicator of H_2 production (Chen et al, 2009). Besides, Hwang et al. (2004) inferred from their findings that the butyric acid production pathway carried the risk of butanol production from the consumption of dissolved hydrogen.

From the literature, there are different viewpoints on ethanol-type fermentation to produce hydrogen. According to Skonieczny and Yargeau (2009), the presence of VFAs and alcohols during anaerobic fermentation by *Clostridia* has been reported in the literature (for instance Fang and Liu, 2002; Hussy et al., 2005), and that the presence of ethanol is undesirable due to its toxic effect on bacteria. In the opinion of Sreethawong et al. (2010), EtOH-type fermentation can consume free electrons that are required to form hydrogen and lead to a higher CO_2 content. On the other hand, solvent fermentation is known to be associated with the early steps of sporulation of *Clostridia* (Rogers and Gottschalk, 1993). Ren et al. (2006) found that H_2 yield was affected by the presence of ethanol and acetate in the liquid phase, and maximum H_2 production rate occurred when the EtOH:HAc (ethanol/acetic acid) ratio was close to 1.0 in a CSTR pilot-scale study using molasses as substrate. They reported that pH 4.5 was suitable for hydrogen production by ethanol-type fermentation because NADH:NAD^+ ratio would become unstable via butyric acid type fermentation, which can readily change to propionic acid type fermentation at higher pH.

1.2 Thermodynamics of Hydrogenase

Hydrogenase is the enzyme responsible for the uptake and evolution of hydrogen and it has been found on the sites of periplasm, cytoplasm, as well as membrane-bound. Hydrogen evolution is achieved by the oxidation of NADH (**Figure 1.2**):

$$NAD^+ \xrightarrow[NADH:Fd\ oxidoreductase]{} Ferredoxin \xrightarrow[hydrogenase]{} H_2$$

Reduction of proton may be accomplished near the external surface of microbes whereas oxidation of NADH takes place inside the cells. In the case of *E. coli*, Padan et al. (1976) reported that internal pH of the cell was constant around 8 while the external pH varied from 5.5 to 9.0. The pH gradient between intra- and extra-cellular conditions has been known to govern the metabolic pathway related to enzymatic activity.

Using the Nernst equation, the redox potential for proton reduction can be described as,

$$E = E_0 + \frac{RT}{2F}\ln\frac{[H^+]^2}{P_{H2}} = E_0 - \frac{2.303RT}{F}\,pH - \frac{RT}{2F}\ln P_{H2} \qquad \textbf{Eq. 1.1}$$

where E_0 is at standard condition for hydrogen (0 V), R is the universal gas constant, F is the Faraday constant, and P_{H2} is the hydrogen partial pressure. For instance, at 25^{o}C and pH 6.0 with 1 atm hydrogen partial pressure, the redox potential of hydrogen is -0.355 V.

For the oxidation of NADH, the redox potential is,

$$E = E_0 + \frac{RT}{2F}\ln\frac{[NAD^+][H^+]}{[NADH]} = E_0 - \frac{2.303RT}{2F}\,pH + \frac{RT}{2F}\ln\frac{[NAD^+]}{[NADH]} \qquad \textbf{Eq. 1.2}$$

where E_0 is -0.113 V, as deduced from a value of -0.320 V at pH 7.0 and 25^{o}C with $[NAD^+]$ = $[NADH]$ (Unden and Bongaerts, 1997). For example, same as the redox potential of hydrogen, the redox potential of NADH would be -0.291 V at pH 6.0 and 25^{o}C when $[NAD^+]$ = $[NADH]$. Hence, hydrogen molecule loses electrons to NAD^+ rather than obtained if both intra- and extracellular pH are the same. Until pH is 3.8, hydrogen evolution is not triggered (**Figure 1.3**).

However, if it is assumed that intracellular pH is maintained neutral (~7.0) with $[NAD^+] = [NADH]$ and the partial pressure of hydrogen is 0.6 atm, the redox potential of NAD^+ becomes -0.320 V (**Eqn 1.2**) and the equivalent potential of hydrogen is reached at pH 5.5 as extracellular pH (**Eqn 1.1**). When pH of extracellular condition becomes lower than 5.5, the redox potential of H_2 is higher than NAD^+, which triggers the hydrogen production. Since it would be impossible to control intracellular pH, the operational pH for a hydrogen-producing reactor is favoured at relatively lower pH when compared to neutral in order to achieve the electron flux from NADH to H_2.

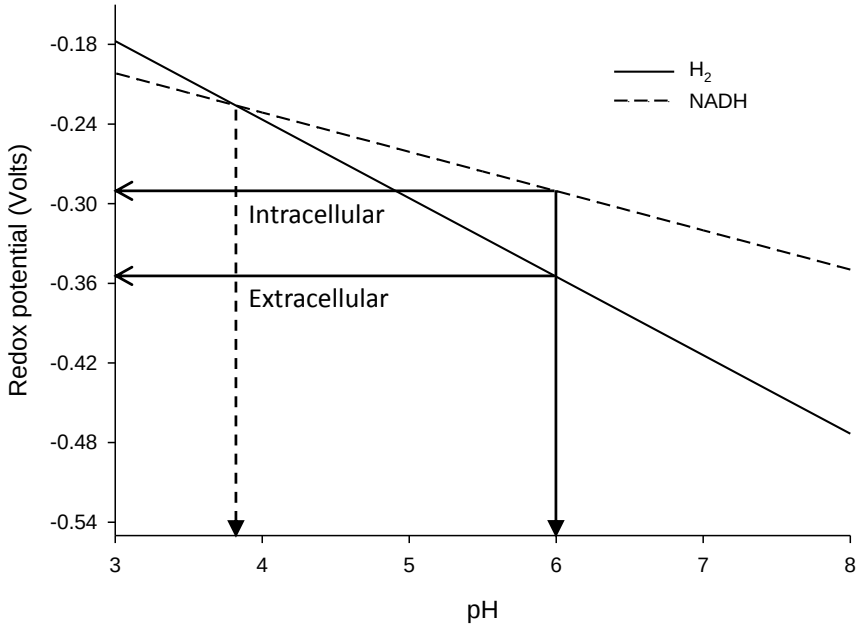

Figure 1.3. Changes of redox potential according to varying pH

Aside from pH control, hydrogen partial pressure also influences the redox potential. The lower the hydrogen partial pressure, the higher the redox potential of hydrogen, which may give us some clues to control hydrogen production during anaerobic

fermentation. In addition, NAD^+/NADH ratio could not always be maintained at 1.0 according to metabolic pathway. Hence, hydrogen productivity is also influenced by metabolic pathway due to varying the redox potential of NAD.

1.3 Maximum Theoretical Yields

In all thermodynamically feasible dark fermentation processes exploited by known microorganisms, hydrogen is only produced in combination with volatile fatty acids (VFA) and/or alcohols, carbon dioxide, and trace amount of methane, carbon monoxide, and/or hydrogen sulfide – never as a single-reduced compound. The maximum theoretical hydrogen yield from complete conversion of glucose to hydrogen and carbon dioxide is 12 mol H_2/mol glucose:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \qquad (\Delta G^0 = +3.2 \text{ kJ}) \qquad \textbf{Eq. 1.3}$$

However, the reaction is not thermodynamically feasible. It is never attained in known biological *in vivo* systems (Westermann et al., 2007) because fermentations have been optimized by evolution to produce cell biomass and not hydrogen. In the absence of external energy, the most common products in the fermentation of carbohydrate are acetate and butyrate through acidogenesis and acetogenesis. Again, using glucose as the model substrate (Nandi and Sengupta, 1998), the reactions proceed as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH \qquad \textbf{Eq. 1.4}$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + CH_3CH_2CH_2COOH \qquad \textbf{Eq. 1.5}$$

According to reactions (1) and (2), the stoichiometric or theoretical maximum yield is 4 mol H_2/mol glucose (544 ml H_2/g hexose) at 25 ℃ in the production of acetic acid, and 2 mol H_2/mol glucose (272 ml H_2/g hexose) in the production of butyric acid, respectively. In addition to these acids, ethanol may also be produced via the following reaction (Hwang et al., 2003 and 2004):

$$C_6H_{12}O_6 + H_2O \rightarrow 2H_2 + 2CO_2 + CH_3COOH + CH_3CH_2OH \qquad \textbf{Eq. 1.6}$$

and the corresponding stoichiometric yield is 2 mol H_2/mol glucose. If sucrose or cellobiose is used as the substrate, the stoichiometric yield would be 8 mol H_2/mol sucrose or cellobiose.

The actual hydrogen yield may be substantially lower than these stoichiometric values for several reasons. Firstly, the sugar may be degraded through other pathways without producing hydrogen. Secondly, a fraction of sugar could be consumed for biomass production. Thirdly, stoichiometric yield is achievable only under near equilibrium conditions, which implies slow production rates and/or very low hydrogen partial pressures (Hallenbeck and Benemann, 2002). Lastly, some hydrogen produced may be consumed for the production of other by-products, such as propionate (Vavilin et al., 1995), as shown in the following reaction:

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2H_2O + 2CH_3CH_2COOH \qquad \textbf{Eq. 1.7}$$

Recommended requirements for economically viable production of hydrogen (USDOE, 2004) would be a yield of 8-12 mol H_2/mol glucose, with reference to corn-based production. This requirement may be somewhat relaxed if low-cost feedstocks such

as organic waste materials are recycled to produce biohydrogen. Therefore, technical barriers and challenges must be overcome via R&D studies to achieve cost-effective production of hydrogen via direct fermentation. Kotay and Das (2008) summarized the techniques that can provide solutions to improve hydrogen production via dark fermentation, which echoed these recommendations: microbial strain selection and augmentation; manipulation of microbial metabolic pathway; refinement of bioreactor technology; hybrid fermentation process and optimization of key operational parameters.

1.4 Pretreatment of Seed Sludge

Application of mixed cultures for hydrogen production requires inhibition or elimination of methanogens. Selection of spore-forming bacteria such as *Clostridium* and *Bacillus* by heat treatment of inoculum and maintenance of low pH (around 4.0-5.7) are the two most commonly used approaches that have been effective for this end (Hallenbeck 2005). Other pretreatment methods involved the use of chemicals such as acid, alkaline, chloroform, bromoethanesulfonate, or iodopropane, and the use of repeated-aeration.

Wang and Wan (2008) concluded that inoculum pretreated by heat shocking was most efficient in the enrichment of hydrogen-producing bacteria among the various pretreatment techniques. Ren et al. (2008) suggested that different pretreatment methods would result in the change in the metabolic pathway – butyric acid, mixed-acids, and ethanol types. They did batch tests using glucose (10,000 mg/L) as the substrate; the observed maximum hydrogen yield after 3 days was similar (189.5 mL versus 180.4 mL H_2) with and without heat-shock pretreatment of the seed sludge obtained from secondary wastewater treatment plant clarifier. Zhu and Béland (2006) found the 2-

bromoethanesulfonic acid and iodopropane pretreatments were outstanding to inhibit methanogenic activity among 6 different pretreatments. Besides, the control gave higher hydrogen yield when compared to heat-shock; whereas, Kawagoshi et al. (2005) observed no differences between non-heat-treated and heat-treated digested sludge. They suggested that other factors can affect the hydrogen production ability besides pretreatment methods.

One of the enzymes involved in *Clostridium* spp., NADH:ferredoxin oxidoreductase, is known to be inhibited by hydrogen partial pressure as low as 60-100 Pa (0.5-0.8 μM) (Angenent et al., 2004; Hallenbeck, 2005). As distinguished from the strictly anaerobic *Clostridium* spp., *Enterobacter aerogenes*, as facultative microbe, is also known as an excellent hydrogen producing bacteria. Its hydrogen evolution mechanism is similar to that of *E. coli* (Nandi and Sengupta, 1998; Kurokawa and Tanisho, 2005). *Enterobacter* spp. could work well under acidic conditions (pH 4.0) and they can tolerate high H_2 partial pressure of 30,000 Pa (230 μM) (Tanisho et al., 1989; Yokoi et al., 1995). Yokoi et al. (1998) demonstrated via batch tests that a co-culture of *Clostridium* spp. and *Enterobacter aerogene* without reducing agents produced more hydrogen when compared to *Clostridium* spp. alone with a reducing agent.

Therefore, in summary, the advantages of inocula pretreatment include the ability to select hydrogen producing bacteria from mixed microbial sources, and helping with recovery from system upset. However, the major disadvantage lies with the fact that only spore-forming hydrogen producing bacteria such as *Clostridia* are selected, while it blocks other non-spore forming H_2-producing microbial strains such as *Enterobacter*. Moreover, it could not eliminate the H_2-consuming homoacetogens. Homoacetogens are able to

convert glucose into acetic acid through both heterotrophic (**Eqn 1.8**) and autotrophic (**Eqn 1.9**) mechanisms.

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \qquad \textbf{Eq. 1.8}$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \textbf{Eq. 1.9}$$

It is unlikely that these pretreatment methods are applicable to full-scale reactors, as they would require high energy consumption. Besides, methanogens can be continuously re-introduced to the reactor since agri-food and municipal organic waste streams ususally contain methanogens (Shizas and Bagley, 2005).

1.5 Process and Operational Parameters

Li and Fang (2007) compiled and analyzed a large number of publications related to fermentative hydrogen production. Their review covered the types of substrates (pure substrates, single substrates in synthetic wastewater, actual wastewater and solid waste), pretreatment conditions for screening hydrogen-producing bacteria from anaerobic sludge or soil, process parameters (pH, temperature, hydraulic retention time, seed sludge, nutrients, inhibitors, reactor design, and the means used for lowering hydrogen partial pressure), and performance parameters (hydrogen yield, production rate and conversion efficiency). It is realized from their review that experimental apparatus ranged from serum bottles to pilot-scale reactors, and some studies were done in continuous operation mode over a long time period while others used batch operation mode. Yet, most of these studies used carbohydrate-rich waste (glucose, sucrose, cellobiose and starch) as feedstock.

Wang and Wan (2009) also summarized the main factors influencing fermentative hydrogen production in their review. The reviewed factors included inocula, substrate, reactor type, nitrogen, phosphate, metal ion, temperature, and pH. Their review at the time showed that there usually existed some disagreements on the optimal condition of a given factor for fermentative hydrogen production, thus more research in this respect is recommended. Subsequently, in more recent research studies, Wu et al. (2009) found the operating conditions of (HRT 12-16 hr, pH 5.0, 37^{o}C) to be optimal for maximum hydrogen production (2.4-3.1 L H_2/L reactor.d) and hydrogen yield (1.57-1.63 mol H_2/mol hexose) when liquid swine manure and glucose was used as the substrate in an anaerobic sequencing batch reactor. For continuous stirred tank reactor, Wu et al. (2010) conducted a number of tests on the operating parameters with glucose as substrate (concentration 14000 mg/L), and found the optimal conditions to be (pH 5.0, HRT 8.3 hr, 33.5^{o}C) for maximum yield of 2.15 mol H_2/mol hexose.

1.5.1 Temperature

Temperature affects hydrogen evolution because the hydrogenase is active in narrow range of temperature. In most studies, the temperature for hydrogen production was set between 30 and 37^{o}C. For single carbohydrate substrates in synthetic wastewaters, the average yields were 1.27, 1.41, and 1.40 mol H_2/mol hexose, respectively, for temperatures in these three ranges. A similar trend was observed for the highest reported yields - 1.96, 2.45, and 2.41 mol H_2/mol hexose, respectively. These results suggest that hydrogen yields and production rates were comparable at mesophilic and thermophilic temperatures, but lower at the ambient temperatures. When actual wastewater was used as

feedstock, the average yields were 0.80, 1.59, and 2.33 mol H_2/mol hexose for 23-26 ℃, 32-37 ℃, and 55-60 ℃, respectively, while the highest yield was 2.52 mol H_2/mol hexose from treating a sugar factory wastewater at 60 ℃ (Ueno et al., 1996). With solid wastes, the average yields were 1.65, and 1.89 mol H_2/mol hexose for 35-37 ℃ and 55 ℃. The highest yield was 3.22 mol H_2/mol hexose from treating a mixed food and paper waste at 55 ℃. Gilroyed et al. (2008) reported maximum hydrogen production was achieved at 52^{o}C over the range from 36^{o}C to 60^{o}C in their batch tests using heat treated cattle manure and small changing temperature induced the shift in microbial metabolic pathways. Shin et al. (2004) compared biohydrogen production from acidogenesis of food waste using pure culture (*Thermoanaerobacterium*) under thermophilic conditions versus using mixed culture under mesophilic conditions, whereby hydrogen yield was observed to be 1.8 mol H_2/mol hexose and 0.05 mol H_2/mol hexose.

These results indicated that in general, hydrogen yield increased with temperature (Chang and Lin, 2004; Yu et al., 2002; Morimoto et al., 2004 and Valdez-Vazquez et al., 2005), but the beneficial effects due to thermophilic conditions were not always observed. Temperature differences might not be the only factor affecting yields reported in different studies, as there are also differences in reactor type, substrate, seed sludge, and other process conditions.

1.5.2 Hydraulic retention time (HRT)

HRT is considered to be a major factor influencing the performance of continuous operation. Shorter HRTs would change the fermentation pattern and suppress the methanogens which generally require relatively longer time to grow compared to the

acidogens. Shorter HRT is also preferred by reason of lower capital cost required. It was widely reported that the H_2 yield increased with decreasing HRT for different types of reactors (Chang and Lin, 2004; Lee et al., 2004; Van Ginkel et al., 2005),; whereas, the results from Wu et al. (2009)'s study demonstrated an optimal HRT amidst a range of HRTs tested. They suggested that the reduction in H_2 yield at long HRTs is probably due to the reuse of H_2 by homoacetogens which produce acetate from dissolved CO_2 in the presence of H_2 (Morinaga and Kawada, 1990). Fan et al. (2006) reported that varying HRTs changed the composition of liquid metabolites and the highest hydrogen production rate was obtained at HRT 18 hr among a range of 8 –48 hr using CSTR and brewery wastewater as substrate, whereas Zhang et al. (2006) optimized the reactor with the shortest HRT of 6 hr to obtain maximum hydrogen production rate and suppression of propionic acid production, though the substrate utilization efficiency was only about 78%.

Most of the solid wastes were treated in slurry form by mixing with water. The optimal HRT of the slurry varied significantly, from 6-9 hr for bean curd waste in a CSTR or a membrane bioreactor (Noike et al., 2003) to 84 hr for organic solid food waste in a semi-continuous reactor (Valdez-Vazquez et al., 2005). Shin and Youn (2005) compared the hydrogen yield at 48, 72, and 120 hr for hydrogen conversion from a food waste, and reported that a very long HRT of 120 hr was correlated with the highest hydrogen yield.

1.5.3 pH

The operating pH plays a major role on the effluent composition of the acidogenic reactor (Donanyos et al., 1985). Many researchers have studied the effects of pH on hydrogen production, including hydrogen content in biogas, hydrogen yield, hydrogen

production rate and the type of metabolites. It may also affect the activity of the Fe-hydrogenase - a gradual decrease in pH can inhibit hydrogen production (Dabrock et al., 1992). Hydrogenase catalyzes the conversion between hydrogen and proton depending on the oxidation state. In terms of thermodynamic aspects, NADH is not able to give electron to proton since hydrogen has very low redox potential (-414 mV) versus NAD (-320 mV) at the standard conditions (P_{H2} = 1 atm, 25^{o}C and pH 7.0) (Tanisho et al., 1989), which implies a positive value in Gibb's free energy. Theoretically, lower pH would lead to smaller redox potential difference between NAD^{+} and H_2. In addition, pH is a crucial factor for the suppression of the hydrogen-consuming methanogens (Chen et al., 2002).

A range of pH (between 5 and 6) is reported to be optimum for fermentation of carbohydrates by mixed bacterial cultures (Fang and Liu, 2002; Lay et al., 1999; Khanal et al., 2004; Chen et al., 2001). For single carbohydrate substrates in synthetic wastewaters, the optimal pH was found to be in the range of 5.2-7.0 with an average of pH 6.0. Optimal pH values for hydrogen conversion when actual wastewater or solid wastes were used as feedstock were all within the range of pH 5.2-5.6. One exception was reported by Fang et al. (2006); they observed an optimal pH of 4.5 for rice slurry with a hydrogen yield of 2.55 mol H_2/mol hexose.

The pH also affects the metabolic pathways in hydrogen production (Lay, 2000). In most studies, butyrate and acetate were the two main products, while low pH seemed to favour butyrate production. Propionate production increased substantially at pH 7.0 and above. Horiuchi et al. (2002) reported that butyrate was predominant at pH 5.0; Kim et al. (2004) also reported that butyrate was the main product at pH 5.5, but butanol became predominant at pH 4.3. Hwang et al. (2004) reported that the main metabolic products

were butyrate at pH 4.0-4.5, ethanol at pH 4.5-6.0, and propionate at pH 5.0-6.0. These studies suggested that pH values around 4.5-5.5 would be favourable for hydrogen production.

Among a large number of research included in the reviews by Li and Fang (2007) and Wang and Wan (2009), some of the studies have been identified to utilize a variety of liquid or solid organic wastes as substrate and different types of inocula in batch tests and ASBR (anaerobic sequencing batch reactor). Further analysis of the reported results in the literature reveals a general trend, as exhibited in **Figure 1.4**. In the literature, hydrogen productivity was reported in terms of H_2 production rate (HPR), H_2 yield or both. Where necessary, the reported H_2 yields have been converted into units of [mol H_2 per mol hexose] before they are presented in **Figure 1.4**. It shows a decrease in H_2 yield with increasing pH, within the range of pH 4.5 and 7.0. The maximum H_2 yield attained was 2.48 mol H_2/mol hexose or 62% of the theoretical maximum yield at pH 4.5 and 5.0 using food waste and bean curd manufacturing waste as substrate in batch tests.

For ASBR operation using cassava wastewater as substrate, the H_2 yield was 42.5% of theoretical maximum value. Similarly, H_2 yields obtained in some of the studies using synthetic substrates (glucose or sucrose) as carbon sources are summarized in **Figure 1.5**. At pH levels above 6, the H_2 yields were greater, being 1.0-2.8 mol H_2/mol hexose, when compared to 0.1-1.2 mol H_2/mol hexose for real wastes as previously presented in **Figure 1.4**. As seen in **Figure 1.5**, when tests were performed using CSTR operation, H_2 yield could reach 70% of theoretical maximum. Batch tests could also achieve up to 60% of the theoretical maximum H_2 yield. However, only 18% of the theoretical maximum yield was attained with ASBR operation.

No definitive correlation between pH level and H_2 yield could be deduced, though Skonieczny and Yargeau (2009) suggested that in general, there appears to be a strong trend of increasing hydrogen production rate with an increase in pH, based on observations from their batch-test study using glucose as substrate and *Clostridium beijerinckii* as the inocula. The ranking of hydrogen productivity in terms of HPR for a wider range of studies using real wastewater and synthetic substrate is shown in **Figure 1.6.** ASBR and CSTR reactors were found to have higher HPR values of 3.5-5.8 L H_2/L reactor.d, as comapored to batch test results. The highest HPR was achieved at pH 5.5 in an ASBR digesting cassava wastewater. Yet, in another study whereby sucrose was used as substrate in an ASBR, a low HPR of ~1.0 L H_2/L reactor.d was observed.

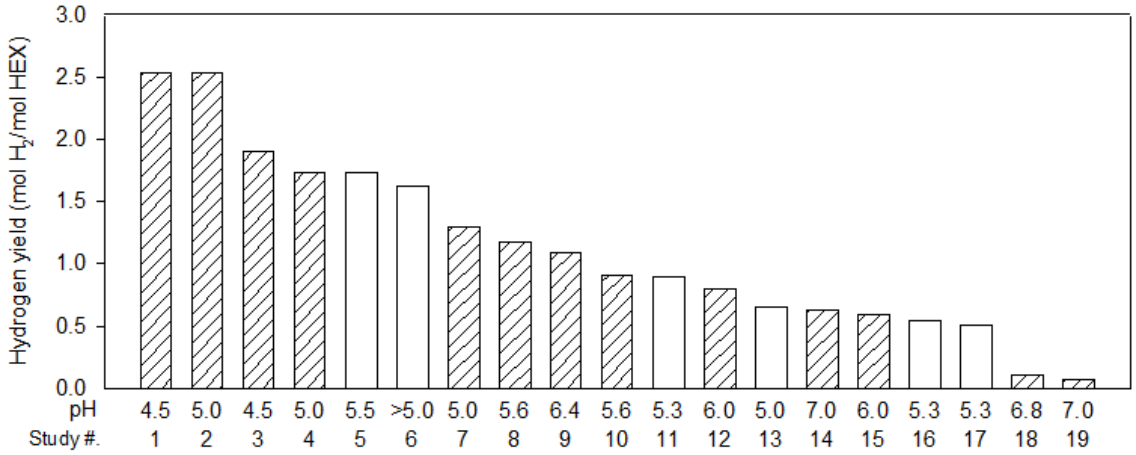

Figure 1.4. Hydrogen yield reported in other studies with real wastewater in batch tests ▨ Batch operation ▭ ASBR

Study #	Authors	Substrates	Inocula
1	Fang et al. 2006	Rice slurry	Anaerobic digested sludge
2	Noike and Mizuno 2000	Bean curd manufacturing waste	Soy bean meal
3	Yang et al. 2007	Cheese powder with additives	Sewage sludge
4	Noike and Mizuno 2000	Wheat bran	Soy bean meal
5	Sreethawong et al. 2010	Cassava wastewater	Cassava treating sludge
6	Wu et al. 2009	Swine manure and glucose	Anaerobic digested sludge
7	Noike and Mizuno 2000	Rice bran	Soy bean meal
8	Lay et al. 1999	Mixed waste	Soy bean meal
9	Van Ginkel et al. 2005	Food processing and domestic wastewater	Soil
10	Lay et al. 1999	Mixed waste	Anaerobic digested sludge
11	Kim et al. 2010	Food waste	Anaerobic digested sludge
12	Logan et al. 2002	Molasses	Soil
13	Saraphirom et al. 2011	Sweet sorghum syrup	Anaerobic digested sludge
14	Okamoto et al. 2000	Rice	Anaerobic digested sludge
15	Logan et al. 2002	Potato	Soil
16	Kim et al. 2010	Food waste	Anaerobic digested sludge
17	Arooj et al. 2008	Corn starch	Sewage sludge
18	Wang et al. 2003	Waste biosolids	Waste biosolids
19	Okamoto et al. 2000	Fats	Anaerobic digested sludge

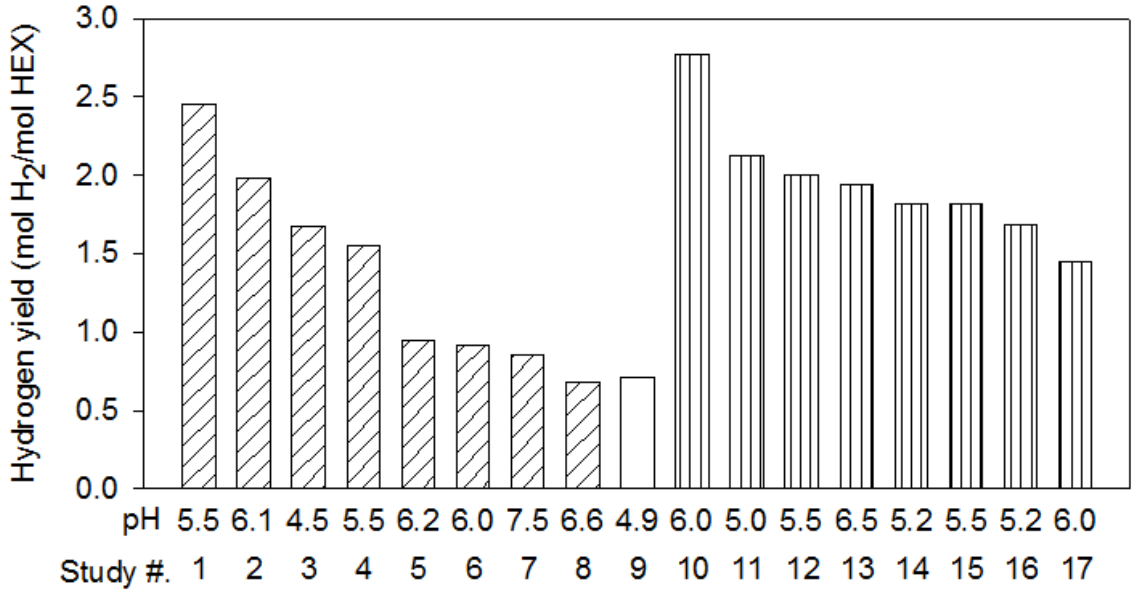

Figure 1.5. Hydrogen yield reported in other studies with synthetic substrates in ASBR, CSTR, and batch reactor - Batch ASBR CSTR

Study #	Authors	Substrates	Inocula
1	Van Ginkel et al. 2001	Sucrose	Compost
2	Wu et al. 2002	Sucrose	Sewage sludge
3	Khanal et al. 2004	Sucrose	Compost
4	Mu et al. 2006	Glucose	Sewage sludge
5	Oh et al. 2003	Glucose	Anaerobic digested sludge
6	Logan et al. 2002	Glucose	Soil
7	Lin and Lay, 2004	Sucrose	Acclimated sewage sludge
8	Liu et al. 2003	Cellulose	Acclimated sludge
9	Chen et al. 2009	Sucrose	Anaerobic digested sludge
10	Hafez et al. 2010	Glucose	Sewage sludge
11	Wu et al. 2010	Glucsoe	Cow dung compost
12	Fang and Liu 2002	Glucose	Sewage sludge
13	Mariakakis et al. 2011	Sucrose	Anaerobic digested sludge
14	Hussy et al. 2005	Sucrose	Anaerobic digested sludge
15	Iyer et al. 2004	Glucose	Soil
16	Hussy et al. 2003	Wheat starch	Anaerobic digested sludge
17	Mizuno et al. 2000	Glucose	Soy bean meal

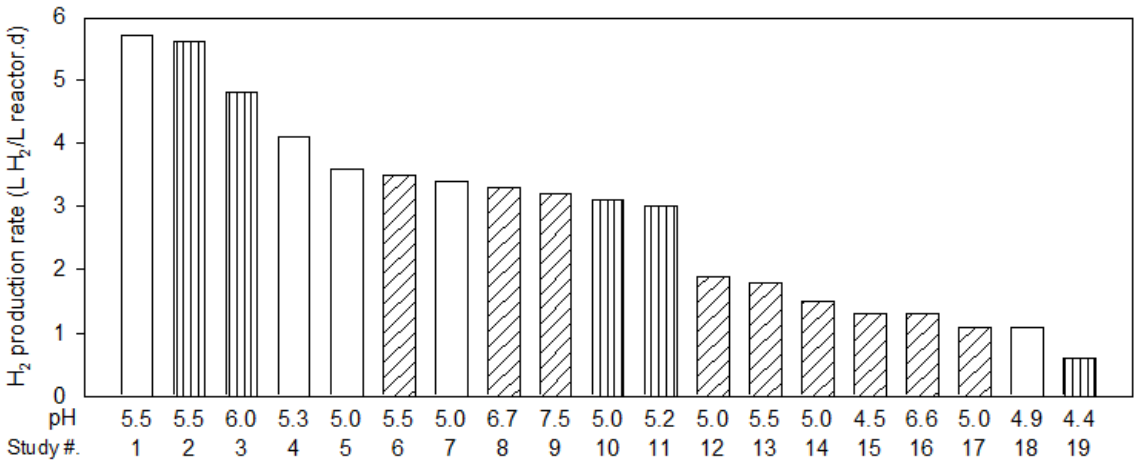

Figure 1.6. Hydrogen production rate reported in other studies in ASBR, CSTR, and batch reactor - Batch ASBR CSTR

Study #	Authors	Substrates	Inocula
1	Sreethawong et al. 2010	Cassava wastewater	Cassava treating sludge
2	Iyer et al. 2004	Glucose	Soil
3	Mizuno et al. 2000	Glucose	Soy bean meal
4	Arooj et al. 2008	Corn starch	Sewage sludge
5	Wu et al. 2009	Swine wastewater with glucose	Anaerobic digested sludge
6	Mu et al. 2006	Glucose	Sewage sludge
7	Saraphirom et al. 2011	Sweet sorghum syrup	Anaerobic digested sludge
8	Wu et al. 2002	Sucrose	Sewage sludge
9	Lin and Lay 2004	Sucrose	Acclimated sewage sludge
10	Fang and Liu 2002	Glucose	Sewage sludge
11	Hussy et al. 2003	Wheat	Anaerobic digested sludge
12	Noike and Mizuno 2000	Wheat bran	Soy bean meal
13	Van Ginkel et al. 2001	Sucrose	Compost
14	Noike and Mizuno 2000	Bean curd manufacturing waste	Soy bean meal
15	Fang et al. 2006	Rice slurry	Anaerobic digested sludge
16	Liu et al. 2003	Cellulose	Acclimated sludge
17	Noike and Mizuno 2000	Rice bran	Soy bean meal
18	Chen et al. 2009	Sucrose	Anaerobic digested sludge
19	Yang et al. 2007	Dry whey permeate powder	Sewage sludge

1.5.4 Reactor type

Many exploratory studies were conducted in batch reactors for simple operation and efficient control. However, industrial operations would require continuous or semi-continuous production processes for practical engineering reasons. Reactors for continuous hydrogen production included the completely mixed, packed-bed, fluidized-bed, sequencing-continuous reactor, trickling biofilter, and membrane bioreactors.

Completely mixed reactor without recycle is relatively simple and known to be applicable for high concentration wastes (including solid wastes). It has relatively long hydraulic retention time (HRT) and the reactor is operated with solids retention time (SRT) practically equal to HRT since the influent and effluent flow continuously. Hence, it is possible to maintain steady-state physiologically. Packed-bed reactor contains some types of packing materials such as ceramic, rock, plastic, slag, and so on. A greater number of microbes are attached and growing on the packing materials in the reactor; hence, packed-bed reactor is able to decouple HRT from SRT and enables high-loading rate to be attained without loss of microbes. Fluidized-bed reactor is very similar to packed-bed reactor except that the microbial bed can move with the fluid flow. A typical fluidized bed reactor is an upflow anaerobic sludge blanket (UASB) reactor, and this has been widely studied for anaerobic digestion. Finally, a semi-continuous reactor is operated repeatedly by cyclic duration and keeps microflora. This makes it different from a batch reactor. SRT is also decoupled from HRT; reaction circumstances during a cycle are changed as microbes grow and intermediates are produced since the reaction phase is operated as batch type. The reactor does not reach steady-state.

Continuous stirred tank reactors (CSTR) have been used by many researchers in their studies on biohydrogen production. CSTRs reach steady-state and show high efficiency and stable performance when the operational conditions are optimized. However, CSTRs have an intrinsic disadvantage to unite HRT and SRT, which may cause wash-out of biomass when the dilution rate (the inverse of HRT) is higher than the microbial growth rate. Besides, CSTRs would not be appropriate for decide operational parameters with respect to microbial growth.

The Anaerobic Sequencing Batch Reactor (ASBR) as an alternative reactor can maintain higher biomass concentration over CSTR since HRT is decoupled from SRT by the *Settle* phase during a cycle. ASBRs may not show higher productivity over CSTRs since it cannot reach steady-state and it is semi-continuous. Moreover, the advantages of sequencing batch reactors include the following: A higher degree of process flexibility with respect to changes in organic loading rate (OLR); a single vessel for reaction and settling (hence, no need for a separate clarifier); relative ease of operation in a semi-continuous mode (hence, more feasible for potential real applications) and lower capital investment (Wu et al., 2009).

However, it has disadvantages such as having an upper limit in OLR and lower biogas production. The reported highest OLR of 19 $kg/m^3.d$ is much lower than 100 $kg/m^3.d$ allowed by upflow anaerobic sludge blanket reactors with continuous mode of operation (Angenent and Dague, 1995), and 103 kg $COD/m^3.d$ reported by Hafez et al. (2010) as optimum for a CSTR coupled with a clarifier for solids.

1.5.5 Hydrogen partial pressure

Hydrogen production is a means by which bacteria re-oxidize reduced ferredoxin and hydrogen-carrying coenzymes, and these reactions are less favourable as the H_2 concentration in the liquid rises (Hawkes et al., 2002). To be more specific, hydrogen synthesis pathways are sensitive to H_2 concentrations and are subject to end-product inhibition. As previously mentioned in Section 1.4, hydrogenase activity is severely inhibited when hydrogen partial pressure is only about 60 -100 Pa (0.5-0.8 μM) (Angenent et al., 2004; Hallenbeck, 2005). Different from the obligate anaerobes, *Clostridium* spp., one of facultative genera, *Enterobacter,* is also known as one of the well-known hydrogen producing bacteria. It has similar hydrogen evolution mechanism with *E. coli* (Nandi and Sengupta, 1998; Kurokawa and Tanisho, 2005). *Enterobacter* spp. tolerate well under acidic conditions (pH 4.0) and high H_2 partial pressure of 30,000 Pa (230 μM) (Tanisho et al., 1989; Yokoi et al., 1995). In order to avoid using reducing agents to remove oxygen in the reactor, a co-culture of *Clostridium* spp. and *Enterobacter aerogene* without reducing agents led to higher hydrogen production than *Clostridium* spp. alone with reducing agents via batch tests (Yokoi et al., 1998).

As H_2 concentration (partial pressure) increases, H_2 synthesis decreases and metabolic pathways shift to produce a larger amount of reduced substrates such as lactate, ethanol, acetone, butanol, or alanine, which can become inhibitive to H_2 production. One method of lowering dissolved H_2 is to sparge the reactor with reducing agents such as nitrogen or argon gas, which not only helps to increase H_2 yield, but also to remove trace amounts of oxygen present in the medium.

All of above concerns are to be kept in mind upon scaling up of the reactor. Ren et al. (2006) reported maximum HPR (H_2 production rate) of 5.57 m^3 H_2/m^3 reactor.d with reactor size of 1.48 m^3 using molasses as feedstock; while, Kim et al. (2010) obtained H_2 yield of 0.54 mol H_2/mol hexose from a 0.15 m^3 reactor using food waste as feedstock. Lin et al. (2011) showed that maximum HPR of 15.6 m^3 H_2/m^3 reactor.d and 1.04 mol H_2/mol sucrose was obtained from 0.4 m^3 reactor. However, H_2 yield reported above was very low compared to lab-scale experiments, which is probably caused by using higher OLR. On the other hand, Chou et al. (2008) compared 10 L to 100 L reactor in order to evaluate the effect of pH and stirring speed. Maximum H_2 production rates were similar between the two reactors but H_2 yield was different; the large reactor exhibited lower H_2 yield which was governed by the stirring speed generating laminar flow. Consequently, these pilot-scale tests indicated that hydrogen production might not be significantly affected by scale-up; however, stirring speed must be considered for gas diffusion.

1.6 Biohydrogen for Fuel Cells

Fuel cells are viewed as environmentally clean and next generation technology. Many companies are trying to increase the efficiency and apply it to diverse fields from stationary power systems to small portable and personal systems. Proton exchange membrane fuel cell (PEMFC) is operated at relatively low temperature, 80^oC and it is compact. However, this type of fuel cell has 40-60% efficiency, and catalyst and membrane are expensive materials. Besides, lots of synthesized water during the reaction causes the efficiency to drop and generate problems (Su et al., 2006). Alternatively, Solid oxide fuel cells (SOFC) typically operate at up to 1000^oC and can use various sources as

fuel such as methane, propane, butane, fermentation gas and so on. It requires high energy to sustain the high temperature. In order to increase the overall efficiency, SOFC-GT system has been developed to use off-gas from SOFC to run a gas turbine (Chan et al., 2002)

Levin et al. (2004) accessed the potential application of biological hydrogen production with the following assumptions: cell efficiency 50%, H_2 utilization 95%, and average cell voltage 0.779 V, which is derived from the equation below:

$$\eta = \mu_f \frac{V_c}{E_f} \qquad \textbf{Eq. 1.10}$$

where η is the cell efficiency, μ_f is the fuel utilization efficiency, V_c is the cell output voltage, and E_f is the theoretical maximum output electricity.

Hence, E_f could be obtained from:

$$E_f = \frac{-\Delta \bar{h}_f}{zF} \qquad \textbf{Eq. 1.11}$$

where $-\Delta \bar{h}_f$ is 285.85 kJ/mol for hydrogen, z is the number of electrons through the electrolyte, z = 2, and F is Faraday's constant (96485 C/mol). Hence, the required amount of hydrogen can be calculated as:

$$n = \frac{PowerOutput(kW)}{2 \times V_c \times F} \qquad \textbf{Eq. 1.12}$$

where n is the amount of required hydrogen in the cell (mole/s). Hence, 1 kW of electricity, for instance, can be generated by 23.9 moles (48.3 g) H_2/hour through PEMFC. Take Ren

et al. (2006)'s pilot-scale test, for example, hydrogen production rate of 5.5 L H_2/L reactor.d was reported with molasses as substrate in a 1.5 m^3 reactor, which is quantitatively equivalent to 28.3 g H_2/hr. This is sufficient to produce 587 W of power based on the assumptions. Furthermore, assuming that an average household in British Columbia uses 13,000 kWh per year (Levin et al., 2004), a 1.5 kW PEMFC (13,140 kWh per year) would be required as the minimum size of fuel cell. This would in turn require a much higher hydrogen production rate of 14 L H_2/L reactor.d, and pose a great challenge to future research work to improve hydrogen yield from real wastes.

1.7 Research Motivation

Based on the literature review, yield improvement is essential towards achieving economic feasibility of biological hydrogen production via dark fermentation. This could be achieved using a variety of biotechnological and engineering strategies, including microbial strain selection and augmentation, manipulation of microbial metabolic pathways, refinement of bioreactor technology and optimization of key bioprocess operational parameters. The focus of this thesis research is on the engineering techniques. The following constraints were considered at the early stage of the research.

1) Organic waste/wastewater from a variety of sources may be utilized as feedstock for biohydrogen production. Wastes that contain high carbohydrate content have been preferred since the biodegradation rate of fats and proteins is generally slower than carbohydrates; besides, some metabolites may exert inhibitory effects on hydrogen production though they are important for microbial activity.

2) Pretreatment of inoculum may favour the selection of the spore-forming *Clostridium* over many other hydrogen producers. However, hydrogen-consuming spore-forming bacteria could still remain in the pretreated inoculum. Pretreatment is not applicable to the non-spore forming bacteria which also possess hydrogen-producing metabolism. Thus, it is not necessary to focus only on techniques that promote the metabolic pathway from *Clostridium*. Besides, it might not be desirable in terms of cost-effectiveness and operational control over the long term.
3) Types of bioreactor – Continuous stirred tank reactor (CSTR) has been the most commonly used type of reactor because of its higher yield, but it is more expensive and may require a clarifier. The trend of operational conditions in bioreactor for hydrogen production has been short hydraulic retention time (HRT) and high organic loading rate (OLR). Typical CSTR has a higher potential of losing its biomass under such trends. Hence, research efforts in recent years have aimed at retaining a higher concentration of biomass in the bioreactor via microbial immobilization, granulating, semi-continuous process, and so on. Anaerobic sequencing batch reactor (ASBR) is a semi-continuous process, and it has some advantages over CSTR.
4) Most previous studies on dark fermentation for biohydrogen reported the hydrogen productivity, such as hydrogen content, production rate and yield under a set of specific operational conditions (pH, temperature, HRT, substrate concentration, OLR, F:M ratio). Accurate predictions of reactor performance based on these results might not be possible due to diverse experimental circumstances. Research methodology which adopts an integrated approach to investigate the effects of key

process parameters on hydrogen production, together with a detailed analysis of the soluble metabolite products as well as identification of the dominant microorganisms, is required. The literature review indicated that such approach has not been used in experimental studies without inoculum pretreatment and few studies are relevant to ASBR.

1.8 Research Objectives

The overall goal of the thesis research is to investigate engineering techniques for enhancing biohydrogen production from the anaerobic fermentation of agri-food wastewater. The specific objectives are as follows:

1) To study the key operational parameters (pH, HRT, OLR, and cyclic duration) in an anaerobic sequencing batch reactor using synthetic wastewater and real wastewater as feedstocks;
2) To determine the feasibility of biohydrogen production without the pretreatment of inoculum;
3) To delineate the most appropriate or optimal operational conditions for hydrogen productivity in terms of various performance indicators;
4) To affirm the metabolic pathway for biohydrogen production via the relationship analysis of the metabolites; and
5) To identify the dominant microorganisms during anaerobic fermentation.

The research methodologies adopted in Chapters 2-5 are pertinent to both objectives #1 and #2. Experimental studies in Chapters 3-5, along with modeling in Chapter 5 are directed towards objective #3, while objective #4 is also addressed in these Chapters. Finally, one major research activity in Chapter 5 is focused on objective #5.

Chapter 2: Technical Feasibility of Anaerobic Fermentation of Dairy Wastewater for Biological Hydrogen Production

2.1 Introduction

In the Lower Fraser Valley of British Columbia, intensive livestock and poultry production has generated excessively large volumes of manure. Managing manure more effectively is becoming more challenging for farmers. The federal-provincial Environmental Farm Planning program is a voluntary process that applies to all types and sizes of farm operations throughout each province, and addresses environmental concerns related to the release of farm waste and wastewater. Manure may be generated in liquid, semi-solid or solid form, depending on the total solids (TS) content. A liquid-solids separation process will produce liquid manure and manure solids. The term "dairy wastewater" may be used interchangeably with "liquid dairy manure" when its TS content is less than 10%, and it usually includes the wastewater from the milking parlor. For dairy farming in the Lower Fraser Valley region, manure needs to be stored in different forms for 5-7 months when crops are not likely to take up the nutrients, or when the risk of manure or manure nutrients entering surface or groundwater is too great (BCMAFF, 2004).

As an alternative to storing manure over an extended period of time, farmers may treat manure using physical, chemical and/or biological methods. Anaerobic digestion technology for methane generation from organic wastes is a viable biological technology with many advantages and environmental benefits. It can address public concerns about water pollution problem, and odour, ammonia and greenhouse gas emissions from manure spreading. A number of medium-to-large scale anaerobic digestion facilities have been installed on dairy farms in various parts of the world in recent years, which use the

methane in biogas for cogeneration of heat and electricity (Tikalsky and Mullins, 2007). In fact, the co-digestion of mixed organic waste streams including manure is increasingly being practiced as part of the solution to climate change problems.

Hydrogen is considered as a viable alternative fuel and 'energy carrier' of the future, which would contribute towards achieving sustainability in the long term. Goodrich (2005) studied the feasibility of using fuel cell technology for a working farm. They had run a 5 kW proton exchange membrane fuel cell (PEMFC) successfully on biogas intermittently and are working towards running the fuel cell on biogas continuously. After gas cleaning, the CO_2 in biogas was reduced from 30% to 3%; also, hydrogen in methane was freed up inside the fuel cell. However, it is desirable to investigate technologies that can modify the anaerobic digestion process to produce hydrogen biologically instead of producing it via methane reformation. When the biohydrogen produced is eventually purified and used in fuel cells for generating power, it releases only water vapour as exhaust rather than CO_2 from the biogas-fed generator. Water vapour is a very effective absorber of long-wave radiation and it could be a powerful greenhouse gas. However, it can be readily removed in the form of precipitation. Hence, it has a short atmospheric lifetime (in the order of days or hours), and does not accumulate in the atmosphere in the same way as other non-condensing greenhouse gases such as carbon dioxide, methane, nitrous oxide, ozone and fluorocarbons. Global warming is primarily due to anthropogenic CO_2, CH_4 and N_2O.

Dairy manure is regarded as an useful renewable energy source and focus to date has been placed on methane production during anaerobic digestion. Besides, it contains various natural microbial communities including hydrogen-consuming bacteria which are

originated from the cow gut and developed during the storage period. Researchers had described the beneficial effects of seed sludge pretreatment using techniques such as heat-shock, acids, alkalis, repeated aeration, and chemicals for selecting spore-forming hydrogen producing bacteria such as *Clostridia* or eliminating hydrogen-consuming bacteria from the sources of mixed microbial communities. However, the major disadvantage of inoculum pretreatment lies with the fact that only spore-forming bacteria are selected, while it blocks other non-spore forming H_2-producing microbial strains such as *Enterobacter* and *Prevotella.* Besides, these techniques did not always lead to higher hydrogen productivity, and homoacetogens that are known to be hydrogen-consuming bacteria had been found in the pretreated inoculum (Zhu and Béland, 2006; Kawagoshi et al., 2005).

Though dairy manure is not suitable for hydrogen production and carbohydrate-rich substrates have been preferred, this material as feedstock has an advantage to evaluate the suppression of methanogenesis only with operational condition (HRT and OLR), but no pH control. Furthermore, the selection of inoculum via pretreatment such as heat and chemicals might not be necessary.

Hence, the objectives of this Chapter were to pave the way to improve hydrogen productivity via finding the inhibitory effects of methanogenesis without any pretreatment of both feedstock and inoculum but only with the manipulation of the operational conditions (HRT and OLR). Moreover, the performance of ASBR (semi-batch experiment) would be assessed.

2.2 Materials and Methods

2.2.1 Experimental apparatus

A lab-scale bioreactor (New Brunswick Scientific Inc., Model BioFlo 3000 fermenter, NJ, USA) with 6 L working volume was operated as an ASBR (Anaerobic Sequencing Batch Reactor). Its advantages over other types of anaerobic fermentation reactors include the requirement of a single vessel for reaction and settling, relative ease of operation and flexibility with respect to the change in organic loading rate (OLR). However, it has disadvantages such as having an upper limit in OLR (the reported highest OLR of 19 $kg/m^3.d$ is much less than 100 $kg/m^3.d$ allowed by upflow anaerobic sludge blanket reactors with continuous mode of operation) and lower biogas production.

Prior to start-up of the reactor, it was sparged with nitrogen gas for 20 min to induce anaerobic condition. These techniques are meant to decrease the partial pressure of hydrogen, which is known to be favourable for hydrogenase enzyme activity and hence improved hydrogen yield. Nitrogen gas was also used in every cycle when the effluent was decanted as displacement gas in order to equalize pressure inside of the reactor. The reactor was perfectly sealed and the head unit contained functional ports such as feed inlet and outlet, pH probe and temperature probe. The amount of biogas produced was recorded daily using the water displacement method and the acidified water, which was maintained at pH less than 3 in order to prevent biogas dissolution (Yu and Fang, 2001).

Agitation or stirring speed is regarded as an important factor to provide complete mixing and help decrease the hydrogen partial pressure in order to enhance biohydrogen production. It may vary with the type of impeller and reactor, and feedstock characteristics,

thus, leading to different Reynolds number, which is a function of liquid density and viscosity, reactor diameter, and flow rate.

Figure 2.1. New Brunswick Scientific Inc., Model BioFlo 3000 fermenter, NJ, USA

Chou et al. (2008) evaluated the effect of stirring and pH on anaerobes converting spent brewery grains to hydrogen using two sizes of reactors. The optimal agitation speed was recommended to be 120 rpm at pH 6. When the stirring speeds of the 10 L bioreactor operating in the batch mode were greater than this value, its mixing condition changed from laminar to turbulent flow. The pilot-scale (100 L) experiment with a sequencing batch mode of operation confirmed that the hydrogen production rate of 20 L H_2/d obtained with laminar flow was significantly more stable and reproducible than with turbulent flow.

Further literature review revealed that the stirring speed for tests using CSTR (size of reactor 1.0-5.0 L) ranged from 200 to 300 rpm (Cheong and Hansen, 2006; Kim et al., 2006; Karlssen et al., 2008; Davila-Vazguez et al., 2009; Wu et al., 2010). An exception was the 100 rpm adopted by Hussy et al. (2005) for two reactor sizes of 2.3 and 9.0 L. By comparison, lower stirring speeds of 90-150 rpm were reported for tests using ASBR or batch reactor (size of reactor 1.3-6.0 L) (Wang et al., 2005; Arooj et al., 2008; Buitrón et al., 2010; Ohnishi et al., 2010). Since a CSTR is operated with continuous flow of the influent, higher agitation speed is generally required to maintain the constant homogenization of the reactor contents, when compared to ASBR or batch reactor which is fed once within a cycle period.

During the experiment in this study, samples were collected from various locations in the ASBR when the agitation speed was set at 120 rpm. They were found to have similar MLSS (mixed liquor suspended solids) values, which further indicate that this stirring speed is adequate to provide complete mixing in the reactor.

As seen in **Figure 2.2**, the main computer monitored pH, temperature, gas flow rate, and agitation speed. Where necessary, pH and temperature could be controlled automatically by the addition of acid/base solutions prepared with 3M HCl and 3M NaOH and water jacket, respectively. The influent and effluent buckets were connected to peristaltic pump (Cole-Parmer Instrument Co.) and the volume was controlled by timer controller, ChronTrol XT (ChronTrol Corporation, 2001). All connected valves and a mixing motor were powered by this timer controller.

The sequence of the ASBR is composed of *Feed* → *React* → *Settle* → *Decant* per cycle. This cycle is continuously repeated so that HRT and SRT can be separated.

Hydrogen production and the degradation of organic wastewater are achieved continuously during the *React* period. There are several parameters for ASBR operation and the estimation of hydrogen productivity, including HRT, OLR, F/M ratio, HPR, and hydrogen yield.

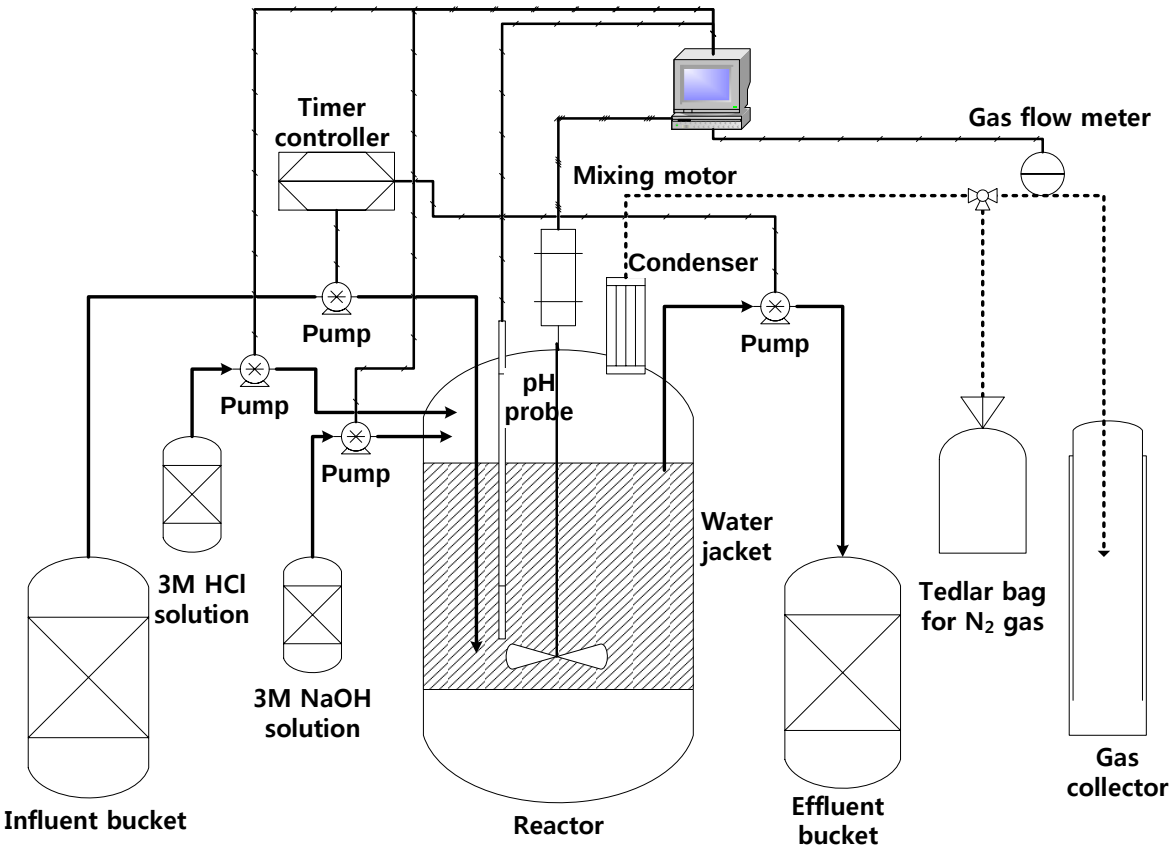

Figure 2.2. Schematic layout of anaerobic sequencing batch reactor

Hydraulic retention time (HRT) is defined by **Eqn 2.1** in a continuous system.

$$HRT = \frac{V_r}{Q} \qquad \textbf{Eq. 2.1}$$

where V_r (L) is the working volume of the reactor and Q (L/d) is the flow rate of influent. HRT can be expressed in units of "d" or "hr".

Cyclic duration is a parameter unique to ASBR, and it is related to flow rate (Q),

$$Q = N_c \times v_{in} \qquad \textbf{Eq. 2.2}$$

where N_c indicates the number of cycles (#cycles/d) and v_{in} is the influent/effluent volume in each cycle (L/cycle).

Organic loading rate (OLR) is a function of HRT and substrate concentration, and it is expressed in units of "g COD or sucrose/L reactor.d", as defined in **Eqn 2.3**.

$$OLR = \frac{S \times Q}{V_r} = \frac{S}{HRT} \qquad \textbf{Eq. 2.3}$$

where S is the influent substrate concentration (g/L). In an experiment, the substrate concentration can be determined when HRT and OLR are specified.

As for the food-to-microorganism ratio (F/M ratio), "food" means the organic substrate contained in the influent and "microorganism" can be represented by the mixed liquor volatile suspended solids (MLVSS). Hence,

$$\text{F/M ratio} = \frac{S \times Q}{V_r \times M} = \frac{OLR}{MLVSS} \qquad \textbf{Eq. 2.4}$$

where MLVSS (M) is the biomass concentration in the reactor (g/L).

Hydrogen production rate (HPR) (L H_2/L reactor.d) can be obtained from the data of biogas collected during one day and knowing the reactor size. After the biogas volume

is recorded periodically, hydrogen content is obtained through gas chromatography and hydrogen volume is determined by **Eqn 2.5**,

$$\text{HPR} = \frac{V_g \times C_{H_2}}{V_r \times t} \qquad \textbf{Eq. 2.5}$$

where V_g is a total biogas volume in certain time, C_{H2} is hydrogen content (%) in biogas, V_r is a working volume of the reactor, and t is a time period of hydrogen production.

Hydrogen yield (Yield) represents the mass of hydrogen (mol) per unit mass of substrate (mol) loaded into the reactor. Therefore, the molar mass of hydrogen produced is divided by the molar mass of substrate loaded, as given in **Eqn 2.6**,

$$\text{Yield} = \frac{(V_g \times C_{H_2}) / 24.2(L/mol)}{S_{mol}} \qquad \textbf{Eq. 2.6}$$

where ($V_g \times C_{H2}$) is the volume of hydrogen produced, and S_{mol} is a molar mass of substrate loaded.

2.2.2 Seed sludge

Seed sludge was obtained from the Department of Civil Engineering's biological nutrients removal (BNR) pilot plant for sewage treatment at the University of British Columbia. This pilot-scale facility implemented the three-stage Bardenpho process which comprised of "anaerobic", "anoxic" and "aerobic" zones. It had a four-step prefermenter equipped with ringlace, and with a capacity of 1350 L aside from the prefermenters and the clarifier. Domestic sewage with a strength of about 360 mg COD/L was fed to the

reactor. Seed sludge was picked up from the anaerobic zone as mixed liquor and screened with 1 mm pore mesh and stored at $4^{o}C$ prior to use in the experiments.

Hence, experiments reported in this Chapter were conducted without pretreatment of inoculum, for avoidance of hydrogen-consuming bacteria. For start-up of the ASBR with dairy wastewater, after the seed sludge was settled for 90 min, the supernatant was thrown away and the seed sludge was washed with tap-water twice since it contained domestic sewage when it was picked up. It was agitated for two days without feeding. Since the seed sludge had been established with low COD concentration (about 360 mg COD/L), a 2-month acclimatization period was applied for the stabilization of the seed sludge with a gradual increasing COD concentration from the similar strength as domestic sewage to high concentration (933 ± 326 mg COD/L) of dairy wastewater. Seed sludge that had been stabilized, as confirmed by active methane production and greater than 80% COD removal efficiency, was placed in the reactor at a mixed liquor volatile suspended solids (MLVSS) level of 11,250 mg /L.

2.2.3 Procedure

The research in this Chapter was conducted using dairy wastewater as substrate, with an aim to determine the effectiveness of inhibiting methanogenesis with the manipulation and control of hydraulic retention time (HRT) and OLR, but without pH and temperature control. Moreover, neither the inoculum nor the substrate received any form of pretreatment, and the reactor was operated under mesophilic temperature regime with less energy consumption as compared to thermophilic temperature. This operation strategy would provide the baseline data for future improvements where necessary. Dairy

wastewater was collected and screened with 1 mm pore mesh after preliminary settling at the UBC Dairy Education and Research Centre, Agassiz, BC, Canada. It contained 11,760 mg/L COD and 1.1% total solids (TS); inert solids were not very high as the fraction of total volatile solids (TVS) was 86% of total solids; pH was 7.4 and alkalinity was 3,750 mg/L expressed as $CaCO_3$.

Table 2.1. Operational sequence of the system

		Acclimatization	Run 1	Run 2	Run 3
HRT	(d)	15.00	3.00	0.67	0.25
Feed	(hr)	0.25		0.08	0.08
React		23.17		7.34	2.34
Settle		0.50		0.50	0.50
Decant		0.08		0.08	0.08

The test series was sub-divided into four stages – Acclimatization run, Run 1, Run 2 and Run 3 (**Table 2.1**). For acclimatization, the reactor was operated for 48 d, with HRT of 15 d and OLR of 0.2 ± 0.1 kg/m^3.d. To delineate the effect of HRT, Run 1 was performed with reduced HRT at 3 d, primarily via reducing the substrate concentration while increasing OLR somewhat to 1.7 ± 0.6 kg/m^3.d. Hence, the step change in HRT from 15 d to 3 d was not expected to induce a shock-loading situation. The strategy of experimental operation was to gradually increase OLR until significant changes of the biogas composition occurred. If there were no changes in the biogas composition with 3 days of the HRT, then OLR would be adjusted to higher value and HRT reduced to smaller values. Shorter HRTs would suppress the methanogens which generally require relatively longer time to grow compared to the acidogens. Therefore, in Run 2, changes in biogas

composition and COD removal efficiency were tracked as HRT was reduced to 0.7 d while OLR was gradually increased to ~ 14.5 kg/m^3.d for 10 days. COD removal efficiency did not drop substantially (that is, by less than ~40%), thus HRT was further decreased to 0.25 d and OLR was further increased to ~32 kg /m^3.d for the duration of 31 days in Run 3 (**Table 2.2**). Run 1 had been operated with 24 hr/cycle, whereas Runs 2 and 3 had been operated with 8 hr/cycle and 3 hr/cycle, respectively, for the shorter HRTs. After each cycle of operation, 2 L of reactor contents was discharged, to be replenished by 2 L of influent wastewater (Run 1); the amount was 3 L for Runs 2 and 3.

Table 2.2. Variation of COD removal efficiency with organic loading rate

	Influent (mg COD/L)	Effluent	COD removal efficiency (%)	OLR (kg/m^3.d)	pH
Acclimatization	3164 ±1163	924 ±253	70.8	0.2 ±0.1	6.0
Run 1	4894 ±1886	1491 ±934	69.5	1.7 ±0.6	6.3
Run 2	7360 ±2516	3435 ±660	53.3	10.9 ±3.7	6.8
Run 3	7192 ±1039	6392 ±1181	11.1	28.3 ±4.1	7.2

Figure 2.3 shows the relationship between COD removal efficiency with hydrogen yield. The opposite trends of H_2 yield versus COD removal efficiency suggested that the amount of COD removed would not be an appropriate parameter for normalizing biohydrogen yield data since complex organic compounds are degraded into volatile fatty acids and alcohols as smaller forms with hydrogen and carbon dioxide but liquid metabolites are counted as COD of effluent.

Overall, temperature of the reactor was not controlled but 27.0 ±0.6^oC could be maintained as following room-temperature of the laboratory as throughout the test.

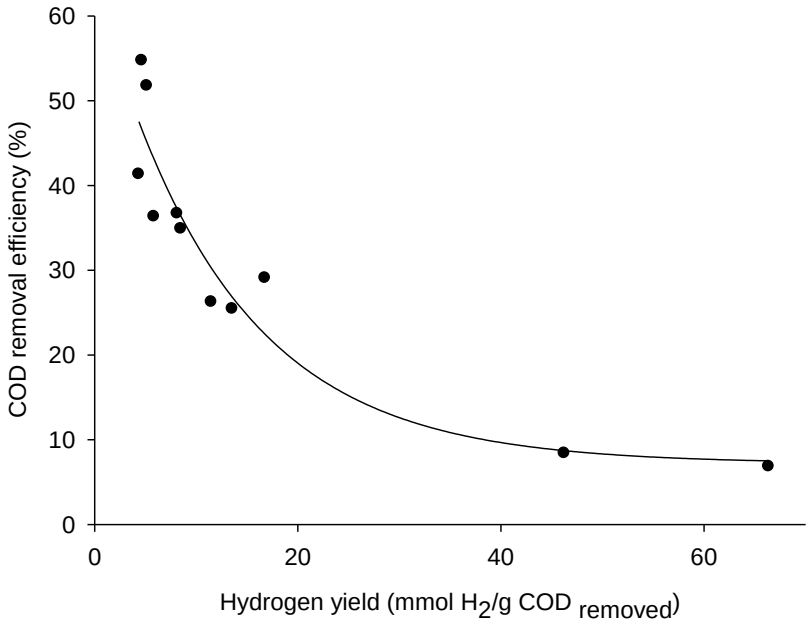

Figure 2.3. The relationship between COD removal efficiency and hydrogen yield

2.2.4 Analytical methods

Biogas produced during the fermentation was measured by a gas chromatography (GC, Varian Inc., CA, USA Model CP-3800) equipped with two-channel thermal conductivity detector (TCD). The columns used for H_2 were 1.0 m x 3.2 mm Hayesep Q (80/100 mesh) and 1.0 m x 3.2 mm Molesieve 5A (60/80 mesh) with argon as carrier gas. For CO_2, CH_4, and N_2, 50 m x 0.32 mm Poraplot Q column was used with helium as carrier gas. The temperature of injector, oven, and detector was kept at 80, 50, 150^{o}C, respectively. Carrier gas flow rate was 40 mL/min. Chemical oxygen demand (COD), total solids (TS), total volatile solids (TVS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and alkalinity were measured by Standard Methods (APHA, 2005).

2.3 Results

In the acclimation period, the bioreactor was initially operated under flexible HRT condition to obtain stable COD removal efficiency. Afterwards, the operational condition was adjusted to relatively long HRT of 15 d and with average OLR of 0.25 kg/m^3.d, without pH and temperature control. Because this stage was carried out to stabilize the bioreactor and to assess the possibility for inhibition of methanogenesis only with operational control, it was necessary to confirm the activity of methanogens as the hydrogen consuming bacteria. Results indicated that biogas content was largely dominated by methane (> 77% in the biogas) along with an average COD removal efficiency of 70.8%, while H_2 content in biogas slowly increased and reached 3.2% towards the end of the period. At this time, the average pH was 6.0.

When HRT was decreased from 15 days to 3 days (Run 1), hydrogen evolution was increased up to 26.1% and methane production decreased temporarily to a minimum of 22.9% for the first 10 days; yet methane production from the bioreactor recovered to greater than 90% thereafter. COD removal efficiency was maintained around 70%. Attempt to increase OLR to obtain more stable and higher volume of hydrogen production under the same HRT was unsuccessful as the H_2 content in biogas remained below 1.0%. The average pH was 6 and this was probably favourable for methanogens. Hence, a 3-day HRT was considered too long for hydrogen production without any other control.

Upon adjusting HRT to 0.7 d (16 hr) and increasing OLR to over 13 kg/m^3.d (Run 2), methane production was suppressed from 95% to a minimum of 66.4%, whereas H_2 content in biogas started to increase. As a result of further changes in HRT and OLR to

0.25 d (6 hr) and 32 kg/m^3.d respectively (Run 3), maximum hydrogen content of 45% was attained and the hydrogen production rate was 0.08 L/L reactor.d (**Figure 2.5**).

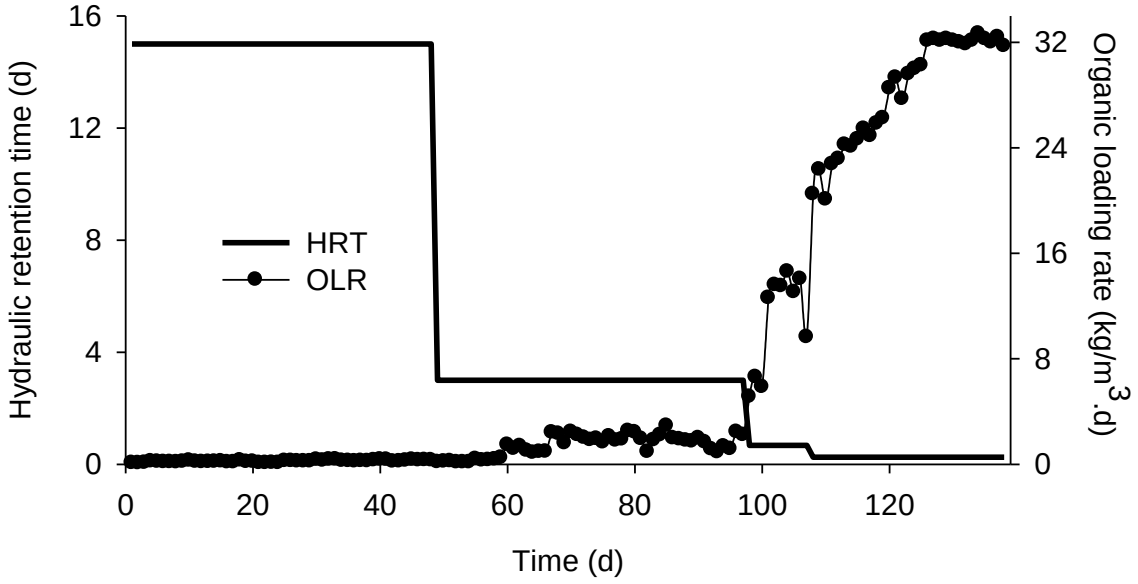

Figure 2.4. Hydraulic retention time and organic loading rate for the various runs

This represents a greater yield compared to that (0.05 L/L reactor.d) deduced from Mohan et al. (2007)'s results with dairy wastewater as substrate and pretreatment of seed sludge. Although an average pH of 7.2 was observed in this stage without pH control, methane activity was inhibited to yield a low CH_4 content of 19.3% .

In this regard, Chang et al. (2004) reported the occurrence of peak hydrogen production at HRT of 8 hr with synthetic substrates (sucrose as carbon source). This could result from the differences between the substrates, as dairy manure contains relatively fewer short-chain carbohydrates than synthetic substrates. Hydrogen evolution during the fermentation process comes from acidogenesis and acetogenesis and volatile fatty acids

(VFAs) are produced as intermediates. Dairy manure contains diverse nutrients and inert solids, including protein and fats which are less favoured than carbohydrates towards the formation of VFAs. It should be noted that COD removal efficiency dropped somewhat to 53.3% for the operating conditions in Run 2 and even more dramatically to 11.1% in Run 3 (**Table 2.2**).

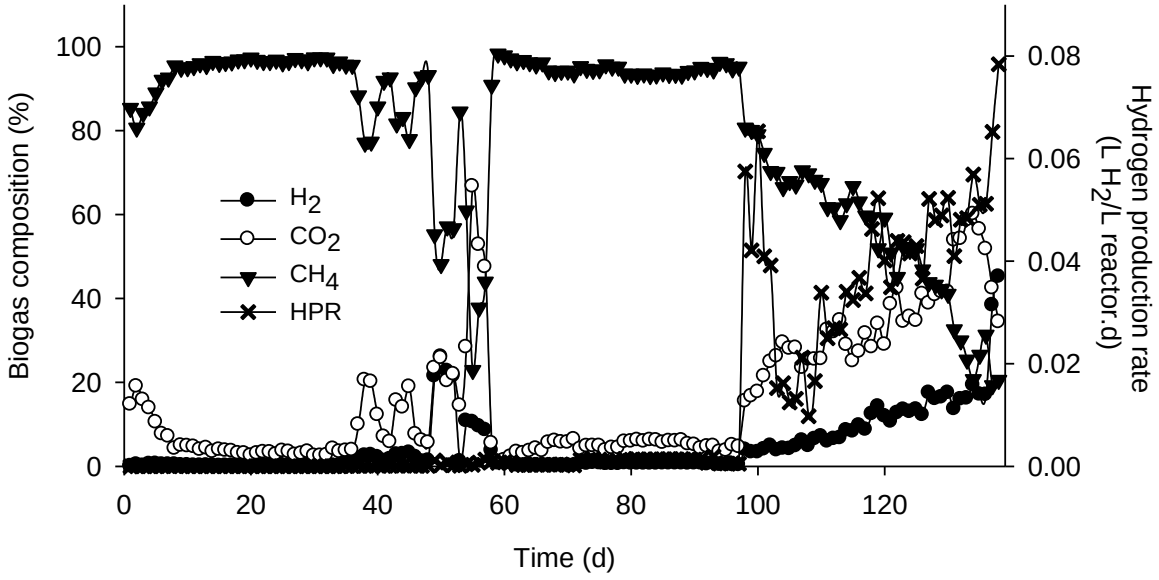

Figure 2.5. Biogas composition and hydrogen production rate with dairy farm wastewater as substrate

As OLR increased from 0 to 32 kg/m^3.d, H_2 and CO_2 contents in the biogas increased in a linear manner, while CH_4 production was suppressed. These results demonstrated that the possibility of inhibiting methanogenesis could be suppressed without pH control and pretreatments, but rather through manipulating HRT and OLR. Nevertheless, the maximum H_2 yield of 0.08 L/L reactor.d derived from dairy wastewater was lower than the yield of biohydrogen from other carbohydrate-rich waste materials to

different extents, such as molasses from sugar refining (5.57 L/L reactor.d; Ren et al., 2006), potato processing and confectioner wastewater (0.13 L and 0.10 L/L reactor.hr, respectively; van Ginkel et al., 2005), as well as other agri-food waste materials such as cornstalks (0.05 L/L reactor.d; Zhang et al., 2007) and sugarcane biomass (1.86 L/L reactor.d; Hafner, 2006). Although these previous studies in the literature used vessels ranging from 250 mL serum bottles to 1.5 m^3 pilot-scale reactors, and some were done in continuous operation mode over a long time period while others used batch operation mode, their common point was the use of carbohydrate-rich waste as feedstock, whereas dairy manure generally contains a relatively larger proportion of protein and lipid.

These protein and lipid except carbohydrates are not preferable for hydrogen production since the hydrolysis of protein has been known to be slower than that of carbohydrates and lipid has very low biodegradability (Menear and Smith, 1973; Pavlostathis and Giraldo-Gomez, 1991; Petruy and Lettinga, 1997; Demirel et al., 2005; Amon et al., 2006).

2.4 Conclusions

Biohydrogen production from dairy wastewater as substrate was studied using an ASBR (Anaerobic Sequencing Batch Reactor) in a series of test that involved different hydraulic retention times (HRTs) under mesophilic temperature conditions. Methanogenesis was relatively well suppressed with manipulation of changes in HRT and organic loading rate (OLR), but without pH and temperature control, or any pretreatment of seed sludge and the substrate. At the shortest HRT of 0.25 d and largest OLR of 32 kg/m^3.d, the maximum hydrogen content was 45.1% and the hydrogen production rate was

0.08 L/L reactor.d. Though hydrogen productivity was low compared to the yield of biohydrogen reported in the literature to different extents (with other carbohydrate-rich or agri-food waste materials as feedstock), the results suggested that it is possible to suppress methane production with HRT and OLR control, and avoid the pretreatment of inoculum, which could potentially benefit scaled-up applications.

The objectives in this Chapter 2 are meant to pave the way for future work on the co-digestion of agri-food waste, in the form of animal wastewater and food processing wastewater, so as to improve the utilization of dairy manure for hydrogen production as renewable bioenergy. VFA analysis is required to find indirectly microbial metabolic pathways as an additional analytical procedure. Molecular biology techniques would be applied to characterize microbial community changes during the process, hence confirming the abundance or lack of dominant hydrogenase-possessing bacterial strains under different operating conditions.

Chapter 3: Effects of Key Operational Parameters on Biohydrogen Production via Anaerobic Fermentation in a Sequencing Batch Reactor[1]

3.1 Introduction

Many researches on hydrogen production by mixed cultures have focused on simple carbohydrate substrates (glucose, sucrose, cellobiose and starch) supplemented with excess nutrients, which mimic carbohydrate-rich synthetic wastewater (Li and Fang, 2007). Among various operational parameters mentioned in literature review, hydraulic retention time (HRT), and organic loading rate (OLR) are considered as important parameters.

The operating pH plays a critical role in governing the metabolic pathways of microbial H_2 production and it affects the effluent composition of the acidogenic reactor. Many researchers have studied the effects of pH (Li and Fang, 2007) on hydrogen production. HRT is considered to be a major factor influencing the performance of continuous operation. Shorter HRTs would change the fermentation pattern and suppress the methanogens which generally require relatively longer time to grow compared to the acidogens. Shorter HRT is also preferred by reason of lower capital cost required. It was widely reported that the H_2 yield increased with decreasing HRT for different types of reactors, whereas the results from Wu et al. (2009)'s study demonstrated an optimal HRT amidst a range of HRTs tested. They suggested that the reduction in H_2 yield at long HRTs is probably due to the reuse of H_2 by homoacetogens which produce acetate from

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[1]A version of chapter 3 has been published except section 3.3.3. Won, S. G. and Lau, A. K. (2011). Effects of key operational parameters on biohydrogen production via anaerobic fermentation in a sequencing batch reactor. *Bioresource Technology, 102*, 6876-6883.

dissolved CO_2 in the presence of H_2. According to Arooj et al. (2008), the advantages of sequencing batch reactors include greater biomass retention (hence, the ability to decouple solids retention time, SRT, from hydraulic retention time, HRT), a higher degree of process flexibility with respect to changes in organic loading rate OLR, a single vessel for reaction and settling (hence no need for a separate clarifier), relative ease of operation and lower capital investment. The semi-continuous mode operation of this process is also considered more feasible for potential real applications and commercialization (Wu et al., 2009).

Hydrogen productivity from ASBR as reported in the literature has been relatively low although ASBR has advantages over other reactor types. Hence, this chapter was conducted to investigate: (1) the effects of key operational parameters in combination on biohydrogen production in an ASBR, using sucrose as the main substrate to mimic carbohydrate-rich wastewater; (2) the relationship between the characteristics of the metabolites from fermentation and hydrogen productivity; (3) the feasibility of hydrogen production via dark fermentation without pretreatment of the seed sludge; and (4) the restoration of hydrogen productivity via biogas recirculation in order to decrease H_2 partial pressure.

3.2 Materials and Methods

3.2.1 Experimental apparatus and procedure

The bioreactor and other experiment apparatus were described in Section 2.2.1 and two sets of tests were conducted with hydraulic retention time (HRT) of 1.25 d and 0.83 d. In each set of lab-scale tests, pH level was controlled at 4.0, 4.5 and 5.0, whereas

temperature was maintained at 28-30^{o}C. The operational sequence for the ASBR was "*Feed* → *React* → *Settle* → *Decant*", with a cyclic duration of 12 hr and 8 hr for the two sets of tests, respectively. pH control was implemented in the tests, using acid and base solutions prepared with 3M HCl and 3M NaOH, respectively. The effect of pH on biohydrogen production was assessed. The amount of biogas produced was recorded daily using the water displacement method.

3.2.2 Substrate and seed sludge

Sucrose-rich synthetic wastewater was used as the substrate. The substrate solution consisted of sucrose as the carbon source. NH_4Cl, K_2HPO_4, KH_2PO_4 were added to give a C:N:P ratio of 200:5:1; and K_2SO_4 was used to provide the right balance with sulfur. Other macro- and micro- nutrients were supplied in the following dosages (in mg/L): $MgCl_2$ 10, $NiCl_2$ 1, $FeCl_2$ 181, $CaCl_2$ 10, $ZnSO_4$ 1 and $CuSO_4$ 5.

Seed sludge (inoculum) was initially obtained from the Department of Civil Engineering's biological nutrient removal pilot plant for sewage treatment at the University of British Columbia. More details have been provided in Section 2.2.2. For this experiment, the inoculum was stored at 4^{o}C prior to use. It was washed with tap-water, and the supernatant was thrown away twice after settling for 90 min. Then, the inoculum was adapted to sucrose-rich synthetic wastewater over a month. After an extended period of acclimatization when the seed sludge had been stabilized, it was placed in the reactor at a mixed liquor volatile suspended solids (MLVSS) level of 11,000 mg/L.

Experiments were conducted to measure biomass concentration during anaerobic fermentation for hydrogen production as a function of COD levels, and results indicated

that optimal biomass growth was attained at COD 13,800 mg/L (**Figure 3.1**). This COD level was used in the subsequent tests.

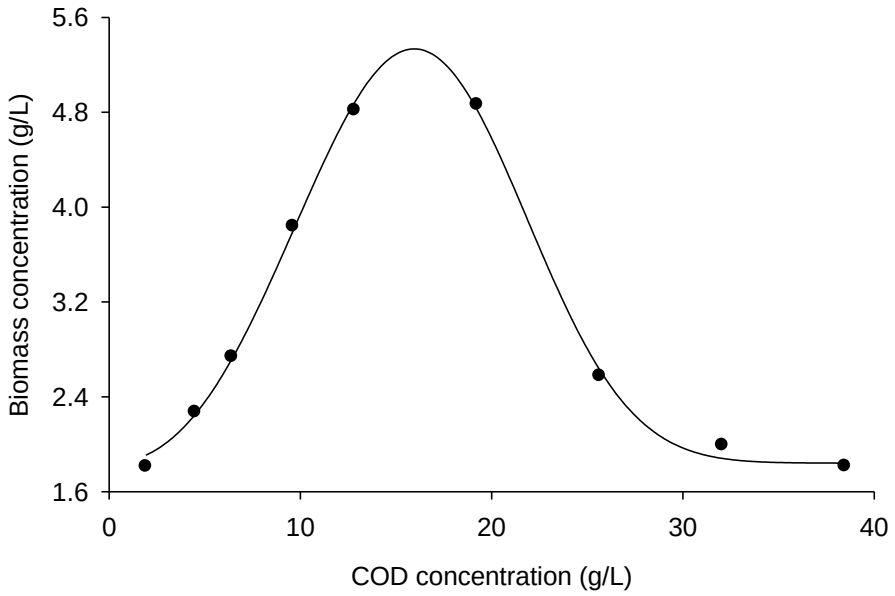

Figure 3.1. Variation of biomass growth with COD concentrations after three weeks

3.2.3 Analytical methods

The biogas produced was sampled and analyzed in a gas chromatography which was described in Section 2.2.4. The analysis of VFAs (acetic acid, propionic acid, and butyric acid) and alcohols (ethanol and butanol) was conducted using the same GC but with a flame ionization detector (FID). The temperature of both the injector and the detector was set up at 230^{o}C. Oven temperature was kept at 80^{o}C for 6 min initially and was elevated to 230^{o}C at a rate of 15^{o}C/min for 10 min. Finally, 230^{o}C was maintained for 14 min. Methanol was used as an internal standard. Chemical oxygen demand (COD), total solids (TS), total volatile solids (TVS), mixed liquor suspended solids (MLSS),

mixed liquor volatile suspended solids (MLVSS) and alkalinity were measured in accordance with Standard Methods (APHA, 2005).

3.3 Results and Discussion

3.3.1 Effects of pH and HRT on hydrogen production rate and yield

3.3.1.1 Hydraulic retention time 1.25 d

In Set I tests, HRT was set at 1.25 d (30 hr) and the COD concentration was fixed at 13,800 mg/L. This provided a fixed OLR of 11.0 kg/m^3.d to the reactor, while pH was maintained at 4.0, 4.5 and 5.0, in turn, for the three runs. As illustrated in **Figure 3.2**, H_2 content varied from 61% to 78%, with an average value of 72.3%. Methane content in the biogas was very low (0–0.5%), suggesting that hydrogen production was effective without the need for pretreating the seed sludge with heat-shock or other methods to inhibit the hydrogen-consuming methanogens. The profiles of hydrogen production rate and hydrogen yield are also shown in the same graph. Hydrogen yield is calculated on the basis of the amount of substrate added into the reactor.

Results are also summarized in **Table 3.1**, for three levels of pH (4.0, 4.5 and 5.0). The performance of hydrogen productivity was best for pH 4.5, with a hydrogen production rate of 3.04 ± 0.66 L H_2/L reactor.d and hydrogen yield of 2.16 ± 0.47 mol H_2/mol hexose over the experimental period of 36 d, though these values are not significantly different from those pertinent to pH 4.0. The average H_2 yield represents 54% of the theoretical maximum possible H_2 content in the biogas, which is 66.7% based on stoichiometry, assuming that all of the sucrose is converted to H_2 and CO_2, while acetate is the only metabolite. However, studies such as Noike and Mizuno (2000) and Hafez et al.

(2010) have measured hydrogen contents between 70% and 80%. At pH 5.0, system performance was significantly lower.

Table 3.1. Set I tests – effect of pH on hydrogen production rate and yield at HRT 1.25 d

Run #	pH	H_2 production rate (L H_2/L reactor.d)	H_2 yield (mol H_2/mol hexose)
1	4.0	2.51 ±0.82	1.78 ±0.59
2	4.5	3.04 ±0.66	2.16 ±0.47
3	5.0	1.73 ±0.25	1.23 ±0.18

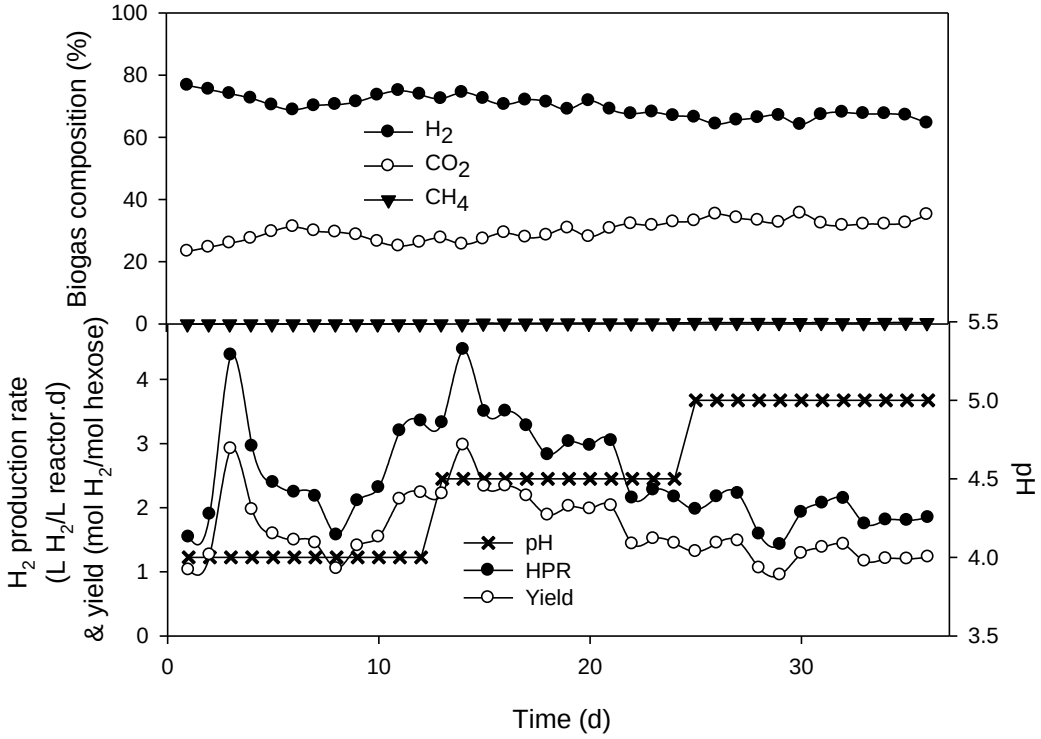

Figure 3.2. Performance of reactor in Set I tests with HRT 1.25 d: (upper) biogas composition; (lower) hydrogen production rate and hydrogen yield

Comparison was made with findings from other studies. Van Ginkel et al. (2001) studied biohydrogen production as a function of pH and substrate concentrations, using sucrose-rich synthetic wastewater (7500 mg COD/L) as substrate in batch experiments and 250 mL serum bottles at 37^{o}C temperature, and compost was used as the inocula. They observed the highest hydrogen production rate to be 0.08 L H_2/L. hr at pH 5.5. Cheong and Hansen (2006) reported higher hydrogen production rates of 0.12–0.22 L H_2/L hr (or, 0.5–1.0 mol H_2/mol hexose upon converting the units) when pH was controlled at an optimal value of 5.7 in their study using glucose as substrate (21,300 mg COD/L) in 1.9 L completely mixed batch reactors and with temperature controlled at 35.5^{o}C. Chen et al. (2009) used a 3 L working volume ASBR in their study and found maximum hydrogen yield to be 1.41 mol H_2/mol sucrose added when the reactor was operated at 35^{o}C and with HRT of 16 hr, sucrose concentration of 25,000 mg/L (expressed as COD), and pH 4.9. Ren et al. (2006) reported that pH 4.5 was suitable for hydrogen production by ethanol type fermentation because NADH/NAD^{+} ratio is unstable via butyric acid type fermentation, which can readily change to propionic acid type fermentation at higher pH. These studies suggested that pH values around 4.5–5.5 would be favourable for hydrogen production.

3.3.1.2 Hydraulic retention time 0.83 d

Set II tests were conducted with HRT reduced to 0.83 d (20 hr). Since the COD concentration remained at 13,800 mg/L, the OLR to the reactor was increased to a fixed value of 16.6 kg/m^3.d. The various runs in these tests involved operating the reactor at pH 4.0, 4.5 and 5.0. It is evident from **Table 3.2** that both hydrogen production rates and hydrogen yields dropped significantly at all pH relative to the results obtained using HRT

of 1.25 d. Methanogens were effectively suppressed as evidenced by an average CH_4 content of 1.3% (**Figure 3.3**). Nevertheless, CO_2 content in biogas remained high at 30–60% compared to the result from **Figure 3.2**, suggesting that techniques to capture or reduce CO_2 formation could be studied in the future for improvement in hydrogen yield such as recirculation of inert gases (N_2 or Ar). An additional run was made with the reactor operated at pH 5.5. Hydrogen production rate was higher at 1.03 ± 0.18 L H_2/L.d, whereas H_2 yield was also higher at 0.49 ± 0.08 mol H_2/mol hexose. Both performance indicators were better than the other pH values (4.0, 4.5 and 5.0), though they were not significantly different versus pH 4.5 in particular.

Based on the observations from these two sets of tests the optimal pH value would vary depending on the HRT; furthermore, (pH 4.5, HRT 1.25 d or 30 hr) constitute the operational conditions for maximum hydrogen production and yield. By comparison, Wu et al. (2009) found the operating conditions of (pH 5.0, HRT 12–16 hr) to be optimal at 37^{o}C for maximum hydrogen production (2.4–3.1 L H_2/L.d) and hydrogen yield (1.57–1.63 mol H_2/mol hexose) when liquid swine manure and glucose was used as the substrate in an ASBR.

It is also useful to compare the yield with a CSTR operation. Wu et al. (2010) conducted a number of tests on the CSTR operating parameters with glucose as substrate (concentration 14,000 mg/L), and found the optimal conditions to be (pH 5.0, HRT 8.3 hr, 33.5^{o}C) for maximum yield of 2.15 mol H_2/mol hexose. This study has demonstrated that it is possible for ASBR to achieve a similar magnitude of hydrogen yield as CSTR. As seen in **Table 3.1**, hydrogen yield of 2.16 ± 0.47 mol H_2/mol hexose was observed under

the conditions of (pH 4.5, ~30^{o}C) using a similar substrate concentration of 13,800 mg/L, though at the expense of longer HRT of 1.25 d.

Table 3.2. Set II tests – effect of pH on hydrogen production rate and yield at HRT 0.83 d

Run #	pH	H_2 production rate (L H_2/L reactor.d)	H_2 yield (mol H_2/mol hexose)
1	4.0	0.46±0.06	0.22±0.03
2	4.5	0.83±0.20	0.39±0.09
3	5.0	0.64±0.07	0.30±0.04

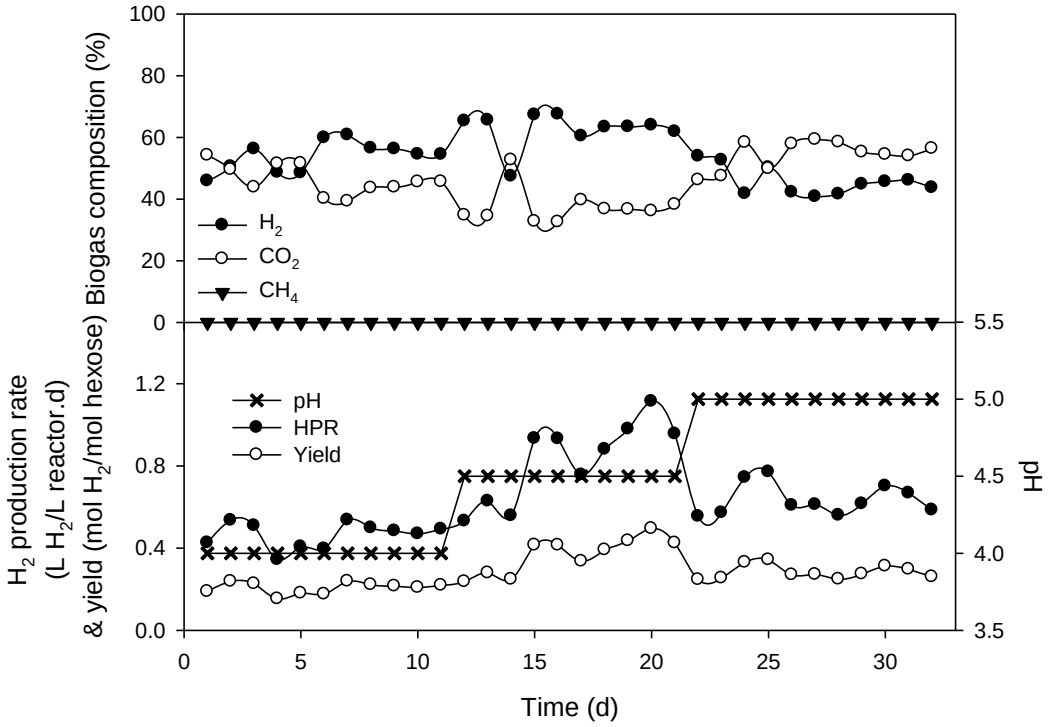

Figure 3.3. Performance of reactor in Set II tests with HRT 0.83 d: (upper) biogas composition; (lower) hydrogen production rate and hydrogen yield

3.3.1.3 Food-to-microorganism ratio for hydrogen production

The food-to-microorganism ratio (F/M) is another key factor that can impact the anaerobic digestion process; a lower F/M ratio would generally result in a greater percentage of the substrate being converted to biogas. For aerobic biological treatment processes, the optimal F/M ratio, in units of [g/g d], for a sequencing batch reactor could be up to an order of magnitude lower than a complete mix reactor (Metcalf & Eddy Inc., 2003). Lay et al. (1999) tested two types of microorganisms; results from their study indicated that a high hydrogenic activity for the pretreated digested sludge was obtained at a high F/M ratio, but that for the hydrogen-producing bacteria was found at a low F/M ratio. Yang et al. (2007) conducted batch H_2 fermentation experiments in 1 L reactors using cheese processing wastewater as substrate and mixed microbial communities under mesophilic conditions. They observed maximum H_2 yields at F/M ratio of 1.0 to 1.5. For CSTR, Hafez et al. (2010) obtained maximum H_2 yield at 2.8 mol H_2/mol glucose with much higher F/M ratio ranging from 4.4 to 6.4 g/g.d. In this study, the influence of F/M ratio on hydrogen production was also observed in both sets of tests.

As shown in **Figure 3.4**, during the Set I tests with HRT 1.25 d (and OLR 11.0 kg/m^3.d), average F/M ratio had lower values (0.78-0.88 g/g.d) as compared to Set II tests with HRT 0.83 d (and OLR 16.6 kg/m^3.d) when F/M ratio had higher values (1.1-4.0 g/g.d). The two OLRs led to differences in the F/M ratio, as F/M is calculated as OLR divided by MLVSS (biomass) concentration. Hence, microbial growth is expected to affect F/M ratio. In both sets of tests, the measured MLVSS concentration was mostly around 13 g/L, except for the operating condition (pH 4, HRT 0.83 d) when MLVSS concentration had a dramatically lower value of 4.0 g/L. Maximum hydrogen production rate occurred at

F/M ratio of 0.84 g/g d. Hence, further tests with the ASBR used in this thesis project were run with F/M ratio maintained at 0.85 and below 1.0 g/g d.

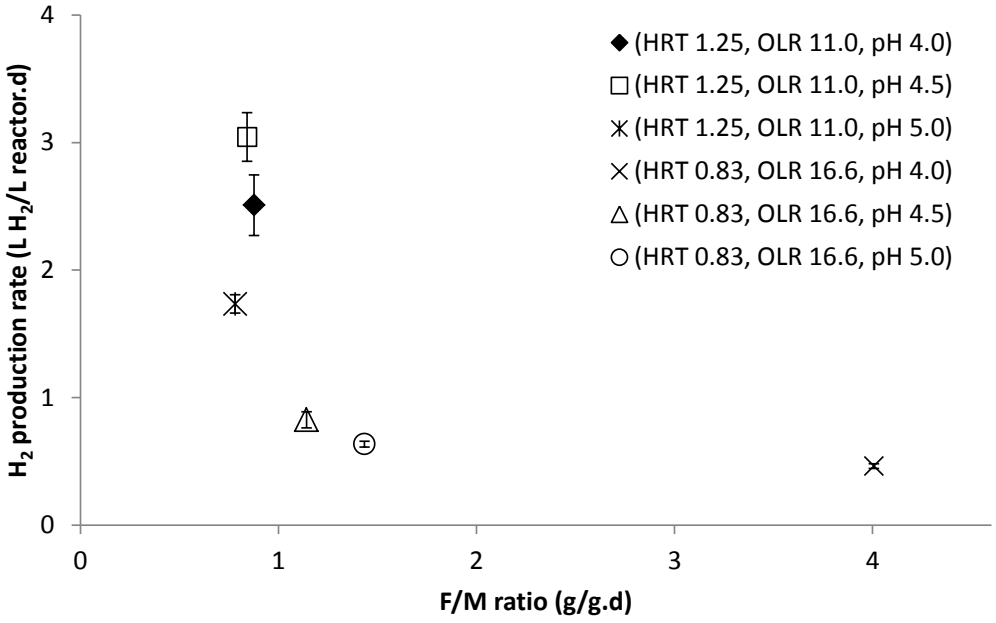

Figure 3.4. The influence of food-to-microorganism ratio on hydrogen production rate

3.3.2 Metabolites concentration

Figure 3.5 (a, b) shows the profiles of the metabolites for Set I tests (HRT 1.25 d) and Set II tests (HRT 0.83 d). The metabolites measured include acetic acid (HAc), propionic acid (HPr), butyric acid (HBu) and ethanol (EtOH). With HRT 1.25 d, the HAc concentration varied from 5-18 mM whereas EtOH concentration varied from 10-30 mM. By comparison, the HAc and EtOH concentration profiles at HRT 0.83 d fluctuated within a much wider range of 10-50 mM and 20-50 mM, respectively. The lower hydrogen productivity at the shorter HRT of 0.83 d may be attributed to the less stable profiles of the metabolites together with a higher HAc concentration, which could be due to the growth of

homoacetogens (Chen et al., 2009). The concentration of propionic acid (HPr) was the highest under pH 5.0 condition for both HRTs of 1.25 d and 0.83 d. This observed trend is in contrast to Dinopoulou et al. (1988)'s findings that the percentage of acetic acid in the VFAs increased with increasing pH, while the percentage of propionic acid decreased accordingly.

Ren et al. (2008) reported that mixed-acid type fermentation was achieved when no pretreatment was applied to the inocula. Based on the volatile fatty acids profiles obtained, Arooj et al. (2008) suggested that the HBu:HPr ratio was the most important parameter to justify hydrogen yield at various HRTs. Wu et al. (2010) reported butyric acid-type fermentation occurring in most tests involved in their study; at pH 5.5, 5.0 and 4.0, the effluent contained mostly butyric acid (43–57%), followed by acetic acid (25–30%). However, from the study by Wu et al. (2009), ethanol and organic acids were the major aqueous metabolites produced during fermentation, with acetic acid accounting for 56–58%. The hydrogen yield was found to be proportional to the HAc:HBu ratio, though they cautioned that other researchers have observed the opposite trends thus rendering the HAc:HBu ratio an insufficient indicator of H_2 production (Chen et al., 2009). In Set I and Set II tests, a trace amount of HBu was detected at HRT 1.25 d (< 1.0 mM) relative to other VFAs and ethanol; the concentration of HBu increased to a high value of 15 mM at (HRT 0.83 d and pH 5.0)

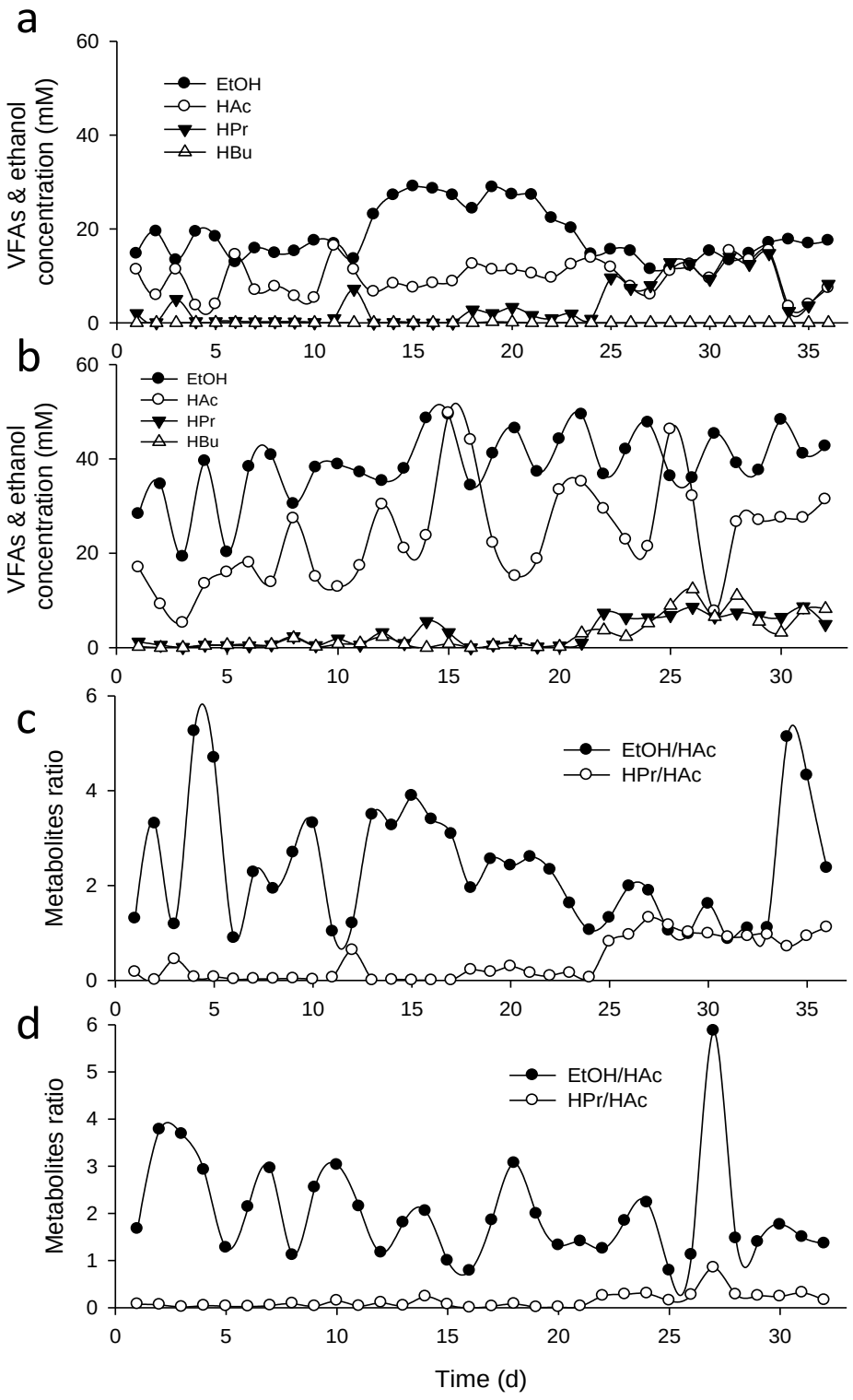

Figure 3.5. Metabolite concentrations and ratio of metabolites:
(a, c) HRT 1.25 d; (b, d) HRT 0.83 d
(EtOH: ethanol; HAc: acetic acid; HPr: propionic acid; HBu: butyric acid)

Two relationships involving the ratio of metabolites, HPr:HAc and EtOH:HAc were derived from the actual data and presented in **Figure 3.5 (c, d)**. When the reactor was operated at HRT 1.25 d, EtOH:HAc ratio varied from 1.0 to 5.5 regardless of pH; however, HPr:HAc ratio had larger values around 1.0 at pH 5.0. Upon reducing the HRT to 0.83 d, values of EtOH:HAc ratio were 1.0-4.0, whereas the HPr:HAc ratio was somewhat smaller.

An attempt was made to correlate hydrogen production with the ratio of metabolites. As seen in **Figure 3.6**, higher levels of propionic acid relative to acetic acid would lead to lower hydrogen content in the biogas and lower hydrogen yield. **Figure 3.6** also illustrates that there exists a threshold value (approximately 1.25) of the EtOH:HAc ratio for effective hydrogen production (greater than 40% H_2 content) when ethanol-type fermentation was present.

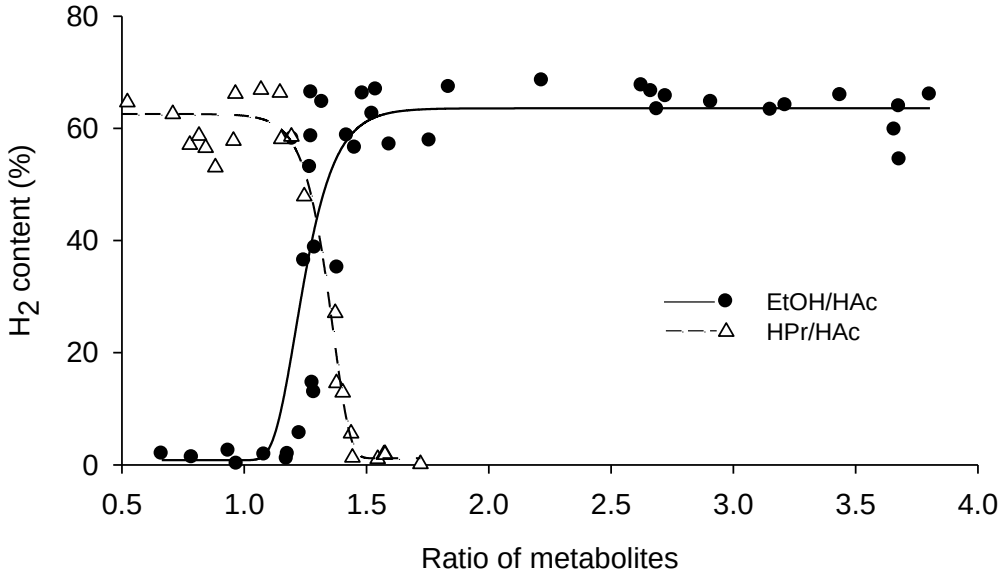

Figure 3.6. Relationship between hydrogen content in biogas and ratios of metabolites

This relationship between hydrogen production and EtOH/HAc ratio contradicts the literature. There are different viewpoints on ethanol-type fermentation to produce hydrogen. Sreethawong et al. (2010) concluded that EtOH-type fermentation can consume free electrons that are required to form hydrogen and lead to a higher CO_2 content. Ethanol production was reported with toxic effects on bacteria (Skonieczny and Yargeau, 2009). Whereas, the observations of Ren et al. (2006) coincided with our results. Ethanol and acetic acid production affected hydrogen yield and maximum hydrogen production rate was achieved at EtOH:HAc ratio of ~1.0 in their pilot-scale study using CSTR and molasses as substrate. Further, ethanol type fermentation led to the better hydrogen production at pH 4.5 rather than butyric acid type fermentation with respect to $NADH/NAD^+$ ratio. The latter type fermentation is unstable and readily changed to propionic acid type fermentation at higher pH.

3.3.3 Restoration of hydrogen productivity with biogas recirculation

As previously mentioned in Sections 1.1 and 1.3, higher propionic acid production among the VFAs and alcohols and homoacetogenic reactions would cause low hydrogen productivity due to hydrogen consumption (Vavilin et al., 1995). The acetogenesis of propionic acid (HPr) in anaerobic fermentation is very low compared to ethanol and butyric acid, causing the accumulation of HPr and lowering the rate of methanogenesis. Besides, propionic acid was believed to be the most toxic volatile fatty acid produced during anaerobic fermentation (Hanaki et al., 1994). Cohen et al. (1980) linked the accumulation of HPr to organic and hydraulic overloadings in the bioreactor. According to Fynn and Syafilla (1990) and Harper and Pohland (1986), higher hydrogen partial pressure

could induce the inhibition of hydrogen production and change the metabolic pattern to produce more propionic acid. Hence, propionic acid production must be controlled along with other parameters to achieve higher biogas productivity, be it hydrogen or methane.

Treatment by CO_2 had been widely used for over a century for the restraint of microbial growth in water and food products such as dairy product, and meat and fish (Donald et al., 1924). Lacoursiere et al. (1986) concluded that carbon dioxide could influence the physiological effects through various enzymatic reactions and may affect the equilibrium of the pathway to produce metabolites using *E. coli*. Hence, it was hypothesized that the intermittent sparging of carbon dioxide into the reactor could induce a stimulating condition for microbial enzymatic activity; it may then change the microbial metabolic pathway to produce other VFAs rather than propionic acid, which may result in the recovery of hydrogen productivity when hydrogen production becomes low. The recirculation of biogas (mostly CO_2) could help flush the residual H_2 out of the reactor's headspace, leading to an increase in the mass transfer rate from liquid to gas phase for H_2 (Kraemer and Bagley, 2006).

Tests were conducted under the same operational conditions (pH 4.5, HRT 24 hr, OLR 10.7 $kg/m^3.d$) as maintained by automatic control. CO_2 has a relatively high solubility in water (under the standard temperature of 25^oC, the solubility of CO_2: 3.4×10^{-2} mol/L at 1 atm (the inverse Henry's law constant) as compared to H_2, 7.8×10^{-4} mol/L at 1 atm) and its solubility increases with increasing pH. Hence, the lower operating pH of 4.5 in this study would reduce the CO_2 solubility in wastewater. Willquist et al. (2009) compared the effects of N_2 and CO_2 stripping at pH 6.5 and 70^oC with *Caldicellulosiruptor saccharolyticus*. Carbon dioxide stripping was found to cause a higher osmotic pressure,

and lead to lower hydrogen productivities and microbial growth rate. However, it may vary with operational conditions and the intensity of CO_2 sparging (continuous or intermittent). Inoculum and substrate composition were the same as section 3.2.2. Analytical methods followed from Section 3.2.3.

After the CO_2 content in the biogas produced had been observed to rise above 90% for a week, the biogas was recirculated into the bioreactor via airstone using a peristaltic pump and with agitation. Recirculation of the biogas lasted for 18–31 min in the beginning of the *React* phase of a cycle on the days shown in **Table 3.3**. At this time, the connection of the biogas collection line to the reactor was opened to atmospheric pressure and the reactor was not pressurized. The volume of the recirculated biogas and flow rate are also shown in **Table 3.3**. Since CO_2 may be harmful for microbial growth (Dixon and Kell, 1989), the second event of biogas recirculation did not occur until one week after the first occasion when hydrogen evolution was confirmed. Thereafter, biogas recirculation took place every 3-4 days upon checking that hydrogen evolution was definitely positive.

Table 3.3. Recirculation of biogas into the bioreactor

Day number	62	68	71	74	78	81
Biogas volume (L)	6.0	7.7	7.4	6.5	10.4	8.7
CO_2 volume (L)	5.9	6.0	5.0	4.7	5.6	2.1
CO_2 flow rate (mL/min)	328	261	227	235	187	81

3.3.3.1 Results

Results of the tests are shown in **Figure 3.7**. As propionic acid concentration and the HPr/HAc (propionic acid-to-acetic acid) ratio increased to 28 mM and beyond 1.0 on day 34, respectively, the $\%H_2$ content in the biogas started fluctuating. Eventually, higher propionic acid concentration inhibited the hydrogen production, and the biogas was comprised of 98.6±0.7% CO_2 from day 56 to day 61. On day 62, CO_2 recirculation into the bioreactor began. After the first event of biogas recirculation, H_2 content in the biogas sharply increased to 50% on day 66 before it declined again. Further recirculation of biogas was carried out every 3-4 days. The HPr concentration began to decrease until the HPr/HAc ratio dropped to below 0.2.

When CO_2 produced is recirculated into the reactor, $CO_{2(g)}$ freely permeates the microbial cell membrane to be dissolved. According to Dixon and Kell (1989), this would stimulate a futile cycle which is the energy (ATP) consuming pathway. Ideally, microorganisms choose the higher ATP production pathway with acetic acid production (theoretical maximum: 4 ATP per glucose molecule). However, microorganisms would tend to choose the less acidic products' pathway such as [acetic acid HAc + ethanol EtOH] rather than [acetic acid HAc + butyric acid HBu] when biogas recirculation is acidifying their environment. As seen in **Figure 3.7**, on day 86, acetic acid concentration acquired an abrupt peak at 50 mM whereas ethanol concentration slightly increased. Thus, it was suggested that biogas recirculation would suppress propionic acid production but stimulate acetic acid and ethanol production.

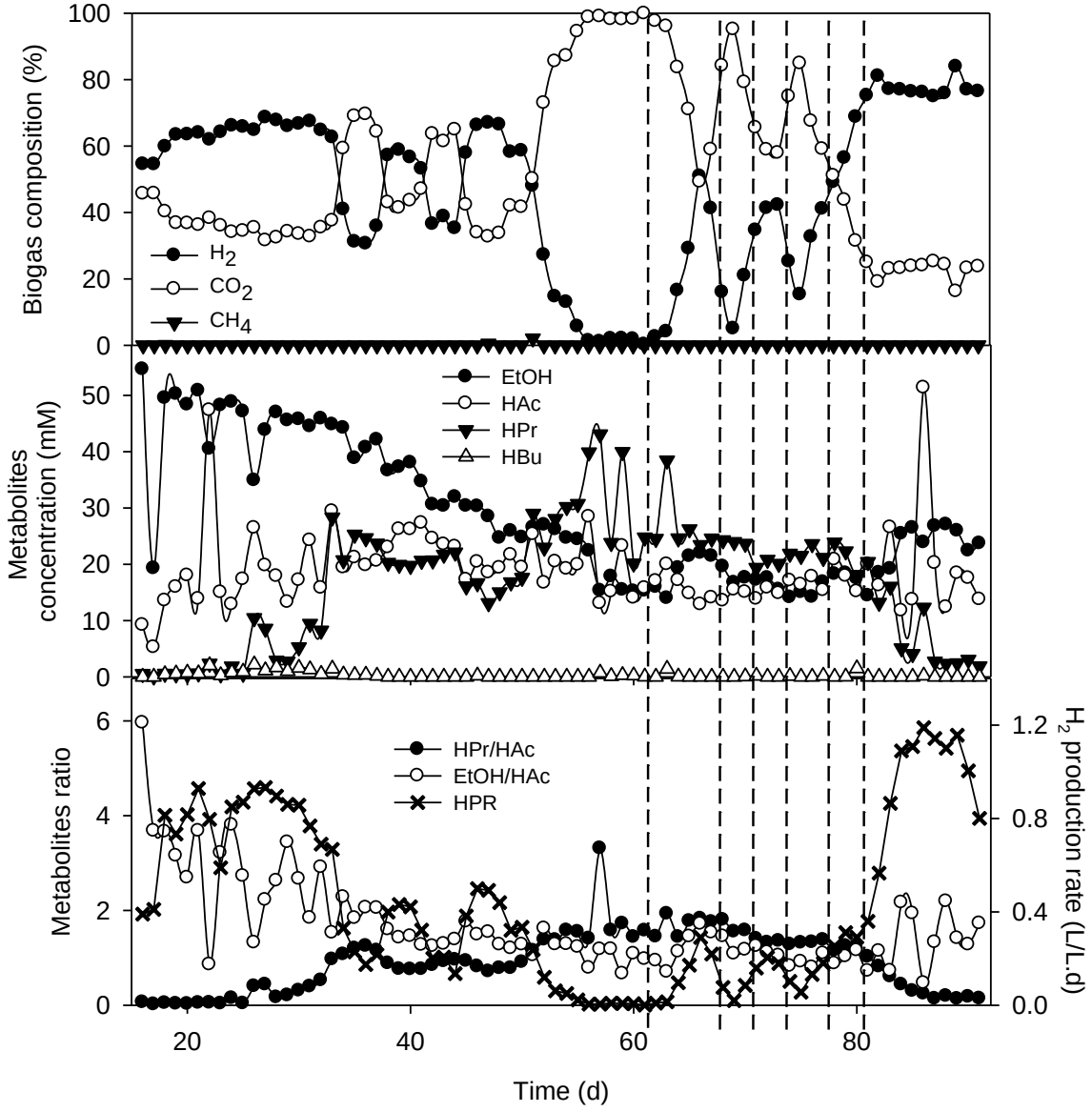

Figure 3.7. Results demonstrating the restoration of hydrogen productivity through the recirculation of biogas produced into the bioreactor
***Vertical dotted lines for the occasions of biogas recirculation**

3.4 Conclusions

Biohydrogen production from sucrose-rich synthetic wastewater as substrate was studied using an anaerobic sequencing batch reactor in tests that involved different pH (4.0,

4.5, 5.0) and hydraulic retention times (HRT: 1.25, and 0.83 d) under mesophilic temperature conditions. Without pretreatment of the seed sludge, it was feasible to inhibit the methanogens activities under appropriate operational conditions.

With a fixed OLR of 11.0 kg/m^3 d, higher hydrogen production rate (3.04±0.66 L H_2/L reactor.d) and yield (2.16±0.47 mol H_2/mol hexose) were achieved when HRT and pH were 1.25 d and 4.5, respectively. The higher hydrogen productivity was found to be associated with a lower F/M ratio of around 0.85 g/g.d and more stable profiles of the metabolites. Two relationships involving the ratio of metabolites were derived from the actual data. Firstly, a propionic acid-to-acetic acid ratio (HPr:HAc) that exceeded 1.2 was observed with respect to a decrease in hydrogen content, and further increase of this ratio halted hydrogen production. Secondly, there exists a threshold ethanol-to-acetic acid ratio (EtOH:HAc) of approximately 1.25 for effective hydrogen production, suggesting that the ethanol-type fermentation may be favoured for hydrogen production.

The recirculation of biogas containing mainly CO_2 into the bioreactor has effectively stimulated and restored hydrogen productivity via reducing the propionic acid production. Since the blowing of other purified inert gases into the reactor may require extra operating costs, CO_2-rich biogas recirculation could also provide one way to recover hydrogen production from system failure.

Chapter 4: Manipulating Cyclic Duration for Optimized Biohydrogen Production

4.1 Introduction

Fermentative hydrogen production is always accompanied with the production of soluble metabolites such as alcohols and VFAs (volatile fatty acids). In theory, maximum yield of hydrogen during dark fermentation can only be achieved with the acetic acid metabolic pathway (Chapter 1). Hydrogen is an abundant and essential element for microbial metabolism. Microbial activity is achieved by electron transfer, and it plays a crucial role for hydrogen production when two protons are reduced by hydrogenase. Electrons released from substrates are taken up by electron sinks such as soluble metabolites, synthesized biomass, and hydrogen. Higher hydrogen production may be achieved when electron sinks other than protons are minimized. Unlike anaerobic fermentation to produce methane, hydrogen is the end product rather than the intermediate product during anaerobic fermentation for hydrogen. Hence, if hydrogen production might play a role as a major electron acceptor, microbial growth and other by-product production would decrease. It could be stressful for microorganisms to survive, which may result in system failure. In order to form a balanced distribution of electrons, it may be necessary to find the optimal operating parameters for hydrogen production in relation to microbial growth. Previous studies have suggested shorter hydraulic retention times (HRT) and higher organic loading rates (OLR) but the relationship between hydrogen production and the operational parameters affecting microbial growth is not very clear (Whang et al., 2011). For instance, pH ranging from 4.5 to 6.0 has been reported, but it could vary

according to the origin of seed sludge, type of feedstock, type of bioreactor, or the other operating parameters.

A major difference between a continuous stirred tank reactor (CSTR) and a sequencing batch reactor for anaerobic fermentation (ASBR) is in the operational parameter "cyclic duration". In an ASBR, cyclic duration is the sum of the time period allocated for each phase of operation *Feed* → *React* → *Settle* → *Discharge* sequenced within one cycle. Since solids/liquid separation occurs during the *Settle* phase and the liquid portion (supernatant) is only decanted at the *Discharge* phase, the seed sludge remains in the reactor and a new cycle begins with the feeding of substrate. Hence, ASBRs are able to keep higher biomass concentration when compared to CSTRs. Cyclic duration is not equal to HRT unless the reactor is in "batch" operation (ref. **Eqn 2.2**). When HRT and OLR are held constant, the influent volume per cycle (which is equal to the effluent volume) is varied according to the changes in cyclic duration. Thus, a longer or shorter cyclic duration may affect microbial growth as well as hydrogen production through product inhibition or substrate inhibition. Literature review indicated that aside from Chen et al. (2009), few studies have been performed to assess the effects of cyclic duration on hydrogen productivity in ASBR.

The objective of this Chapter was to investigate the effects of pH and cyclic duration on hydrogen production under constant hydraulic retention time HRT and organic loading rate OLR. Biomass growth characteristics were also observed. The effective ranges of food-to-microorganism ratio (F/M) were delineated through the analysis of the relationship between the observed biomass growth and hydrogen productivity.

4.2 Materials and Methods

4.2.1 Seed sludge and feedstock

Inoculum was originated from the pilot-scale anaerobic treatment tank for sewage, which was operated by the Department of Civil Engineering at the University of British Columbia. During last three years, the inoculum had been kept with the operation using dairy wastewater and carbohydrate-rich synthetic wastewater as feedstock without pretreatments under specified operating conditions (Chapters 2 and 3) for hydrogen production. Observations indicated that methanogenic reaction had been relatively well suppressed without pretreatment of the inoculated seed sludge. Experiments were also conducted without pretreatment of inoculum in this Chapter.

Synthetic wastewater was prepared with sucrose as a major carbon source. Nitrogen and phosphate were added in the form of NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, and Na_2HPO_4 to generate a C:N:P ratio of 200:5:1. The concentrations [mg/L] of other trace minerals are as follows: $MgCl_2 \cdot 6H_2O$, 10; $NiCl_2 \cdot 6H_2O$, 1; $ZnSO_4$, 1; $FeCl_2$, 181; $CuSO_4 \cdot 5H_2O$, 5; $CaCl_2 \cdot 6H_2O$, 10; $Na_2MoO_4 \cdot 2H_2O$, 1. The prepared substrate had a COD (chemical oxygen demand) concentration of 10,340 mg/L.

4.2.2 Bioreactor and operational procedure

The experimental apparatus was described in Chapter 3, Section 3.2.1. Experiments were conducted with OLR held constant at 10.3 $kg/m^3.d$ and HRT held constant at 24 hr (1 day). However, pH (4, 5, and 6) and cyclic duration (4, 8, and 12 hr) were varied in a 3×3 factorial experiment. Indeed, the statistical method of factorial design of experiments shall be able to avoid systematic errors with randomly allocation of runs

and an estimate of the experimental error (Zinatizadeh et al., 2011). If cyclic duration were longer than 12 hr under the constant HRT of 24 hr, the decanting volume would be greater than 50% of the reactor; this could probably cause an unintended loss of biomass during the *Discharge* phase and a negative impact on hydrogen productivity. The volume of settled biomass after the 30-min *Settle* phase should not be less than one-third of the reactor volume, though this is somewhat dependent on the status of flocculation of biomass (higher or lower settlability). Otherwise, increased duration of the *Settle* phase can help minimize the volume of settled biomass; whether this is preferable depends on the cost-effectiveness. In this study, Run numbers were assigned according to pH (from 4 to 6) and cyclic duration (from 4 to 12 hr), as indicated in **Table 4.2**. The runs were not randomized and it could be exposed to the possibility of systematic errors due to carry-over effects of biomass concentration. In order to minimize these possible effects, the interval between pH changes was given a week in length; besides, a time period of three days was allocated for stabilization between the changes in cyclic duration.

As shown in **Table 4.1**, the ratio of influent volume (per cycle) to working volume of reactor (r_{tv}) varies with different cyclic durations. In Chapter 3, after a 30-min *Settle* phase, the biomass was observed to be well-flocculated and settle to below 50 percent of the working volume of 5 L. It shall be noted that a test with longer cyclic duration in this Chapter would require higher influent volume. This might in turn lead to an increase in the volume of the settled biomass before decanting. Hence, the working volume was increased to 6 L. Cyclic duration is independent of HRT and OLR. For instance, when cyclic duration was 8 hr, the ASBR was operated with 3 cycles/day and the influent volume was

2 L/cycle; by comparison, when cyclic duration was shortened to 4 hr, the influent volume was reduced to 1 L/cycle with the same substrate concentration.

Temperature was again maintained at 31^{o}C. pH was monitored and automatically controlled to the setpoints using 3 M NaOH and 3 M HCl. Changes in cyclic duration were controlled by a digital timer (XT Series Timer, ChronTrol corporation, USA).

Table 4.1. Operational conditions with varying cyclic durations

pH	Cyclic duration (hr)	Influent volume (L/cycle)	Duration of each phase (min)				r_{tv}*
			Feed	React	Settle	Discharge	
4, 5, 6	4 (6 cycles/d)	1	10	180	30	15	0.17
4, 5, 6	8 (3 cycles/d)	2	10	420	30	15	0.33
4, 5, 6	12 (2 cycles/d)	3	10	660	30	15	0.50

*r_{tv} is the ratio of the influent volume (per cycle) to the working volume of the reactor.
HRT = 24 hr; OLR = 10.3 kg/m^3.d

4.2.3 Analysis

All analytical methods for biogas and liquid samples from the bioreactor were described in Chapter 3, Section 3.2.3.

4.3 Results and Discussion

4.3.1 Effects of pH and cyclic duration on hydrogen productivity

As seen in **Figure 4.1**, methanogenesis was relatively well suppressed in all of the experimental runs without pretreatment of inoculum. CH_4 content was less than 1% (v/v) in the biogas, whereas H_2 content exceeded 50% except for Run 7. The highest H_2 content of 72% was obtained in Run 9 (pH 6; cyclic duration 12 hr) and Run 8 (pH 6; cyclic

duration 8 hr) (**Table 4.2**). The lowest value of 24% H_2 content was from Run 7 (pH 6; cyclic duration 4 hr), while at the same time the CO_2 content reached a highest level of 75% among all runs. It caused very low hydrogen productivity in terms of HPR and H_2 yield at 0.26 ± 0.35 L H_2/L reactor.d and 0.25 ± 0.34 mol H_2/mol sucrose, respectively. Besides, H_2 content had a large standard deviation. The high CO_2 content could be due to the activity of the homoacetogens; hydrogen consumption doubles over CO_2 consumption when acetic acid is produced via homoacetogenic reaction (**Eqn 1.9**). By comparison, when the ASBR was operated at pH 4 or pH 5, percent hydrogen contents were not pronouncedly different as cyclic duration varied from 4 hr to 12 hr.

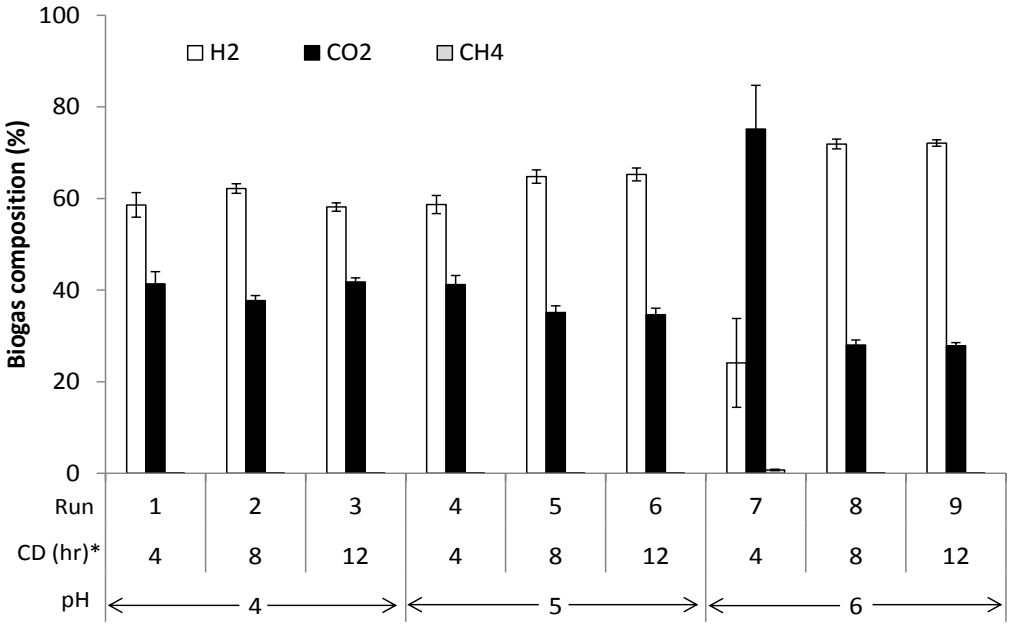

Figure 4.1. Biogas composition according to the operational condition.
***CD: Cyclic duration**

Hydrogen production rate (HPR, in units of L H_2/L reactor.d) represents the efficiency of the bioreactor to produce hydrogen regardless of organic loading. Maximum HPR of 2.2-2.3 L H_2/L reactor.d was achieved in Run 9 (pH 6; cyclic duration 12 hr) and

Run 6 (pH 5; cyclic duration 12 hr). For both pH 5 and pH 6, longer cyclic durations led to higher HPR. A similar trend was not observed at pH 4, whereby changes in cyclic duration had little impact on hydrogen production rate; moreover, the lower HPR (1.15-1.35 L H_2/L reactor.d) might be due to less hydrogenase activity at this low pH level.

Table 4.2. Hydrogen productivity and biomass concentration in response to varying pH and cyclic duration

Run	pH	Cyclic duration	Hydrogen content	Hydrogen production rate	Hydrogen Yield	Biomass Concentration
		(hr)	(%)	(L H_2/ L reactor.d)	(mol H_2/ mol sucrose)	(g MLVSS/L)
1	4	4	58.6 ±7.6	1.35 ±0.33	1.38 ±0.14	6.3 ±0.04
2	4	8	62.2 ±3.0	1.30 ±0.19	1.76 ±0.23	5.6 ±1.24
3	4	12	58.1 ±2.8	1.15 ±0.15	1.32 ±0.11	4.6 ±0.39
4	5	4	58.7 ±6.6	1.06 ±0.46	1.06 ±0.24	6.5 ±0.64
5	5	8	64.8 ±4.8	1.93 ±0.45	2.03 ±0.44	7.6 ±0.09
6	5	12	65.3 ±4.7	2.22 ±0.40	2.17 ±0.35	10.0 ±1.56
7	6	4	24.1 ±32.1	0.26 ±0.35	0.25 ±0.34	15.5 ±1.76
8	6	8	71.9 ±3.5	2.05 ±0.74	1.55 ±0.45	13.5 ±1.11
9	6	12	72.1 ±2.3	2.28 ±0.60	2.01 ±0.43	12.5 ±0.82

Results in terms of hydrogen yield [mol H_2/mol sucrose] exhibit similar trends as HPR with respect to the effects of pH and cyclic duration, and maximum H_2 yield of 2.2 mol H_2/mol sucrose was obtained in Run 6 (pH 5; cyclic duration 12 hr). In fact, according to **Figure 4.2,** pH 5.0~5.5 and cyclic duration longer than 9 hr could effectively generate higher H_2 yield. If cyclic duration were longer than 12 hr under the constant HRT of 24 hr,

the loss of biomass may occur when the reactor is in *Discharge* phase, which results in the underestimation of hydrogen productivity due to the lower level of biomass concentration.

Based on ANOVA results (**Table 4.3**), cyclic duration alone as well as the (pH × cyclic duration) interaction have significant influence on hydrogen productivity in terms of HPR and H_2 yield ($p < 0.05$). An increase in cyclic duration would induce increased hydrogen productivity. Run 7 results were relatively far-off from all other runs, and the reason for these anomalous results could not be ascertained though homoacetogenesis might be possible as mentioned earlier. However, the influence of pH alone on HPR and H_2 yield is statistically insignificant ($p > 0.05$) regardless of the system performance pertinent to Run 7. For instance, the operational conditions (pH 5; cyclic duration 12 hr) and (pH 6; cyclic duration 12 hr) did not give rise to different HPR and H_2 yield.

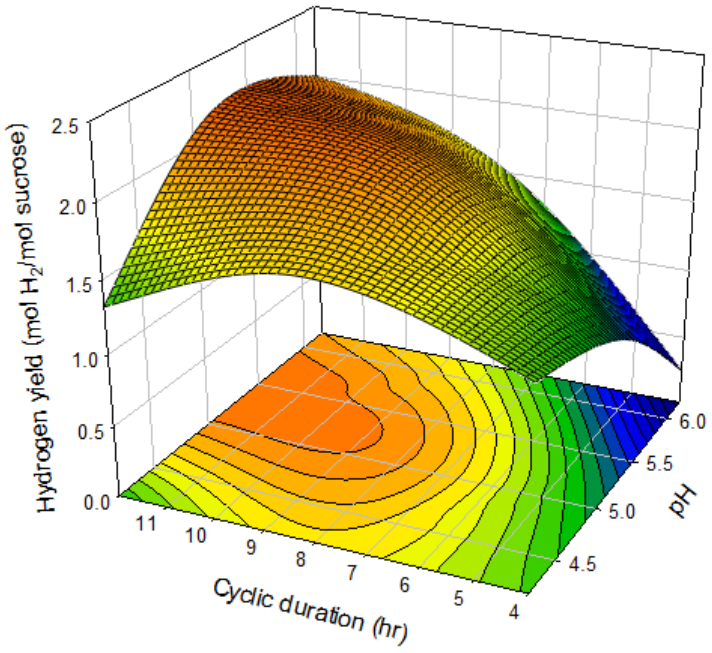

Figure 4.2. Variation of hydrogen yield with pH and cyclic duration

Table 4.3. ANOVA Results – Effects of pH and cyclic duration on hydrogen productivity in terms of %H_2 content, HPR and H_2 yield

	p-values		
	%-H_2	HPR	H_2 Yield
pH	0.682	0.309	0.051
CD*	0.108	0.019	0.001
pH×CD	0.089	0.024	0.002

*CD: Cyclic duration

Lin and Jo (2003) observed different trends of hydrogen productivity with varying *React* periods; much higher H_2 yield was associated with a longer *React* period. Their results are in close agreement with this study. Sreethawong et al. (2010) reported higher hydrogen productivity with shorter cyclic duration when OLR was 30 kg/m^3.d. However, the opposite effect was realized when OLR was 15 kg/m^3.d (a value similar to this study); thus, implying that the optimal cyclic duration could depend upon other major operational conditions such as OLR.

The higher hydrogen production rate and yield with cyclic duration of 8-12 hr could be explained by the ratio of influent volume (or effluent volume)-to-working volume of reactor, r_{tv} in each cycle. Cyclic duration of 4, 8, and 12 hr corresponds to r_{tv} of 0.17, 0.33, and 0.50, respectively (**Table 4.1**). Higher r_{tv} ratios would allow longer time for microorganisms to function in the biological reaction. It also means influent volume per cycle is larger, and the residual soluble metabolite products (SMP) would be lower and even minimized at the beginning of the *React* phase.

Figure 4.3 depicts the relationship between H_2 yield and r_{tv} under each pH condition in this study. For pH 4, higher H_2 yield was obtained at r_{tv} 0.33; but for pH 5 and

6, a higher r_{tv} 0.5 induced higher H_2 yield. Moreover, at pH 5, there is no statistically significant difference between r_{tv} 0.33 and 0.5, which corresponds to cyclic duration of 8 hr and 12 hr ($p > 0.05$). Therefore, it may be concluded that higher H_2 yield could be achieved with cyclic duration of 8-12 hr in this study. For comparison purposes, calculations were then performed using experimental data reported in the literature, and the following findings were obtained. Badiei et al. (2011) achieved higher hydrogen productivity at r_{tv} of 0.33, using palm oil mill effluent as feedstock. Chen et al. (2009) and Sreethawong et al. (2010) reported higher hydrogen productivity for sucrose and cassava wastewater at pH 5.5, with r_{tv} of 0.25 amidst a range of r_{tv} values. Yet, Saraphirom et al. (2011) and Buitrón et al. (2010) reported better hydrogen production with a higher value of 0.5 for r_{tv}, using sweet sorghum syrup at pH 5.0 and tequila vinasses at pH 5.5 as feedstock, respectively. Hence, the optimal value of r_{tv} would be dependent on the characteristics of the feedstock and again, other major operational factors such as pH, HRT and OLR.

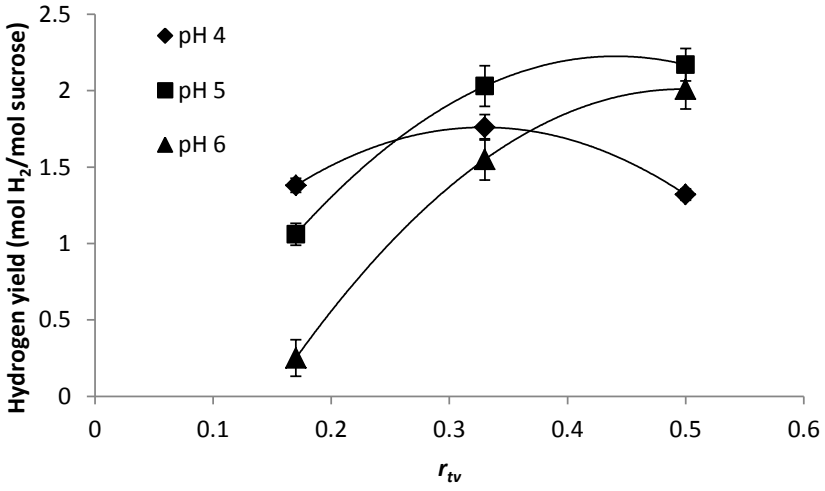

Figure 4.3. The relationship between hydrogen yield and r_{tv} with varying pH
***Error-bars indicate the standard errors of means.**

4.3.2 Effects of pH and cyclic duration on microbial growth

Microbial growth is essential in order to operate the bioreactor but it can become a major electron-acceptor consuming electrons, which results in lower hydrogen productivity. Microbial growth may be represented by a change in the biomass concentration.

The highest biomass concentration of 15.5 g MLVSS/L was achieved in Run 7 (pH 6; cyclic duration 4 hr), whereas Run 3 (pH 4; cyclic duration 12 hr) had the lowest biomass concentration of 4.6 g MLVSS/L. It shall be noted that biomass concentration was not controlled in these tests. A closer to neutral value of pH is known to be favourable for microbial biomass growth. It is expected that microbial growth varied inversely as pH, as demonstrated by the biomass concentration data in **Table 4.2**. Previous studies (Desvaux et al., 2001 and Ray et al., 2010) have also suggested that higher pH (~7) and the presence of adequate substrate would promote microbial metabolism (electron flux) that favours microbial growth, including the non-hydrogen producing bacteria.

The variation of cyclic duration from 4 hr to 12 hr has mixed effects on biomass concentration and hence microbial growth. Biomass concentration increased with shorter cyclic duration at pH 4 and pH 6, but the opposite trend was seen for pH 5. Lin and Jo (2003) increased the *React* period in their study (at pH 6.7) and observed lower microbial growth. Sreethawong et al. (2010) showed that biomass concentration at (pH 5.5, cyclic duration 4 hr, OLR 15 $kg/m^3.d$) induced 2.5 times higher microbial growth as compared to a longer cyclic duration of 6 hr. The opposite phenomenon demonstrated by the data derived from the pH 5 tests might be due to a shift in the microbial communities in the reactor.

Maximum biomass concentration was achieved in Run 7 (pH 6; cyclic duration 4 hr), yet this set of operational conditions caused the lowest HPR of 0.26 L H_2/L reactor.d, suggesting that microbial growth could be inversely proportional to hydrogen productivity with respect to cyclic duration. **Figure 4.4** illustrates the variation of hydrogen production rate with changes in biomass concentration. When biomass concentration fell within the range of 8-13 g MLVSS/L, higher hydrogen production rate was achieved. Nevertheless, a further increase in biomass concentration to beyond 13 g MLVSS/L resulted in a substantial decrease in hydrogen production rate. Therefore, the operational conditions that favour higher biomass concentration did not always lead to higher hydrogen production. In this regard, Chen et al. (2009) has also demonstrated that the best growth condition of microbes is not necessarily accompanied by the best hydrogen productivity.

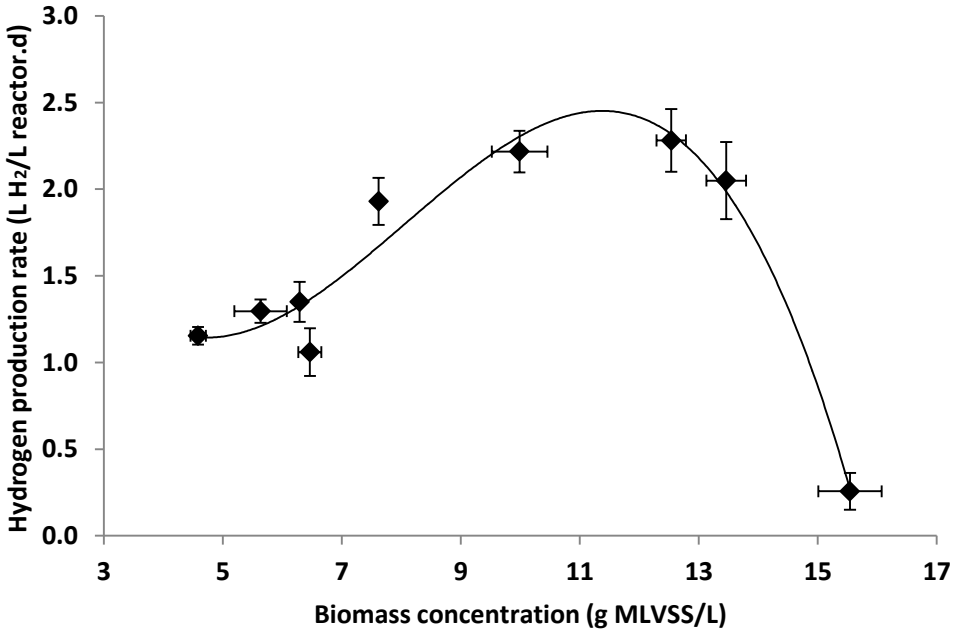

Figure 4.4. Hydrogen production rate according to varying biomass concentration

The concentrations of the soluble metabolites are plotted along with biomass concentration versus cyclic duration in **Figure 4.5.** The ASBR was operated under pH 4 conditions. Biomass (MLVSS) concentration had a positive correlation with ethanol concentration (EtOH) when cyclic duration was increased from 4 hr to 12 hr, whereas butyric acid concentration (HBu) showed the opposite pattern. In this case, the pronounced increase in HBu concentration could have inhibited biomass growth. Chin et al. (2003) and Zheng et al. (2005) have also discussed the inhibition due to HBu over EtOH in their studies when the reactor was operated at pH 6.

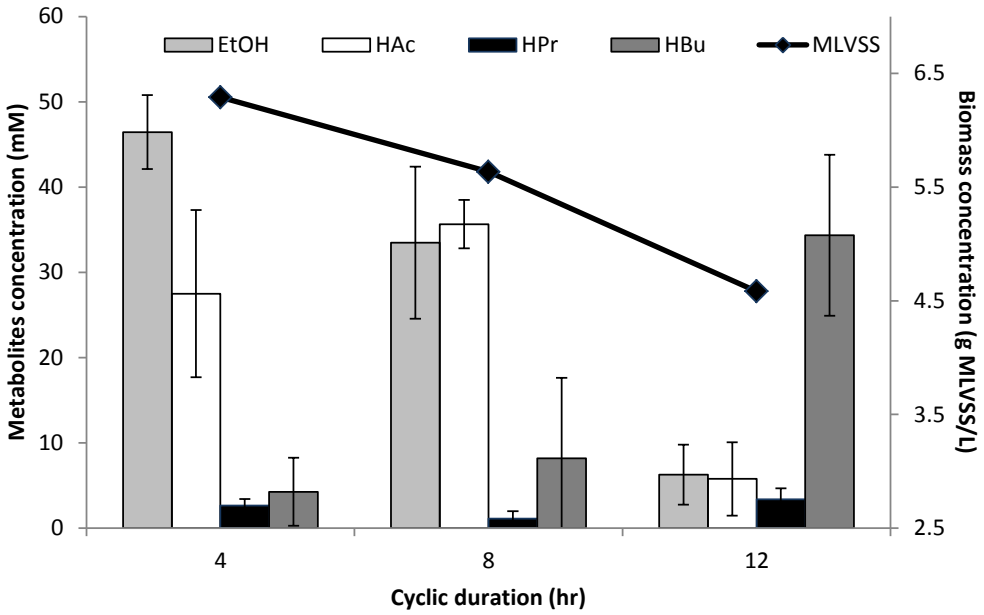

Figure 4.5. The trends of soluble metabolites and biomass concentration against the changes in cyclic duration at pH 4

4.3.3 Food-to-Microorganism ratio

In order to improve hydrogen production and scale up the bioreactor, it is important to find a proper food-to-microorganism ratio (F/M ratio) for the design and

operation of bioreactors. In this Chapter, OLR (10.3 kg/m^3.d) and HRT (24 hr) were held constant while pH and cyclic duration varied in three levels. Thus, biomass concentration was allowed to change freely according to particular combinations of pH and cyclic duration.

Figure 4.6 shows the variations of hydrogen production rate along with ethanol (EtOH) and butyric acid (HBu) concentrations relative to changes in the F/M ratio. The highest hydrogen production rate occurred at F/M ratio of 0.84, which was slightly less than the F/M ratio of 0.96 deduced in Chapter 3 (Section 3.3.1). Apparently, the trend of ethanol production followed the curve of hydrogen production rate. Whereas, butyric acid production was suppressed when F/M ratio was below 1.5. At F/M ratio greater than 1.5, HBu production started to increase sharply while EtOH production experienced a large decrease, implying a possible change of the microbial metabolic pathway from ethanol-type fermentation to butyric acid-type fermentation. This observation is in line with the results obtained by Ren and Gong (2006), who reported that an initial F/M ratio of 1.6 during start-up of a CSTR which induced acidification in the reactor. Furthermore, they recommended a F/M ratio of 1.0 which is more favourable for ethanol production.

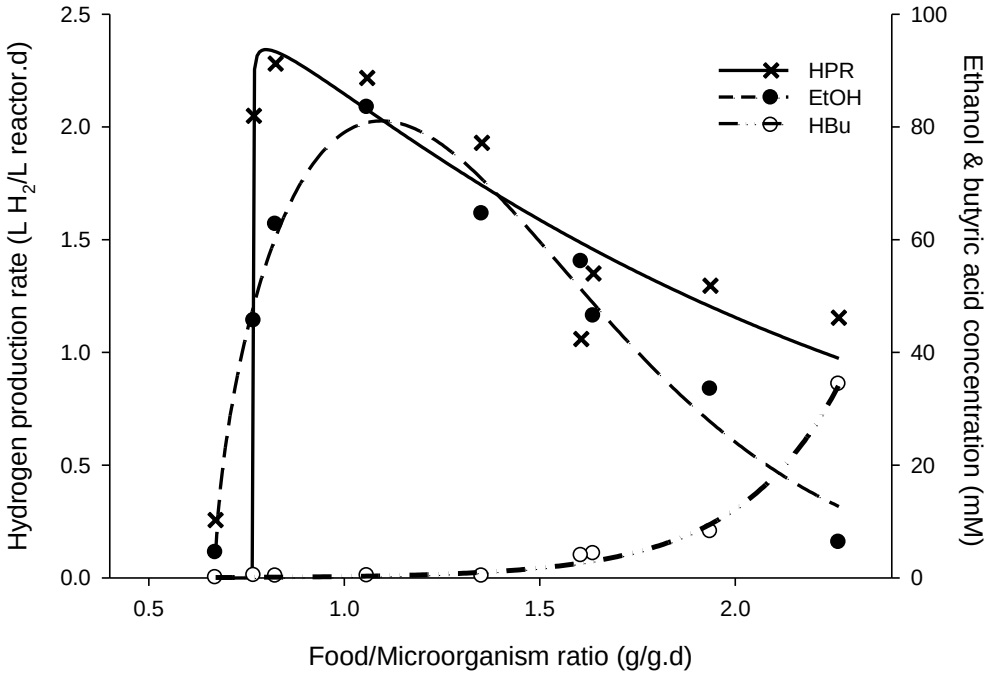

Figure 4.6. Hydrogen production rate and ethanol and butyric acid production according to varying food to microorganism ratio

4.4 Conclusions

Anaerobic sequencing batch reactor (ASBR) was operated in semi-batch (or semi-continuous) mode. The reactor has a unique operational parameter, cyclic duration, which is not found in continuous stirred tank reactor (CSTR) or upflow anaerobic sludge blanket (USAB). Cyclic duration could control the substrate loading per cycle and influent volume according to the changes in cyclic duration when HRT and OLR are held constant. Hence, the effects of cyclic duration and pH were investigated in a 3×3 factorial experiment (pH range 4, 5, 6 and cyclic duration range 4, 8, 12 hr), while OLR and HRT were maintained at constant values of 10.3 kg/m^3.d and 24 hr, respectively.

Cyclic duration corresponded to the ratio of influent volume (per cycle) to working volume of the reactor (r_{tv}); it strongly influenced microbial growth and hydrogen

productivity. An increase in cyclic duration means increased hydrogen production. Based on ANOVA, the influence of cyclic duration as well as the interaction of (pH × cyclic duration) on HPR ($p < 0.05$) and H_2 yield ($p < 0.005$) was statistically significant though pH alone has insignificant influence on hydrogen productivity. For both pH 5 and 6, longer cyclic duration (12 hr) led to maximum H_2 production rate of 2.2-2.3 L H_2/L reactor.d and yield of 2.0-2.2 mol H_2/mol sucrose. Besides, the influence of (pH 5; cyclic duration 8 hr) on hydrogen yield was not statistically different with cyclic duration 12 hr. Thus, it may be concluded that cyclic duration 8-12 hr corresponding to r_{tv} 0.33-0.5 was found to achieve higher H_2 yield in this study.

Due to no control of biomass concentration in the experiment, shorter cyclic duration had the benefit of the resulting higher biomass concentration, except for pH 5. Greater hydrogen production rate was observed with biomass concentration ranging from 8 to 13 g MLVSS/L (pH 5-6; cyclic duration 8-12 hr), but a substantial drop in hydrogen production was shown when biomass concentration went beyond 13 g MLVSS/L. The highest hydrogen production rate was observed at F/M ratio of 0.84, and the variations of hydrogen production rate were relative to ethanol concentration according to F/M ratio. Microbial metabolic pathway was shifted from ethanol-type fermentation to butyric acid-type fermentation when F/M ratio was over 1.5. Consequently, higher biomass concentration did not always lead to higher hydrogen production and ethanol production was closely related to hydrogen production. By taking biomass concentration into consideration, the proper cyclic duration would be 8-12 hr in order to obtain stable hydrogen productivity.

Chapter 5: Investigating the Combined Effects of pH, Hydraulic Retention Time, and Organic Loading Rate in Anaerobic Fermentation of Sugar Refinery Wastewater and Kinetic Modeling

5.1 Introduction

Most of the effluent/wastewater streams from food processing operations contain indigenous hydrogen-consuming bacteria (methanogens and homoacetogens) and higher fractions of insoluble COD, proteins, fats, and ligno-cellulosic matters. Hence, they have low hydrogen production potential when compared to sucrose as the major carbon source for substrate. As mentioned in Chapter 1, previous studies on biohydrogen production applied different methods to pretreat the inocula (seed sludge). This would lead to non-hydrogen producing bacteria being continuously re-introduced into the bioreactor while attempting to suppress the hydrogen-consuming bacteria. Results of biohydrogen production using dairy wastewater as substrate have been presented in Chapter 2, whereby higher protein and fat contents were found to be unfavourable for hydrogen production. Furthermore, it suggested that hydrogen productivity is dependent upon the content of carbohydrate in wastewater when anaerobic fermentation is operated under short hydraulic retention time HRT and higher organic loading rate OLR conditions.

As an application of anaerobic sequencing batch reactor (ASBR) to produce hydrogen from real wastewater, sugar refinery wastewater stream may be a good substrate since its composition was known to be mostly carbon source with some trace minerals. The main objective of Chapter 5 was therefore to determine the optimal factors for biohydrogen production from anaerobic digestion of sugar refinery wastewater. In Chapter 3, with sucrose-rich synthetic wastewater used as the substrate for anaerobic fermentation,

the key operating parameters investigated include pH (4.5, 5.0, 5.5), HRT (10, 20, 30 hr) and OLR (7, 11, 15 kg/m^3.d). These operational parameters which represent three independent variables were again investigated for the anaerobic fermentation of sugar refinery wastewater. Experimental results are reported in terms of hydrogen content (%), hydrogen production rate (L H_2/L reactor. d), and hydrogen yield (mol H_2/mol sucrose).

Kinetic modeling was applied to analyze the experimental data, with an aim to assist with the scale-up of the hydrogen-producing bioreactor. The modified Gompertz model was adopted to build up the empirical model, and then applied to predict hydrogen production during a cycle in each run of the ASBR experiment.

5.2 Materials and Methods

5.2.1 Seed sludge and substrate

Pretreatment of the seed sludge was not conducted, as reported earlier in Section 2.2.2 and subsequently other Chapters. Samples of sugar refinery wastewater were collected from Roger Sugar's refining facility in Vancouver, BC, Canada. Before each run, a new batch of wastewater was characterized. As indicated in **Table 5.1**, when the wastewater had a relatively low chemical oxygen demand (COD), it did not possess sufficiently high strength for use in the experiment; hence supplementary sugar (sucrose) was mixed with the wastewater in order to establish a target substrate concentration. The substrate concentration, *S* [mg/L], was adjusted according to the required levels of HRT and OLR, based on the relationship *OLR = S/HRT* (Chapter 2, **Eqn 2.2**).

Nitrogen and phosphate were added in the form of NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, and Na_2HPO_4 to generate a C:N:P ratio of 200:5:1 and the concentrations [mg/L]

of other trace minerals are as follows [$MgCl_2 \cdot 6H_2O$, 10; $NiCl_2 \cdot 6H_2O$, 1; $ZnSO_4$, 1; $FeCl_2$, 181; $CuSO_4 \cdot 5H_2O$, 5; $CaCl_2 \cdot 6H_2O$, 10; $Na_2MoO_4 \cdot 2H_2O$, 1].

Table 5.1. Characteristics of sugar refinery wastewater

Parameters	Value*
Colour	Dark/light Brown
pH	4.7 – 5.2
TS	3840 – 5780
TSS	30 – 170
TDS	3610 – 5210
VS	560 – 6470
COD	572 – 6612
NH_4^+-N	3.7 – 10.1
P	2.0 – 4.0
Cu	0.02 – 0.04
Ni	0.01 – 0.03
Ca	0.02 – 219

* Units in [mg/L] except for pH and colour

5.2.2 Experimental apparatus

All experimental apparatus was described in Section 2.2.1.

5.2.3 Analytical methods

All analytical methods as reported in Section 3.2.3 were followed, except for COD test. It was not adopted as an indicator of substrate degradation efficiency in this Chapter because COD is not able to distinguish carbohydrate utilization between influent and

effluent. Rather, carbohydrate analysis was performed using the phenol-sulfuric acid technique (Dubois et al., 1956). Substrate utilization is represented in each cycle and used in estimating the removal efficiency of the wastewater. Thus, the initial carbohydrate concentration in the reactor at the beginning of the cycle, C_{in} can be expressed as follows:

$$C_{in} = \frac{(S_{in} \times v_{in}) + \{S_{ef-1} \times (V_r - v_{in})\}}{V_R} \quad \textbf{Eq. 5.1}$$

where S_{in} is the initial carbohydrate concentration of the substrate, S_{ef-1} is the residual carbohydrate concentration in the effluent from a previous cycle, v_{in} is the influent volume in each cycle, and Vr is the reactor's working volume. Then, carbohydrate degradation efficiency η_{cu} (%) may be calculated knowing the carbohydrate concentration in the effluent.

$$\eta_{cu} = \frac{C_{in} - S_{ef}}{C_{in}} \times 100 \quad \textbf{Eq. 5.2}$$

where S_{ef} is the carbohydrate concentration in the effluent.

5.2.4 Experimental design and procedure

5.2.4.1 Central composite design

Response Surface Methodology (RSM) may be used to determine the effects of individual variables and their interactions. It is particularly useful for developing empirical models and investigating uncertain phenomena. RSM is a statistical method introduced by Box and Wilson in 1951. Since then, it had been employed in many fields for designing experiments, improving the efficiency, evaluating the effects of diverse factors, and

finding an optimal condition as desired. RSM approach has also been applied to optimizing the operational conditions for H_2 production with other by-products (Wang et al., 2005 and Karlsson et al., 2008). In this study, the effects of the parameters (pH, HRT and OLR) were investigated by RSM. The experiments could be designed with a few techniques in RSM procedure. The Central Composite Design as a fractional factorial design is useful for describing the effects of parameters and their interactions on a response with a second-order polynomial equation. It could be an effective alternative to a full factorial design which requires a lot of resources to obtain the data (Kincl et al., 2005; Rigas et al., 2000; Myer and Montgomery, 2002).

For this experiment, the designed RSM procedure was a Central Composite Design with three independent variables, pH (x_1), HRT (x_2), and OLR (x_3). Each independent variable had three levels. A total of 18 combinations (including three replicates of the centre point) were chosen in random order according to the central composite configuration for the three factors. The variables are coded (x_i) in advance to compare the significance of their effects on response according to **Eqn 5.3**,

$$x_i = \frac{X_i - X_0}{\Delta X} \qquad \textbf{Eq. 5.3}$$

where X_i is the uncoded value of the independent variable, X_0 is the value of X_i at the centre point, and ΔX is the step change value. Based on literature review relevant to real wastewater, the centre points of the variables (pH, HRT, and OLR) were approximately 5.5, 16 hr and 11 kg/m^3.d, respectively. pH value lower than 4.0 had been found to be the operational limit for microbial activity and hence hydrogen production. The centre points of pH, HRT, and OLR were assigned values of 5.0, 20 hr and 11 kg/m^3.d, respectively,

taking into consideration the results from the previous experiments in Chapter 3. Cyclic duration affects the throughput (inflow and outflow that take place in one cycle of ASBR operation). In this experiment, cyclic duration was fixed at 6 hr. It would allow enough microbial growth and avoid product inhibition based on the experience gained from Chapter 4. The complete set of experimental runs is shown in **Table 5.2.** Each run was conducted over a time period of 2 weeks.

The observed responses to be measured will represent hydrogen productivity. The mathematical relationship between the coded levels of the independent variables, x_1 (pH), x_2 (HRT), and x_3 (OLR) and the responses to these variables was approximated as a second-order polynomial equation as follows:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \qquad \textbf{Eq. 5.4}$$

where y is the predicted response, b_0 is the offset term, $b_{1,\,2,\,and\,3}$ are the linear coefficients, $b_{11,\,22,\,and\,33}$ are the squared coefficients, and $b_{12,\,13,\,and\,23}$ are the interaction coefficients.

The adequacy of the model was determined through analysis of variance (ANOVA). The coefficients of the response surface equation (**Eqn 5.4**) were also determined using regression analysis. Both ANOVA and regression analysis were performed using JMP® 10.0.0 (SAS Inc.). Furthermore, contour plots were generated for demonstrating the response with two parameters at a time and helping to decide the optimal values of each variable.

Table 5.2. Operational procedure with coded and uncoded values from central composite design

Run	x_1	x_2	x_3	pH	HRT (hr)	OLR ($kg/m^3.d$)
1	-1	-1	-1	4.5	10	7
2	-1	-1	1	4.5	10	15
3	-1	0	0	4.5	20	11
4	-1	1	-1	4.5	30	7
5	0	-1	0	5.0	10	11
6	0	0	-1	5.0	20	7
7	0	0	1	5.0	20	15
8	0	0	0	5.0	20	11
9	-1	1	1	4.5	30	15
10*	0	0	0	5.0	20	11
11*	0	0	0	5.0	20	11
12*	0	0	0	5.0	20	11
13	0	1	0	5.0	30	11
14	1	-1	-1	5.5	10	7
15	1	-1	1	5.5	10	15
16	1	0	0	5.5	20	11
17	1	1	-1	5.5	30	7
18	1	1	1	5.5	30	15

* Triplicate runs for the centre-point

5.2.4.2 Kinetic modeling

The Monod kinetics model has been widely used to explain and predict microbial growth under growth limiting substrate conditions (Lyberatos and Skiadas, 1999). However, anaerobic fermentation with mixed culture and sufficient substrates might not be explained by the Monod model. Biohydrogen production in anaerobic fermentation is achieved during the acidogenic and acetogenic phases so that the metabolites and end-products could inhibit microbial growth and products formation. Some metabolites may also be degraded or formed further with hydrogen consumption or generation. Hence, these complicated pathways can make kinetic modeling difficult to implement (Fang and Yu, 2002). An alternative modeling approach is adopted in this Chapter.

The Gompertz model as developed by B. Gompertz in 1825 and modified by Zwietering et al. (1990) had some advantages over other kinetic models with *Lactobacillus plantarum*. Some researchers have used this modified Gompertz model for microbial growth, substrate utilization, metabolites production, as well as cumulative hydrogen production. The growth rate is assumed to be of first order. The primary form of Gompertz model is:

$$y = a \cdot \exp[-\exp(b - ct)] \qquad \textbf{Eq. 5.5}$$

Through the second derivative of the function with respect to *t*, the modified Gompertz equation which considers the inflection point of the curve is obtained as follows (Zwietering et al., 1990):

$$y = A \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\} \qquad \textbf{Eq. 5.6}$$

where λ is defined as the lag time, A is the asymptote on the typical microbial growth curve, t is time and e =2.71828. Van Ginkel et al. (2005) have used the modified Gompertz equation to analyze the cumulative biogas production curves over the course of their batch experiments.

The above equation may be further differentiated, so that microbial hydrogen production potential (A) and production rate (μ_m), and the maximum production rate at time t could be predicted.

$$\frac{dy}{dt} = \mu_m \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right] + \left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right] + 1\right\} \qquad \textbf{Eq. 5.7}$$

The cumulative hydrogen production curve was plotted from data relevant to a cycle of each experimental run. This would enable kinetic models of hydrogen production to be developed, for evaluating hydrogen productivity under different operational conditions. Based on the modified Gompertz model, regression lines were computed along with the correlation coefficient using SigmaPlot 10.0 (Systat Software, Inc.); hence, the potential of hydrogen production, lag time, and hydrogen production rate were evaluated. For this work, samplings were automatically performed every 30 minutes during the 6-hr cycle duration.

5.2.4.3 Microbial identification

Characterization of the microbial communities involved in biological hydrogen production is an important task. It can confirm the abundance or lack of dominant hydrogenase-possessing bacterial strains under different operating conditions. Moreover, if

the mechanisms of the dominant microbial species can be defined by means of microbial identification, it would be helpful towards building an effective strategy for the operation of the ASBR, scaling-up of the reactor, and further defining the relationship between the different species where desirable.

A variety of molecular biology techniques to assess microbial diversities such as PCR-DGGE (Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis), FISH (Fluorescence In-Situ Hybridization), and microarray have been developed and widely used for some time. Advances in sequencing techniques have evolved over the past decade, which can compensate for the weaknesses such as missing unknown genes in the microbial community and limit of band resolution, resulting in the underestimation of the true bacterial diversity (Chojnacka et al., 2011).

Here, pyrosequencing technique was used to investigate the changes of the microbial community with respect to variations in pH, which is regarded as the most significant factor affecting hydrogen productivity when compared to HRT and OLR. Thus, samples were collected from experimental runs with three different levels of pH (4.5, 5.0, and 5.5) and having a range of bioreactor performance. For microbial analysis, the MLSS (mixed liquor suspended solids) sample was collected at the completion of the *React* phase of the reactor and it was centrifuged at 10,000 *g*. DNA was extracted from the sample using the bead beating method and PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Then, it was stored at -20^{o}C after the intensity of the extracted DNA had been confirmed using a spectrophotometer (Nano Drop 2000: Thermo Scientific). Finally, the DNA samples were sent to Génome Québec Innovation Centre for

pyrosequencing, and followed by Phylogenetic analysis via OTUs (operational taxonomic units).

5.3 Results and Discussion

The optimization of the operational parameters - pH, HRT, and OLR for the ASBR was to be based on each response that is related to hydrogen productivity, which includes hydrogen content (%), hydrogen production rate HPR (L H_2/L reactor.d), and hydrogen yield (mol H_2/mol sucrose). **Table 5.3** provides a summary of the experimental results (responses).

Table 5.3. Responses according to the changes of operational conditions

Run	pH	HRT (hr)	OLR ($kg/m^3.d$)	H_2 (%)	HPR (L H_2/ L reactor.d)	Yield (mol H_2/ mol sucrose)
1	4.5	10	7	59.9±8.7	0.86±0.23	1.43±0.29
2	4.5	10	15	73.6±4.2	1.82±0.48	1.48±0.38
3	4.5	20	11	57.8±8.0	2.18±0.52	1.29±0.25
4	4.5	30	7	53.8±9.3	1.19±0.24	1.43±0.39
5	5.0	10	11	16.1±5.5	0.24±0.08	0.11±0.03
6	5.0	20	7	15.9±3.7	0.41±0.19	0.16±0.05
7	5.0	20	15	57.8±2.9	1.53±0.36	0.70±0.01
8	5.0	20	11	14.3±2.3	0.41±0.10	0.12±0.02
9	4.5	30	15	45.7±21.5	0.60±0.34	0.52±0.29
10	5.0	20	11	15.3±2.4	0.34±0.13	0.11±0.03
11	5.0	20	11	22.0±7.4	0.38±0.14	0.20±0.08
12	5.0	20	11	22.4±2.3	0.41±0.06	0.21±0.04
13	5.0	30	11	41.1±10.8	1.09±0.36	0.62±0.19
14	5.5	10	7	4.1±4.0	0.09±0.08	0.05±0.04
15	5.5	10	15	71.8±10.5	2.11±0.31	0.95±0.13
16	5.5	20	11	0.6±0.5	0.01±0.01	0.01±0.00
17	5.5	30	7	0.3±0.5	0.00±0.01	0.00±0.01
18	5.5	30	15	57.7±3.9	1.44±0.20	0.94±0.08

5.3.1 Hydrogen content

Based on the hydrogen content (% H_2) in the biogas produced from the 18 runs, a quadratic second-order polynomial equation was obtained below, along with the coefficients:

$$H_2(\%) = 20.728 - 15.619x_1 - 2.689x_2 + 17.27x_3 + 2.009x_1x_2 + 14.954x_1x_3 - 4.003x_2x_3 + 6.194x_1^{\,2} + 5.635x_2^{\,2} + 13.881x_3^{\,2}$$

($p = 0.005$; $R^2 = 0.89$) **Eq. 5.8**

where H_2 is in its original unit (%) and $x_{1,\,2,\,and\,3}$ are the coded values (-1, 0, +1). When the p-value derived from ANOVA is generally less than 0.05, a statistical significant regression is obtained. Here, the quadratic model is able to describe the response surface of hydrogen content as **Eqn 5.8** with a regression coefficient (R^2) of 0.89. The overall p-value of 0.005 at 95% confidence level also indicates that the empirical equation is statistically significant. ANOVA was further performed to determine the p-values of the coefficients in **Eqn 5.8**. For the variables x_1 (pH), x_3 (OLR) and the interaction x_1x_3 (pH×OLR), the p-value were below 0.05, suggesting that pH and OLR were the most significant factors that influence percent hydrogen purity in the biogas produced. However, other terms in **Eqn 5.8** that are less significant can not be dropped out of the equation lest inaccurate predictions would result.

Based on the experimental data, the two-dimensional contour plots for hydrogen content against OLR and HRT under each pH condition (4.5, 5.0, and 5.5) are shown in **Figure 5.1.** Hydrogen content was more than 50% when OLR was no less than 14.5 kg/m^3.d for virtually all pH and HRTs. The highest hydrogen content (~75%) was

achieved at shorter HRT of 10 hr and higher OLR of 15 kg/m^3.d when pH was 4.5. Changes in pH may cause changes in the microbial communities. As pH was increased to 5.5, changes in OLR exerted a stronger influence on the hydrogen content than changes in HRT, as indicated by the steeper gradients in OLR (y-direction). This is in agreement with the ANOVA results that x_2 (HRT) is not a very significant factor that affects H_2 content.

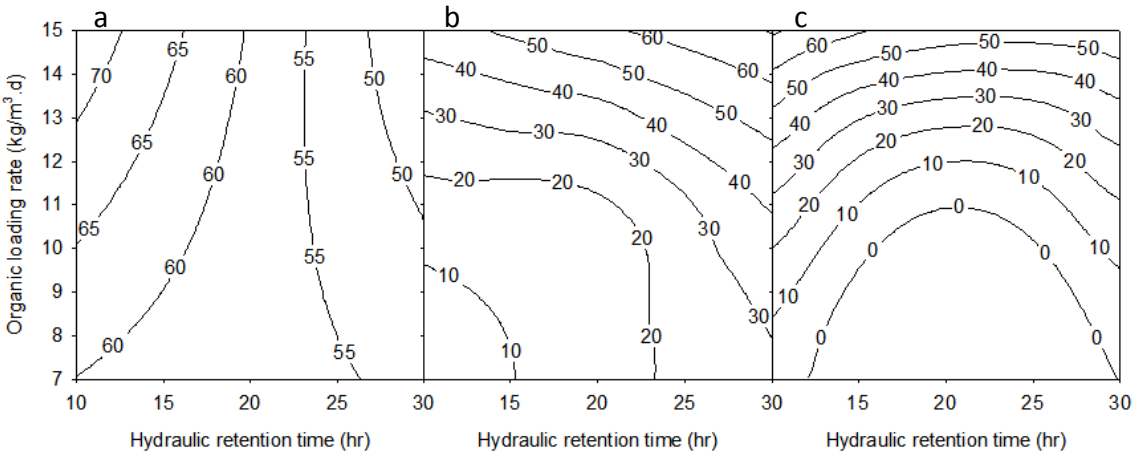

Figure 5.1. Contour plots of hydrogen content (%) against OLR (kg/m^3.d) and HRT (hr) - (a) pH 4.5; (b) pH 5.0; (c) pH 5.5

5.3.2 Hydrogen production rate HPR

The three variables, pH (x_1), HRT (x_2), and OLR (x_3), for hydrogen production rate (L H_2/L reactor.d) were also evaluated in the same manner as hydrogen content. Again, based on the measured HPR from the 18 runs, a second-order polynomial equation with the specific coefficients was obtained by nonlinear regression analysis as shown below:

$$HPR = 0.565 - 0.300x_1 - 0.080x_2 + 0.497x_3 + 0.018x_1x_2 + 0.386x_1x_3 - 0.268x_2x_3 + 0.349{x_1}^2 - 0.080{x_2}^2 + 0.225{x_3}^2$$

($p = 0.161$; $R^2 = 0.70$) **Eq. 5.9**

where *HPR* is in original unit (L H_2/L reactor. d) and $x_{1,\ 2,\ and\ 3}$ are coded values (-1, 0, +1). As shown in **Eqn 5.9**, the overall p-value (ANOVA) is greater than 0.05 while R^2 value is less than 0.80, implying that the equation does not provide a good fit to the HPR data, and the correlation between HPR and (pH, HRT, OLR) as a whole is not strong. Further statistical analysis for the coefficients suggested OLR has stronger influence on HPR ($p < 0.05$) than HRT; nevertheless HRT is not an insignificant factor. **Figure 5.2** illustrates the OLR and HRT effects on HPR for pH 4.5, 5.0 and 5.5 in the form of contour plots. The hydrogen production rate reached 2.2 ± 0.5 L H_2/L reactor.d at pH 4.5, in combination with HRT 18 hr and OLR 11.5 kg/m^3.d as the optimal conditions. In the case of pH 5.0, the optimal combination involves a longer HRT (30 hr) and higher OLR (15 kg/m^3.d). Yet, a shorter HRT (10 hr) together with the same OLR (15 kg/m^3.d) was the most favourable condition when pH is 5.5. Hence, the optimal values of HRT and OLR for hydrogen production rate depend on pH.

Ueno et al. (1996) used sugary wastewater as substrate in a CSTR to study biohydrogen production at thermophilic temperature of 60^{o}C. With pH maintained at 6.8, HRT 12 hr and OLR 19.7 kg/m^3.d, the maximum HPR was 4.4 L H_2/L reactor.d. They observed an increase in the formation of VFAs but a reduction in hydrogen production with an increase in the HRT. Wu et al. (2009) investigated hydrogen production from swine wastewater at pH 5.0 and 37^{o}C in an ASBR, and reported that HPR increased from 1.2 to 3.6 L H_2/L reactor.d when HRT was lowered from 24 hr to 8 hr. The effect of HRT on hydrogen production rate was revealed to have compatibility between ASBR and CSTR when compared to other literature.

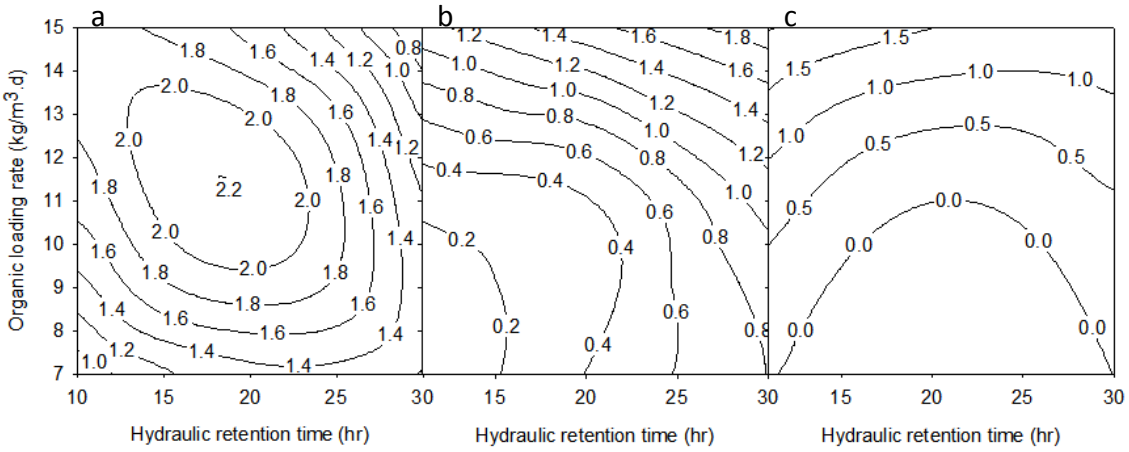

Figure 5.2. Contour plots of hydrogen production rate (L H_2/L reactor.d) against OLR (kg/m^3.d) and HRT (hr) - (a) pH 4.5; (b) pH 5.0; (c) pH 5.5

5.3.3 Hydrogen yield

Hydrogen yield (mol H_2/mol sucrose) was estimated in a similar way by statistical methods. Thus, regression analysis was applied, resulting in the following second-order polynomial equation.

$$Yield = 0.212 - 0.420x_1 - 0.051x_2 + 0.151x_3 + 0.113x_1x_2 + 0.338x_1x_3 - 0.115x_2x_3 + 0.385{x_1}^2 + 0.103{x_2}^2 + 0.163{x_3}^2$$

($p = 0.005$; $R^2 = 0.89$) **Eq. 5.10**

where *Yield* is in its original unit (mol H_2/mol sucrose) and $x_{1,\ 2,\ and\ 3}$ are coded values (-1, 0, +1). As shown in **Eqn 5.10**, the overall p-value of 0.005 from ANOVA is low, whereas R^2 value of 0.89 is closer to 1.0. Hence, this regression model is deemed adequate to explain hydrogen yield in response to the three variables. Each coefficient of the equation was also evaluated by statistical analysis. The coefficients of x_1 (pH), x_1^2 (pH^2), and x_1x_3 (pH×OLR) being -0.420, 0.385, and 0.338, respectively, show the highest statistical significance in

hydrogen yield with the corresponding p-values of 0.001, 0.0425, and 0.0066 (all less than 0.05). Thus, higher hydrogen yield could be achieved at the lower pH of 4.5 and the lower OLR of 7 kg/m^3.d.

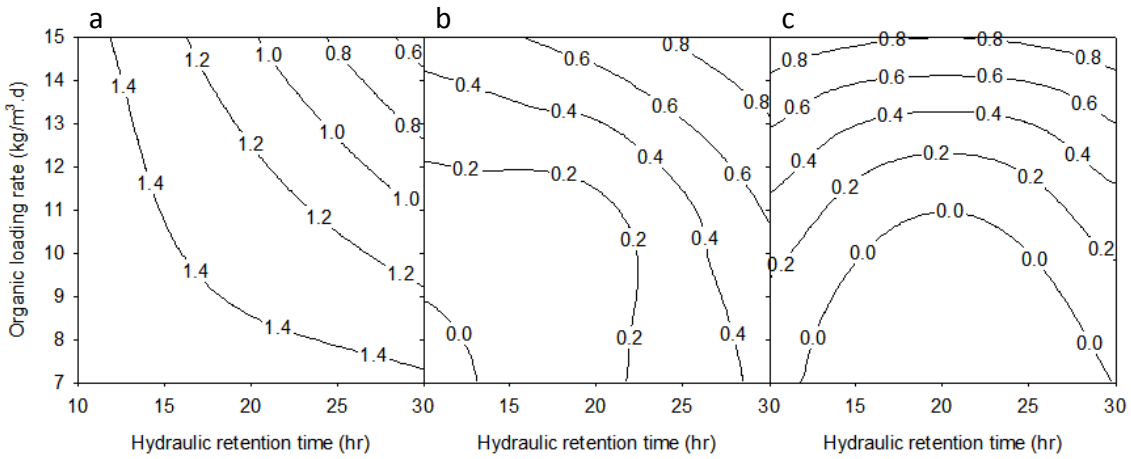

Figure 5.3. Contour plots of hydrogen yield (mol H_2/mol sucrose) against OLR and HRT - (a) pH 4.5; (b) pH 5.0; (c) pH 5.5

Hydrogen yield was displayed in the contour plots (**Figure 5.3**) against OLR and HRT for each level of pH. The optimal values of OLR and HRT in combination for maximum hydrogen yield may be deduced. When pH was 4.5, an increase in HRT and OLR caused a reduction in hydrogen yield, and this is in contrast to the bioreactor performance at pH 5.0. At pH 5.5, higher OLR led to higher yield; however, hydrogen yield was not sensitive to changes in HRT. These observations might be attributed to changes in the microbial communities in response to changes in pH.

The substrate degradation efficiencies in the experiment were determined to be 92±10%. Generally, the values were somewhat lower for pH 4.5, having an average of 82%. The performance indicator "Yield" is in units of [mol H_2/mol sucrose added], but it

can also be expressed in units of [mol H_2/mol sucrose consumed]. Owing to the high substrate degradation efficiencies observed for all runs, the differences in "Yield" as expressed in one unit versus the other are not pronounced.

5.3.4 Optimization of the operational condition

The objective of this chapter was to delineate the optimal operational conditions in an ASBR for maximizing hydrogen productivity from the anaerobic fermentation process. Hydrogen content (%H_2), hydrogen production rate (HPR) and hydrogen yield are the responses to pH, HRT, and OLR that are considered to be the key operational parameters. Hydrogen content represents the degree of hydrogen purity in the biogas produced; it is more directly related to HPR. In turn, HPR is a performance indicator for the efficiency of hydrogen production for a given size of the bioreactor. Furthermore, the performance of the anaerobic fermentation process in terms of the efficiency of substrate utilization is quantified as hydrogen yield.

The relationships between %H_2, HPR, and hydrogen yield are depicted in **Figure 5.4**. Although the correlations have a R^2 value greater than 0.80, this reaffirms the fact that HPR and yield depend on other factors such as biogas volume aside from hydrogen content (**Eqns 2.5** and **2.6)**.

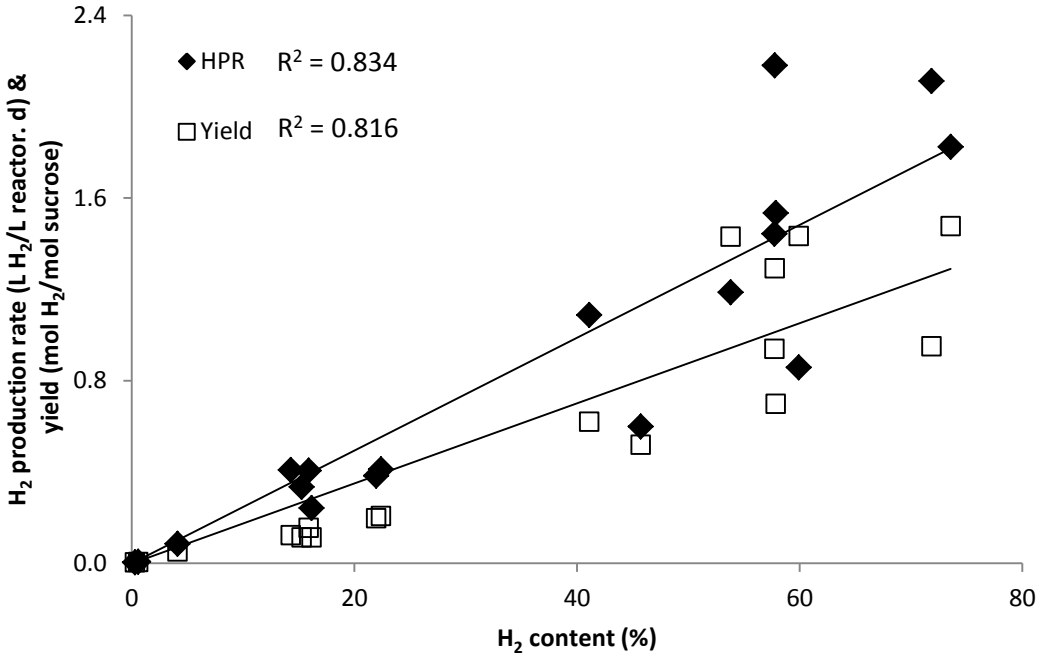

Figure 5.4. Correlation between hydrogen content and hydrogen productivity

Statistical T-tests were performed for three pairs of experimental conditions (Run 2 vs. Run 3; Run 2 vs. Run 15; and Run 3 vs. Run 15) which had high values of hydrogen content, hydrogen production rate and hydrogen yield. The findings are as follows. Run 2 and Run 15 are significantly different in terms of yield ($p < 0.001$), but not for $\%H_2$ ($p > 0.5$) and HPR ($p > 0.01$). Run 3 and Run 15 are significantly different in terms of $\%H_2$ and yield ($p < 0.001$), but not for HPR ($p > 0.5$). Run 2 and Run 3 are significantly different in terms of $\%H_2$ ($p < 0.001$), but not for HPR ($p > 0.01$) and yield ($p > 0.1$).

If the primary criteria for system performance assessment are hydrogen content and hydrogen production rate, then the short HRT of 10 hr in combination with the high OLR of 15 kg/m^3.d would be required. Examination of the experimental data in **Table 5.3** reveals that when pH and HRT are held constant, the high OLR of 15 kg/m^3.d would lead to higher HPR. Specifically, at pH 4.5 and HRT 10 hr, approximately 90% of maximum

hydrogen yield (1.86 mol H_2/mol sucrose) could be attained when OLR is increased from 7 to 15 kg/m^3.d. The experimental results associated with pH 5.0 did not show any maximum responses of hydrogen productivity versus both pH 4.5 and 5.5. Hydrogen yield can be considered another criterion for system performance. From the experimental data, higher hydrogen yields of 1.29 ± 0.25 to 1.48 ± 0.38 mol H_2/mol sucrose were possible when the bioreactor was operated at pH 4.5. Therefore, at this point, the optimal operational conditions could be narrowed down to (pH 4.5, HRT 10 hr, and OLR 15 kg/m^3.d) and (pH 5.5, HRT 10 hr, and OLR 15 kg/m^3.d), considering that Run 2 and Run 15 had similar system performance in terms of %H_2 and HPR, and Run 2 had higher yield.

5.3.5 Inhibitory effect on hydrogen producing activity

Figure 5.5 illustrates the averaged biogas composition for the complete set of experimental Runs #1-18. Methanogenesis was relatively well suppressed despite the fact that no pretreatment was applied to the inoculum. Overall, pH 4.5 is a very severe condition for methanogens. In Runs 16 and 17, at pH 5.5, methanogenesis was sufficiently activated to produce methane (10-20% CH_4 content in the biogas) while hydrogen was depleted. In contrast with a higher level of OLR (15 kg/m^3.d) but at the same pH 5.5, the inhibitory effect for methanogenesis was demonstrated in Runs 15 and 18, when CH_4 content was near 0% while hydrogen production became activated. Thus, depending on OLR, the activity of methane producing bacteria was not necessarily suppressed, even though the range of pH 5.0-5.5 is generally known to be rather unfavourable condition for methanogens. This phenomenon was supported by Williams and Crawford (1985) who

showed the acid-tolerant strains of methanogens could produce methane even when pH dropped to 5.0 or below.

At lower levels of OLR (7 and 11 kg/m^3.d), higher percentage of CO_2 was evolved at pH 5.0 and pH 5.5, versus pH 4.5. The higher content of carbon dioxide in biogas might come from homoacetogenesis, in which hydrogen molecule is consumed by two-fold more than carbon dioxide molecule to produce acetic acid (Chapter 1, **Eqn 1.9**).

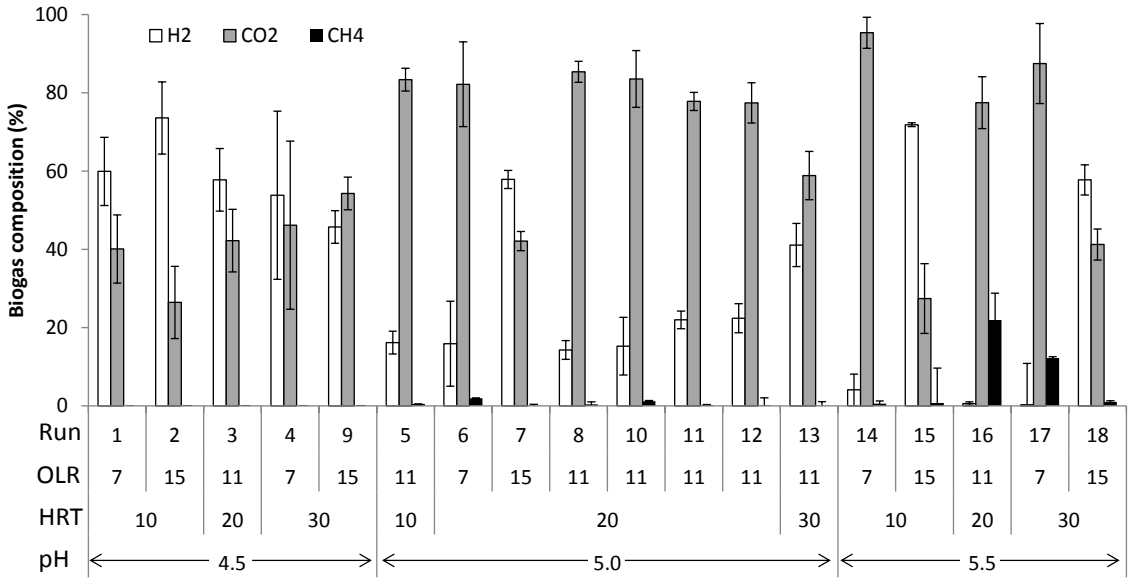

Figure 5.5. Biogas composition of the experimental Runs #1-18

As for the effect of HRT, results from all runs indicated that a short HRT of 10 hr was very effective in suppressing methanogenic activity regardless of pH and OLR. Runs having low hydrogen productivity along with no methane production were probably caused by homoacetogens. When comparing Runs 14 and 15 (shortest HRT; low and high OLR), it can be seen that higher OLR suppressed both homoacetogenesis and methanogenesis. The acetic acid (HAc) pathway can lead to the theoretical maximum yield

of hydrogen at 4 mol H_2/mol glucose. However, high percent HAc in the metabolites could be attributed to the hydrogen-consuming pathway (homoacetogenesis). In this aspect, Luo et al. (2011) concluded that the inhibitory effects of hydrogen-consuming activity by methanogens and homoacetogens were not achieved by pretreatments of inoculum but by fermentation conditions in their long-term tests, and this may be caused by acid-tolerant or spore-forming acetogens (Göβner et al., 2008).

The investigation of soluble metabolite products (SMP) during biohydrogen formation process is a useful tool to trace the microbial metabolic pathway, and it may be used to explain the inhibitory effect of hydrogen producing pathway. VFAs and alcohols are major metabolites; propionic acid and butanol are the two substances known to induce poor hydrogen production. However, it shall be noted that the abundance of acetic acid during fermentation does not guarantee higher hydrogen production with respect to the theoretical maximum yield, as explained above. Hence, the ratios of soluble metabolites are not always adequate to support the conclusion that higher hydrogen production is achieved; some cases are proven for better hydrogen production when homoacetogenesis is excluded.

Electron transfer that occurs during the anaerobic fermentation process results in the release of electrons from the substrate, which could find three possible sinks - soluble metabolites, microbial growth, and hydrogen. **Figure 5.6** shows the volumetric distribution (v/v %) of the soluble metabolites (HAc, HPr, HBu, and EtOH) which are directly related to H_2 production.

As described in Section 3.3.3, the higher production of propionic acid would also hinder hydrogen productivity. When the bioreactor was operated at pH 5.5 (Runs 16 and

17), there is a remarkable augmentation in HPr production and $\%H_2$ was much suppressed. In contrast, for Runs 15 and 18, HPr production was much lower and $\%H_2$ reached 70-80%.

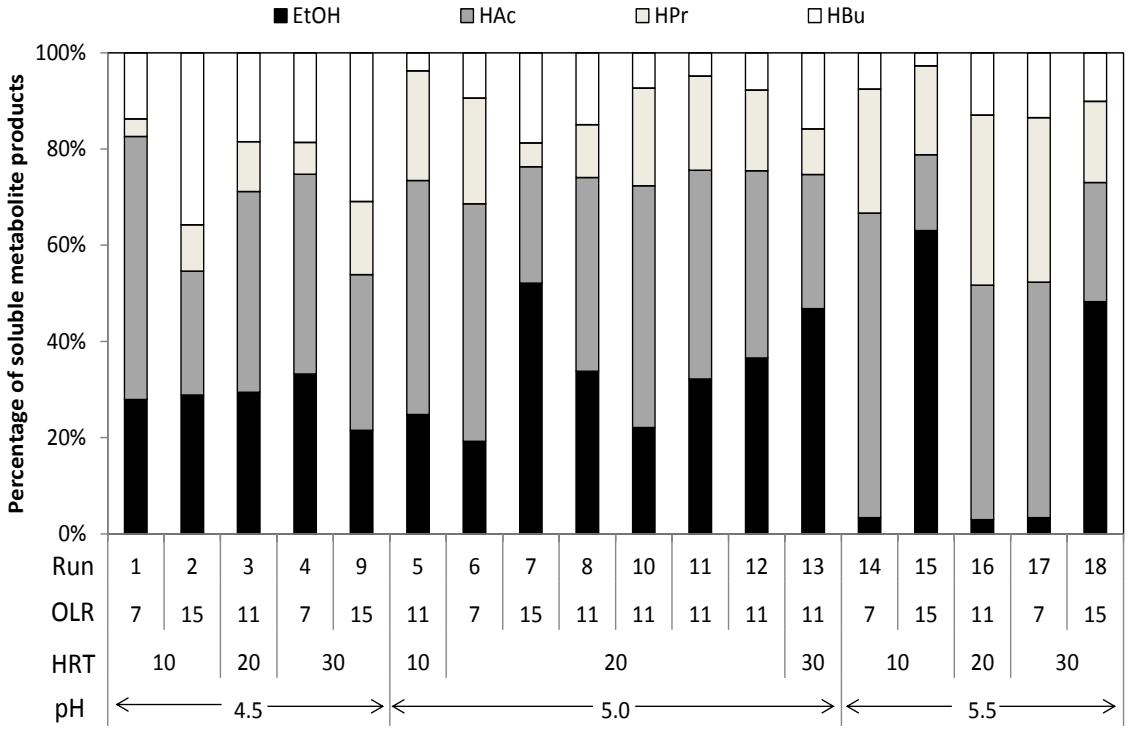

Figure 5.6. Percent distribution of soluble metabolite products for all experimental runs

Among the SMPs, ethanol was considered a major by-product, while acetic acid was the basic metabolite during biohydrogen production (Ren et al., 2007). Theoretically, both the butyric acid (HBu) type fermentation and ethanol (EtOH) type fermentation produces 2 mol H_2/mol glucose. However, Wang et al. (2008) reported that the addition of ethanol caused the inhibition of hydrogen production to a much less extent when compared

to the addition of acetic acid, propionic acid, and butyric acid in their test for inhibitory effects of soluble metabolites production.

The concentrations of the metabolites are shown in **Table 5.4**. Based on the average values, the ethanol/acetic acid (EtOH/HAc) ratio is found to be highly proportional to hydrogen productivity in terms of $\%H_2$ content ($R^2 = 0.92$) when only pH 5.0 and 5.5 data are included in the linear regression analysis, as illustrated in **Figure 5.7**. In particular, when the EtOH/HAc ratio reached 2.0, hydrogen content was above 50%. However, pH 4.5 condition did not generate a similar and clear trend of results; the EtOH/HAc ratio has a lower and narrower range of values (0.5-1.4) and yet hydrogen content can still generally exceed 50%.

In fact, on the basis of further correlation analysis, the EtOH/HAc ratio is also highly correlated to hydrogen production rate and hydrogen yield ($R^2 = 0.95$ and 0.85, respectively), again for pH 5.0 and 5.5 only. It shall be noted that all three system performance indicators have no correlation with the other SMP ratios - HBu/HAc, HPr/HAc, and HBu/HPr, for all pH (4.5, 5.0 and 5.5).

The hydrogen productivity, in terms of hydrogen content, of synthetic sugar wastewater (Chapter 3) differs from that of real sugar refinery wastewater (this Chapter) with respect to the EtOH/HAc ratio. A threshold EtOH/HAc ratio of 1.25 was deduced for synthetic wastewater with pH ranging from 4.0 to 5.5. However, real sugar refinery wastewater may introduce different microbial communities, thus resulting in different SMP concentrations in response to changes in operational conditions, noticeably pH. This could be due to higher acetic acid production by homoacetogenesis as compared to the results obtained using synthetic sugar wastewater as substrate.

Table 5.4. The concentrations and ratios of soluble metabolite products (SMPs)

Run	HAc	HBu	HPr	EtOH	HBu /HAc	HPr /HAc	EtOH /HAc
	(mmol/L)						
1	12.0±3.0	3.0±3.4	0.8±1.5	6.1±3.2	0.25	0.07	0.51
2	9.2±3.8	12.8±5.4	3.4±1.9	10.3±2.8	1.39	0.37	1.12
3	29.3±2.2	13.0±0.8	7.3±1.1	20.6±1.8	0.44	0.25	0.70
4	21.5±5.7	9.6±4.5	3.4±2.5	17.2±4.5	0.45	0.16	0.80
5	7.2±2.8	0.6±0.9	3.4±1.6	3.7±0.8	0.08	0.47	0.51
6	13.9±3.7	2.7±1.4	6.2±1.9	5.4±0.9	0.20	0.45	0.39
7	10.9±4.1	8.4±2.6	2.2±2.0	23.4±4.3	0.77	0.20	2.15
8	10.1±4.4	3.8±2.8	2.8±2.1	8.5±4.4	0.38	0.28	0.84
9	33.3±8.2	31.9±16.6	15.6±3.0	22.1±3.4	0.96	0.47	0.66
10	13.1±2.9	1.9±0.7	5.3±1.7	5.8±1.8	0.15	0.40	0.44
11	11.5±2.1	1.3±0.8	5.2±0.9	8.5±1.1	0.11	0.45	0.74
12	13.1±3.1	2.6±1.4	5.6±2.0	12.3±1.2	0.20	0.43	0.94
13	14.5±3.2	8.2±2.1	4.9±1.4	24.2±1.4	0.57	0.34	1.67
14	3.4±1.8	0.4±0.7	1.4±0.9	0.2±0.2	0.12	0.41	0.06
15	0.7±0.5	0.1±0.1	0.8±0.3	2.7±1.2	0.14	1.14	3.86
16	6.9±2.2	1.8±1.1	5.0±1.4	0.4±0.3	0.26	0.72	0.06
17	5.7±3.5	1.6±1.5	4.0±2.3	0.4±0.4	0.28	0.70	0.07
18	7.2±1.6	2.9±1.3	4.9±0.9	14.0±2.4	0.40	0.68	1.94

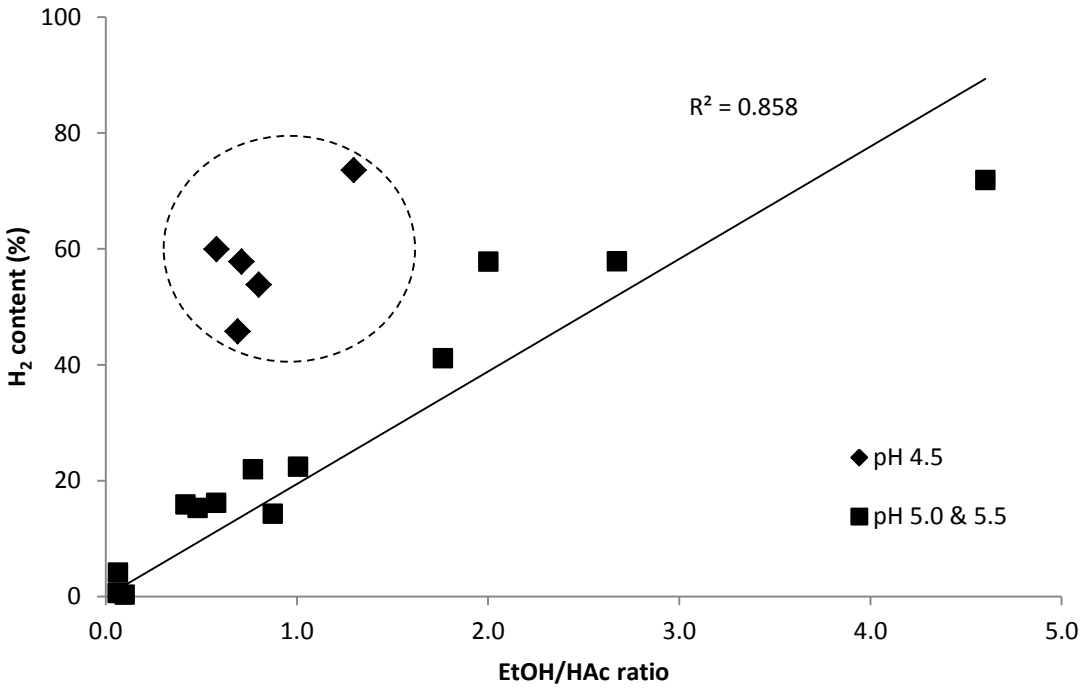

Figure 5.7. The relationship between hydrogen content and the ethanol-to-acetic acid ratio

5.3.6 Modified Gompertz model

In this section, the trend of hydrogen evolution in each cycle was analyzed using the modified Gompertz model, with an aim to determine whether the empirical modeling results could support the findings.

To begin with, the operational conditions associated with the highest cumulative hydrogen production were identified for each pH (4.5, 5.0 and 5.5). Regression curves in the form of **Eqn 5.6** were then fitted to these data, as shown in **Figure 5.8**. The regression curve of Run 2 (pH 4.5, HRT 10 hr and OLR 15 kg/m^3.d) showed the highest hydrogen production potential of 2593 mL as compared to 703 mL and 1099 mL for Run 7 (pH 5.0, HRT 20 hr and OLR 15 kg/m^3.d) and Run 15 (pH 5.5, HRT 10 hr and OLR 15 kg/m^3.d), respectively. **Table 5.5** summarizes the values of the model parameters for all runs, as

derived from curve fitting using nonlinear regression. The lag time of 0.01 hr for Run 15 is seen to be much shorter than the lag time of 0.9 hr for Run 2. Furthermore, the regression curve of Run 2 does not exhibit an asymptote since the reaction time of 5.2 hr in each cycle was too short. In contrast, the regression curve of Run 15 reaches the asymptote within 2.5 hr and no further reaction for hydrogen production occurs. This could be beneficial in terms of saving time and resources and preventing possible deterioration in hydrogen production through, for instance, methanogenic reaction. The elapsed time required to reach the maximum hydrogen production rate, μ_m was derived from the derivative *dy/dt* as 3.05, 2.35, and 0.75 hr, for pH 4.5, 5.0 and 5.5, respectively (**Figure 5.9**).

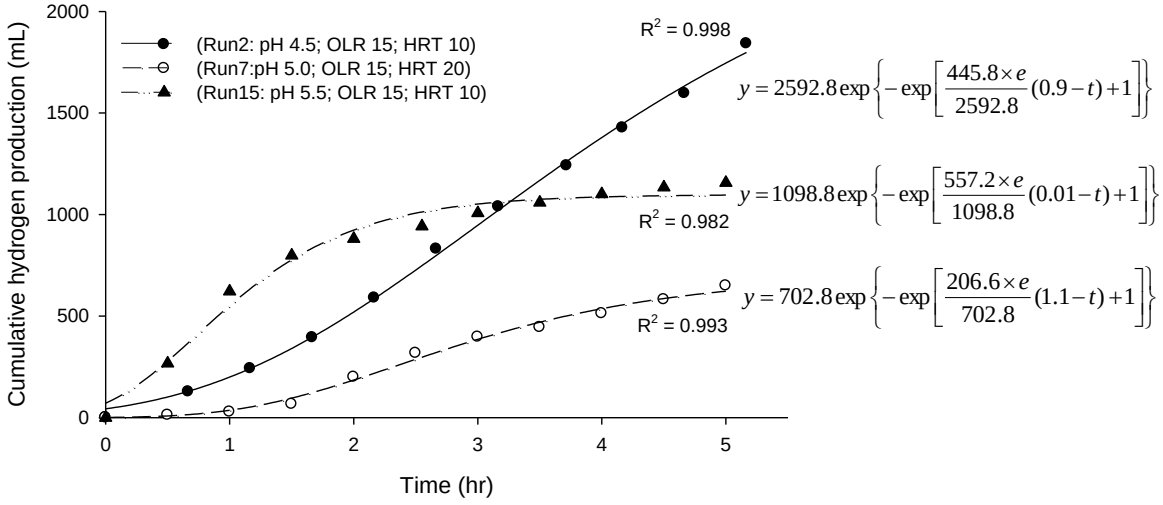

Figure 5.8. Cumulative hydrogen production (*y*) curves fitted by the modified Gompertz model
***Experimental data: ●, ○, ▲ and model curves: , ——, − −, −··**

Table 5.5. Summary of modified Gompertz Model parameters for all runs

Run	pH	OLR ($kg/m^3.d$)	Max. H_2 production potential (A) (mL)	H_2 production rate (μ_m) (mL/hr)	Lag time (λ) (hr)
1	4.5	7	1965	343.3	1.6
2	4.5	15	2593	445.8	0.9
3	4.5	11	837	195.4	0.3
4	4.5	7	792	158.9	1.1
5	5.0	11	158.5	19	1.51
6	5.0	7	123.5	25.4	0.31
7	5.0	15	703	206.6	1.1
8	5.0	11	144	32	0.09
9	4.5	15	1132	177	0.4
10	5.0	11	84.5	15.3	0.3
11	5.0	11	125.5	24.4	0.08
12	5.0	11	99.5	17.5	0.29
13	5.0	11	477	108.9	0.7
14	5.5	7	N/A*	N/A	N/A
15	5.5	15	1099	557.2	0.01
16	5.5	11	9.3	2.2	0.9
17	5.5	7	N/A	N/A	N/A
18	5.5	15	987	333.2	0.3

*N/A: not available (**Appendix C**)

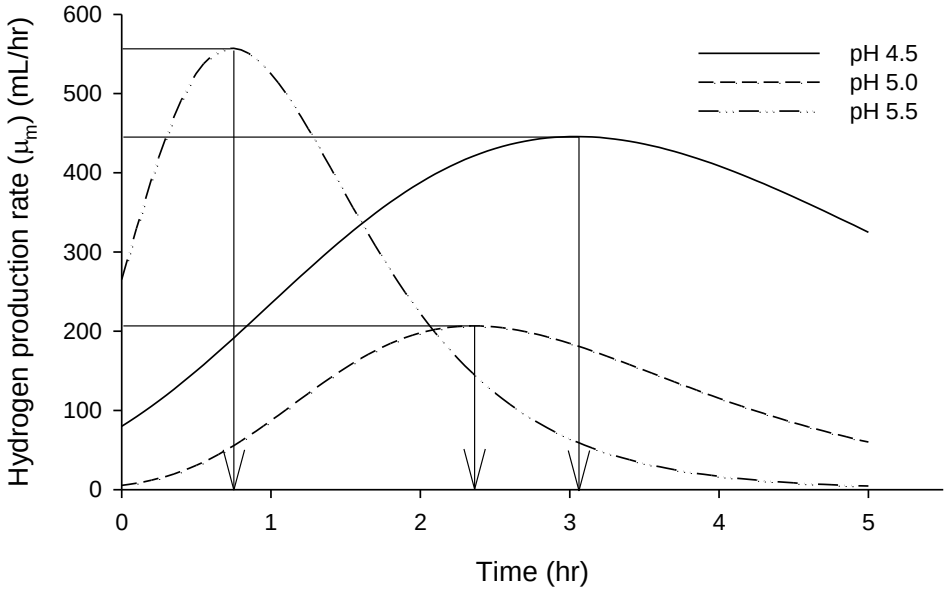

Figure 5.9. Elapsed time to reach the predicted maximum hydrogen production rate

The modified Gompertz model explains the trend of hydrogen evolution within a cycle. Among the model parameters, the predicted values of H_2 production rate (mL H_2/hr) and maximum H_2 production potential (L) could be relevant to the experimental data of H_2 production rate (L H_2/L reactor.d) and yield (mol H_2/mol sucrose). Hence, linear regression was performed to determine the degree of correlation between the predicted values and the actual data. Results are depicted in **Figure 5.10** along with the R^2 values, being 0.89 and 0.95, respectively.

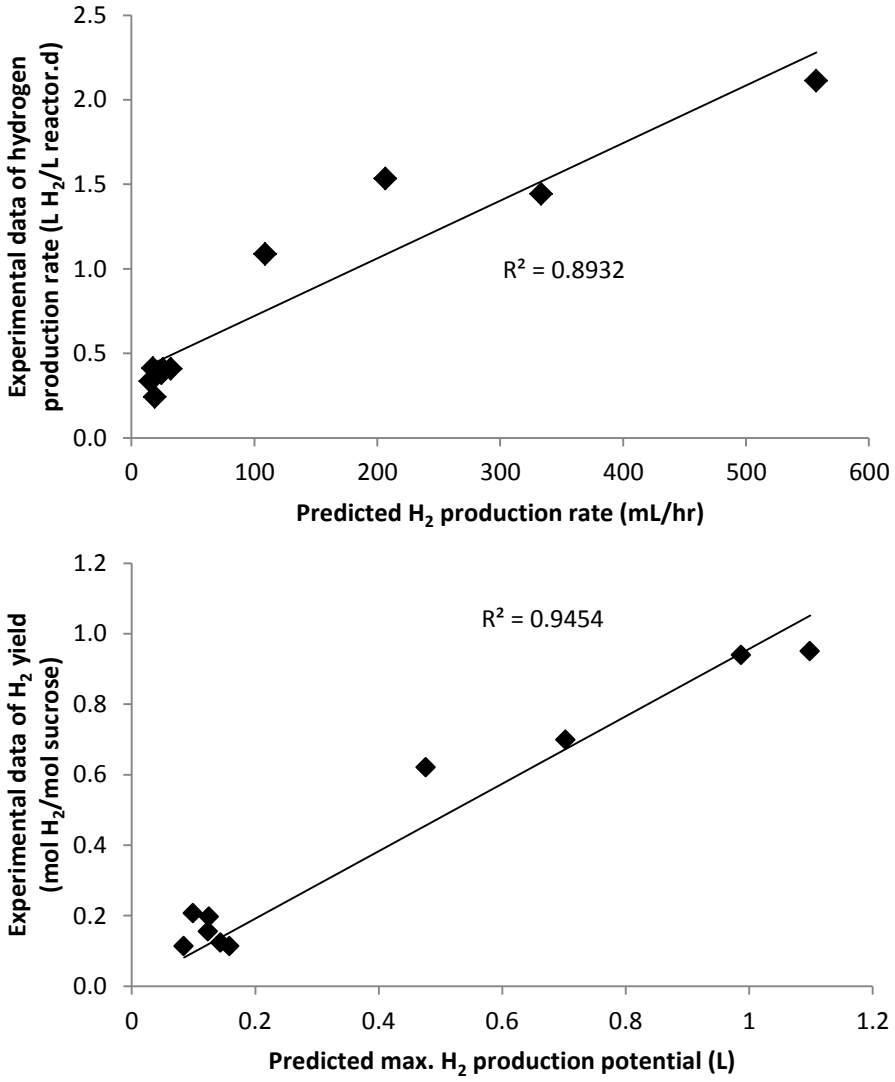

Figure 5.10. Regression of experimental data versus predicted values from the modified Gompertz model

5.3.7 Dominant microorganisms

Previous studies on anaerobic fermentation for biohydrogen have presented the characteristics of soluble metabolite products during hydrogen evolution. In these studies, the operational conditions pH, OLR, and HRT are different; moreover, as summarized in the literature review in Chapter 2, the experiments have used inocula which originated

from diverse sources such as anaerobic digester sludge, river sediments, animal manure, and so on. The genus *Clostridium* is known to be a major type of hydrogen-producing bacteria. It produces butyric acid as a main soluble metabolite in the metabolic pathway for hydrogen production. Pretreatments of inoculum were practiced in many of these studies using techniques such as heat-shock, acid/alkali treatment, and repeated aeration for suppressing the hydrogen-consuming bacteria in the mixed microflora, while facilitating the selection of spore-forming *Clostridium* species. Nevertheless, Kim et al. (2006) found hydrogen-consuming spore-forming bacteria in pretreated microflora and suggested that it is not necessary to accept only the metabolic pathway from *Clostridium* for hydrogen production since various other microbial genera, the non-spore-formers, also possess hydrogen producing metabolism. Besides, the pretreated inoculum is susceptible to contamination since the waste/wastewater stream as substrate usually contains endogenous microorganisms including the hydrogen-consuming bacteria. According to Ohnishi et al. (2010), pretreatment of inoculum may not be desirable in terms of cost-effectiveness and operational control over the long term.

Thus, diverse microbial genera are taking part in hydrogen production. The hydrogen-producing mechanism of obligate anaerobic microorganisms is different from that of the facultative microorganisms. Hence, in mixed culture with real wastewater, it can be difficult to line up the metabolic pathway(s) used for hydrogen production with the soluble metabolites produced. With this in mind, taxonomic analysis using DNA sequencing techniques was performed, and the results are presented below.

Table 5.6. Arranged OTUs with major taxonomic branches for the four samples obtained from the various experimental runs

Taxon	Run3	Run9	Run13	Run15
Bacteria (kingdom)	5684	5722	5460	5489
Actinobacteria (phylum)	796	19	527	271
Bifidobacteriales (class)	766	6	2	48
Bifidobacterium (genus)	766	6	2	48
Propionibacteriales (class)	24	12	525	139
Propionibacterium (genus)	24	12	525	106
Bacteroidetes (phylum)	3834	544	2140	1058
Bacteroidia (class)	3834	528	2135	601
Prevotella (genus)	3834	528	2133	600
Firmicutes (phylum)	1034	5141	2692	3913
Clostridia (class)	852	65	2312	3865
Clostridium (genus)	507	51	545	1859
Ethanoligenens (genus)	52	10	1583	277
Incertae Sedis (genus)	244	3	148	701
Bacilli (class)	181	5076	379	46
Lactobacillus (genus)	43	4941	313	16

Table 5.6 shows the OTUs (operational taxonomic units) along with the major taxonomic branches and the microbial diversity was very wide. The *Bacteria* kingdom (99.5%) occupied the reactor in the experiments, and the remaining being *Archaea* 0.2% and *Eukaryota* 0.2%. The two dominant phyla *Bacteroidetes* (33.9%) and *Firmicutes*

(57.2%) were affiliated in the Bacteria kingdom while two other phyla existed as *Actinobacteria* (7.2%) and *Proteobacteria* (0.5%); uncultured soil bacteria made up 1.2%. Under the phylum *Actinobacteria*, the two classes *Bifidobacteriales* (mostly genus *Bifidobacterium*) and *Propionibacteriales* (mostly genus *Propionibacterium*) occupied 51.4% and 43.8%, respectively. On the other hand, the *Bacteroidetes* phylum was basically made up of the genus *Prevotella* (93.7%). The *Firmicutes* phylum was composed of mostly the *Clostridia* class (55.5%) and the *Bacilli* class (44.5%). Furthermore, the *Clostridia* class was affiliated with mainly three genera, *Clostridium* (41.8%), *Ethanoligenens* (27.1%), and *Incertae Sedis* (15.5%).

5.3.7.1 pH 4.5

Two samples were collected for from Run3 (pH 4.5, HRT 20 hr, OLR 11 kg/m^3.d) and Run 9 (pH 4.5, HRT 30 hr, OLR 15 kg/m^3.d). As an extension to the data displayed in **Table 5.6,** the percent distribution of the major bacterial genus is shown in **Figure 5.11** and **Figure 5.12**, respectively for Run 3 and Run 9. Evidently, the dominant genus in Run 3 was *Prevotella* spp.

For the Run 3 operating conditions, hydrogen productivity was quite high with 58% H_2, HPR 2.18 L H_2/L reactor.d and yield 1.29 mol H_2/mol sucrose. According to Takahashi and Yamada (2000), sucrose fermentation by *Prevotella* sp. produced succinic acid and acetic acid, and the metabolic pathway for acetic acid production is favourable for hydrogen production. *Bifidobacterium* spp., the lactic acid producing bacteria were also found in the Run 3 sample. Lactic acid production is known to inhibit hydrogen production and rendering it unstable (Ohnishi et al., 2010; Noike et al., 2002). Ohnishi et al. (2010)

conducted a sequencing batch experiment using food waste slurry as substrate and without inoculum pretreatment. Their operating conditions were (37^{o}C, pH 6.0, and HRT 48 hr). Results showed that lactic acid producing bacteria were prevalent in their bioreactor; nevertheless, lactate consumption and continual hydrogen production were observed. The authors attributed this phenomenon to the presence of *Megasphaera elsdenii* as the dominant hydrogen producing bacteria, which utilized the lactic acid produced, whereas *Clostridium* spp. were not detected. Other researchers such as Baghchehsaraee et al. (2009) also found hydrogen production to be promoted by lactic acid along with some symbiotic activity.

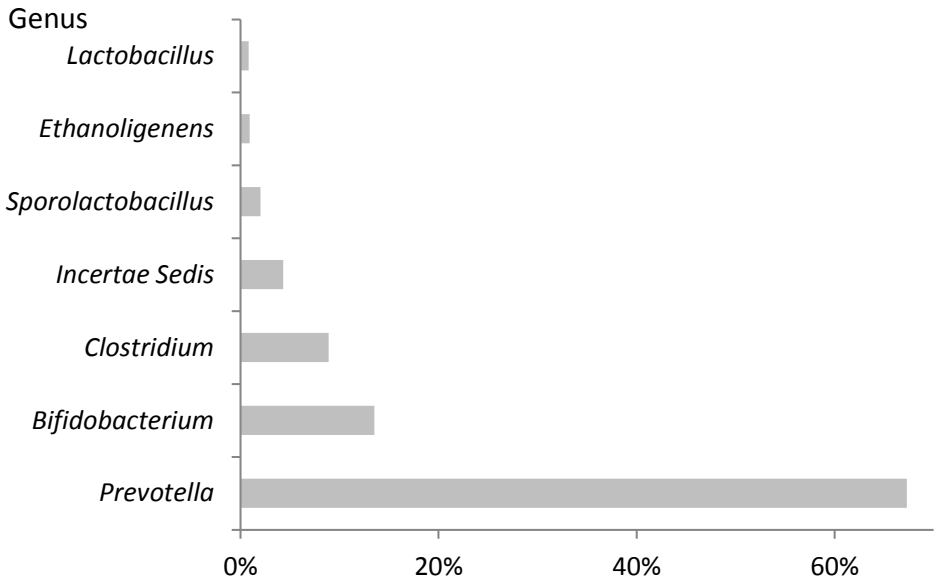

Figure 5.11. Percentage of bacterial genus in pH 4.5 [Run3] (below 0.5% excluded)

In Run 9, the reactor was dominated by *Lactobacillus* spp., which have lactic acid metabolic pathway, and unrelated to hydrogen-producing metabolism. Under the same pH 4.5 as Run 3, but with higher OLR and longer HRT, the growth of *Clostridium* spp. was

inhibited, neither was *Prevotella* abundant. Higher concentration of butyric acid was produced among the soluble metabolite composition, which is similar to the findings by Cheng et al. (2010). Their results indicated that lactate and acetate as intermediate products were utilized to form butyrate and eventually a small amount of hydrogen production. Different HRTs and OLRs affect the dominant microorganisms and hydrogen production. Shorter HRT and lower OLR (Run 3) gave rise to higher hydrogen productivity with *Clostridium* spp. The higher concentration of soluble metabolites in Run 9 did not lead to greater hydrogen productivity versus Run 3.

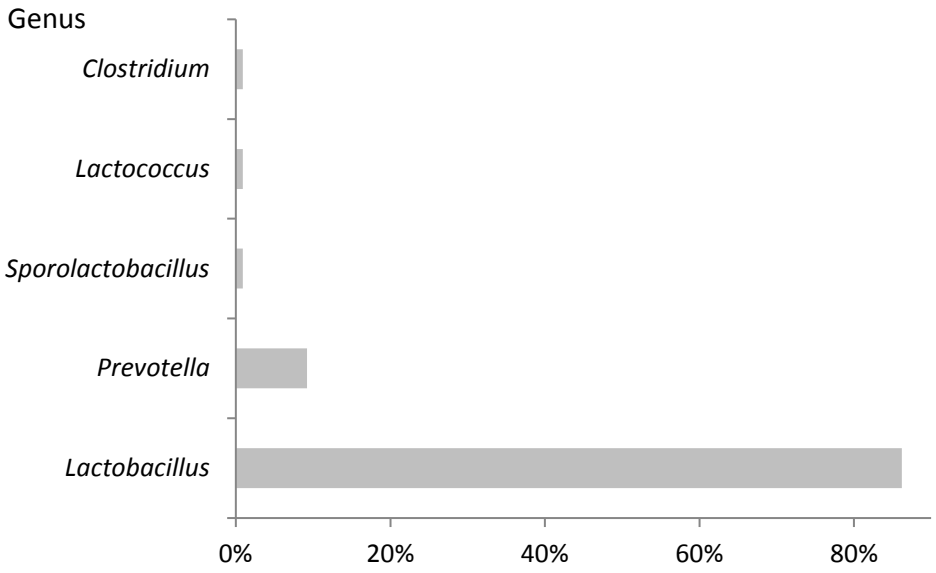

Figure 5.12. Percentage of bacterial genus in pH 4.5 [Run9] (below 0.5% excluded)

5.3.7.2 pH 5.0

Figure 5.13 shows that the genera *Prevotella* and *Ethanoligenens* are the dominating microorganisms in the Run 13 sample. *Ethanoligenens* spp. are known to have ethanol production capability during anaerobic fermentation. These bacteria are non-spore-

forming, gram-positive, and obligate anaerobes. Their optimal growth condition is at relatively low pH (4.5–5.0) under mesophilic temperature. They consume diverse-sized carbon molecules and produce ethanol, acetic acid, hydrogen and carbon dioxide as major products.

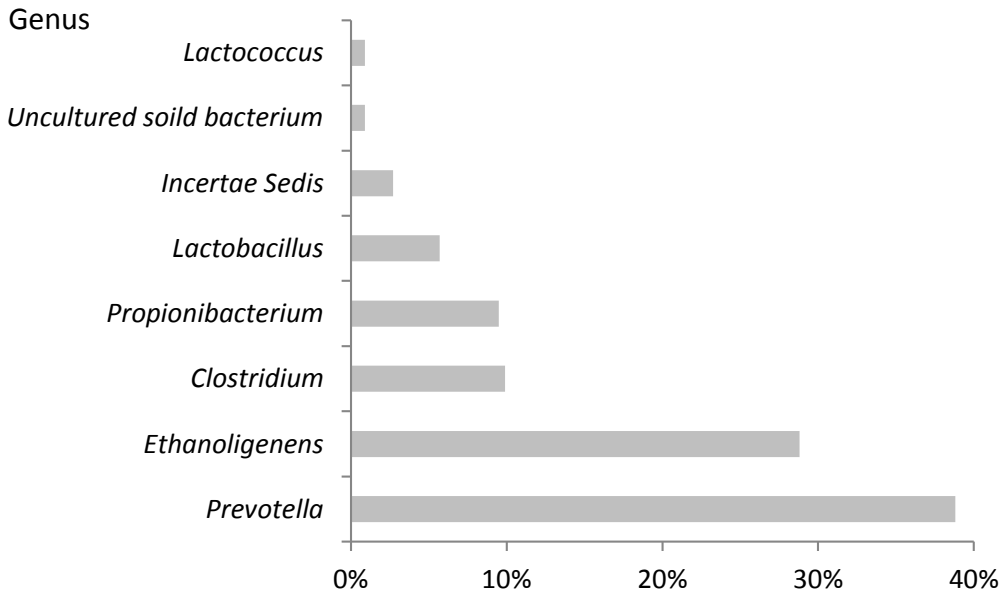

Figure 5.13. Percentage of bacterial genus in pH 5.0 [Run13] (below 0.5% excluded)

According to the reported results by Xing et al. (2006), *Ethanoligenens* spp. produced 2.81 mol H_2/mol glucose in pure culture when the EtOH/HAc ratio was 1.64 (i.e., 1.13 mol EtOH and 0.69 mol HAc per mol glucose). In their study of dark fermentation for biohydrogen in a 30 L lab-scale CSTR, Mariakakis et al. (2011) found H_2 yield attaining a maximum of 1.72 mol H_2/mol hexose (or 3.44 mol H_2/mol sucrose) for HRT 38 hr and OLR 20 kg/m^3.d, and no hydrogen production could be established for OLR lower than 10 kg/m^3.d. Biohydrogen production was associated to mixed butyric acid/ethanol types fermentation, brought about by both *Clostridium* spp. and *Ethanoligenens* spp. as the

dominant microbial genera in the process, and in the absence of lactate as intermediate metabolite products. Cheng et al. (2010) also revealed a metabolic pathway for hydrogen production with butyric acid which was synthesized from lactic acid and acetic acid. Mariakakis et al. (2011) reported *Prevotella* spp. to be the main species in some of their tests; however, H_2 yield only reached 0.78 mol H_2/mol hexose.

5.3.7.3 pH 5.5

Based on literature review, the optimal pH for *Clostridium* spp. was above 5.0 (Li and Fang, 2007; Wang and Wan, 2009) when the inoculum was pretreated for the selection of spore-forming bacteria. In the present study, *Clostridium* spp. was found to be the dominant bacteria for Run 15 (pH 5.5, HRT 10 hr, OLR 15 kg/m3.d) and active hydrogen evolution was achieved.

As seen in **Figure 5.14,** this higher pH condition led to vigorous growth of diverse microorganisms. It shall be noted that besides pH, the governing factors for the growth of *Clostridium* spp. and hydrogen production also include HRT and OLR. *Uncultured Veillonellaceae* which have been shown to possess some capability of using lactate as substrate (Bädaich et al., 1990) were also identified in this sample. The microbial genera *Prevotella* spp. were less abundant. Examples of previous studies that observed the presence of *Prevotella* spp. include Arooj et al. (2008) (ASBR; synthetic substrate at pH 5.3), Ohnishi et al. (2010) (ASBR; food waste slurry at pH 6.0), and Mariakakis et al. (2011) (CSTR; synthetic wastewater at pH 6.5).

With the exception of Run 18, other tests conducted at pH 5.5 (Runs 14, 16 and 17) showed that hydrogen productivity was very low. This can be attributed to long HRT

and/or lower OLR operational conditions, despite pH 5.5 being favourable for *Clostridium* spp. Ethanol production among soluble metabolites other than volatile fatty acids could be contributed by *Ethanoligenens* spp. Very trace amounts of the methanogenic bacteria, *Methanobacterium* spp., was observed only at pH 5.0 and 5.5.

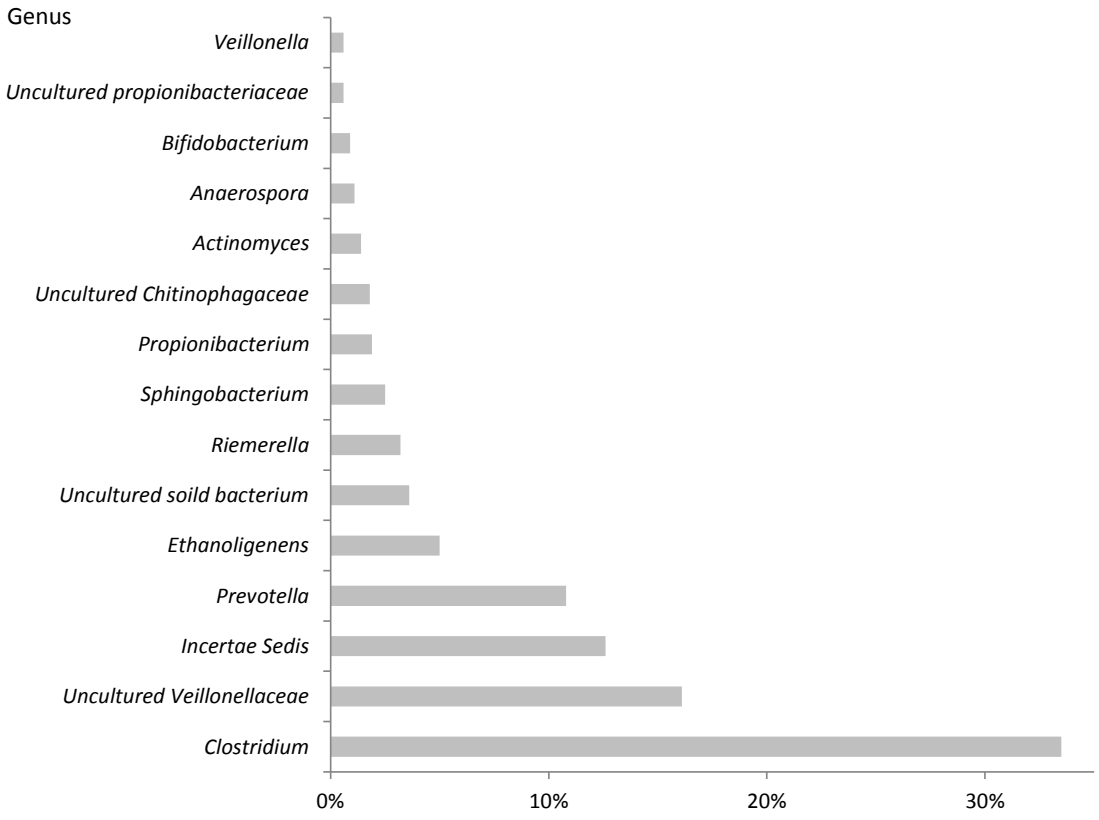

Figure 5.14. Percentage of bacterial genus in pH 5.5 [Run15] (below 0.5% excluded)

Overall, **Figure 5.15** summarizes the trends of the dominant or major bacterial genera found in the four samples from the four experimental runs. The classification of bacterial species was established in affiliation with the various pH conditions (4.5, 5.0, and 5.5). As mentioned previously, pH 5.5 (Run15) was regarded as the optimal pH for *Clostridium* spp., whereas pH 5.0 (Run 13) was optimal for *Prevotella* and *Ethanoligenens*

spp. As for pH 4.5, long HRT and high OLR conditions (Run 9) were favourable for *Lactobacillus* spp., whereas shorter HRT and lower OLR at the same pH (Run 3) were not preferred by *Lactobacillus* spp., but rather *Prevotella* spp.

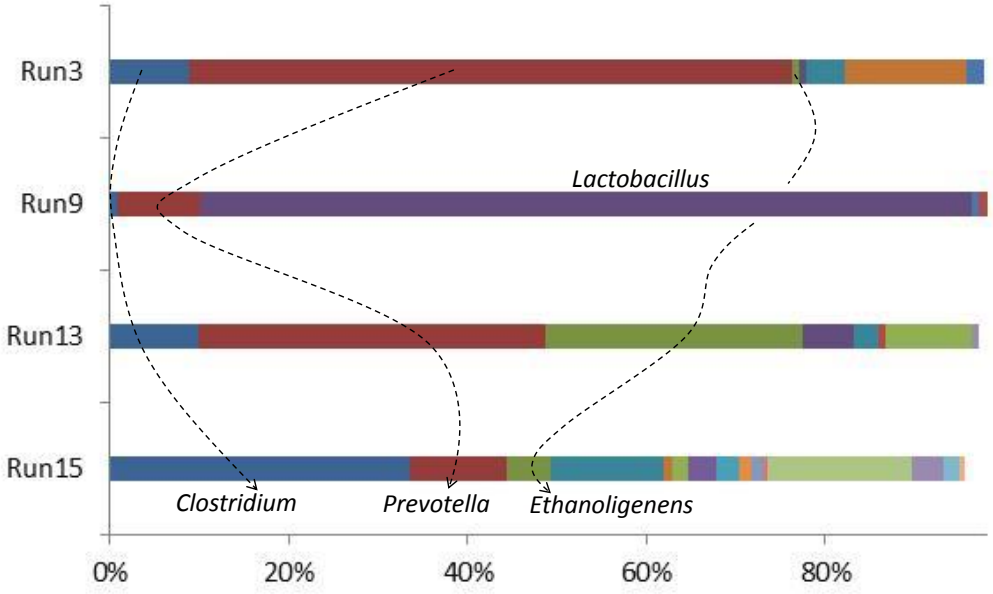

Figure 5.15. Varying microbial communities at each experimental run with different operational conditions

Table 5.7 summarizes the percent distributions of the major hydrogen-producing bacterial genera in the ASBR along with the results pertinent to hydrogen productivity. Both Run 3 and Run 15 gave rise to higher HPR and yield, which appears to correlate better with the high ratios of *Clostridium* to *Ethanoligenens* spp. (10.0 and 6.6 for Run 3 and Run 15, respectively), rather than with the EtOH/HAc ratio (a low value of 0.70 for Run 3 versus a high value of 3.86 for Run 15, as previously noted). Moreover, the high $\%H_2$ attained in Run 15 could be associated with the relatively high *Clostridium* to *Prevotella* spp. ratio of 3.1.

Table 5.7. The concentration and ratio of major microbial genus for hydrogen production along with hydrogen productivity

	Unit	Run3	Run9	Run13	Run15
H_2 content	%	57.8 ±8.0	45.7 ±21.5	41.1 ±10.8	71.8 ±10.5
H_2 production rate	L H_2 /L reactor.d	2.2 ±0.5	0.6 ±0.3	1.1 ±0.4	2.1 ±0.3
H_2 yield	mol H_2 /mol sucrose	1.3 ±0.3	0.5 ±0.3	0.6 ±0.2	1.0 ±0.1
Clostridium /Prevotella		0.13	0.10	0.26	3.10
Clostridium /Ethanoligenens		10.00	N/A	0.34	6.63
Clostridium	g/L	1.8	0.2	2.14	3.38
Prevotella	g/L	13.64	2.08	8.39	1.09
Ethanoligenens	g/L	0.18	0	6.23	0.51

Total biomass concentration was measured as the mixed liquor volatile suspended solids (MLVSS). Results indicated that the MLVSS concentration was 19.4 ±6.0 g/L for all experimental runs, and it has no correlation with hydrogen productivity. On the other hand, the measured values of food-to-microorganism (F/M) ratio were 0.62 ±0.28 g/g.d, and Run 15 has the highest F/M ratio of 1.5 g/g.d. Yang et al. (2007) conducted batch H_2 fermentation experiments using cheese processing wastewater as substrate and mixed microbial communities under mesophilic conditions. They observed maximum H_2 yields at F/M ratio of 1.0 to 1.5.

Based on ANOVA, a strong relationship was established between the F/M ratio and the key operational parameters pH, HRT and OLR. The overall p-value is 0.003, and R^2 is 0.90. As for the individual parameters, the p-values are 0.044, 0.023 and 0.0006 for pH, HRT and OLR, respectively.

Considering the results derived from the modified Gompertz model, a lower F/M ratio would be preferred from the viewpoint of microbial activity at lower pH (4.5); however, a higher F/M ratio might be favourable for higher pH (5.0 and 5.5). A good case lies with Run 15, whereby the high F/M ratio at pH 5.5 and short HRT is associated with near-complete substrate degradation and relatively high hydrogen production rate.

5.4 Conclusions

In this Chapter, an ASBR was operated with sugar refinery wastewater as the substrate. Three major operational parameters (pH, HRT, and OLR) were assessed using a Central Composite Design and response surface methodology (RSM), with an aim to delineate the most appropriate or optimal operating conditions for hydrogen productivity. The experimental design involved three levels of pH (4.5, 5.0, and 5.5), HRT (10, 20, and 30 hr), and OLR (7, 11, and 15 $kg/m^3.d$) as independent variables.

As result of statistical analysis, the favoured values of HRT and OLR for system performance indicators, hydrogen content (% H_2), hydrogen production rate (HPR; L H_2/L reactor.d) and yield (mol H_2/mol sucrose) were found to be dependent on pH. In comparison to pH and OLR, the influence of HRT was less significant for H_2 content and yield, whereas OLR has much impact on HPR.

Hydrogen productivity was low when the ASBR was operated at pH 5.0. The relationships (H_2 content versus HPR), and (H_2 content versus yield) depend on other factors such as biogas volume, as expected. As for optimizing the operational conditions, HRT and OLR converged to 10 hr and 15 $kg/m^3.d$, respectively, when H_2 content and HPR were used as the major criteria for system performance assessment. This set of HRT and

OLR values was also applicable to H_2 yield, which was stipulated as another criterion for system performance, but it was not definitive with respect to pH 4.5 versus pH 5.5.

Methanogenesis that is fatal on H_2 production was activated in several runs at pH 5.5. On the other hand, the higher content of CO_2 along with virtually zero CH_4 content in the biogas was attributed to homoacetogenesis. The inhibitory effect on both methanogenesis and homoacetogenesis could have occurred at a higher level of OLR (15 $kg/m^3.d$). Higher propionic acid production among the VFAs induced a substantial decrease in H_2 production; but variations of HRT and OLR at the same pH inhibited propionic acid production. All system performance indicators had no correlation with SMP ratios except the ethanol-to-acetic acid ratio (EtOH/HAc), which was found to be highly proportional to $\%H_2$ for pH 5.0 and 5.5. Nevertheless it did not exhibit a threshold value as observed for synthetic sucrose wastewater in Chapter 3. Again, this could be due to homoacetogenesis with the introduction of real sugar refinery wastewater into the reactor.

The modified Gompertz model was well fitted to the experimental data collected during a cycle ($R^2 > 0.98$). Maximum H_2 production potential was obtained at a lower pH of 4.5, but maximum H_2 production rate and much shorter lag time were associated with pH 5.5. The predicted values of maximum hydrogen production potential and production rate correlated linearly with the experimental data of H_2 yield and HPR (R^2 = 0.95 and 0.89, respectively). Microbial activity was likely more vigorous at pH 5.5, which could be beneficial to operation in terms of saving time and resources. Consequently, it is possible that the operational setting of (pH 5.5, HRT 10 hr, OLR 15 $kg/m^3.d$) could lead to more active H_2 production over the setting of (pH 4.5, HRT 10 hr, OLR 15 $kg/m^3.d$).

Without pretreating the inoculum in this study, the microbial analysis results showed diverse microbial communities taking part in the biohydrogen production process. pH was a critical factor, but the variations of HRT and OLR also played a role. Greater percent distribution of *Clostridium* was observed at pH 5.5; besides, the higher proportion of *Clostridium* spp. over the other bacterial species such as *Prevotella* and *Ethanoligenens* was conducive for H_2 productivity.

Chapter 6: Conclusions and Recommendations

6.1 Conclusions

The overall goal of the thesis research is to investigate engineering techniques for enhancing biohydrogen production from the anaerobic fermentation of agri-food wastewater. The specific objectives are: 1) to study the key operational parameters (pH, HRT, OLR, and cyclic duration) in an anaerobic sequencing batch reactor (ASBR) using carbohydrate-rich synthetic wastewater and real wastewater as feedstocks; 2) to determine the feasibility of biohydrogen production without the pretreatment of inoculum; 3) to delineate the optimal operational conditions for hydrogen productivity in terms of various performance indicators; 4) to conduct the relationship analysis of the metabolites; and 5) to identify the dominant microorganisms during fermentation.

In Chapter 1, the principles and other fundamental aspects of biological hydrogen production using anaerobic fermentation were reviewed. Microbial metabolic pathways and the key enzymes involved in hydrogen production were introduced, along with the relationship between soluble metabolite products such as volatile fatty acids (VFAs) and alcohols and hydrogen production. Literature review also covered the pretreatment of inoculum using various techniques in order to avoid hydrogen-consuming bacteria. The effects of temperature, hydraulic retention time, pH, reactor type, and hydrogen partial pressure on hydrogen productivity were discussed. Results from a range of biohydrogen production research studies were summarized in graphical form, with emphasis on pH as a major factor that governs microbial pathway. Most studies have been carried out using continuous stirred tank reactors rather than anaerobic sequencing batch reactors which exhibited lower hydrogen productivity.

Experimental studies started with dairy wastewater as the substrate with an aim to determine the feasibility of biohydrogen production during anaerobic fermentation in an ASBR for Chapter 2. Anaerobic sewage sludge without any pretreatments was inoculated into the bioreactor, which was operated with varying hydraulic retention time (HRT) and organic loading rate (OLR) but without pH control under mesophilic temperature range. Although methane production was activated at the initial acclimation period, the manipulation of hydraulic retention time (HRT) and organic loading rate (OLR) provided some inhibitory effect on methane production; methane content in the biogas was substantially reduced to around 20%. With OLR 13 kg/m^3.d and HRT 16 hr as operating conditions, the hydrogen content started to increase and maximum H_2 content of 45% was achieved at the shortest HRT 6 hr and the highest OLR 32 kg/m^3.d, but hydrogen production rate was only 0.08 L H_2/L reactor.d. Nevertheless, the results are encouraging in terms of the feasibility of hydrogen production without the need for the pretreatment of inoculum.

In Chapter 3, sucrose-rich synthetic wastewater was used as feedstock in a series of tests in order to find the optimal operational conditions. The ASBR was operated with the combinations of pH (4.0, 4.5 and 5.0) and HRT (1.25 and 0.83 d) at constant substrate concentration of 13,800 mg COD/L under mesophilic temperature of 28-30^oC. Hydrogen content (%H_2), hydrogen production rate (HPR) and hydrogen yield were measured as the performance indicators. Hydrogen content represents the degree of hydrogen purity in the biogas produced; it is more directly related to HPR. In turn, HPR is a performance indicator for the efficiency of hydrogen production for a given size of the bioreactor.

Furthermore, the performance of the anaerobic fermentation process in terms of the efficiency of substrate utilization is quantified as hydrogen yield.

Without the pretreatment of inoculum, methanogenesis was again effectively inhibited. For a constant OLR of 11.0 kg/m^3.d, the maximum hydrogen production rate and hydrogen yield were 3.04 ± 0.66 L H_2/L reactor.d, and 2.16 ± 0.47 mol H_2/mol hexose respectively, when HRT was 30 hr and pH was 4.5. There exists a threshold ethanol-to-acetic acid ratio of approximately 1.25 for effective hydrogen production and it was suggested that the ethanol-type fermentation may be favoured for hydrogen production; whereas a propionic acid-to-acetic acid ratio of 1.2 and above led to decreased hydrogen content in the biogas produced. In addition, the appropriate food-to-microorganism ratio was found to be 0.84. The recirculation of biogas containing mainly CO_2 into the bioreactor was able to reduce propionic acid production and hence restore hydrogen productivity, which may be due to changes in the microbial metabolic pathway. This technique may result in lower operating costs as compared to blowing other purified inert gases into the reactor in order to recover hydrogen production from system failure.

In Chapter 4, the main objective was to investigate the effect of cyclic duration (CD) as another operational parameter which is unique to sequencing batch reactor, along with varying pH, on hydrogen production. HRT and OLR were fixed at 24 hr and 10.3 kg/m^3.d, respectively. Again, sucrose-rich synthetic wastewater was used as feedstock. Cyclic duration of 4, 8, and 12 hr was evaluated, in combination with pH of 4, 5, and 6 in a 3x3 factorial experiment. With a fixed HRT, cyclic duration corresponds to the ratio of influent volume per cycle to the working volume of the reactor (r_{tv}). Increased hydrogen production was observed with an increase in cyclic duration. The influences of CD and

pH×CD interaction were statistically significant with respect to hydrogen production rate ($p < 0.05$) and yield ($p < 0.005$) based on ANOVA. Maximum hydrogen production rate of 2.2-2.3 L H_2/L reactor.d and yield of 2.0-2.2 mol H_2/mol sucrose were achieved at pH 5 and pH 6. Upon comparing the two sets of operating conditions (pH 5, CD 8 hr) and (pH 5, CD 12 hr), their effects on hydrogen productivity were not statistically significant. Thus, effective hydrogen yield could be achieved at cyclic duration of 8-12 hr (r_{tv} = 0.33-0.5) in this study. Biomass concentration was not controlled in the experiment; higher hydrogen production rates were observed with biomass concentration ranging from 8-13 g MLVSS/L (pH 5-6, CD 8-12 hr).

Moreover, the highest hydrogen production rate was associated with a food-to-microorganism (F/M) ratio of 0.84; the same result was derived from the experiments in Chapter 3. The shift of major soluble metabolite production from ethanol to butyric acid occurred when F/M ratio was above 1.5. Thus, it may be concluded that higher microbial growth was not necessarily accompanied with higher hydrogen production and ethanol production was closely related to hydrogen production. In consideration of biomass concentration, cyclic duration of 8-12 hr would be appropriate for stable hydrogen productivity.

The main objective of Chapter 5 was to assess and delineate the most appropriate or optimal operating conditions in an ASBR, using sugar refinery wastewater as substrate. The key operational parameters, pH (4.5, 5.0 and 5.5), HRT (10, 20 and 30 hr) and OLR (7, 11 and 15 kg/m^3.d) were investigated as three independent variables using a Central Composite Design and response surface methodology (RSM). Based on ANOVA, the influence of HRT was less significant for H_2 content and yield in comparison to pH and

OLR, whereas OLR has much impact on HPR. Hydrogen-consuming activity (methanogenesis and homoacetogenesis) was deduced in several runs, but a higher level of OLR (15 kg/m^3.d) showed the inhibitory effect. The favoured conditions of HRT and OLR for hydrogen productivity were dependent on pH level. Higher hydrogen productivity was obtained at HRT 10 hr and OLR 15 kg/m^3.d, but at this point it was not definitive with respect to pH 4.5 versus pH 5.5. Curve fitting was then applied to the experimental data using the modified Gompertz model; results indicated a reasonably good fit with a coefficient of determination (R^2) greater than 0.98. Maximum hydrogen production potential was obtained at a lower pH 4.5; however, maximum hydrogen production rate and much shorter lag time were associated with pH 5.5 which may have advantages in terms of saving time and resources. Consequently, it may be concluded that more effective hydrogen production could be achieved at pH 5.5 than pH 4.5, while HRT and OLR were maintained at 10 hr and 15 kg/m^3.d, respectively.

Further findings from Chapter 5 are summarized below. All system performance indicators have no correlation with ratios of soluble metabolite products, except for the ethanol-to-acetic acid ratio (EtOH/HAc) which was found to be highly proportional to hydrogen content for pH 5.0 and 5.5. Nevertheless, a threshold EtOH/HAc value could not be determined, in contrast to the findings of Chapter 3; this could be due to homoacetogenesis, with indigenous microorganisms from real sugar refinery wastewater. Identification of microbes was done using DNA sequencing techniques along with taxonomic analysis. Sampling from the hydrogen-producing reactor indicated that diverse microbial communities contributed to the hydrogen production process. Variations in pH as well as HRT and OLR induced changes in the dominant microorganisms. Even without

pretreatment of inoculum which is meant to select spore-forming hydrogen-producing bacteria such as *Clostridium*, a higher proportion of *Clostridium* spp. over the other bacterial species such as *Prevotella* and *Ethanoligenens* was observed at pH 5.5, and this is compatible with the high hydrogen productivity observed in the experiments. Hence, (pH 5.5, HRT 10 hr and OLR 15 $kg/m^3.d$) was delineated as the optimal operational conditions for an ASBR working with sugar refinery wastewater as the substrate.

6.2 Recommendations for future research

Using the anaerobic sequencing batch reactor (ASBR) in this thesis research, the operational parameters for hydrogen production were evaluated and optimized. Though ASBR as a hydrogen producing reactor did not demonstrate superiority over a continuous stirred tank reactor, it has potential for cost-effective biohydrogen production.

Pretreatment of inocula has the primary purpose of selection for *Clostridium* spp. which could have superior hydrogen productivity compared to other bacteria species but this technique might not be desirable from the cost-effectiveness and operational control points of view. Hence, it would be particularly useful to track the dynamic changes of the microbial species concomitantly with optimizing HRT and OLR in a hydrogen-producing reactor in future studies. This would contribute towards making the best possible decision about the operational conditions for the ASBR.

In order to overcome the barriers of commercializing biological hydrogen production, various aspects need to be considered. These are centred around microbial genetic modification via pure cultures, as well as process engineering.

1) Through the genetic modification of microorganisms, it can be expected to have over-expression of hydrogenase which has a capacity to endure higher hydrogen partial pressure and control of metabolic pathway with focus on hydrogen production. The modified bacterial strains should have tolerance for external microbial communities since the substrate may be continuously contaminated by introducing indigenous microbes in the organic waste streams. Otherwise, pretreatment of feedstock must be carried out to eliminate external microorganisms, for instance, using UV light or probiotics to gain control of the bioreactor prior to feeding.
2) CO_2-rich biogas recirculation technique for restoring greater hydrogen productivity should be subject to further testing, such as on pilot-scale, in order to develop a standard protocol. Appropriate techniques for gas separation or extraction from the bioreactor, which can improve hydrogen production by reducing hydrogen partial pressure, should also be investigated.
3) Cyclic duration in ASBR is a specific operational condition which affects hydrogen productivity via microbial growth. The period of cyclic duration must be controlled by the end-point of hydrogen evolution during a cycle (that is, asymptote in the curve of cumulative hydrogen production) since it may cause insufficient or excess reaction time and unnecessary reaction. If real-time control can be applied, at least one of the factors must be automated via the signals of microbial status, so that the productivity of the bioreactor and feedstock utilization can be maximized.
4) Overall, hydrogen-producing reaction was found to be related to ethanol production aside from the production of acetic acid. Ethanol derived from organic waste

sources is also a great energy source besides hydrogen; hence this combination of products would be promising alternatives to fossil fuel in terms of sustainability.

5) In the longer term, the aim of the research on dark fermentation would lie with demonstrating the feasibility of co-generation of power in the range of 0.5 to 3 kW via fuel cells that utilize the biohydrogen or biomethane produced, with application on farm or off-farm, for instance, in home appliances.

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Appendices

Appendix A. Summary of hydrogen yield reported in the literature

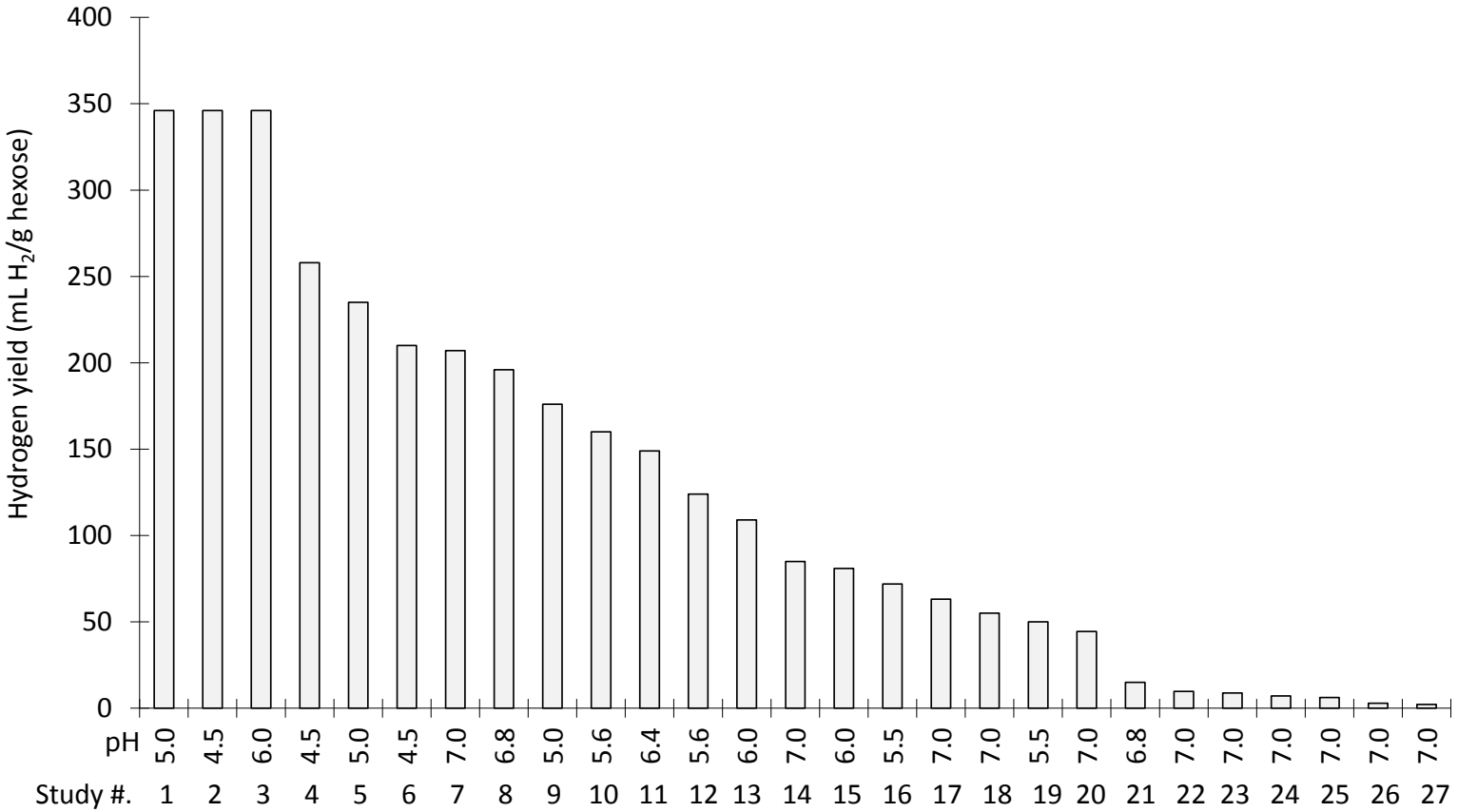


Figure A1. Hydrogen yield reported in other studies with real wastewater using batch reactor

Table A1. List of literatures for Figure A1

Study #	Authors	Substrates	Inocula
1	Noike and Mizuno 2000	Bean curd manufacturing waste	Soy bean meal
2	Fang et al. 2006	Food waste	Anaerobic digested sludge
3	Mizuno et al. 2000	Bean curd manufacturing waste	Soy bean meal
4	Yang et al. 2007	Cheese powder with additives	Sewage sludge
5	Noike and Mizuno 2000	Wheat bran	Soy bean meal
6	Fang et al. 2006	Food waste	Anaerobic digested sludge
7	Lay et al. 2004	Food waste	Compost
8	Wang et al. 2003	Filtered leachate of waste biosolids	Waste biosolids
9	Noike and Mizuno 2000	Rice bran	Soy bean meal
10	Lay et al. 1999	Mixed waste	Soy bean meal
11	Van Ginkel et al. 2005	Food processing and domestic wastewater	Soil
12	Lay et al. 1999	Mixed waste	Anaerobic digested sludge
13	Logan et al. 2002	Molasses	Soil
14	Okamoto et al. 2000	Rice	Anaerobic digested sludge
15	Logan et al. 2002	Potato	Soil
16	Tang et al. 2008	Cattle manure	Sewage sludge
17	Okamoto et al. 2000	Carrot	Anaerobic digested sludge
18	Okamoto et al. 2000	Cabbage	Anaerobic digested sludge
19	Massanet-Nicolau et al. 2008	Enzyme and heat treated primary sludge	Heated sewage sludge
20	Lay et al. 2003	Carbohydrate-rich high solid organic waste	Compost
21	Wang et al. 2003	Waste biosolids	Waste biosolids
22	Okamoto et al. 2000	Fat	Anaerobic digested sludge
23	Okamoto et al. 2000	Chicken skin	Anaerobic digested sludge
24	Okamoto et al. 2000	Lean meat	Anaerobic digested sludge
25	Okamoto et al. 2000	Egg	Anaerobic digested sludge
26	Lay et al. 2003	Fat-rich high solid organic waste	Compost
27	Lay et al. 2003	Protein-rich high solid organic waste	Compost

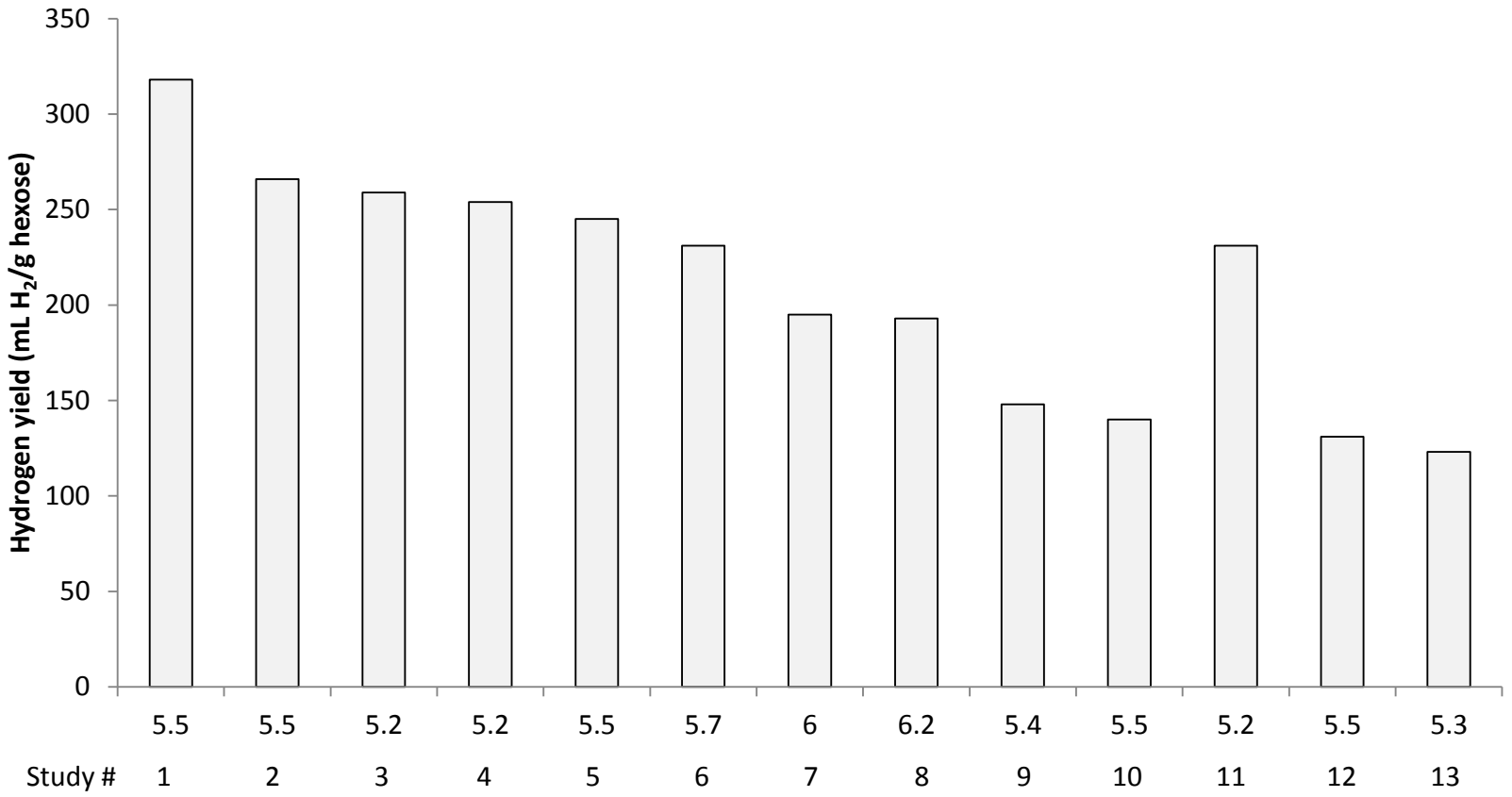


Figure A2. Hydrogen yield reported in other studies using continuous stirred tank reactor

Table A2. List of literatures for Figure A2

Study #	Authors	Substrates	Inocula
1	Van Ginkel et al. 2005	Glucose	Soil
2	Fang et al. 2002	Sucrose	Sewage sludge
3	Hussy et al. 2005	Sucrose	Anaerobic digested sludge
4	Hussy et al. 2003	Wheat	Anaerobic digested sludge
5	Iyer et al. 2004	Glucose	Soil
6	Lin and Chang 1999	Glucose	Sewage sludge
7	Mizuno et al. 2000a	Glucose	Soy bean meal
8	Lin and Chang 2004	Glucose	Sewage sludge
9	Shin et al. 2004	Sucrose	Anaerobic digested sludge
10	Zhang et al. 2004	Glucose	Soil
11	Hussy et al. 2005	Sugarbeet wastewater	Anaerobic digested sludge
12	Noike et al. 2003	Bean curd manufacturing waste	Sewage sludge
13	Arooj et al. 2008	Starch	Anaerobic digested sludge

Appendix B. Propionic acid-to-acetic acid ratio

Chapter 3: Tests using carbohydrate-rich synthetic wastewater as substrate

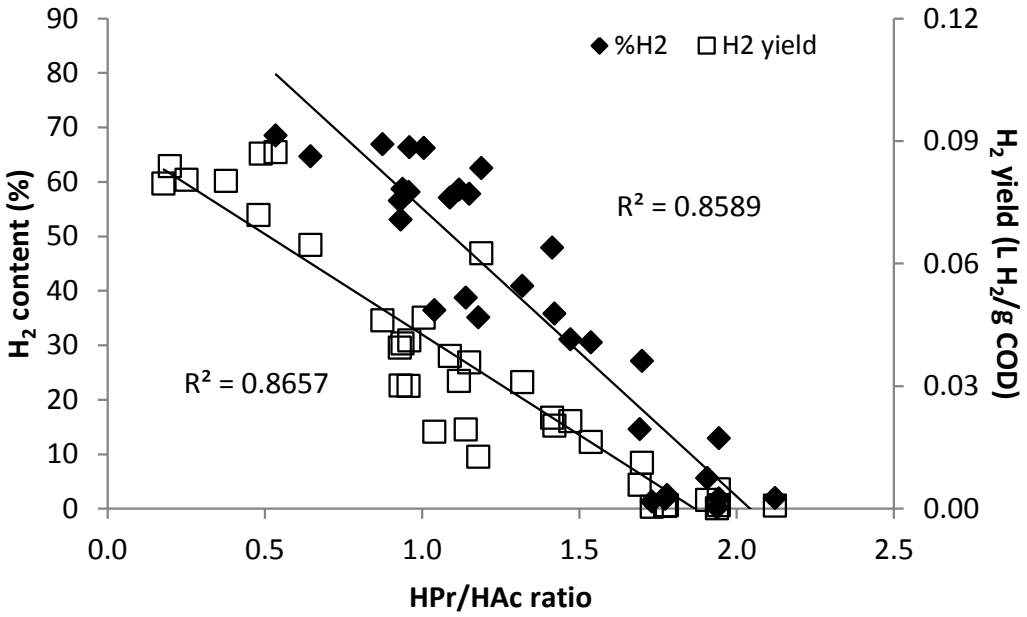


Figure B1. The relationship between hydrogen productivity and the propionic acid-to-acetic acid (HPr/HAc) ratio

Appendix C. Characteristics of products and operational conditions

Chapter 4: Biogas composition, metabolites concentration and hydrogen productivity versus cyclic duration at each pH level (Fixed HRT 24 hr and OLR 10.3 kg/m^3.d)

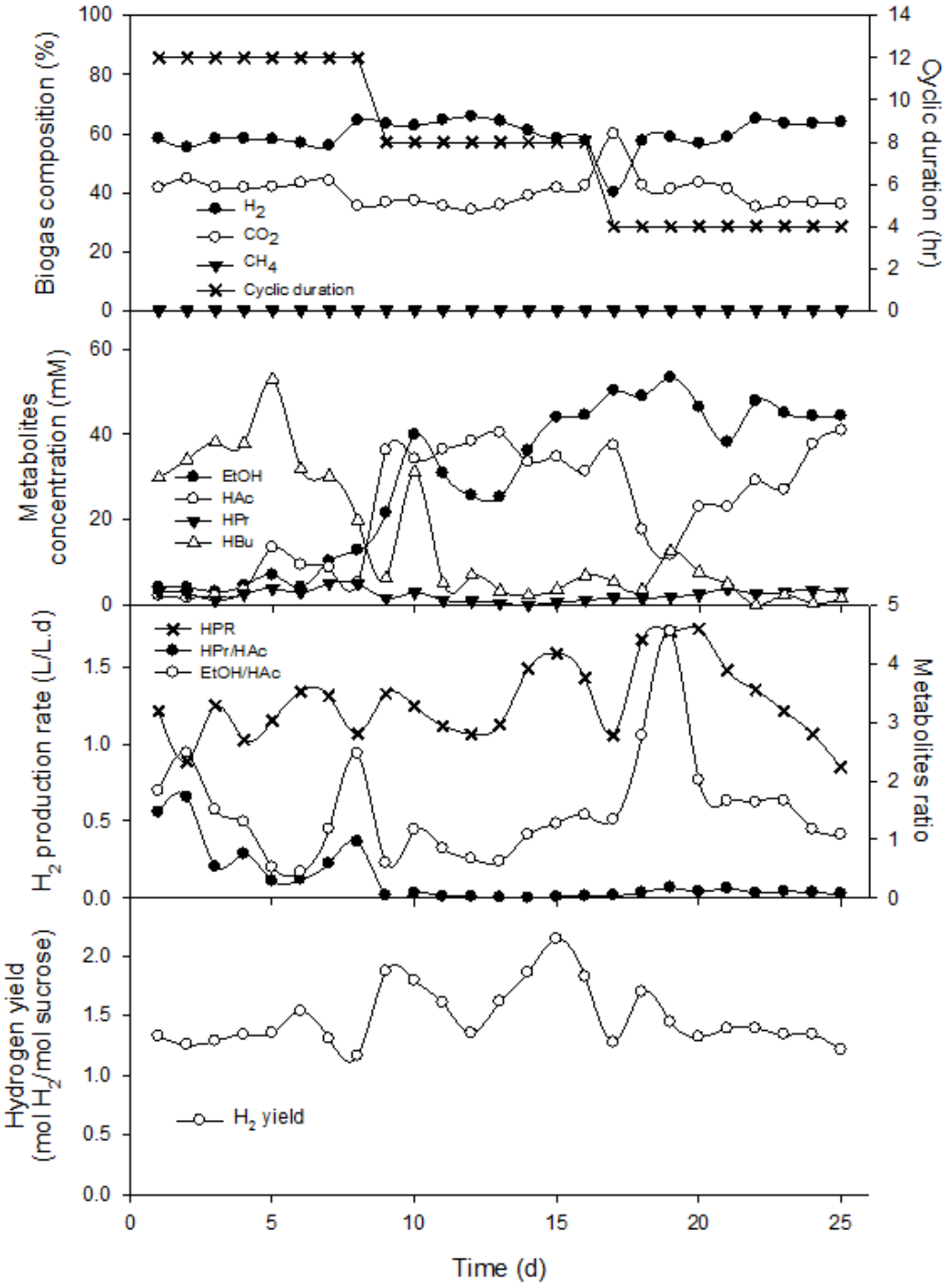


Figure C1. pH 4.0

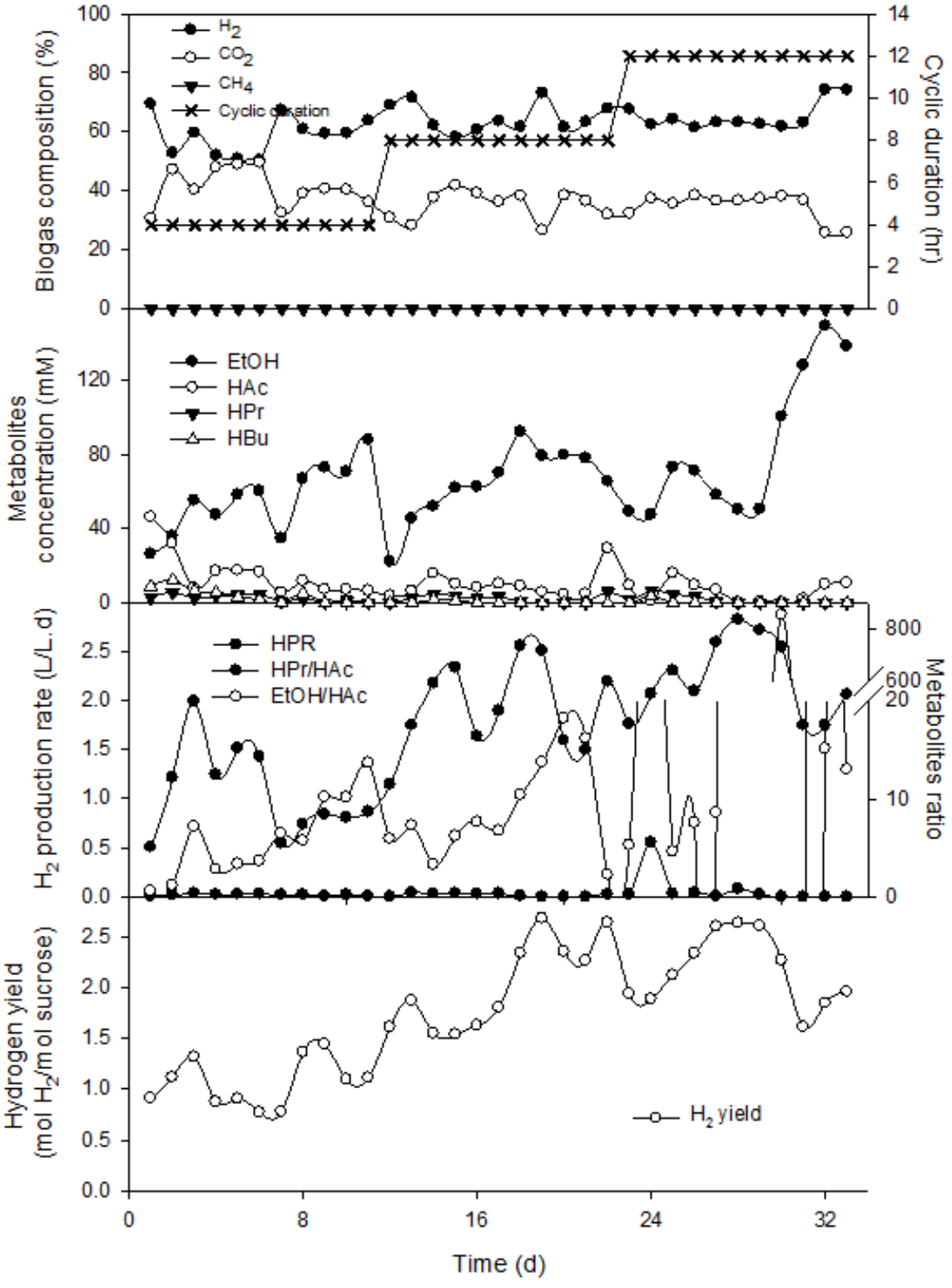


Figure C2. pH 5.0

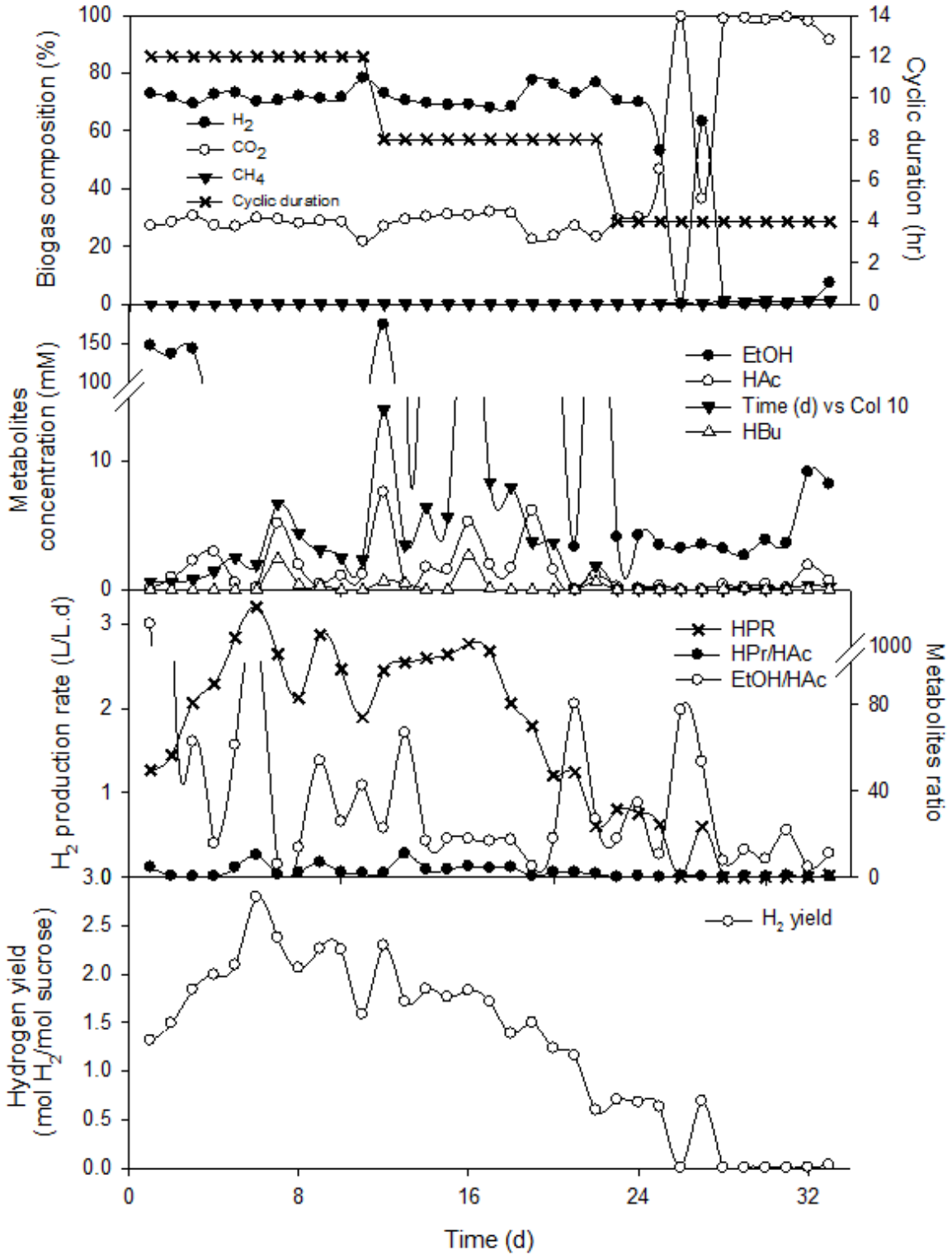


Figure C3. pH 6.0

Appendix D. Characteristics of gas production and soluble metabolite products

Chapter 5: Biogas composition, metabolites concentration and hydrogen productivity versus operational conditions.
During the experiment, a track study was conducted within a cycle in each run in order to build the modified Gompertz model.

Table D1. Characteristics of gas production and SMP (soluble metabolite products) at pH 4.5

Run	HRT	OLR	Biogas composition (%)			HPR	Yield	EtOH	HAc	HPr	HBu
	hr	kg/m^3.d	H_2	CO_2	CH_4	(L H_2/ L reactor.d)	(mol H_2/ mol sucrose)	mM			
1	10	7	59.9 ±8.7	40.1 ±8.7	0.0 ±0.0	0.86 ±0.23	1.43 ±0.29	6.1 ±3.2	12.0 ±3.0	0.8 ±1.5	3.0 ±3.4
4	30	7	53.8 ±9.3	46.2 ±9.2	0.0 ±0.0	1.19 ±0.39	1.43 ±0.39	17.2 ±4.5	21.5 ±5.7	3.4 ±2.5	9.6 ±4.5
3	20	11	57.8 ±8.0	42.2 ±8.0	0.0 ±0.0	2.18 ±0.52	1.29 ±0.25	20.6 ±1.8	29.3 ±2.2	7.3 ±1.1	13.0 ±0.8
9	30	15	45.7 ±21.5	54.3 ±21.5	0.0 ±0.0	0.60 ±0.34	0.52 ±0.29	22.1 ±3.4	33.3 ±8.2	15.6 ±3.0	31.9 ±16.6
2	10	15	73.6 ±4.2	26.4 ±4.2	0.0 ±0.0	1.82 ±0.48	1.48 ±0.38	10.3 ±2.8	9.2 ±3.8	3.4 ±1.9	12.8 ±5.4

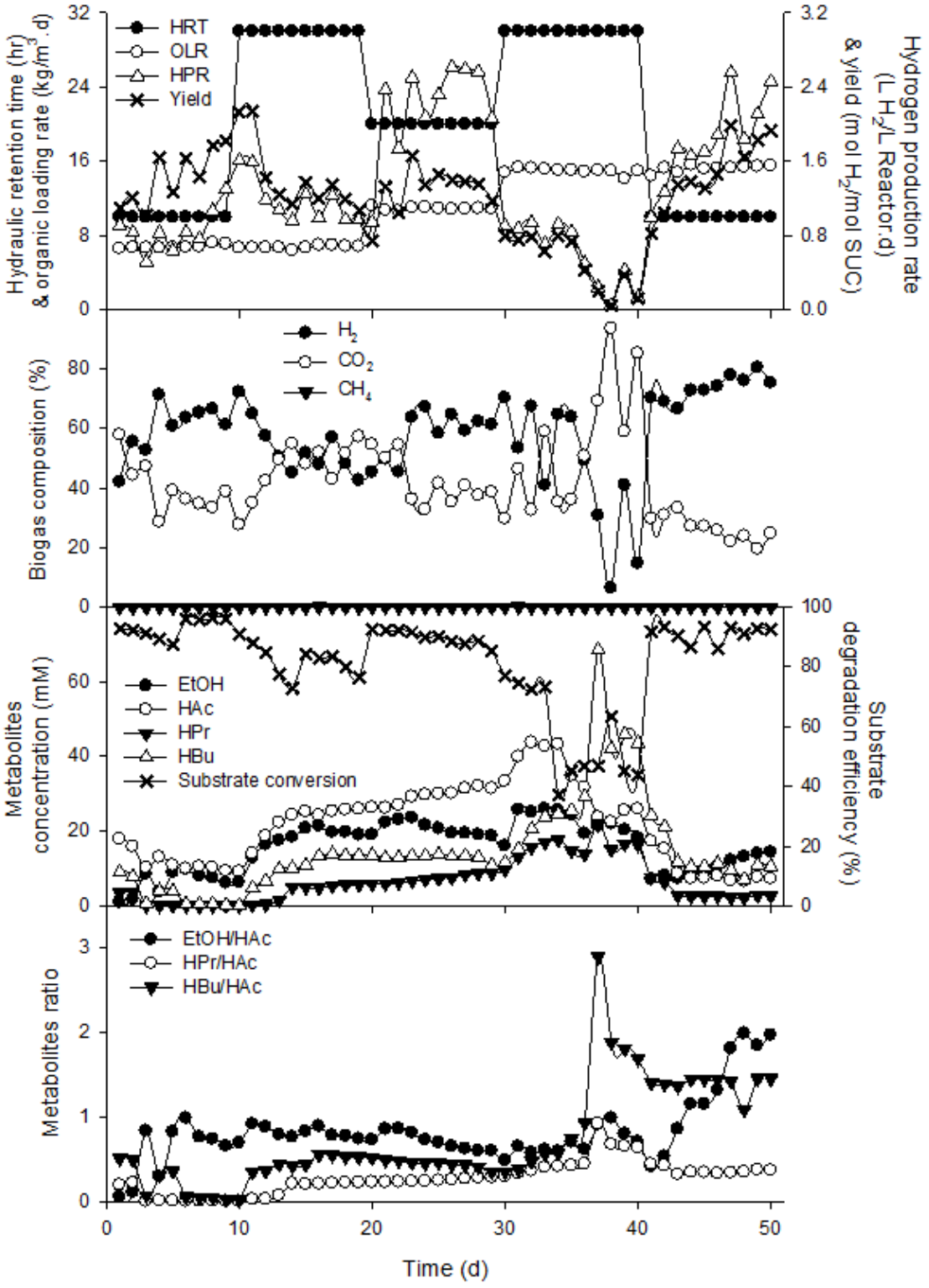


Figure D1. Five runs at pH 4.5, and various combinations of OLR (7.0, 11.0, and 15.0 kg/m^3.d) and HRT (10, 20, and 30 hr)

Table D2. Characteristics of gas production and SMP (soluble metabolite products) at pH 5.0

Run	HRT	OLR	Biogas composition (%)			HPR	Yield	EtOH	HAc	HPr	HBu
	hr	$kg/m^3.d$	H_2	CO_2	CH_4	(L H_2/ L reactor.d)	(mol H_2/ mol sucrose)	mM			
7	20	15	57.8 ±2.9	42.1 ±2.9	0.0 ±0.0	1.53 ±0.36	0.70 ±0.09	23.4 ±4.3	10.9 ±4.1	2.2 ±2.0	8.4 ±2.6
13	30	11	41.1 ±10.8	58.8 ±10.8	0.1 ±0.1	1.09 ±0.36	0.62 ±0.19	24.2 ±1.4	14.5 ±3.2	4.9 ±1.4	8.2 ±2.1
8	20	11	14.3 ±2.3	85.4 ±2.5	0.4 ±0.1	0.41 ±0.10	0.12 ±0.02	8.5 ±4.4	10.1 ±4.4	2.8 ±2.1	3.8 ±2.8
10	20	11	15.3 ±2.4	83.5 ±2.7	1.2 ±0.1	0.34 ±0.13	0.11 ±0.03	5.8 ±1.8	13.1 ±2.9	5.3 ±1.7	1.9 ±0.7
11	20	11	22.0 ±7.4	77.8 ±7.3	0.2 ±0.1	0.38 ±0.14	0.20 ±0.08	8.5 ±1.1	11.5 ±2.1	5.2 ±0.9	1.3 ±0.8
12	20	11	22.4 ±2.3	77.4 ±2.3	0.2 ±0.1	0.41 ±0.05	0.21 ±0.04	12.3 ±1.2	13.1 ±3.1	5.6 ±2.0	2.6 ±1.4
6	20	7	15.9 ±3.7	82.2 ±5.2	1.9 ±0.2	0.41 ±0.19	0.26 ±0.05	5.4 ±0.9	13.9 ±3.7	6.2 ±1.9	2.7 ±1.4
5	10	11	16.1 ±5.5	83.4 ±6.1	0.5 ±0.1	0.24 ±0.08	0.11 ±0.03	3.7 ±0.8	7.2 ±2.8	3.4 ±1.6	0.6 ±0.9

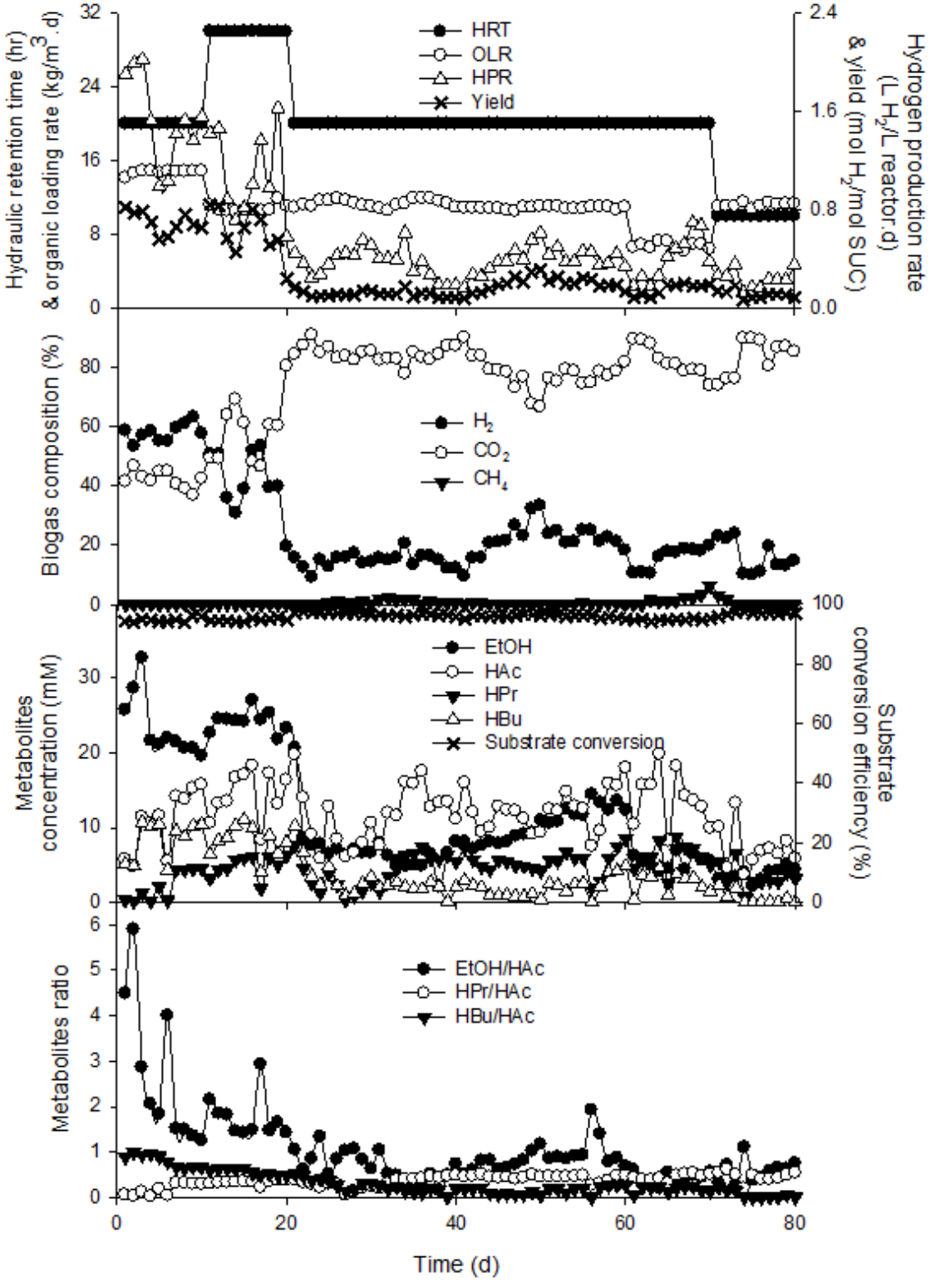


Figure D2. Eight runs at pH 5.0, and various combinations of OLR (7.0, 11.0, and 15.0 kg/m^3.d) and HRT (10, 20, and 30 hr)

Table D3. Characteristics of gas production and SMP (soluble metabolite products) at pH 5.5

	HRT	OLR	Biogas composition (%)			HPR	Yield	EtOH	HAc	HPr	HBu
Run	hr	$kg/m^3.d$	H_2	CO_2	CH_4	(L H_2/ L reactor.d)	(mol H_2/ mol sucrose)	mM			
14	10	7	4.1 ±4.0	95.3 ±4.0	0.6 ±0.7	0.09 ±0.08	0.05 ±0.04	0.2 ±0.2	3.4 ±1.8	1.4 ±0.9	0.4 ±0.7
17	30	7	0.3 ±0.5	87.5 ±8.9	12.2 ±8.9	0.00 ±0.01	0.00 ±0.01	0.4 ±0.4	5.7 ±3.5	4.0 ±2.3	1.6 ±1.5
16	20	11	0.6 ±0.5	77.5 ±6.6	21.9 ±6.9	0.01 ±0.01	0.01 ±0.00	0.4 ±0.3	6.9 ±2.2	5.0 ±1.4	1.8 ±1.1
15	10	15	71.8 ±10.5	27.4 ±10.2	0.7 ±0.3	2.11 ±0.31	0.95 ±0.13	2.7 ±1.2	0.7 ±0.5	0.8 ±0.3	0.1 ±0.1
18	30	15	57.7 ±3.9	41.2 ±4.0	1.0 ±0.3	1.44 ±0.20	0.94 ±0.08	14.0 ±2.4	7.2 ±1.6	4.9 ±0.9	2.9 ±1.3

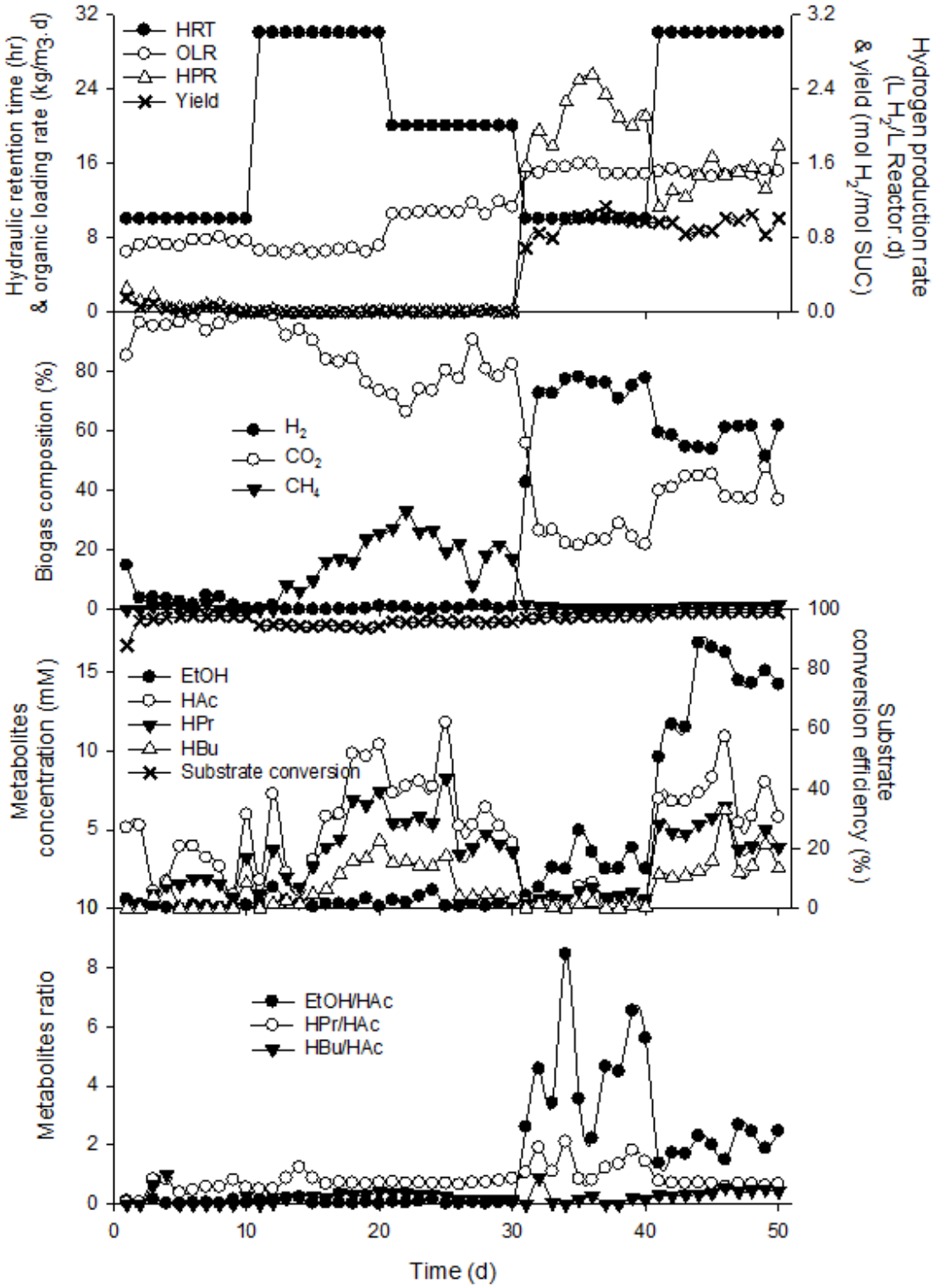


Figure D3. Five runs at pH 5.5, and various combinations of OLR (7.0, 11.0, and 15.0 kg/m^3.d) and HRT (10, 20, and 30 hr)

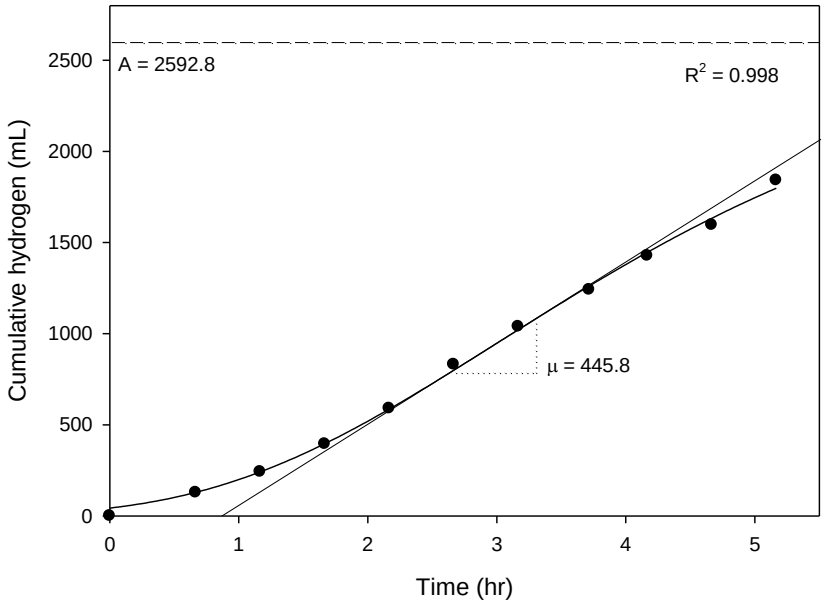
Table D4. Summary of modified Gompertz Model parameters for all runs

Run	pH	OLR ($kg/m^3.d$)	Max. H_2 production potential, A (mL)	H_2 production rate μ_m (mL/hr)	Lag time, λ (hr)
1	4.5	7	1965	343.3	1.6
2	4.5	15	2593	445.8	0.9
3	4.5	11	837	195.4	0.3
4	4.5	7	792	158.9	1.1
5	5.0	11	158.5	19	1.51
6	5.0	7	123.5	25.4	0.31
7	5.0	15	703	206.6	1.1
8	5.0	11	144	32	0.09
9	4.5	15	1132	177	0.4
10	5.0	11	84.5	15.3	0.3
11	5.0	11	125.5	24.4	0.08
12	5.0	11	99.5	17.5	0.29
13	5.0	11	477	108.9	0.7
14	5.5	7	N/A*	N/A	N/A
15	5.5	15	1099	557.2	0.01
16	5.5	11	9.3	2.2	0.9
17	5.5	7	N/A	N/A	N/A
18	5.5	15	987	333.2	0.3

*N/A indicates that the modified Gompertz model was not applicable to the data.

Figure D4. Track study results

Run2: pH 4.5, HRT 10 hr, OLR 15.1 kg/m^3.d



$$y = 2592.8\exp\left\{-\exp\left[\frac{445.8\times e}{2592.8}(0.9-t)+1\right]\right\}$$

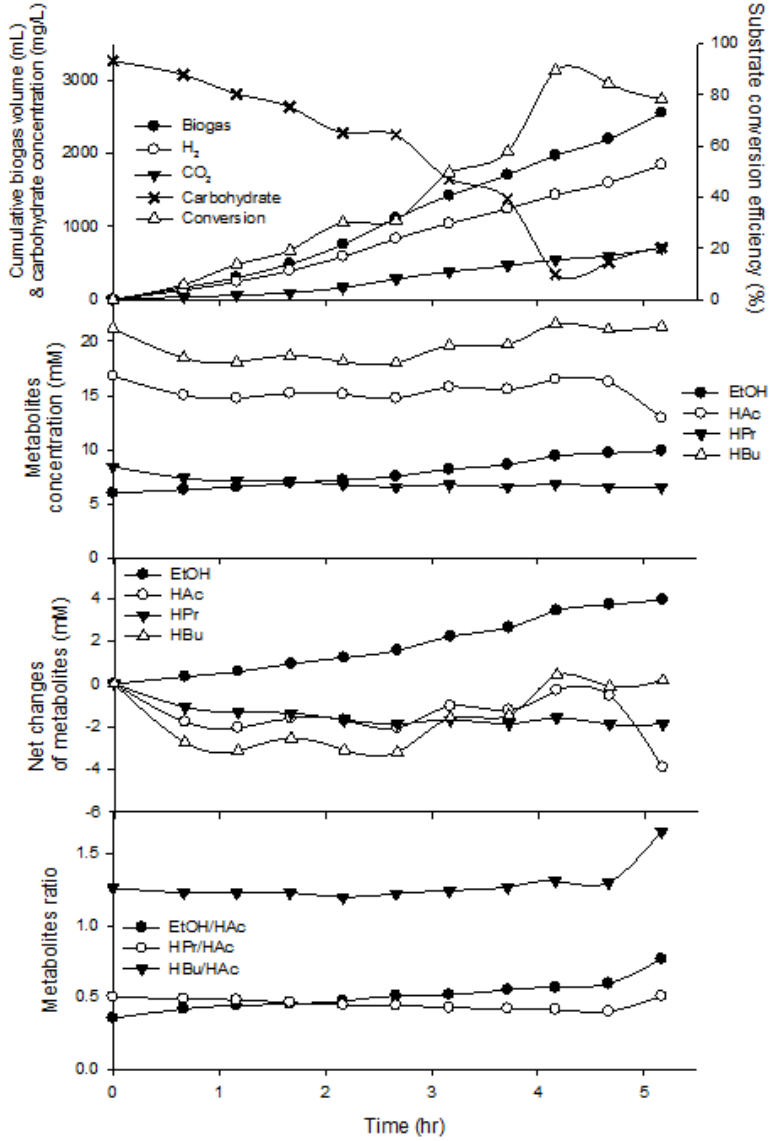
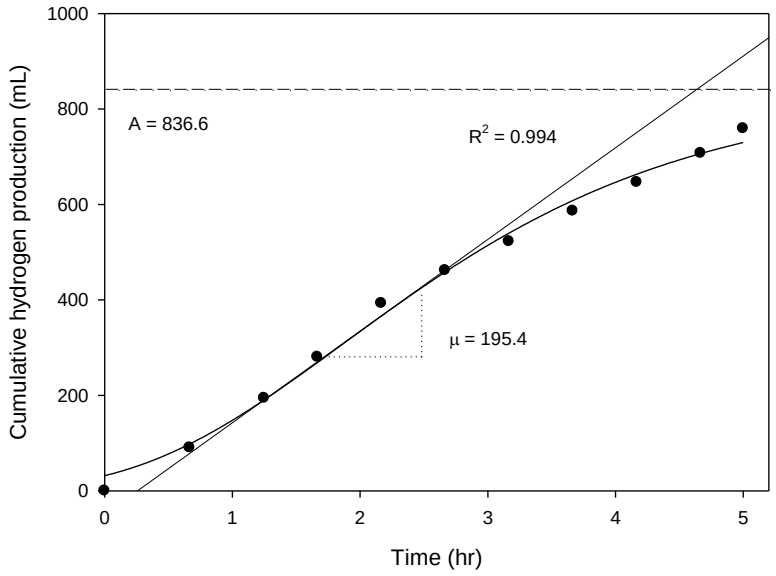


Figure D5. Track study results

Run3: pH 4.5, HRT 20 hr, OLR 11.0 kg/m^3.d



$$y = 836.6 \times \exp\left\{-\exp\left[\frac{195.4 \times e}{836.6}(0.3 - t) + 1\right]\right\}$$

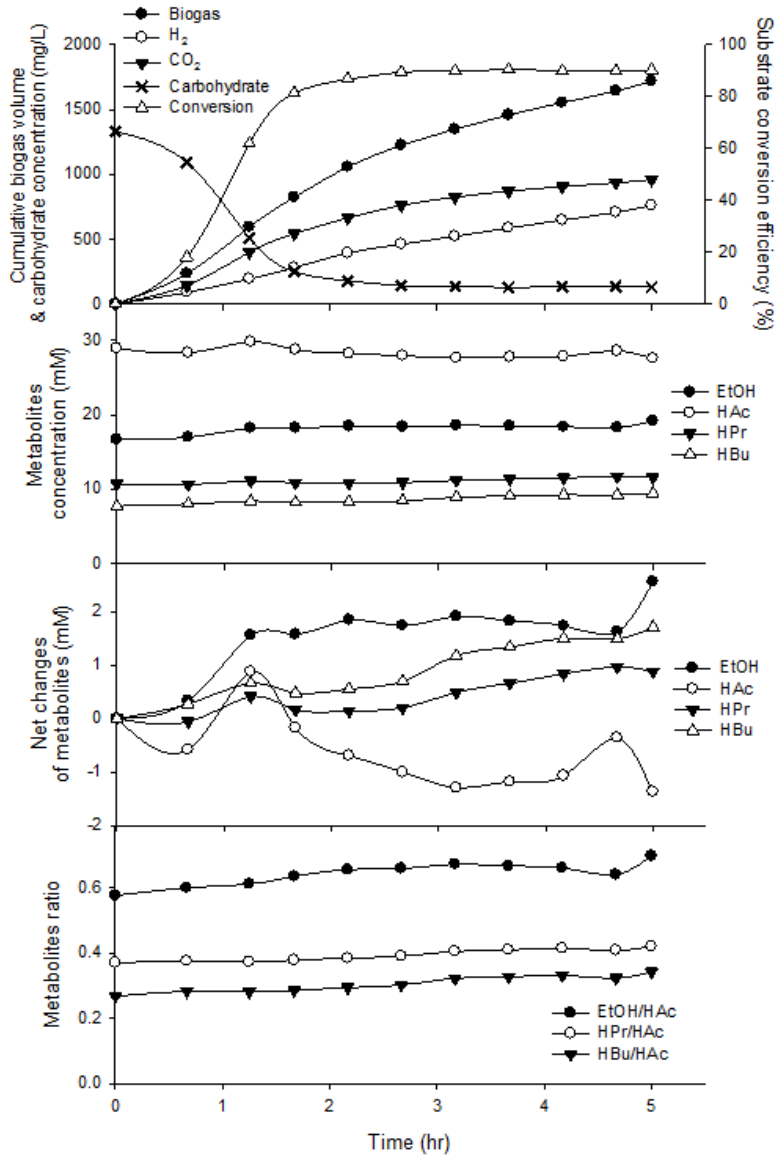
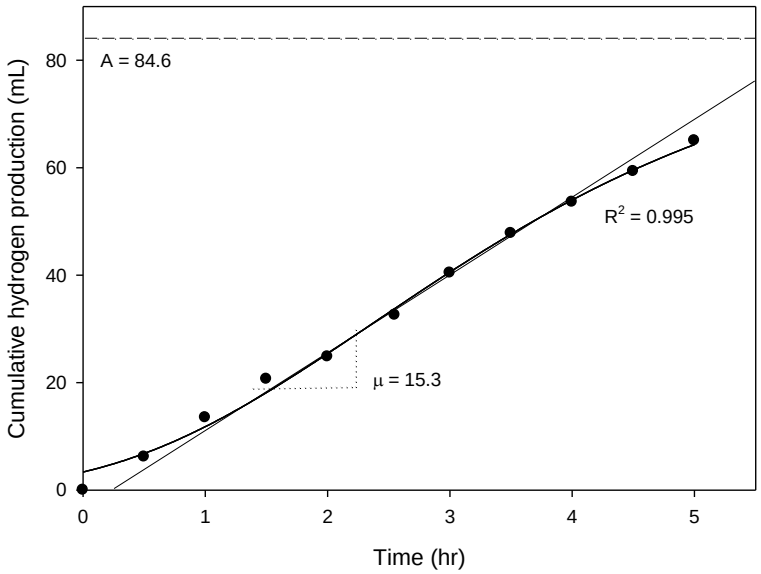


Figure D6. Track study results

Run10: pH 5.0, HRT 20 hr, OLR 11.4 kg/m^3.d



$$y = 84.6 \times \exp\left\{-\exp\left[\frac{15.3 \times e}{84.6}(0.3 - t) + 1\right]\right\}$$

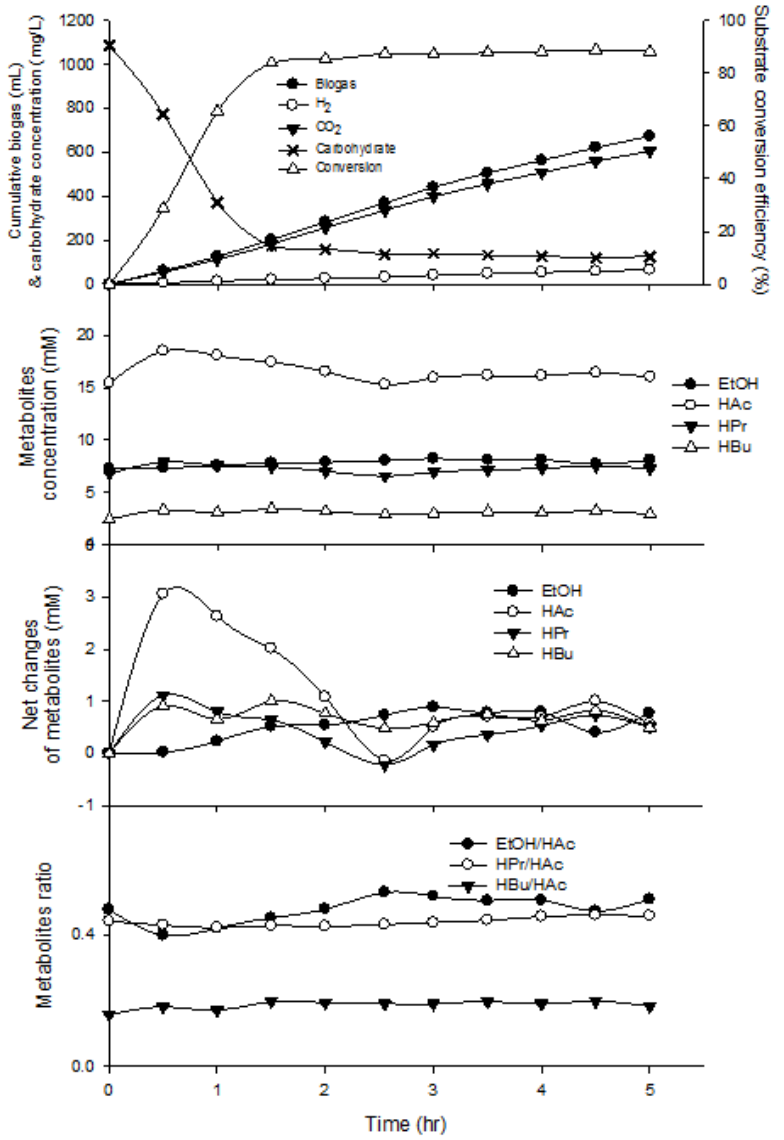
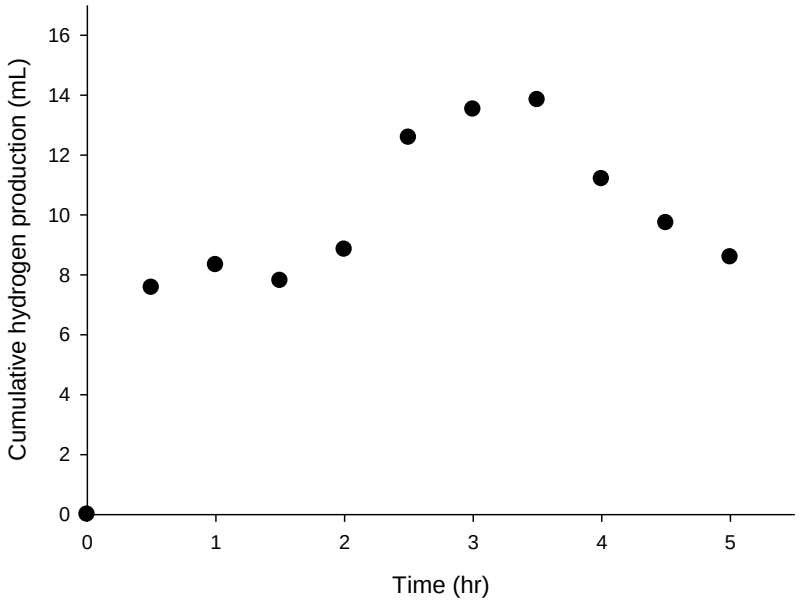


Figure D7. Track study results

Run14: pH 5.5, HRT 10 hr, OLR 7.7 kg/m^3.d



*Unavailable to be applied to modified Gompertz model

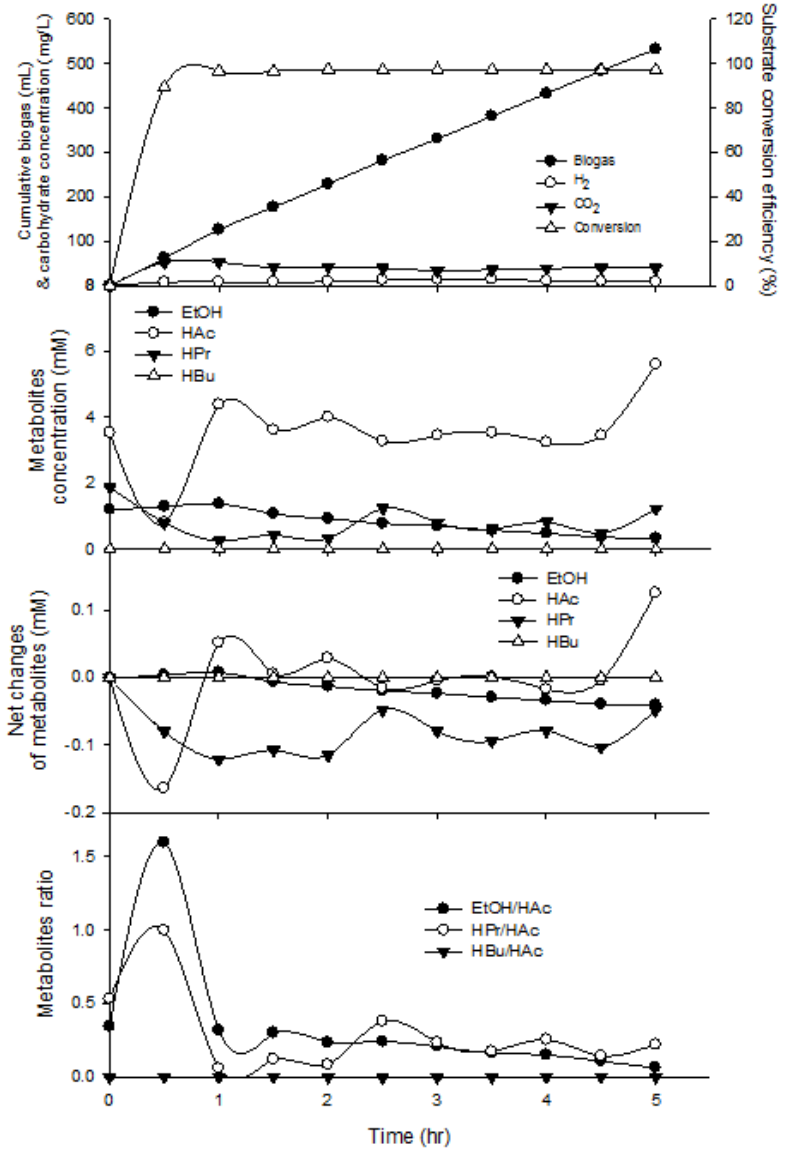
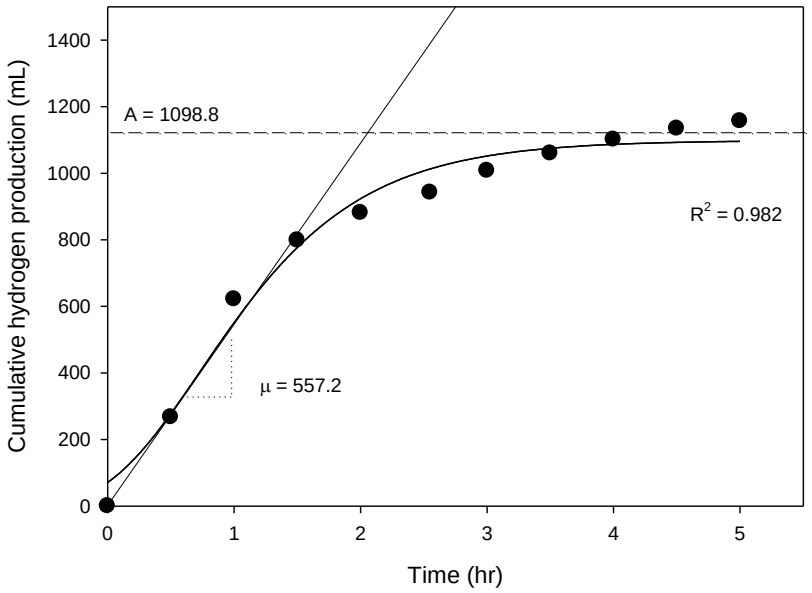
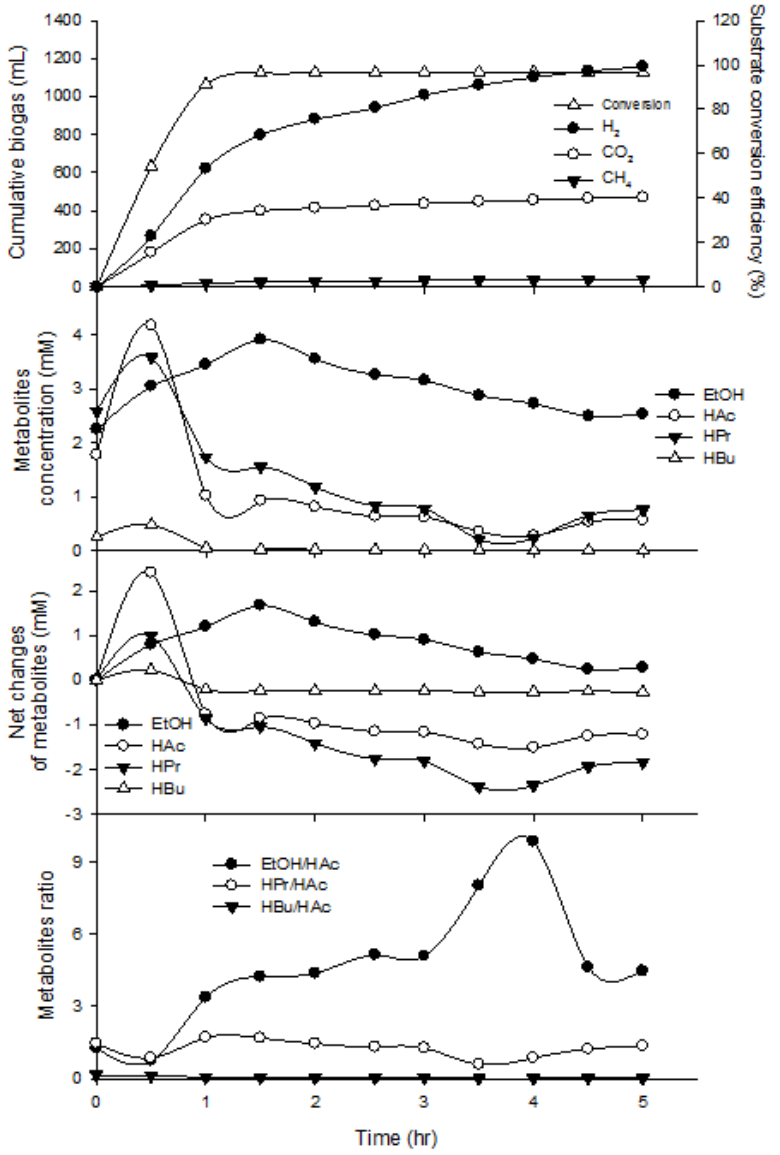


Figure D8. Track study results

Run15: pH 5.5, HRT 10 hr, OLR 15.2 kg/m^3.d



$$y = 1098.8 \exp\left\{-\exp\left[\frac{557.2 \times e}{1098.8}(0.01 - t) + 1\right]\right\}$$



Appendix E. Sample analysis of variance results

Chapter 4: 3×3 factorial

pH (x_1): (4, 5, 6) coded as (-1, 0, 1)

Cyclic duration (x_2): (4, 8, 12 hr) coded as (-1, 0, 1)

Table E1. ANOVA Results for hydrogen production rate and yield

	H_2 production rate (L H_2/L reactor.d)		Yield (mol H_2/mol sucrose)	
R^2	0.941		0.992	
p-value (overall)	0.046		0.002	
	Estimate	Prob>t	Estimate	Prob>t
Intercept	1.984	0.002	2.027	<.0001
pH (x_1)	0.131	0.309	-0.110	0.051
CD (x_2)	0.498	0.019	0.467	0.001
pH^2 (x_1^2)	-0.338	0.167	-0.374	0.009
pH×CD (x_1x_2)	0.555	0.024	0.454	0.002
CD^2(x_2^2)	-0.372	0.139	-0.415	0.006

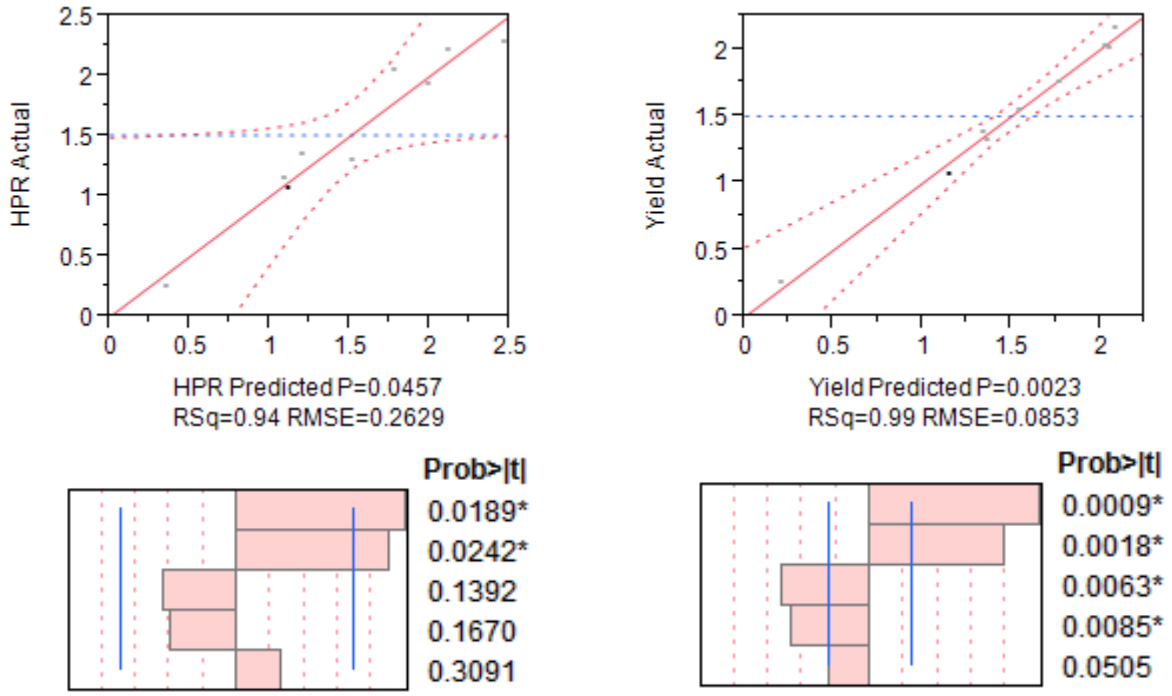


Figure E1. Actual vs predicted values of hydrogen production rate and yield with the coefficient estimations of operating parameters

Chapter 5: Central Composite Design

pH (x_1): (4.0, 4.5, 5.0) coded as (-1, 0, 1)

HRT (x_2): (10, 20, 30 hr) coded as (-1, 0, 1)

OLR (x_3): (7, 11, 15 kg/m^3.d) coded as (-1, 0, 1)

Table E2. ANOVA Results for hydrogen content and yield

	H_2 content (%)		Yield (mol H_2/mol sucrose)	
R^2	0.892		0.892	
p-value (overall)	0.005		0.005	
	Estimate	Prob>t	Estimate	Prob>t
Intercept	20.728	0.002	0.212	0.074
pH (x_1)	-15.619	0.003	-0.420	0.001
HRT (x_2)	-2.689	0.499	-0.051	0.554
OLR (x_3)	17.270	0.002	0.151	0.107
pH×HRT (x_1x_2)	2.009	0.649	0.113	0.259
pH × OLR (x_1x_3)	14.954	0.008	0.338	0.007
HRT×OLR (x_2x_3)	-4.003	0.374	-0.115	0.252
pH^2 (x_1^2)	6.194	0.421	0.385	0.043
HRT^2 (x_2^2)	5.635	0.462	0.103	0.535
OLR^2 (x_3^2)	13.881	0.094	0.163	0.336

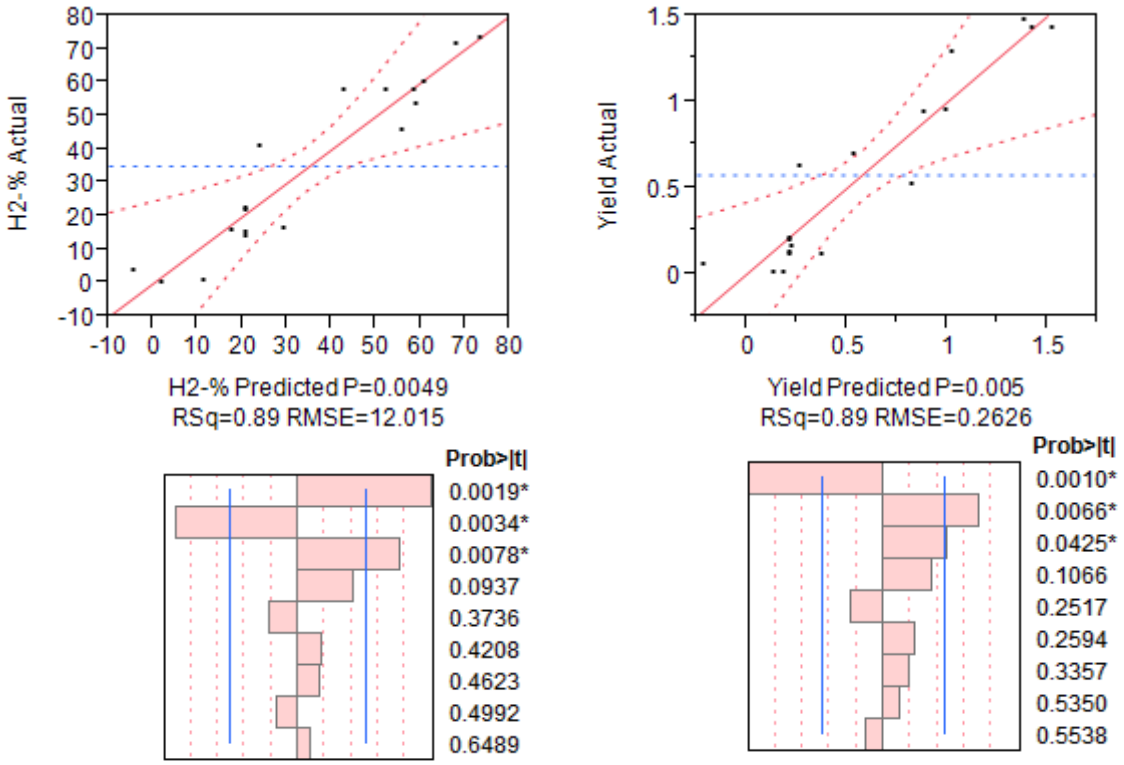


Figure E2. Actual vs predicted values of hydrogen content and yield with the coefficient estimations of operating parameters

Appendix F. Genome sequencing results

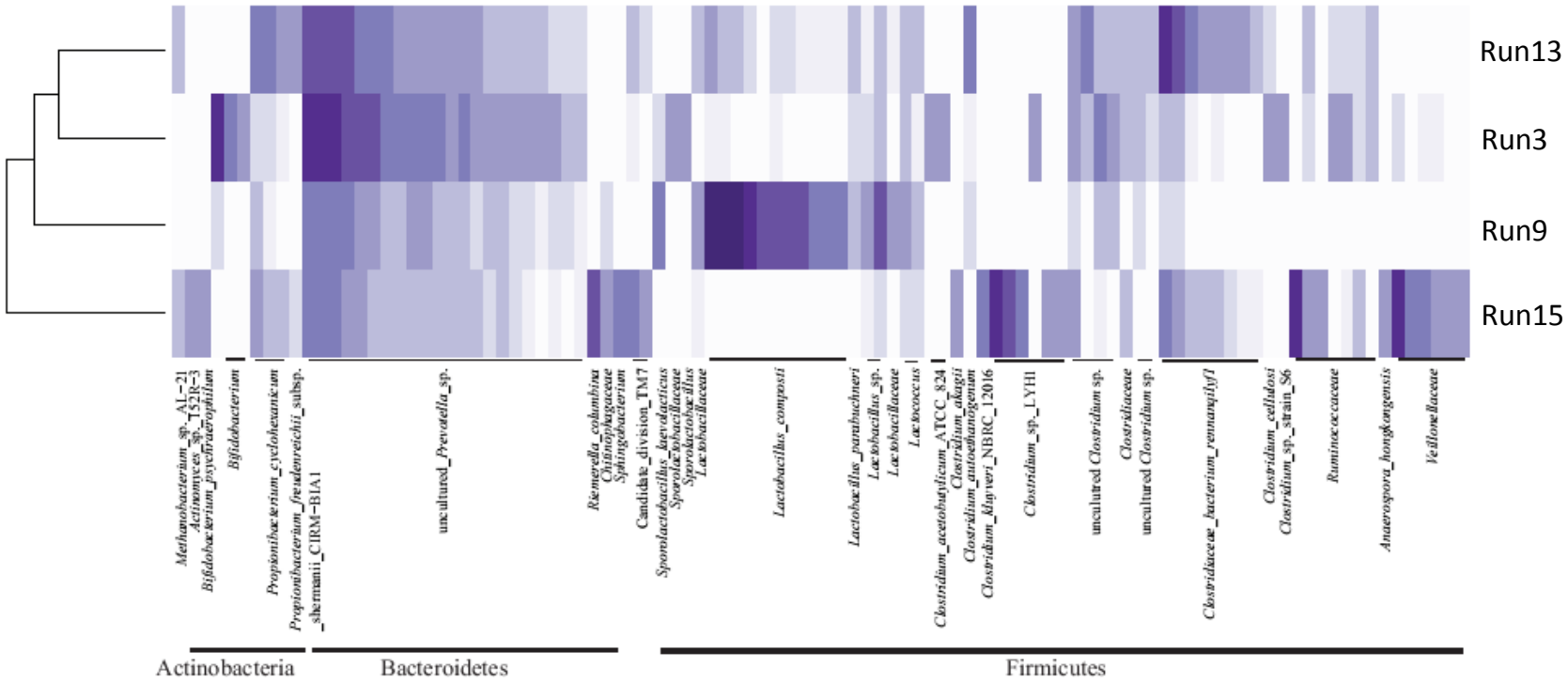


Figure F1. Heatmap of the top 100 most highly represented operational taxonomic units (OTUs) found in the pyrotag sequences from the hydrogen reactor. Intensity of the colour is proportional to the $\log_2$ of the number of reads contained in each OTU normalized with respect to the same number of total reads per sample.

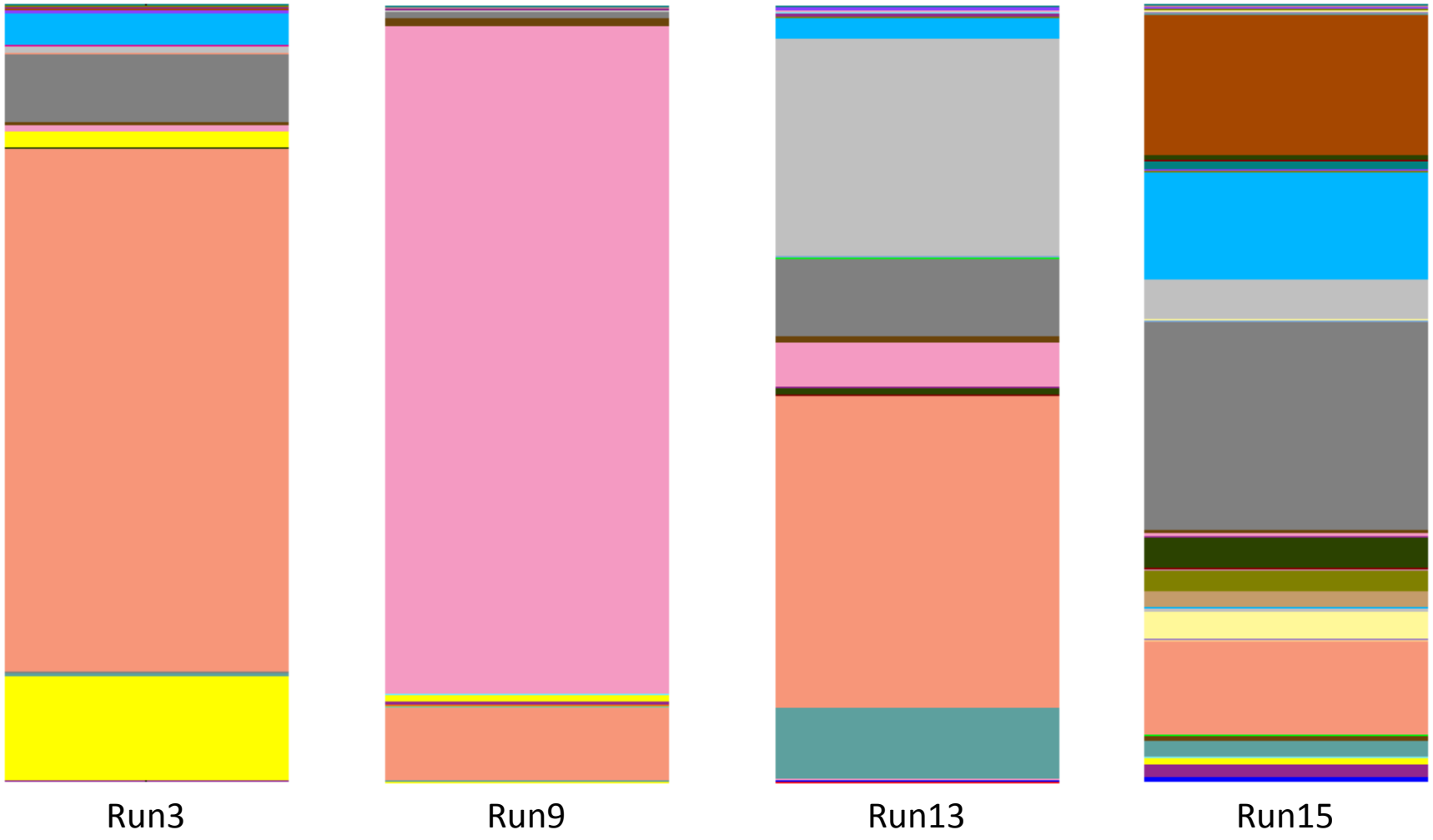


Figure F2. Genus summary from the hydrogen-producing reactor

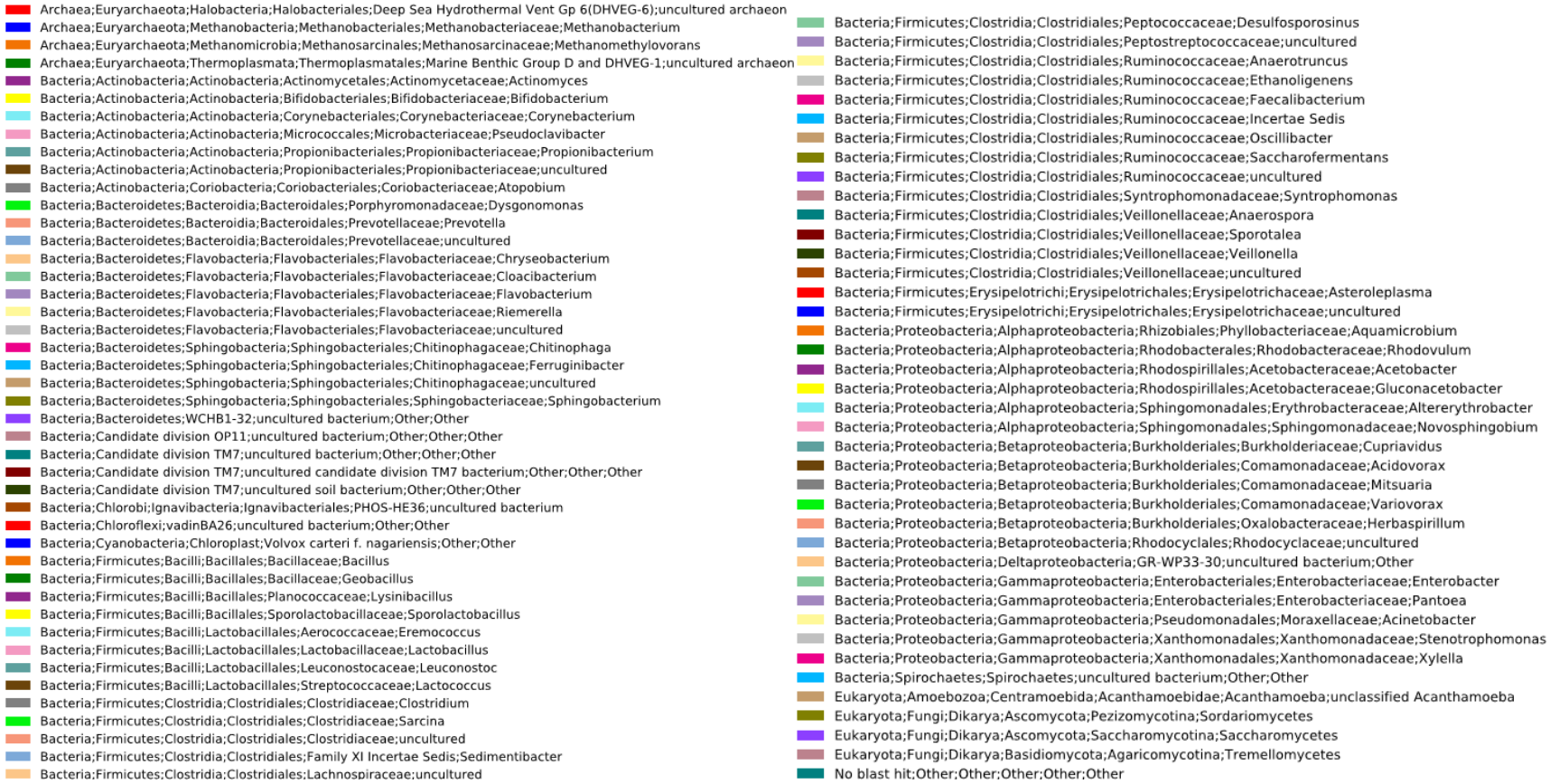


Figure F3. The legend of genus summary