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Impact of chemical stressors on hydrogen metabolism

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IMPACT OF CHEMICAL STRESSORS ON HYDROGEN METABOLISM

By

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DECLARATION OF CO-AUTHOURSHIP/ PREVIOUS PUBLICATION

I. CO-AUTHORSHIP DECLARATION

I hereby declare that this dissertation incorporates material that is the result of joint research:

It incorporates the outcome of laboratory work, which was done by Dr. Subba Rao Chaganti under the supervision of Dr. Daniel Heath; the contribution of co-authors is limited to the provision of microbiological results which are included in chapters 4, 5, 6, 7 and 8 of the dissertation.

In all cases, the experimental design was proposed by the author and approved by Dr. Jerald A. Lalman. The key ideas, primary contributions, engineering laboratory work, data analysis and interpretation, were performed by the author.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my dissertation, and have obtained written permission from each of the co-author(s) to include the above material(s) in my dissertation.

I certify that, with the above qualification, this dissertation, and the research to which it refers, is the product of my own work.

II. Declaration of previous publication

Results from chapters 4, 5 and 8 of this dissertation have been submitted for publication in International Journal of Hydrogen Energy (IJHE), respectively. Draft copy of the manuscript for Chapters 4, 5 and 8 was provided by the author to Dr. Jerald A. Lalman and he has reviewed and provided significant changes with the manuscript for submission. I would like to thank Dr. Jerald A. Lalman in this regard. The results from chapter 5 are accepted for publication in IJHE (Volume 39, Issue 1, pages 249-57; DOI: http://dx.doi.org/10.1016/j.ijhydene.2013.10.084). Results from chapter 8 are also accepted for publication in IJHE and is currently in press, corrected proof; DOI: (http://dx.doi.org/10.1016/j.ijhydene.2014.04.115). No part of this dissertation except chapters 4, 5 and 8 has been published/ submitted for publication.

I certify that the above material describes work completed during my registration as graduate student at the University of Windsor.

I declare that, to the best of my knowledge, my dissertation does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my dissertation, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. I declare that this is a true copy of my dissertation, including any final revisions, as approved by my dissertation committee and the Graduate Studies office, and that this dissertation has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

The effects of chemical stressing agents on H_2 metabolism were evaluated using thermodynamic, biochemical, genomic and statistical methods. The objectives of this study were to examine the role of homoacetogens and hydrogenotrophic methanogens exposed to different stress treatments under various fermentation conditions. Negligible H_2 consumption was observed at mesophilic and thermophilic temperature (at pH 4.5) when combined with the addition of 2 g L^{-1} linoleic acid (LA). Genomic analysis revealed that LA-treated cultures were dominated by *Clostridium* sp. whereas control cultures were dominated by homoacetogens and methanogens. Lauric acid (LUA), LA, fish oil and furfural affected H_2 consumption similarly to BES. The H_2 consumption (%) of the control and chemically treated cultures revealed the following trend: Control > Fish oil = LA = Furfural > BES > LUA. Treatment with different stressing agents also resulted in the formation of diverse fermentation metabolites.

The long-term effects of different culture pretreatments under mesophilic condition resulted in higher mean H_2 yields compared to the yields from cultures incubated at thermophilic condition (after 5 glucose additions). Hydrogen consumption studies using long term stress treated cultures showed lower consumption at thermophilic temperature than at mesophilic temperature. Uptake hydrogenase activities correlated positively with the H_2 consumption data. Genomic analysis indicated that both methanogens and homoacetogens were present in control cultures, but they were absent from the pretreated cultures. Studies conducted in anaerobic sequencing batch reactors revealed that lowering the HRT from 37.5 h to 7.5 h reduced the methane yield and increased the H_2 yield. Higher H_2 yields were obtained in cultures operated at thermophilic temperature compared to mesophilic and psychrophilic temperature using corn stalk (CS) as substrate. Cultures fed CS liquor showed lower levels of specific methanogenic activities than cultures fed pure sugars.

The results from these studies indicate that all the chemical stressing agents investigated were active against H_2 consumers (methanogens in particular). In addition to different stress treatments, proper control of operational parameters such as pH, HRT and

temperature is required to minimize H_2 consumption and maximize H_2 production in dark fermentation process.

DEDICATION

I dedicate this dissertation to my beloved and wonderful family

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Nomenclature

ABFBR	Anaerobic biofilm fluidized bed reactor
Acetyl-CoA	Acetyl Co-enzyme A
A-D	Anderson-Darling
AD	Anaerobic digestion
ADP	Adenosine diphosphate
AGFBR	Anaerobic granular fluidized bed reactor
16S rRNA	16S ribosomal RNA
ATP	Adenosine triphosphate
AnSBR	Anaerobic sequencing batch reactor
AFBR	Anaerobic fluidized bed reactor
BES	Bromoethane sulfonic acid
BOD	Biological oxygen demand
C 12	Lauric acid
C 18:2	Linoleic acid
CH_4	Methane
CIGSBR	Carrier induced granular sludge bed bioreactor
COD	Chemical oxygen demand
CO_2	Carbondioxide
CSTR	Continuously stirred tank reactor
CTAB	Cetyl trimethyl ammonium bromide
EDTA	Ethylene diamine tetra acetic acid
EtOH	Ethanol
FBR	Fluidized bed reactor
Fd_{red}	Reduced ferredoxin

Fe^{2+}	Iron ion (Ferrous)
GC	Gas chromatography
H_2	Hydrogen
H_2/CO_2	Hydrogen/Carbon dioxide
HAc	Acetic acid
HBu	Butyric acid
HCl	Hydrochloric acid
HFr	Formic acid
HLa	Lactic acid
HMF	5-Hydroxy methyl furfural
HPB	Hydrogen producing bacteria
HPLC	High performance liquid chromatography
HPr	Propionic acid
HRT	Hydraulic retention time
IC	Ion chromatography
IC-CSTR	Immobilized cell – continuously stirred tank reactor
K_m	Substrate concentration at half of maximum reaction velocity
LA	Linoleic acid
LCFA	Long chain fatty acid
LUA	Lauric acid
LDH	Lactate dehydrogenase
KOH	Potassium hydroxide
MBR	Membrane bioreactor
MFA	Metabolic Flux analysis

Mg^{2+}	Magnesium ion
MPB	Methane producing bacteria
NaCl	Sodium chloride
NAD^+	Oxidized form of nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
$NADP^+$	Oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
O_2	Oxygen
OD	Optical density
ODW	Oven dry weight
pK_a	Acid dissociation constant
PAC–EGSBR	Powdered active carbon assisted – expanded granular sludge bed reactor
PBR	Packed bed reactor
PCA	Principal component analysis
PrOH	Propanol
SCOD	Soluble chemical oxygen demand
SRB	Sulfate reducing bacteria
SRT	Solids retention time
TBR	Tricking bed reactor
T-RFLP	Terminal fragment length polymorphism
TSS	Total suspended solids
UASBR	Up-flow anaerobic sludge blanket reactor

VFAs	Volatile fatty acids
V_{max}	Maximum hydrogen uptake velocity
VSS	Volatile suspended solids
$\Delta G^{o'}$	Gibbs free energy of the reaction
ΔpH	pH gradient between intracellular and extracellular pH.

CHAPTER 1: INTRODUCTION

1.1 Need for alternative renewable energy

Since the beginning of the 21st century, population growth and industrialization have driven our exponential demand for energy supplies. The depletion of fossil fuel reserves has forced scientists to search for cleaner and renewable alternative fuels (Antonopoulou et al., 2007). Over the past century, fossil fuels have been used to satisfy human needs. However, there is growing concern that the world's fossil fuel reserves are declining and existing inventories will disappear within the next 50 to 100 years. Humans face the possibility of an oil shortage within the next 60 years (Table 1.1). With the increasing concerns of energy security and pollution, hydrogen (H_2) is an emerging energy alternative to fossil fuels. Biodiesel is another emerging renewable energy source while bioethanol production is a well established commercial technology in many industrialized countries.

The potential impact of H_2 use on human society has driven some researchers to evaluate the need for a future H_2 economy (Milciuviene et al., 2006; Winter, 2005). Based on the current energy utilization rate, the demand is projected to outgrow supply by 2050 (Holmes and Jones, 2003). The current global energy consumption is approximately 500 EJ (1 EJ = 10^{18} Joules (J) = 24 million tons of oil consumption) and the energy demand by 2050 is projected to be 600 to 1100 EJ. Solving this looming energy crisis is one of the greatest challenges facing humankind and a solution requires developing sustainable solutions to replace depleting fossil fuel reserves.

Table 1.1 Estimated lifetime for fossil fuels (adapted from (Lodhi, 1997)).

Type of Fossil fuel	Annual consumption rate	Estimated lifetime (years)
Oil	2×10^9 barrels	60
Natural gas	2×10^{12} m^3	120
Coal	3×10^9 metric tons	> 120

1.2 Environmental and health hazards

A major problem with the extensive usage of fossil fuels is their impact on the environment. Global warming and acid rain, global impacts arising from fossil fuel use, have affected the earth's climate and negatively impacted terrestrial and aquatic global ecosystems (Hansen et al., 1981). Fossil fuel combustion produces oxides of carbon, sulfur, and nitrogen, as well as soot and fine-particulate ash. Carbon monoxide (CO) produced along with carbondioxide (CO_2) during the combustion of fossil fuels is also a major contributor to global warming. Global warming has increased the average Earth's surface temperature by 0.5 to 1 °C when compared with data from the 19th century. The increased accumulation of greenhouse gases (GHG) such as CO_2, methane (CH_4), nitrous oxide (N_2O) and chlorofluorocarbons (CFC) has resulted in the depletion of stratospheric ozone. The atmospheric concentrations of CO_2, CH_4 and N_2O have increased by over 31 %, 151 % and 17 % respectively, since the pre-industrial period.

Carbon dioxide accounts for 55 % of anthropic GHG emissions and has a global warming potential (GWP) of 1 (Table 1.2). Methane accounts for about 15 % of anthropic GHG emissions. The major sources of CH_4 emissions (two-thirds) result from human activities, such as farming, mining of natural gas, and also prairie-related activities (e.g. tall grass burning). The remaining one-third of the CH_4 emissions is attributed to plant and animal matter fermentation. Methane has a GWP value of 25, which indicates it is 25 times greater when compared to CO_2. CFC has the highest GWP when compared to the GWP of other GHG. The economic impact due to the environmental damage caused by GHG emissions was estimated at $ 236 billion per year or $ 460 per capita per year (Barbir et al., 1990). According to Barbir et al. (1990), the estimated cost is forecasted to grow higher with increasing environmental damage caused by GHG emissions.

Fossil fuel combustion has exerted a deleterious effect on human society and our well-being. About 40 % of annual deaths are linked directly to environmental degradation (Pimentel et al., 2007) and poor air quality due to fossil fuel combustion. The air-borne particulates released from automobile exhaust cause 20 % of the lung cancer deaths in the

USA (Pearce, 2002). Air pollution annually accounts for about 3 million deaths worldwide (WHO, 2002).

1.3 Hydrogen as a green fuel

Hydrogen (H_2) can be produced from both non-renewable (e.g. coal, nuclear energy) and renewable (e.g. sun, water, winds, biomass, tides) energy sources. Utilization of non-renewable resources to produce H_2 generates byproducts which are not environmentally friendly. In comparison to using non-renewable feedstocks, H_2 production from renewable residues have lesser impacts on the environment. Ultimately, the research goal is to develop and use H_2 as an energy carrier from renewable feedstocks (Da Silva et al., 2005; Sherif et al., 2005; Tsai, 2005). Hydrogen generation using renewable energy sources has been the subject of basic and applied research for several decades. Biological H_2 production employs microorganisms to produce H_2. Some of these microorganisms have the ability to split water (H_2O) into H_2 and oxygen (O_2) molecules while others have the ability to biologically degrade organic materials into H_2 (Levin et al., 2004).

Unlike hydrocarbons, H_2 is a clean (Kovács et al., 2006) and environmentally friendly fuel (Shin et al., 2010). Biohydrogen production is carbon neutral and water is the only combustion byproduct. Due to its low density, H_2 has a high energy value (122 kJ g^{-1}) which is 2.75 times higher than other hydrocarbons (Han and Shin, 2004). Hydrogen can be made from renewable sources such as organic wastes and lignocellulosics and it can be used directly in fuel cells to produce electricity (Benemann, 1996; Lay et al., 1999). Hydrogen has been an unrealized "fuel for the future" for over 30 years, but there are indications that H_2 is finally becoming an important component of the global energy economy (Logan et al., 2002). Hydrogen gas is widely used in various industries for diverse purposes, such as the hydrogenation of fats and oils to produce margarine in the food industry, and the desulfurization and reformulation of gasoline in oil refineries (Kapdan and Kargi, 2006). Hydrogen has found a place in fueling residential housing and is also used as a fuel in buses. Hydrogen has been manufactured from non-renewable resources such as natural gas or petroleum hydrocarbons, using steam reformation.

However, in order for H_2 to become a sustainable and greener energy source, it should be produced microbially.

Table 1.2 Key green house gases and their global warming potential.

GHG	Contribution towards anthropic GHG emission (%)	Global warming potential (GWP)
CO_2	55	1[b]
CH_4	15	25[c]
N_2O	5	298[c]
CFC[a]	15	140 to 23900[c]
Ozone	10	NA[d]

Note: [a]CFC = Chlorofluorocarbon; [b]GWP of CO_2 is taken as the reference gas to study the GWP of other key gases; [c]GWP of gas is relative to CO_2; [d]NA = not available.

Biological H_2 production employs different groups of microorganisms that can be classified into: (1) cyanobacterial and algal cultures that produce H_2 via biophotolysis (Asada and Miyake, 1999), (2) photosynthetic bacteria that produce H_2 by photo-fermentation of organic materials, and (3) dark fermentative bacteria that generate H_2 as an intermediate in the anaerobic digestion of organic materials (Hallenbeck and Benemann, 2002). Photosynthetic organisms have the ability to use light energy and split water into H_2 and oxygen (O_2). Hydrogen can also be produced using photosynthetic or chemosynthetic fermentative bacteria. Some species of cyanobacteria are able to utilize organic compounds under dark conditions (i.e., without light) and generate H_2 by anaerobic fermentation (Carrieri et al., 2008).

Anaerobic fermentative bacteria can produce H_2 without using photo-energy through dark fermentation (Shin et al., 2010), which reduces the production cost of H_2 when compared with the cost of light fermentation processes. Hydrogen can also be produced from low value woody organic residues containing celluloses and hemi-celluloses. Non-woody materials (agriculture crops) containing starches can also serve as feedstocks for producing H_2. The maximum theoretical H_2 yield is 12 mol H_2 mol^{-1} hexose if all the electron equivalents are utilized, 4 mol H_2 mol^{-1} hexose if acetate is the only reduced carbon byproduct or 2 mol H_2 mol^{-1} hexose if butyrate is the only reduced carbon byproduct (Logan et al., 2002; Nandi and Sengupta, 1998).

1.4 Biological hydrogen production methods

Hydrogen can be generated using dark fermentation, light fermentation, water photolysis (by both direct and indirect methods) or by using a combination of dark fermentation and light fermentation. In laboratory scale studies, bio-hydrogen (bio-H_2) can be produced continuously (Manish and Banerjee, 2008). However, there are several bottlenecks when applying these methods to producing H_2 on a large scale (Kopke et al., 2010; Krupp and Widmann, 2009; Tian et al., 2010; Vatsala et al., 2008). Bio-H_2 production is mainly carried out using bacteria that contain hydrogenases which catalyze Eq. 1.1.

$$2\ H^+ + 2\ e^- \leftrightarrow H_2 \qquad (1.1)$$

Three hydrogenase enzymes responsible for molecular H_2 production include nitrogenase, Fe-hydrogenase, and Ni-Fe-hydrogenase.

1.4.1 Biophotolysis

The photo-fermentation process uses the enzyme, nitrogenase, to produce H_2 under nitrogen-deficient conditions, whereas Fe-hydrogenase is used during biophotolysis. During indirect biophotolysis, the H_2 and O_2 evolving reactions are carried out separately, and are coupled to CO_2 fixation or evolution. Among the strains of bacteria that can perform biophotolysis, cyanobacterial strains are the only bacteria which can mediate oxygenic photosynthesis. During oxygenic photosynthesis, they harvest light energy and store it within the cell in the form of carbohydrates. Under certain conditions, these microorganisms use carbohydrates to make produce H_2 (Allahverdiyeva et al., 2010). The overall mechanism of cyanobacterial hydrogen production is shown in Eq. 1.2 and 1.3.

$$12\ H_2O + 6\ CO_2 + h\nu \rightarrow C_6H_{12}O_6 + 6\ O_2 \qquad (1.2)$$

$$C_6H_{12}O_6 + 12\ H_2O + h\nu \rightarrow 12\ H_2 + 6\ CO_2 \qquad (1.3)$$

Where $h\nu$ represents light energy (h is Planck's constant and ν is the frequency of light).

1.4.2 Photo fermentation

Photosynthetic bacteria are able to produce H_2 under nitrogen deficient conditions. They are unable to split water molecules on their own, so they use simple organic compounds, such as acetic acid, as the electron donor for splitting water molecules. These electron equivalents are transferred to the nitrogenase enzyme using ferredoxin. Under nitrogen deficient conditions, the nitrogenase enzyme produces H_2 gas by reducing protons using energy in the form of ATP. The overall mechanism for H_2 production by photo fermentation is given below (Eq. 1.4).

$$C_6H_{12}O_6 + 6\ H_2O + h\nu \rightarrow 12\ H_2 + 6\ CO_2 \qquad (1.4)$$

1.4.3 Dark fermentation

The dark fermentation process is one of the most widely used methods for H_2 production because the rate of H_2 production is greater when compared to other methods. Facultative anaerobes, such as *Enterobacter*, are generally employed for producing H_2 using dark fermentation conditions. However, maintaining a strict anaerobic environment requires adding reducing equivalents. Many researchers have studied H_2 production using fermentative bacteria, such as the *Clostridium* species (Jo et al., 2008), *Enterobacter* species (Shin et al., 2007) and *Escherichia* species (Yoshida et al., 2005). Hydrogen production by dark fermentation is usually mediated via the degradation of pyruvate (Hallenbeck, 2009; Hallenbeck and Benemann, 2002). Pyruvate degradation is performed with the assistance of either pyruvate-formate lyase or pyruvate ferredoxin oxido-reductase.

1.5 Major bottleneck in dark fermentative H_2 production

The principal bottleneck in H_2 production via dark fermentation is due to the syntrophic association between H_2 consumers and H_2 consumers. In order to maximize H_2 production, the selective inhibition of H_2 consuming consortia is necessary. The major H_2 consumers present in mixed anaerobic cultures are homoacetogens, hydrogenotrophic methanogens and sulfate reducers. Methanogens out-compete sulfate reducers in environments that are limited in sulfate. Successful H_2 production via dark fermentation

involves selective inhibition of all types of H_2 consumers to prevent H_2 loss in dark fermentation. An improved understanding inhibiting homoacetogenic and methanogenic growth will be useful in achieving higher H_2 yields.

1.6 Research objectives

The main research objective of the work presented in this thesis is to examine the activity of H_2 consumers (methanogens and homoacetogens) under different stress conditions. Studies were conducted in batch reactors (160 mL with a working volume of 50 - 75 mL) and in sequencing batch reactors (with a working volume of 5 L). The effects of different types of process parameters, such as pH, temperature and hydraulic retention time (HRT), on H_2 production and consumption were studied to evaluate the roles of H_2 consumers in mixed anaerobic culture. In order to scale-up H_2 production to an industrial scale, the substrates used for H_2 production must be derived from cheap, renewable sources such as lignocellulosic biomass. Throughout the thesis, the main focus was to study changes observed in diversity and composition of the mixed culture under various conditions, and correlation of these changes with the by-products of fermentation. To achieve these objectives, the research was divided into five phases:

Phase I: Statistical optimization of hydrogen consumption in mixed anaerobic culture using the Box- Behnken Design

The effects of multiple process parameters were examined using response surface methodology (RSM) tools such as the Box-Behnken Design (BBD). The combined effects of key process parameters, such as pH (4.5, 6.0, and 7.5), temperature (21 °C, 37 °C, and 53 °C) and various linoleic acid (LA) concentrations (0, 1000, and 2000 mg L^{-1}), on H_2 metabolism were studied in batch reactors containing mixed anaerobic cultures. The results of a principal component analysis (PCA) of the metabolite by-products and microbial diversity were used to understand the process performance under different stress conditions.

Phase II: Comparison of specific and non-specific chemical inhibitors on hydrogen metabolism

This phase of the study compared the effectiveness of different types of chemical stressors such as 2-bromoethane sulfonate (BES), linoleic acid (LA), lauric acid (LAU), furfural, and fish oil (which is rich in eicosapentanoic acid (EPA) and docosahexanoic acid (DHA)) on the inhibition of H_2 consumption in mixed anaerobic cultures. The relative effectiveness of different types of chemical stressors was compared using PCA of fermentation metabolites together with PCA of microbial diversity after exposure to different types of chemicals. These batch experiments were conducted using batch reactors at an initial pH of 5.5 and cultures maintained at 37 ^{o}C.

Phase III- Long-term impact of stressing agents on fermentative H_2 production at mesophilic (37 °C) and thermophilic (55 °C) temperatures.

Pretreatment is used to selectively enrich spore-forming H_2 producing communities in mixed culture. A detailed analysis (flux balance analysis (FBA), hydrogenase activity assay and microbial diversity analysis) of the long-term effects for different pretreatment methods on H_2 consumers applied at mesophilic or thermophilic temperature ranges has not been reported. In order to examine the long-term effects of different pretreatment methods on H_2 production, repeated cultivations (each lasting for 96 hrs) were conducted using batch reactors. Acid, alkali, heat, loading shock, BES and LA pretreatment were compared for their effectiveness on H_2 production over long periods (25 days). Pure glucose was used as the substrate for the different pretreatment condition. The initial pH was maintained at 5.5 before the initiation of the experiment. The hydrogenase activities (both H_2 uptake and H_2 evolution) were performed for the long term stress treated cultures (H_2 fed cultures). The FBA together with the PCA of metabolites and population diversity observed for the 1st and 5th glucose feedings were used to study the activity of homoacetogens and methanogens over the experimental period. In order to study the long-term effects of the various pretreatment methods on H_2 consumption, the inocula from the 5th glucose feeding were used to conduct H_2 consumption studies. PCA of the microbial diversity observed after exposure to different stressors were conducted to

compare the long-term efficacy of different pre-treatments on suppressing microbial H_2 consumption.

Phase IV - Effect of HRT and LA on mesophilic H_2 production in an anaerobic sequencing batch reactor (ASBR) using glucose as the substrate.

The effects of hydraulic retention time (HRT) and LA treatment on mesophilic H_2 production was studied in ASBR using glucose as the substrate. The main purpose of this phase of the study is to quantify the effect of HRT combined with LA treatment on H_2 production. Flux balance analysis (FBA) was conducted using the fermentation metabolites collected under each experimental condition to assess the substrate electron equivalents diverted to homoacetogens and methanogens. Changes in the composition of the microbial community for each experimental condition were analyzed using terminal restriction fragment length polymorphism (T-RFLP) analysis. The PCA of fermentation metabolites and microbial diversity were used to evaluate the effects of HRT and LA on H_2 metabolism.

Phase V – Statistical optimization of H_2 production using steam exploded corn stalk hydrolysate using a mixed anaerobic culture in anaerobic sequencing batch reactor

This phase evaluated the feasibility of using biomass residues, such as corn stalks, for H_2 fermentation using mixed culture in batch and sequencing batch reactors. This phase of a steam exploded experimentation was carried out in anaerobic sequencing batch reactors (ASBRs) using corn stalk liquor as the feedstock. The effect of pH (4.5, 5.5, and 6.5), temperature (21, 37, and 53 °C) and HRT (6h, 12h, and 18 h) were studied using a fractional factorial design (FFD). Quantification of homoacetogenic and methanogenic electron fluxes was also carried out using FBA. In order to study the effect of these process parameters on H_2 consumption, specific methanogenic activity (SMA) tests were conducted in batch reactors using the inocula samples collected at the end of each experimental condition. This study provided information regarding the effect of fermentation inhibitors on the activity of H_2 consumers (particularly methanogens) in mixed anaerobic cultures.

1.7 References:

Allahverdiyeva, Y., Leino, H., Saari, L., Fewer, D.P., Shunmugam, S., Sivonen, K., Aro, E.M. (2010). Screening for biohydrogen production by cyanobacteria isolated from the Baltic Sea and Finnish lakes. *Int J Hydrogen Energy*, **35**(3), 1117-27.

Antonopoulou, G., Ntaikou, I., Gavala, H.N., Skiadas, I.V., Angelopoulos, K., Lyberatos, G. (2007). Biohydrogen production from sweet sorghum biomass using mixed acidogenic cultures and pure cultures of *Ruminococcus albus*. *Global NEST J*, **9**(2), 144-51.

Asada, Y., Miyake, J. (1999). Photobiological hydrogen production. *J Biosci Bioeng*, **88**(1), 1-6.

Barbir, F., Veziroglu, T.N., Plass, H.J. (1990). Environmental damage due to fossil fuels use. *Int J Hydrogen Energy*, **15**(10), 739-49.

Benemann, J. (1996). Hydrogen biotechnology: Progress and prospects. *Nature Biotechnol*, **14**(9), 1101-3.

Carrieri, D., Ananyev, G., Dismukes, G.C. (2008). Renewable hydrogen production by cyanobacteria: Nickel requirements for optimal hydrogenase activity. *Int J Hydrogen Energy*, **33**(8), 2014-22.

Da Silva, E.P., Neto, A.J.M., Ferreira, P.F.P., Camargo, J.C., Apolinario, F.R., Pinto, C.S. (2005). Analysis of hydrogen production from combined photovoltaics, wind energy and secondary hydroelectricity supply in Brazil. *Solar Energy*, **78**(5), 670-677.

Hallenbeck, P.C. (2009). Fermentative hydrogen production: Principles, progress, and prognosis. *Int J Hydrogen Energy*, **34**(17), 7379-89.

Hallenbeck, P.C., Benemann, J.R. (2002). Biological hydrogen production; fundamentals and limiting processes. *Int J Hydrogen Energy*, **27**(11-12), 1185-93.

Han, S.K., Shin, H.S. (2004). Biohydrogen production by anaerobic fermentation of food waste. *Int J Hydrogen Energy*, **29**(6), 569-77.

Hansen, J., Johnson, D., Lacis, A., Lebedeff, S., Lee, P., Rind, D., Russell, G. (1981). Climate impact of increasing atmospheric carbon-dioxide. *Science*, **213**(4511), 957-966.

Holmes, B., Jones, N. (2003). Brace yourself for the end of cheap oil. *New Sci*, **179**(2406), 9-10.

Jo, J.H., Lee, D.S., Park, J.M. (2008). The effects of pH on carbon material and energy balances in hydrogen-producing *Clostridium tyrobutyricum* JM1. *Bioresour Technol*, **99**(17), 8485-91.

Kapdan, I.K., Kargi, F. (2006). Bio-hydrogen production from waste materials. *Enzyme Microbial Technol*, **38**(5), 569-82.

Kopke, M., Held, C., Hujer, S., Liesegang, H., Wiezer, A., Wollherr, A., Ehrenreich, A., Liebl, W., Gottschalk, G., Durre, P. (2010). *Clostridium ljungdahlii* represents a microbial production platform based on syngas. *PNAS*, **107**(29), 13087-92.

Kovács, K.L., Maróti, G., Rákhely, G. (2006). A novel approach for biohydrogen production. *Int J Hydrogen Energy*, **31**(11), 1460-8.

Krupp, M., Widmann, R. (2009). Biohydrogen production by dark fermentation: Experiences of continuous operation in large lab scale. *Int J Hydrogen Energy*, **34**(10), 4509-16.

Lay, J.J., Lee, Y.J., Noike, T. (1999). Feasibility of biological hydrogen production from organic fraction of municipal solid waste. *Water Res*, **33**(11), 2579-86.

Levin, D.B., Pitt, L., Love, M. (2004). Biohydrogen production: prospects and limitations to practical application. *Int J Hydrogen Energy*, **29**(2), 173-85.

Lodhi, M.A.K. (1997). Photovoltaics and hydrogen: Future energy options. *Energy Convers Manage*, **38**(18), 1881-93.

Logan, B.E., Oh, S.E., Kim, I.S., Van Ginkel, S. (2002). Biological hydrogen production measured in batch anaerobic respirometers. *Environ Sci Technol*, **36**(11), 2530-5.

Manish, S., Banerjee, R. (2008). Comparison of biohydrogen production processes. *Int J Hydrogen Energy*, **33**(1), 279-86.

Milciuviene, S., Milcius, D., Praneviciene, B. (2006). Towards hydrogen economy in Lithuania. *Int J Hydrogen Energy*, **31**(7), 861-6.

Nandi, R., Sengupta, S. (1998). Microbial production of hydrogen: An overview. *Crit Rev Microbiol*, **24**(1), 61-84.

Pearce, F. (2002). Big City Killer: cigarettes don‟t get you the traffic pollution will. *New Sci*, 8.

Pimentel, D., Cooperstein, S., Randell, H., Filiberto, D., Sorrentino, S., Kaye, B., Nicklin, C., Yagi, J., Brian, J., O'Hern, J., Habas, A., Weinstein, C. (2007). Ecology of increasing diseases: Population growth and environmental degradation. *Hum Ecol*, **35**(6), 653-68.

Sherif, S.A., Barbir, F., Veziroglu, T.N. (2005). Wind energy and the hydrogen economy - review of the technology. *Sol Energy*, **78**(5), 647-60.

Shin, J.H., Hyun Yoon, J., Eun Kyoung, A., Kim, M.S., Jun Sim, S., Park, T.H. (2007). Fermentative hydrogen production by the newly isolated *Enterobacter asburiae* SNU-1. *Int J Hydrogen Energy*, **32**(2), 192-9.

Shin, J.H., Yoon, J.H., Lee, S.H., Park, T.H. (2010). Hydrogen production from formic acid in pH-stat fed-batch operation for direct supply to fuel cell. *Bioresour Technol*, **101**(1 SUPPL.), S53-S58.

Tian, X., Liao, Q., Zhu, X., Wang, Y., Zhang, P., Li, J., Wang, H. (2010). Characteristics of a biofilm photobioreactor as applied to photo-hydrogen production. *Bioresour Technol*, **101**(3), 977-83.

Tsai, W.T. (2005). Current status and development policies on renewable energy technology research in Taiwan. *Renew Sust Energ Rev*, **9**(3), 237-53.

Vatsala, T.M., Raj, S.M., Manimaran, A. (2008). A pilot-scale study of biohydrogen production from distillery effluent using defined bacterial co-culture. *Int J Hydrogen Energy*, **33**(20), 5404-15.

Winter, C.J. (2005). Into the hydrogen energy economy - milestones. *Int J Hydrogen Energy*, **30**(7), 681-5.

Yoshida, A., Nishimura, T., Kawaguchi, H., Inui, M., Yukawa, H. (2005). Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. *Appl Environ Microbiol*, **71**(11), 6762-8.

CHAPTER 2: LITERATURE REVIEW

2.1 History of anaerobic digestion

Anaerobic digestion (AD) is an old technology for treating organic waste. Historical evidence shows that biogas was used for heating bath water in Assyria during the 10^{th} century BC and in Persia during the 16^{th} century (Lusk, 1998). By the end of the 17^{th} century, Jan Baptista Van Helmont found that flammable gases arise from decaying organic matter (Lusk, 1998). Also in 1776, Count Alessandro Volta showed that there was a positive correlation between the amount of decaying matter and the amount of flammable gases produced (Lusk, 1998). In 1808, Sir Humpry Davy applied AD methods to produce CH_4 from cattle manure (Lusk, 1998). The first digestion plant was built in Bombay, India in 1859 (Meynell, 1976). By 1895, AD techniques slowly spread across England, and the recovery of biogas from a sewage treatment facility was used to fuel road lamp burners (McCabe and Eckenfelder, 1957).

Initially, AD took place in anaerobic ponds. Later, with better understanding of AD process control, CH_4 production was carried out using sophisticated equipment and closed temperature-controlled reactors. However, the further development of CH_4 production suffered a setback with the surplus amounts of low cost coal and petroleum available at that time. This led to a decline in research and development of the AD process. After World War II, however, there were fuel shortages in Europe that once again led to further development and improvement of the AD process.

2.2 Anaerobic digestion process - An overview

The process of AD converts a variety of organic substrates, such as glucose, into CH_4 and CO_2. AD is used to treat a wide variety of industrial effluents. There is a large group of microorganisms involved in AD because the process involves a sequence of reactions in which the byproducts of one of the reactions serves as the substrate for the consequent reaction. Specific microbial communities are responsible for carrying out different stages of the AD process. The major by-products of AD include CH_4, CO_2, water, and microbial biomass. The AD process can be divided into four stages: 1. Hydrolysis, 2.Acidogenesis, 3.Acetogenesis, and 4.Methanogenesis (Figure 2.1).

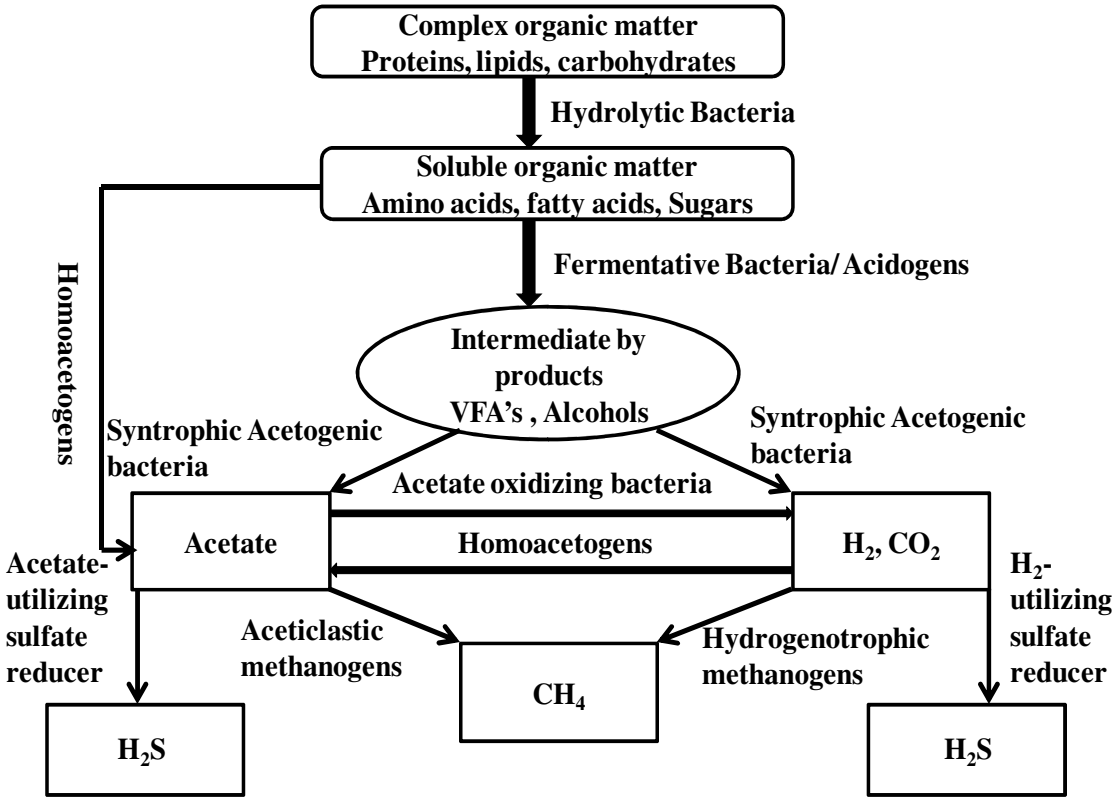

Figure 2.1 Anaerobic digestion (AD) process.
Note: H_2S = hydrogen sulfide; CO_2 = carbondioxide; CH_4 = methane; VFAs = volatile fatty acids.

2.2.1 Hydrolysis

Hydrolysis is the first reaction step in the AD process. Hydrolysis is a chemical process in which the addition of water to the organic compound (parent molecule) results in the addition of a hydrogen ion (H^+) to the parent molecule, leaving behind the hydroxyl ion (OH^-). Under normal circumstances, the reaction between the water molecule and the organic compound is less thermodynamically favorable and the reaction does not proceed easily.

Hydrolysis is an important process in plants and animals for maintaining energy metabolism within the cells. Energy is required by all living cells for two purposes; namely, for the formation of small molecules or macromolecules and to aid in the transport of ions across the cell membrane. The energy attained from the hydrolysis process cannot be used directly for these purposes; rather, it undergoes a series of

reactions before it is stored in the form of adenosine triphosphate (ATP). During hydrolysis, complex organic polymers are converted into monomers. Polysaccharides are converted into glucose by the enzyme, amylase. Similarly, cellulases, proteases and lipases catalyze the hydrolysis of cellulose, proteins and lipids.

Factors affecting the rate of hydrolysis include pH, type of substrate, substrate solubility and temperature. Lipids and proteins are less soluble than carbohydrates in the aqueous phase due to their hydrophobic nature. The hydrolysis of proteins is difficult when compared to the hydrolysis of hemicelluloses because proteins have very complex molecular structures. Protein hydrolysis occurs only when their quaternary or tertiary protein structure unfolds to expose their amide bonding structure. Proteases cannot act on all types of proteins because their action is stereo selective. This reaction requires proper positioning of the amide group for catalysis. Each enzyme has a specific crevice known as the active site where the protein moiety binds. Proteins that cannot fit themselves into the site are not hydrolyzed. This specificity enables the biological system to function normally, and protects the integrity of proteins, such as hormones, from non-specific reactions to hydration.

2.2.2 Acidogenesis

Acidogenesis is the second step in the AD process. During this stage, hydrolyzed byproducts are acted upon by "acidogens" to produce low molecular byproducts, such as VFAs (e.g. acetic acid, propionic acid, and butyric acid), alcohols, aldehydes, and gases (e.g. H_2, CO_2, and ammonia (NH_3)). Acidogens are strict anaerobic organism and compounds such as oxygen or nitrate can impair acidogenesis. The presence facultative bacteria, along with acidogens, in mixed microbial community enable utilization of oxygen if it is present. During acidogenesis, the pH is approximately 4.0 and as a result of H_2 accumulation is coupled with the formation of reduced VFAs. Hydrogen utilization by hydrogenotrophic methanogens, sulfate reducing bacteria (SRB), and/or homoacetogen influences byproduct distribution patterns. The syntrophic association between the H_2 consumers and fermentative bacteria results in surplus energy supply per unit of substrate. Selected acidogenic reactions with their Gibb's free energy values are provided in Table 2.1.

Table 2.1 Selected acidogenesis reactions and their Gibbs free energy values.

Reaction stoichiometry	Gibbs free energy, (ΔG) kJ mol^{-1}	Eq.No.
$C_6H_{12}O_6 + 4\ H_2O \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + 4\ H_2 + 4\ H^+$	-206	2.1
$C_6H_{12}O_6 + 5\ H_2O \rightarrow CH_3CH_2COO^- + 3\ HCO_3^- + 5\ H_2 + 4\ H^+$	-177.9	2.2
$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOO^- + 2\ H^+$	-198.5	2.3
$C_6H_{12}O_6 + 2\ H_2O \rightarrow CH_3CH_2CH_2COO^- + 2\ HCO_3^- + 2\ H_2 + 3\ H^+$	-253.8	2.4
$C_6H_{12}O_6 + H_2 \rightarrow\ CH_3CH_2\ CH_2CH_2COO^- + HCO_3^- + H_2O + 2\ H^+$	-225.4	2.5

2.2.3 Acetogenesis

The utilization of the acetyl CoA pathway is a unique feature of acetogens. Acetogens are strict anaerobes which use the acetyl-CoA (Wood-Ljungdahl) pathway to fix CO_2 (For example, *Clostridium aceticum; C. thermoaceticum*). *C. thermoaceticum* are acetogens which are capable of fixing CO_2 to acetic acid via the Wood-Ljungdahl pathway. *Acetobacterium* sp. and *Clostridium* sp. comprise most of the known acetogenic species. Non-acetogens, such as methanogens and sulfate reducers, use metabolic pathways similar to the acetyl-CoA pathway for either the assimilation of CO_2 into the biomass or for the oxidation of acetic acid (Drake et al., 1994; Fuchs, 1986; Fuchs, 1994). During homoacetogenesis CO_2 is reductively reduced into acetyl-CoA and subsequently converted into acetic acid, cell mass and energy. *C. aceticum*, a spore-former, is able to grow utilizing H_2-CO_2 (Table 2.2) (Wieringa, 1936; Wieringa, 1939; Wieringa, 1941). Acetogens are also able to ferment substrates glucose as well as carboxylic acid, alcohols and aromatic compounds (Pierce et al., 2008). For example, *C. thermoaceticum* is able to ferment 1 mol of sugar into 3 mol of acetic acid.

Table 2.2 Selected acetogenic reactions and their Gibbs free energy values.

Reaction stoichiometry	Gibbs free energy, ΔG (kJ mol^{-1})	Eq.No.
$CH_3CH_2COOH + 2\ H_2O \rightarrow CH_3COOH + 3\ H_2 + CO_2$	+76.2	2.6
$CH_3CH_2CH_2COOH + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ H_2$	+48.4	2.7
$CH_2CHOHCOOH + 2\ H_2O \rightarrow CH_3COOH + HCO_3^- + 2H_2$	-4.2	2.8
$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2\ H_2$	+9.6	2.9
$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$	-104	2.10

Bacteria utilizing CO_2 as the terminal electron acceptor and H_2 as the electron donor to produce acetic acid using the Wood-Ljungdahl pathway are phylogenetically diverse and are known as "homoacetogens". Homoacetogens were present in habitats such as rumen and paddy fields (Henderson et al., 2010; Xu et al., 2009). Although they scavenge H_2, they cannot out-compete methanogens for H_2. This is mainly due to thermodynamic limitations, with the homoacetogens having less Gibb's free energy available than the methanogens (Eq. 2.10 and Eq. 2.12) (Table 2.2 and 2.4). However, in low temperature environments where methanogens are suppressed, homoacetogens play an important role in H_2 oxidation. Acetogenic bacteria are an extremely versatile group of microorganisms because of the following characteristics (Schlegel and Bowien, 1989):

1. **Diversity of habitats:** They are present in a wide range of habitats such as freshwater sediments, animal manure, sewage sludge, hindguts, rhizospheres, etc (Schlegel and Bowien, 1989).

2. **Trophic characteristics:** They are involved in numerous energy conserving and terminal electron accepting processes, in addition to their ability to oxidize a wide range of substrates (Schlegel and Bowien, 1989).

3. **Ecological functions:** They compete with primary fermenters for monomeric substrates, but they also compete with secondary fermenters for lactic acid, ethanol and H_2. They are also able to link themselves in syntrophic associations with aceticlastic methanogens and with syntrophic acetogens (Schlegel and Bowien, 1989).

4. **Commercial applications:** Numerous studies have evaluated the commercial application of acetogens to produce acetic acid (Schlegel and Bowien, 1989).

2.2.3.1 Wood-Ljungdahl Pathway

The Wood-Ljungdahl pathway of acetic acid synthesis (Fig. 2) is comprised of two branches. One of branch assembles the methyl unit of the acetyl group while the other synthesizes the carbonyl group and assembles the acetyl-CoA molecule from these two units and the coenzyme.

2.2.3.1.1 Methyl branch of the pathway

The methyl branch of the pathway begins with the reduction of CO_2 to formic acid ($HCOO^-$). This reaction is catalyzed by the enzyme, formate dehydrogenase. Steps 2 to 5 in the pathway shown in Figure 2.2 involve the cofactor tetrahydrofolate (H_4F).

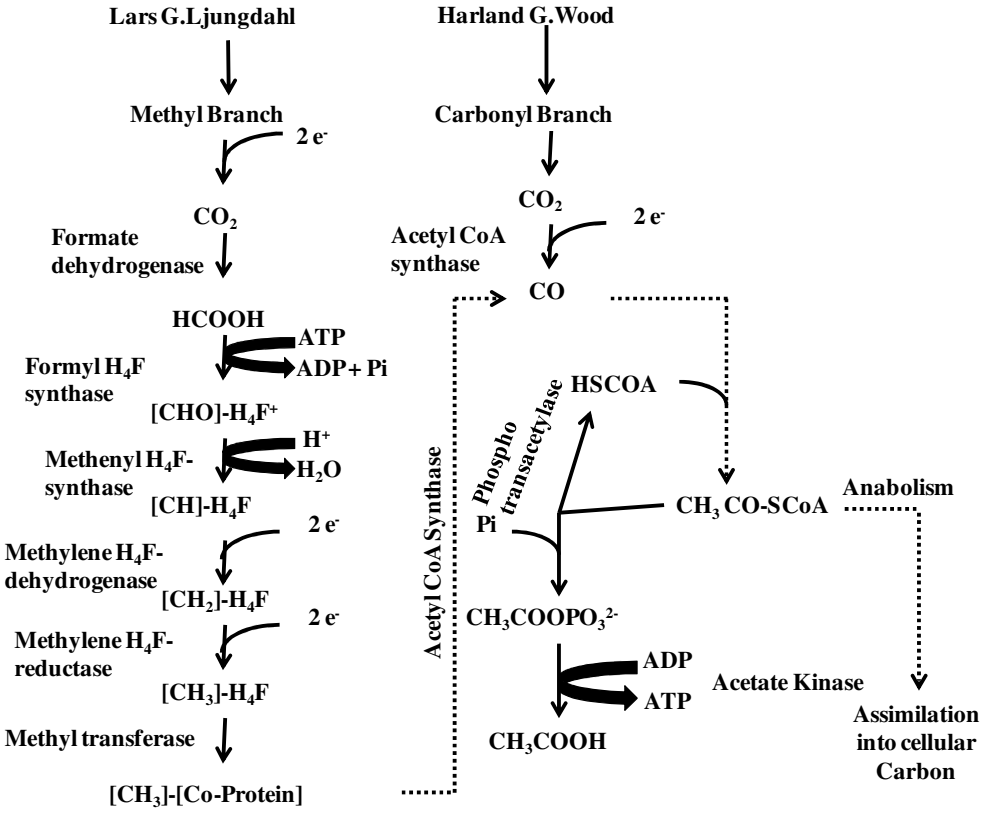

Figure 2.2 Wood-Ljungdahl pathway (Modified from Ragsdale and Pierce (2008)).
Note: H_4F – tetrahydrofolate; HSCoA- Coenzyme A; Pi – Inorganic phosphate; e^- - electron; Co-protein- Corrinoid coenzyme; ATP- Adenosine triphosphate; ADP- Adenosine diphosphate.

Formate is bound to the carbon 10 (C10) position of the H_4F molecule (Step 2) as a formyl group. This reaction involves the utilization of ATP. Steps 3 to 5 reduce the bound formyl group into a methyl group. The methyl group is then transferred to the cobalt-amide group of the corrinoid coenzyme, which then merges with the carbonyl branch through acetyl CoA synthase to form acetyl CoA. Step 5 in the pathway, although

not clearly understood, is sufficient to produce a sodium potential across the membrane. This addition to the cell membrane potential is utilized to produce additional cellular energy (ATP) via the cell electron transport system. If CO is available to the system, then the next step (or first step of the carbonyl branch of the pathway) will be the conversion of CO to CO_2 by the enzyme, carbon monoxide dehydrogenase. This reaction produces the reducing equivalents, which are utilized in the carbonyl branch of the pathway.

2.2.3.1.2 Carbonyl branch and Acetyl CoA synthesis

The carbonyl branch of the Wood-Ljungdahl pathway (Figure 2.2) begins with the reduction of CO_2 by binding CO with the enzyme, carbon monoxide dehydrogenase (CODH). In some acetogens, CODH directly binds with CO which reacts with the methyl group of the Corrinoid coenzyme to form acetyl CoA. A portion of this acetyl CoA is utilized for cellular anabolism and the remainder proceeds to form acetic acid.

Acetogens can utilize a wide variety of electron donors and produce a wide range of byproducts (Table 2.3). According to several reports, acetogenic growth is affected if there is no endogenous supply of CO_2 (Andreesen et al., 1970; Hsu et al., 1990; Matthies et al., 1993; Savage et al., 1987). If CO_2 is available the reaction proceeds to acetic acid production via the Wood-Ljungdahl pathway. In the presence of glucose and CO_2, acetic acid is the only byproduct (Slobodkin et al., 1997). Many acetogens can utilize one or more terminal electron acceptors. For example nitrate is used by *Moorella thermoacetica*, which converts it to nitrite and ammonium (Fröstl et al., 1996; Seifritz et al., 1993). Some closely related acetogens, such as *Moorella glycerini and Moorella thermoacetica*, differ in their ability to use alternative electron acceptors, such as nitrate (Andreesen et al., 1970).

Table 2.3 List of some of the redox couples used by the acetogens.

List of electron donors for acetogenesis	List of electron acceptors for acetogenesis	End products formed
H_2,CO_2, formic acid, alcohols, glycolate, glyoxylate, oxalate, hexoses, pentoses	CO_2, fumarate, nitrate, nitrite, thiosulfate, dimethulsulfoxide, pyruvate, acetaldehyde	Acetic acid, succinate, nitrite, ammonium, sulfide, dimethylsulfide, lactic acid, ethanol

2.2.3.2 Energy metabolism of acetogens

Acetogens that are grown chemoorganotrophically show high growth yields in the range of 50 g cells mol^{-1} glucose (*C.thermoaceticum*) to 68 g cells.mol $fructose^{-1}$ (*A.woodii*) (Schlegel and Bowien, 1989). Chemolithotrophic growth of acetogens (grown on H_2/CO_2) yields about 5 g cells mol acetic $acid^{-1}$, indicating that approximately 0.5 ATP becomes available during the formation of acetic acid from 4 mol of H_2 and 2 mol of CO_2. This raises an interesting question, "how is this ATP synthesized?" (Schlegel and Bowien, 1989).

ATP is utilized in the reaction involving formyl tetrahydrofolate synthetase (Figure 2.2; methyl branch). This spent ATP is then regained during the reaction catalyzed by acetic acid kinase (Figure 2.2; carbonyl branch). None of the other reactions in the Wood-Ljungdahl pathway involve either ADP or ATP. Therefore ATP synthesis by substrate-level phosphorylation is also not possible. Thus, electron transport phosphorylation might be the source for ATP generation (Schlegel and Bowien, 1989). There are three other reactions involved in this pathway, which are of bioenergetic significance. They are the CO dehydrogenase reaction, the methylene-H_4folate dehydrogenase reaction, and the methylene-H_4folate reductase reaction (Schlegel and Bowien, 1989). ATP should be generated in the methylene-H_4folate reductase reaction. However, not much information is known about this mechanism. The discovery of the presence of a Na^+/H^+ antiporter in *C.thermoaceticum* led researchers to compare acetogens to methanogens, which use the energy generated by transmembrane flux of the Na^+ gradient to drive certain thermodynamically unfavorable reactions involved in their energy metabolism (Schlegel and Bowien, 1989).

2.2.4 Methanogenesis

Methanogenesis is the final step in the AD process. The products of acetogenesis (mainly acetic acid) are converted into CH_4 and CO_2 by the actions of bacteria known as "methanogens". Methanogens are more sensitive to environmental conditions compared to other groups of organisms involved in the AD process. Methanogens grow slowly, and are classified into two types; namely, aceticlastic methanogens and hydrogenotrophic

methanogens based on the type of substrate used by the organism (Demirel and Scherer, 2008). Methanogens utilizing H_2 as the electron donor for the production of CH_4 are referred to as hydrogenotrophic methanogens, whereas organisms which utilize acetic acid as the electron donor source are known as aceticlastic methanogens. About 70 % of the CH_4 production in anaerobic reactors is produced as a result of decarboxylation of acetic acid by aceticlastic methanogens. The remaining methane production is generated by hydrogenotrophic methanogens as a result of CO_2 reduction with H_2. The growth rate of aceticlastic methanogens is slower than the growth rate of hydrogenotrophic methanogens because the free energy due to the formation of CH_4 from acetic acid is low compared to the free energy due to the reduction of CO_2 with H_2 (Table 2.4). Methanogens also act on other types of substrates, such as formic acid, methanol and methyl amines, but these substrates account for only minor quantities of CH_4. Excessive accumulation of acetic acid results in a drop in the pH level, which is a clear indicator of instability in the methanogenesis process (Ahring et al., 1995). Some of the methanogenesis stoichiometric reactions, along with their Gibbs free energy values, are given below (Table 2.4). In order to maintain a steady state, both the acid production rate (i.e., production rate of VFAs by acidogenesis and hydrolysis) and acid consumption rate (as a result of acetogenesis and methanogenesis) should be maintained in the proper balance. In cases of imbalance, either H_2 or acetic acid accumulate in the system, and result in process inhibition.

Table 2.4 Selected methanogenesis reactions and their Gibbs free energy values.

Methanogenic substrate	Stoichiometric reaction	ΔG kJ mol^{-1}	Eq.No.
Acetic acid	$CH_3COO^- + H^+ \rightarrow CO_2 + CH_4$	-27.5	2.11
Hydrogen	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-139.1	2.12
Methanol	$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 4\ H_2O$	-544.8	2.13
Methanol/Hydrogen	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-149.8	2.14
Formic acid	$4\ CHOO^- + 2\ H^+ \rightarrow CH_4 + CO_2 + 2\ HCO_3^-$	-302.6	2.15
Formic acid	$CHOO^- + 3\ H_2 + H^+ \rightarrow CH_4 + 2\ H_2O$	-178.3	2.16
Carbon monoxide	$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ H_2O$	-185.9	2.17

The pathway leading to methanogenesis in hydrogenotrophic methanogens (Figure 2.3) involves CH_4 formation from C1-compounds, and is initiated by the enzyme, formyl methanofuran (MFR) dehydrogenase. The action of this enzyme results in the formation

of N-Carboxymethanofuran, which is reduced to form formyl-methanofuran (formyl-MFR). The nucleophilic attack of the uncharged amino group in the methanofuran portion of the N-Carboxymethanofuran molecule with CO_2 results in the formation of carbamate in a spontaneous and reversible manner. This reaction requires reducing equivalents, which are donated by H_2 via hydrogenase or by formic acid via formate dehydrogenase. This methanofuran group (MFR) is replaced by tetrahydromethanopterin (H_4-MPT) to form formyl- H_4-MPT. This byproduct then undergoes a series of reactions, which are reversible, to form methyl H_4-MPT. The intermediates in this pathway are methenyl tetrahydromethanopterin and methylene tetrahydromethanopterin, which are catalyzed by methenyl tetrahydromethanopterin cyclohydrolase (Ermler et al., 1999; Vaupel et al., 1996), methylene tetrahydromethanopterin dehydrogenase (Enssle et al., 1991), and methylene tetrahydromethanopterin reductase (Ermler et al., 2000; Ma and Thauer, 1990; Tebrommelstroet et al., 1990; Tebrommelstroet et al., 1991).

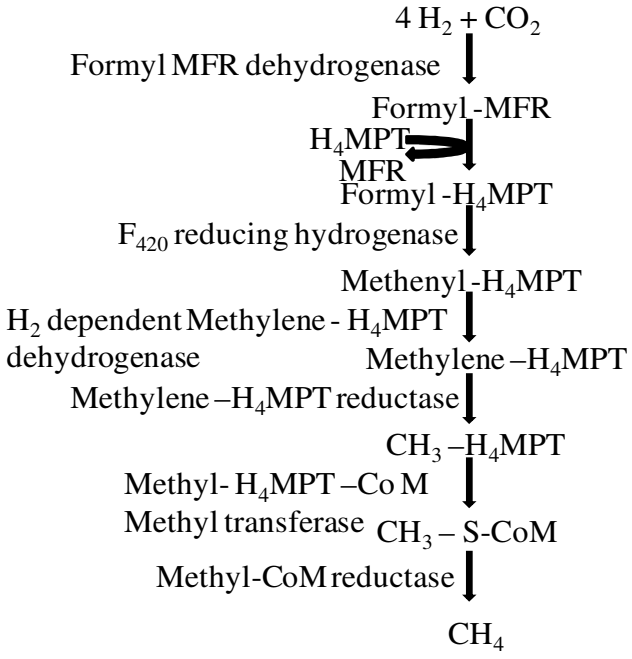

Figure 2.3 Pathway of hydrogenotrophic methanogens (Modified from Reeve et al. (1997)).
ABBREVIATION: MFR = Methanofuran, H_4MPT = Tetrahydromethanopterin dehydrogenase, CoM = Coenzyme M.

The reducing equivalents for the reduction of methenyl tetrahydromethanopterin to methylene tetrahydromethanopterin are provided by reduced coenzyme F_{420}, which reacts with H_2 via F_{420} reducing hydrogenase. This enzyme is present in the cytoplasm of methanogens and contains three different subunits and several iron-sulfur clusters. In some hydrogenotrophic methanogens, another dehydrogenase is present, which directly utilizes molecular H_2 to provide reducing equivalents for the reduction of methenyl H_4-MPT to methylene H_4-MPT. The unique feature of this dehydrogenase is that it does not contain either Fe/S clusters or Ni/Fe clusters, which are present in all hydrogenases. The membrane-bound methyl transferase is involved in the transfer of the methyl group from methyl tetrahydromethanopterin to coenzyme M at this point, and creates a sodium ion gradient across the membrane. The enzyme, methyl coenzyme M reductase, catalyzes this exergonic reaction.

Methanogens are widespread in anoxic environments devoid of nitrate, sulfate and light, but with sufficient carbon-rich, organic compounds. Methanogens are present in the fresh water sediments of lakes and rivers, swamps, rice fields, anaerobic treatment plant digesters, tundra soils, swamps and within the intestinal tracts of termites and ruminants. Methane is one of the major sources of bio-elemental flux at a global level. Methane is ranked second as a major source of greenhouse gas emissions, and contributes 16 % of the greenhouse effect (Demeyer and Fievez, 2000). Only 30 % of the methane emissions come from natural sources, whereas the remaining 70 % is produced as a result of human activities. Methane is the final end-product of the anaerobic food chain, and H_2 is formed as an intermediate byproduct in anaerobic digestion; therefore, consumption of this H_2 is another important function of methanogens. The H_2 partial pressure needs to be maintained at a specific level in order to breakdown organic matter. This is achieved mainly with the help of H_2 scavengers, which will consume the H_2 produced by the system. Methanogens also play a major role in maintaining low H_2 partial pressures. In methanogenic environments, the H_2 partial pressure is kept below 10 Pa. This, in turn, helps in the release of electrons from NADH as H_2, which results in the formation of acetyl-CoA from the intermediate byproducts of the fermentative pathway by syntrophic organisms, and thereby a net gain of ATP through substrate level phosphorylation

(Conrad, 1999; Schink, 1997). Most of the natural gas reserves (from fossil fuels) are generated as a result of methanogenesis.

Methanogens form the most diverse group of Euryarchaeota with their genomic DNA G+C content ranging from 22 to 68 mol %. Methanogenic species show a broad spectrum of structural morphology. Some of their morphological forms include regular and irregular cocci, rods of varying length, spirilla and unusual flattened plate forms. Furthermore, methanogens differ from one another in terms of cell morphology. They are either surrounded by pseudomurein, lipopolysaccharide or protein subunits, and are not surrounded by a murein layer as are most bacteria. This unique feature of methanogens makes them resistant to the antibiotics that affect the synthesis of the bacterial cell wall.

2.2.4.1 Energy metabolism of methanogens

The first step in hydrogenotrophic methanogenesis from H_2/CO_2 involves overcoming an energy barrier because of the positive free-energy change associated with the conversion of CO_2, H_2 and MFR to formyl MFR (Figure 2.4) (Schlegel and Bowien, 1989). This pathway to CH_4 production includes many reactions with small free-energy changes, but the last step in the pathway has a negative Gibb's free energy value that is more than the positive free energy value from the preceding steps (Schlegel and Bowien, 1989). These complex influences on free-energy make the study of the bioenergetics of CH_4 production by methanogens from H_2/CO_2 more difficult. Thus, a simplified process, involving studies with the addition of uncouplers to abolish the CO_2 reduction process, has been examined (Schlegel and Bowien, 1989).

A major break-through was achieved when studies of *Methanosarcina barkeri* were carried out in the presence of methanol and H_2 (Blaut and Gottschalk, 1985). The cell suspension converted these substrates to methane via methyl-coenzyme M, and this reaction was coupled with ATP synthesis suggesting that ATP is synthesized by a chemiosmotic mechanism in this type of fermentation. Blaut and Gottschalk (1985) conducted an experiment to examine this hypothesis (i.e., that ATP generation in methanogens is mostly due to a chemiosmotic mechanism). They performed the experiment using a cell suspension of *M. barkeri* and tetrachlorosalicylanilide (TCS), a

protonophore, which dissipates the transmembrane electrochemical H^+ gradient (Schlegel and Bowien, 1989). The intracellular ATP content decreased irrespective of CH_4 formation which continued at an increased rate. In a second set of experiments, these researchers added N, N-dicyclohexylcarbodiimide (DCCD), and an inhibitor of ATP synthase, instead of TCS. This approach resulted in inhibition of CH_4 formation and ATP synthesis, but left the electrochemical proton potential unaffected. Based on these findings, they concluded that CH_4 synthesis from methanol and H_2 gives rise to the electrochemical proton potential (proton motive force) used by the enzyme, ATP synthase, for ATP formation (Schlegel and Bowien, 1989). If ATP is formed as a result of a chemiosmotic mechanism, such as substrate level phosphorylation, rather than enzymatic (ATP synthase) activity then a decrease in ATP should not have occurred in the presence of DCCD. A type of ATP synthase (F_1F_0 type ATPase) was later identified in *M. barkeri* (Inatomi, 1986*)*. Methane production by ruminants has received global attention due to its possible impact on global warming and green house gas emissions. In the past two decades, research programs in Oceania, North America, and Europe have developed several ways to re-direct the electron equivalents to other reductive substrates in order to reduce CH_4 synthesis (Doreau et al., 2011). Methane production can be inhibited only for short periods with these methods, however, and then the methanogens revert to their initial levels of CH_4 synthesis through a variety of mechanisms.

2.3 Factors affecting biological H_2 production

There are several factors affecting biological H_2 production, namely: pH (Van Ginkel et al., 2001), temperature (Valentine et al., 2000), substrate concentration (Van Ginkel et al., 2001), microbial source (Nandi and Sengupta, 1998), H_2 partial pressure (Ahring and Westermann, 1988), and volatile fatty acids concentration (Yusoff et al., 2010).

2.3.1 Volatile fatty acids

Volatile fatty acids (VFAs), along with H_2, are the major by-products formed during anaerobic fermentation. VFA concentration is a very important influence on the H_2 production process in mixed anaerobic culture and at high levels CH_4 formation is inhibited. The undissociated form of these fatty acids disturbs the intracellular process as

a result of disruption of the cell membrane, which alters the intracellular pH and disrupts the proton pump present in the cell membrane. Acetic acid at higher concentrations is inhibitory to the degradation of VFAs (Ahring and Westermann, 1988; Kaspar and Wuhrmann, 1978). Propionic acid, together with acetic acid, are inhibitory to the degradation of propionic acid in mesophilic propionic acid acclimated sludge (Fukuzaki et al., 1990). Inhibition of aceticlastic methanogenesis by VFAs has been studied by many researchers. Acetic acid at high concentrations (greater than 6000 mg L^{-1}) is inhibitory to aceticlastic methanogens. Propionic acid (at concentrations between 1800-2200 mg L^{-1}) is inhibitory to hydrogenotrophic methanogenesis (Barredo and Evison, 1991). A recent study by Van Ginkel and Logan (2005), using heat treated inoculum at pH 5.5 and 37 °C, showed that H_2 production was greatly affected by the presence of undissociated forms of acetic acid and butyric acid in the reactor. They also reported that H_2 production was inhibited more strongly by the formation of VFAs from initially high substrate concentrations rather than from the addition of external VFAs into the system. Butyric acid was also found to be more inhibitory to H_2 production than acetic acid when using heat-treated, mixed anaerobic sludge at an initial pH of 6.0 (Zheng and Yu, 2005).

2.3.2 pH

H_2 production by dark fermentation is strongly affected by process parameters, such as the operational pH, which has a strong effect on hydrogenase activity (Dabrock et al., 1992), the metabolic pathway of H_2 production (Lay, 2000), and the suppression of H_2-consuming methanogenic activity (Chen et al., 2002). The effect of pH on biohydrogen production has been studied by many researchers; the optimum pH varies for different substrates (Li and Fang, 2007). Thus, pH is one of the most influential factors that can be adjusted to maximize biohydrogen production (Khanal et al., 2004; Mu et al., 2006). Excess production of VFAs might result in an inhibitory effect on hydrogen fermentation (Zhang et al., 2006; Zheng and Yu, 2005). The undissociated forms of the fatty acids that have accumulated inside the microorganism permeate freely inside the plasma membrane, which interrupts the metabolism of the cell (Zhang et al., 2006). The toxicity observed under low pH conditions is often associated with the presence of undissociated VFAs (van Lier et al., 2001). Lowering pH levels will result in the growth of filamentous

bacteria while raising pH levels result in the buildup of ammonia. The methanogenic activity will be reduced at pH values lower than 6.3 and will be increased at pH levels above 7.6 (Jung et al., 2000). Therefore, reducing pH values below neutral levels has a significant effect on biogas production. For example, the substrate removal efficiency of an acidogenic pilot-scale reactor was increased from about 50 % to 80 % when the reactor's pH was maintained at 6.0-7.0 using HCl. When the pH of the acidogenic reactor was uncontrolled, the pH increased to 8.2 and the acid conversion rate decreased compared to that of the pH-controlled system (Jung et al., 2000).

Staley et al. (2011) found that methanogenesis occurred in refuse with high VFA concentrations and acidic pH values ranging from 5.5 to 6.5. Their study showed that *M. barkeri*, which is tolerant to low pH levels, was present in refuse. The pH level is one of the most important environmental factors inhibiting the growth of methanogens during the anaerobic digestion process, but the optimum range can vary for different organisms. For example, the optimum pH range for *Methanosaeta* sp. ranged from 6.8 to 8.2. *Methanosaeta* sp. uses acetic acid as its sole energy source for CH_4 production (Brummeler et al., 1985; Huser et al., 1982). *Methanosarcina* sp. was found to have a broader range of optimal pH levels, ranging from 6.0 to 8.0 (Yang and Okos, 1987), and is capable of growing on a wide variety of organic substrates (such as H_2/CO_2, methanol, methylamines and acetic acid). Enriched cultures of methanogens also showed a broader operating range, with pH levels between 5.1 and 7.1 (Vandenberg et al., 1976). The operational pH range in industrial and municipal anaerobic digesters was 6.0 - 7.0; the rate of CH_4 production, in particular, decreased for pH below 6.0 (Brummeler et al., 1985; Speece, 1996). Speece (1996) recommended the addition of various alkaline substances to maintain an operational pH range of 6.8 - 7.0 for the successful operation of anaerobic digesters. Many researchers have investigated the ability of methanogens to thrive under acidic conditions (Duval and Goodwin, 2000; Goodwin and Zeikus, 1987; Horn et al., 2003; Maestrojuan and Boone, 1991; Patel et al., 1990). Methanogenesis was observed in peats and bogs where, in general, the pH is often less than 5.0 (Duval and Goodwin, 2000). The main reason for methanogenesis at such low pH ranges was due to the occurrence of micro niches and also due to the higher internal cytoplasmic pH of methanogens. These micro niches are often the source of the atmospheric CH_4 generated

from H_2/CO_2 (Goodwin and Zeikus, 1987). Many people have tried to isolate successful strains of methanogens, which are capable of growing at low pH levels (Horn et al., 2003). Isolates of *Methanosarcina* and *Methanobacterium* are capable of growth and CH_4 production at pH values between 4.68 and 5.0 (Maestrojuan and Boone, 1991; Patel et al., 1990). Furthermore, the pH of the operating system influences the composition of the biogas. At neutral pH, the mixture of CO_2 / carbonic acid is converted to either carbonate or bicarbonate (Snoeyink and Jenkins, 1980). At an acidic pH range, the production of carbonates is inhibited. This specifically affects the activity of methanogens using H_2/CO_2 since they use these substrates for CH_4 rather than carbonate production. Increasing the amount of soluble CO_2 available (as carbonic acid), increases the production of CH_4. Decreasing the pH suddenly from 7.0 to 4.5 resulted in longer periods of inhibition of CH_4 production than applying a stepwise drop in pH (from 7.0 to 4.5) in 0.5 increments (Snoeyink and Jenkins, 1980). The amount of aqueous CO_2 decreased by > 40 % for each 0.5 increment over the pH range from 5.0 to 7.0 (Jain and Mattiasson, 1998). These results show that mixed cultures of methanogens have the ability to acclimate to acidic environments with lower pH values.

2.3.3 Temperature

Temperature is one the most important factors controlling the course and rate of anaerobic digestion (Golueke, 1958). Increases in temperature result in increased digestion rates, at least until the process nears pasteurization temperature. Temperature has been broadly classified into four ranges, namely: (1) Psychrophilic (< 25 °C), (2) Mesophilic (25- 40 °C), (3) Thermophilic (45- 60 °C), and (4) Hyper-thermophilic (> 65 °C). For temperatures between 5 and 35 °C, anaerobic digestion processes often obey Vant-Hoff's rule (i.e., the rate of the reaction doubles for every 10 °C rise in temperature). Temperature affects the rate of all of the enzyme-catalyzed reactions. Since all of the microbial processes are enzyme-catalyzed processes, temperature changes play a major role in driving the reactions forward or backward. The optimum temperature range for mesophilic digestion was found to be 30-40 °C and to be 50-60 °C for thermophilic digestion (Golueke, 1958). Consequently, most of the studies conducted previously fall into the mesophilic range or into the upper end of the thermophilic range (> 55 °C).

The advantages of digestion under thermophilic conditions are higher substrate conversion rates associated with higher specific microbial growth rates, but these are frequently offset by higher death rates than organisms maintained under mesophilic conditions (Duran and Speece, 1997; Van Lier, 1995). An important advantage of thermophilic digestion in waste treatment is that it enables greater destruction of pathogens. Most of the vegetative pathogenic bacteria, such as *Escherichia coli* and *Enterococci*, were destroyed during thermophilic digestion, and during mesophilic digestion with thermophilic pre-treatment (Larsen et al., 1994). Studies conducted by Mackie and Bryant (1995) concluded that thermophilic (60 °C) digestion of cattle waste is more stable than mesophilic (40 °C) digestion when hydraulic retention times (HRTs) are varied. In general, the disadvantages of thermophilic digestion are related to lower process stability compared with the stability of mesophilic digestion (Buhr and Andrews, 1977; VanLier et al., 1996; Wiegant and Zeeman, 1986).

Thermophilic digestion also results in poorer effluent quality (Duran and Speece, 1997). Lower growth yields for thermophilic bacteria combined with their higher growth rates often result in longer start-up times. Thermophilic digestion is more susceptible to toxicity and changes in operational parameters. According to the studies conducted by Ahring et al. (2001), increase in operational temperature (from 55 to 65 °C) resulted in an imbalance between the fermenting, H_2-producing bacteria (acidogens, which produce H^+ ions) and the H_2-consuming bacteria (acetogens and methanogens, which consume H^+ ions). Another major drawback to the application of thermophilic digestion is higher operating costs due to the higher energy requirements compared with those of mesophilic digestion. Altogether, these disadvantages result in lower net energy production from the thermophilic process compared with the mesophilic process.

The effects of temperature change depend strongly on the degree of fluctuation, the duration of the shock, and also on the composition of the microbial community. Speece (1983) postulated that anaerobic digestion is sensitive to a sudden change in environmental conditions. The psychrophilic digestion process is less preferred than the mesophilic and thermophilic digestion processes because of the low conversion rate of organic matter into biogas, which occurs due to the limited activity of microorganisms at

low temperatures. Consequently, larger retention times are required in psychrophilic digesters, and hence, very large quantities of tanks and reactors are necessary.

Methanogens comprise a group of bacteria present in wastewater sludge that are responsible for CH_4 production from the degradation of organic matter. Most methanogens belong to species found in the mesophilic temperature range, so quickly acclimate to conditions in this temperature range and exhibit a higher degree of conversion of organic substrates. The behavior of methanogens under mesophilic temperature conditions has direct implications for biogas plants, which maintain stable operating conditions. The stable nature of mesophilic biogas plants makes the digestive process more balanced, and also resistant to inhibition due to the presence of chemicals such as ammonia.

The type of substrate and its concentration, together with an adaptation period, affected the temperature dependence of methanogenic activity. The adaptation of the biomass in a leacheate-fed up-flow anaerobic sludge-blanket reactor (UASBR) maintained at 20 °C for 4 months resulted in a 7-fold increase in CH_4 production at 11 °C, and a 5-fold increase at 22 °C. The activity specifically due to methanogens using a H_2/CO_2 substrate was lower than it was for acetic acid under the same conditions (Chin et al., 1999). The reduced methanogenic activity observed with H_2/CO_2 may be due to a mass transfer limitation. The formation of a thick granular sludge and lower substrate concentrations are two factors that affect mass transfer limitations.

The temperature dependence of methanogenic activity also depends upon the type of biomass; granular sludge has higher levels of activity specific to CH_4 production than suspended cultures. Thermophilic digestion of a VFA-mixture in a UASBR was only slightly affected by temperature change at low organic loading rates (around 20 kg COD m^{3-1} d^{-1}). At higher COD loading rates (45 - 90 kg COD m^{3-1} d^{-1}), temperature changes had a strong effect and resulted in higher effluent VFA concentrations. The results obtained from several experiments indicate that completely mixed systems are more sensitive to temperature variation compared to fixed-bed systems (VanLier et al., 1996; Wheatley, 1990). These researchers observed a drop in substrate affinity with decreasing temperature. Furthermore, in a pure culture of methanogens, *M. barkeri*, there was an

increase in substrate affinity caused by a temperature shift (Westermann et al., 1989). A drop in the threshold acetic acid concentration occurred when the incubation temperature was decreased from 55 °C to 30 °C. Similar effects were observed in the case of nitrifying bacteria (Wijffels et al., 1995).

2.3.4 Hydraulic retention time (HRT)

The hydraulic retention time (HRT), also known as the hydraulic residence time, is a measure of the average length of time a soluble organic compound spends in a particular bioreactor. HRT is defined as the volume of the bioreactor (V) divided by the influent feed flow rate (Q):

$$HRT = V/Q$$

Where, using SI units, the volume is described in m^3 and the influent feed flow rate in m^3 h^{-1}. Thus, HRT is expressed in units of time (hours or days).

Anaerobic digestion of decomposable organic sludge is affected by operational parameters, such as HRT. Changes in HRT affect the activity specific to methanogenic micro-organisms; this process recovers slowly after the upset condition. Changes in HRT are often associated with changes in VFAs production, gas composition and the pH of the operating system, but do not directly affect the current metabolic status of active organisms within the system. The magnitude of CH_4 generation by the anaerobic digestion process is significantly affected by HRT. The main advantage of reducing the HRT is higher organic loading rates; it is possible to increase the organic loading rate when the HRT is lowered (Wang et al., 1997). Lowering the HRT increases the rate of hydrolysis and acidogenesis, resulting in a drop in pH to below neutral (i.e., pH < 7.0) (Kaosal and Sohgrathok, 2012). Non-methanogenic microorganisms responsible for hydrolysis and acidogenesis are able to adapt under acidic conditions (low pH), while the activity of methanogenic microorganisms is inhibited at the same pH levels. Kaosal and Sohgrathok (2012) studied the influence of HRT on biogas production from frozen seafood wastewater, using HRT of 10, 20 and 30 days. A HRT of 20 days maximized the production of biogas, with CH_4 accounting for 64.6 % of the biogas produced. At the lowest HRT tested (10 days), the VFA levels in the effluent reached the highest level

observed (compared to the levels observed for longer HRTs), causing the methanogenic process to shift in this reactor to hydrolysis or to an acidogenic process (Kaparaju et al., 2009). These researchers also concluded that the HRT of a continuously stirred tank reactor (CSTR) operation significantly affects the efficiency of biogas production.

HRT adjustments can be used as a tool to select microbial populations whose growth rates are able to match the dilution rates maintained in the bio-reactor (Tijhuis et al., 1994). HRT is one of the most important process control parameters affecting the continuous production of H_2. Proper control of HRT helps in eliminating the non-spore forming methanogens, which have longer doubling times and will get washed out. The reported optimum HRTs for H_2 production vary, from 8 h for sucrose (Chen and Lin, 2003), 12 h for glucose (Lin et al., 2006), and 18-24 h for brewery wastewater (Fan et al., 2006; Yu et al., 2003). Inconsistent findings such as these are often associated with variations in: the experimental conditions, the nature of active microbial populations associated with a particular type of inoculum source, and the type of substrate used. For example, propionic acid producers are often very sensitive to changes in HRT. Zhang et al. (2006) observed that propionic acid producers were not identified at low HRT (6 h), which is consistent with the ongoing absence of propionic acid from the VFAs found in the analysis. Cha and Noike (1997) noticed that propionic acid producers were not observed in a chemostat using starch as the substrate when the HRT was decreased from 48 h to 12 h. Lin and Chang (2004) also observed decreased propionic acid production with a 6 h HRT. These studies provide evidence that HRT affects propionic acid producers in mixed anaerobic cultures.

Chu et al. (2009) observed a common pattern amongst cells quantified with probe Clost IV (targeting genus *Clostridium* based on 16S rRNA). The number of hydrogen producing bacteria (HPB) increased with decreasing HRT. The number of HPB at a 6 h HRT was found to be $9.2*10^8$ cells mL^{-1} compared to $6.2*10^7$ cells mL^{-1} at a 14 h HRT. The researchers also concluded that HRT seemed to have little impact on the total number of bacteria, whereas the percentage of HPB increased to 97 % at HRT of 6 h from 10 % at HRT of 14 h.

Proper optimization of HRT is required for the continuous H_2 production in bioreactors at high dilution rates. Failure to operate the bioreactor at optimum HRT will often result in the washout of biomass, resulting in operational instability and inefficient biohydrogen production (Chen et al., 2006; Zhang et al., 2006). CSTR operation is considered to be in a steady-state condition when the fermentation by-products are stable with variation of less than 15 % (Mu et al., 2006; Shin et al., 2004; Yusoff et al., 2009). Researchers have also studied the effect of different HRTs on the CO_2/H_2 ratio. The theoretical value of this ratio should fall between 0.5 - 1.0 (Wang et al., 2007). For a HRT of 5 days, the ratio was 10.1 but gradually decreased to 2.3 as HRT was lowered. This decrease in the CO_2/H_2 ratio indicated that the concentration of biohydrogen increased with a decrease in HRT. Higher H_2 yields as well as an increased production rate were observed at low HRTs if the reactor was operated at optimum pH, temperature and biomass concentration (Hawkes et al., 2002; Shin et al., 2004). The ratio of butyric acid (HBu)/acetic acid (HAc) is also a useful indicator of biohydrogen production during the acidogenesis phase (Chen et al., 2001; Das and Veziroğlu, 2001). Researchers have reported different optimal HBu/HAc ratios, depending upon the types of substrates and cultures used. For instance, Chen et al. (2001) reported an optimum ratio of 5.0 using glucose as the substrate, but Kim et al. (2008) obtained a ratio of about 4.0 using the same substrate. Variation in HRTs has an effect on the HBu/ HAc ratio.

2.3.5 Hydrogen partial pressure (P_{H2})

The partial pressure of H_2 (P_{H2}) in the reactor headspace during fermentation has a marked effect on H_2 production. The fermentation pathway shifts from acid production to alcohol production once the P_{H2} in the headspace is above a certain threshold level (Doremus et al., 1985). This, in turn, affects the H^+/H_2 redox potential, thus inhibiting the flow of electrons from reduced ferredoxin to molecular H_2 (via hydrogenase). Under such conditions, the electrons generated from substrate oxidation are diverted to NADPH generation (by ferredoxin oxidoreductase) resulting in increased production of alcohols (butanol and ethanol). Moreover, the acetogenesis of propionic acid and butyric acid requires the maintenance of low P_{H2} level (ΔG = +76.2 and +48.4 kJ for the acetogenesis

of propionic acid and butyric acid). Lower P_{H2} levels also result in the conversion of ethanol to acetic acid (ΔG = +9.6 kJ).

There are several strategies described in the literature to prevent the deleterious effect of H_2 accumulation in the reactor headspace. Sparging with an inert gas such as N_2 will help to remove H_2 from the headspace. Increases in H_2 yields of about 80 % were observed with N_2 sparging compared to the control condition (without sparging) (Mizuno et al., 2000). The major disadvantage of the sparging process is that it dilutes the H_2 gas, which makes the recovery process uneconomical. The yields obtained from the batch fermentation process are low compared to the yields report for many of the semi-continuous or continuous processes. This may be due to the increase in P_{H2} that develops during the batch fermentation process. Logan et al. (2002) reduced the headspace pressure to examine the effects of pressure release on increasing overall biohydrogen production. Continuous pressure release from the headspace (respirometric method- which involves continuous gas release using a bubble measuring device) was found to be more effective than intermittent pressure release (Owen method- which involves removing headspace gas using a tight syringe at regular intervals). With the respirometric method, the pressure in the headspace is always maintained at the ambient pressure. Another study (Valdez-Vazquez et al., 2005) used the intermittently vented solid substrate for anaerobic H_2 generation (IV-SSAH) procedure to prevent the increase in P_{H2}. This procedure involves flushing the headspace of the reactor with N_2 once a specific H_2 level is reached. After releasing the headspace gas H_2 production was monitored without further addition of substrate which was followed by flushing with N_2 gas again for the second time. This procedure was also repeated for the third time in a similar fashion. The researchers found that the cumulative H_2 production of the three release cycles was almost double that obtained during the first cycle alone.

Another strategy used selective permeable membranes to separate H_2 from the reactor headspace. The permeability characteristics of the silicone rubber membrane used in this study were 4.58 x 10^{-8} cm^2/s. kPa (35 °C) for H_2 and 2.60 x 10^{-7} cm^2/s. kPa (35 °C) for CO_2 (Liang et al., 2002). However, the use of a membrane in the interior of the reactor had no significant difference (< 10 %) between the H_2 production of the test reactor with

membrane separation and the control reactor without membrane separation. To date, sparging has been found to effectively increase H_2 yields, reduce the P_{H_2}, and increase gross H_2 production. Further research on this topic is needed, however, to overcome the limitations of this process.

2.4 Role of hydrogenases in biological H_2 production

There are a variety of microorganisms capable of producing H_2 via dark fermentation of which strict anaerobes of the genus *Clostridium* have been studied in greater detail. These bacteria can reversibly reduce protons formed during fermentation to H_2, a reaction catalyzed by hydrogenases. The direction of the reaction is highly dependent upon the oxidation-reduction (redox) potential of the components that can interact with hydrogenase (Vignais and Colbeau, 2004). Nitrogenases, as well as hydrogenases, play an important role in the reduction of H^+ to H_2 under photo heterotrophic conditions. Since 1931, when hydrogenase was first described by Stephenson and Stickland, extensive research has been conducted on this enzyme (Mertens et al., 2003). Hydrogenases are more efficient than nitrogenases with turnover rates that are 1000 times higher than that of nitrogenases (McKinlay and Harwood, 2010). These enzymes are found in diverse organisms, including methanogens, acetogens, sulfate-reducing bacteria, anaerobic archea, rhizobia, protozoa, fungi, and some green algae. Numerous studies have been conducted on biohydrogen production by the genus, *Clostridium*. However, only a few of these focused on the role of hydrogenases (Morimoto et al., 2005; Wang et al., 2008).

Better understanding of these *Clostridial* hydrogenases is necessary for sustainable H_2 production applications. Fermentative hydrogen production from glucose begins with the oxidation of glucose into two molecules of pyruvate, which is further oxidized into two molecules of acetyl-CoA, and ultimately to acetic acid. Hydrogenases play an important role in the occurrence of the above reaction steps by reacting with reduced ferredoxin, which makes these reactions more favorable thermodynamically. For example, H_2 is generated during pyruvate oxidation by the enzyme, Pyruvate: ferredoxin oxido reductase (PFOR). Another pathway to H_2 production occurs during the intial step of glycolysis, in which the cytosolic hydrogenase coupled to NADH: ferredoxin oxido reductase uses NADH as the electron donor to reduce protons to molecular H_2 (Vardar-Schara et al.,

2008). Hydrogen is also generated by trimeric bifurcating hydrogenase, which simultaneously oxidizes NADH and ferredoxin under low P_{H_2} (Schut and Adams, 2009).

The lack of experimental data on the biochemical properties and function of *Clostridial* [Fe-Fe] hydrogenases make these enzymes a "black-box" in fermentative H_2 technology. Understanding the role of these hydrogenases would help to overcome several problems in developing this technology; in particular, examination of their capacity to degrade cellulose. One approach to the study of hydrogenases is to design a 'knockout', a mutant strain lacking an uptake hydrogenase, thereby preventing the oxidation of the H_2 produced. Another approach is to screen for novel hydrogenase enzymes with high turnover rates and with a wide range of redox partners, as well as low sensitivity to O_2 inactivation. Integration of these novel enzymes into non-H_2 producing hosts could increase H_2 yields to a commercially competitive level. *Clostridia* are very well known for their ability to degrade cellulose, the most abundant polymer on earth. Transition to a H_2-fuel economy will not take place unless the H_2 is produced from renewable resources, such as lignocellulosic materials. Efficient degradation of lignocellulosic biomass is required to maximize H_2 production (Liu et al., 2008). Understanding the structure and function of hydrogenases in some of the cellulose-degrading *Clostridium* species, such as *C. cellulolyticum*, *C. phytofermentans* or *C. thermocellum*, will help in the development of large-scale H_2 production. With the advancements in genomics, the genome sequences of 450 hydrogenases are available and this has led researchers to categorize these enzymes (Meyer, 2007). Despite their diversity (in size, structure, electron donors, etc.), these hydrogenases have been grouped into three main categories, namely: [Ni-Fe] hydrogenases, [Fe-Fe] hydrogenases, and [Fe] hydrogenases, depending on the presence of metal atoms in their active centers as described below.

[Ni-Fe] Hydrogenases contain a Ni atom within their active centre and comprise one of the largest families of hydrogenases. [Ni-Fe] hydrogenases are less sensitive to inhibition by CO and O_2 than other hydrogenases (Cammack et al., 2001). They can be dimeric, trimeric or tetrameric in their structure, and are further divided into four classes based on their function:

Class I [Ni-Fe] hydrogenases are uptake hydrogenases, which catalyze the uptake of molecular H_2. They are found in *Wolinella succinogens*, *Aquifex aeolicus*, and *Thiocapsa roseopersicina* as well as some *Desulfovibrio* sp. This class of membrane-bound hydrogenases links the oxidation of H_2 to the reduction of various electron acceptors, such as NO_3^-, SO_4^{2-}, fumarate, CO_2 (during anaerobic respiration) or O_2 (during aerobic respiration) (Sargent et al., 2006). Energy is generated through ATP synthesis via proton motive force. These enzymes are generally characterized by the presence of a long signal peptide at the N terminus of the subunit.

Class II [Ni-Fe] hydrogenase does not contain a signal peptide at the N terminal region of the smaller subunit. This class includes both: (1) cytoplasmic H_2 sensors and (2) cyanobacterial uptake [Ni-Fe] hydrogenases. The cytoplasmic hydrogenases remain in the cytoplasm of the cell, sensing the presence of H_2 in their environment. Once H_2 is detected, these enzymes trigger a cascade of cellular reactions controlling the synthesis of hydrogenases (Kleihues et al., 2000). This type of hydrogenase is usually found in *Bradyrhizobium japonicum*, *Rhodobacter eutropha*, *Rhodobacter capsulatus* and *Rhodobacter palustris*. These enzymes are less sensitive to O_2 than other types of hydrogenase. Cyanobacterial uptake [Ni-Fe] hydrogenases are induced under N_2 fixing conditions and are present in cyanobacteria, such as *Nostoc* and *Anabaena variabilis* (Happe et al., 2000; Oxelfelt et al., 1998).

Class III [Ni-Fe] hydrogenases are bidirectional hydrogenases, which function reversibly and can reoxidize several cofactors, such as NAD, NADP and Cofactor 420 (F_{420}). They are mainly found in archea, such as *Methanothermobacter marburgensis* and *Methanosarcina mazei*. Their main function is to reduce S^o to H_2S using NADPH as the electron donor, and to generate reducing equivalents for heterodisulfide reductase (Ma et al., 2000).

Class IV [Ni-Fe] hydrogenases catalyze H_2 formation from reduced ferredoxin generated during the oxidation of acetic acid to CO_2 (Künkel et al., 1998). The majority of these enzymes are found in the Archea family, including *Methanosarcina barkeri*, *Methanothermobacter marburgensis* and *Pyrococcus furiosus*. They also catalyze the reduction of protons from water, thereby disposing of excess reducing equivalents

generated during anaerobic oxidation of single carbon compounds, such as CO and formic acid (Fox et al., 1996; Sauter et al., 1992).

[Fe-Fe] hydrogenases, unlike their [Ni-Fe] counterparts, are monomeric in nature and vary in their size. They are the only type of hydrogenases present in eukaryotes (Vignais and Colbeau, 2004). They are also found in anaerobic prokaryotes, such as *Clostridia* and sulfate reducers (Atta and Meyer, 2000). They are mainly involved in H_2 production, but there are reports suggesting that they play a role in proton oxidation in *D.vulgaris*, *Eubacterium acidaminophilum*, and as an "electron valve" that enables algae to survive under anaerobic conditions (Graentzdoerffer et al., 2003; Happe et al., 2002; Pohorelic et al., 2002).

[Fe] hydrogenases were first found in the hydrogenotrophic methanogen, *Methanothermobacter marburgensis* (Vignais and Billoud, 2007), and were originally identified as "metal free hydrogenases" or "iron sulfur cluster free hydrogenases" (Shima and Trauer, 2007). These hydrogenases do not catalyze the reversible oxidation of molecular H_2.

Successful H_2 production via dark fermentation requires inhibition of the uptake hydrogenases present in the H_2 consumers present in mixed anaerobic culture. Several studies conducted with mutant strains deficient in uptake hydrogenases showed increased production of H_2 (Kars et al., 2008; Öztürk et al., 2006). Chemical mutagenesis of the uptake hydrogenase (hup) gene in *Anabaena variabilis* resulted in increased production of H_2 (4 to 7 fold increase) compared to production in the wild strain (Mikheeva et al., 1995). Correlating the level of hydrogenase activity with the acetic acid/butyric acid (HAc/HBu) ratio provides better understanding of biohydrogen production from a substrate under particular fermentation conditions (Pendyala et al., 2012). One such study determined the level of hydrogenase gene (hyd) expression using real-time transcriptase polymerase chain reaction (RT-PCR) (De Sá et al., 2011). The levels of hyd gene expression in heat-treated sludge was found to be about 3 times higher than in untreated control sludge. The HAc/HBu ratio in heat-treated sludge was 7, and the correlation between hyd gene expression and the HAc/HBu ratio indicated a clear relationship between hyd gene expression and H_2 production in heat-treated sludge. Pendyala et al.

(2012) compared the activity of uptake hydrogenases with the HAc/HBu ratio in order to obtain a clear indication of the H_2 consumers present in mixed microbial community samples, which had undergone various pre-treatments.

2.5 Reactors for biological H_2 production

Commercial production of H_2 on an industrial scale requires a continuous or semi-continuous fermentation process (Hallenbeck and Ghosh, 2009). An optimized reactor needs to be developed, which can be operated continuously and can provide reliable, stable performance over long periods and can withstand temporary fluctuations in operational parameters. These capabilities will increase the volumetric H_2 production rates (Hallenbeck and Ghosh, 2009). The ability of many types of continuous reactors to meet the above process requirements have been studied (Table 2.5). The continuous stirred tank reactor (CSTR) is the most preferred reactor compared to other type of reactor configurations (Table 2.5) for biological H_2 production because it allows for better control of process parameters, such as pH and temperature (Li and Fang, 2007). The H_2 yield and production rate varies in CSTRs due to differences in reactor configurations and process parameters.

The continuous production of H_2 has been extensively studied using CSTR (Li et al., 2008; Shen et al., 2009; Xu et al., 2010; Yuan et al., 2010). Difficulty in maintaining higher levels of microbial biomass at shorter HRT is often encountered in CSTR which leads to operational instability, and limits the H_2 production rate (de Amorim et al., 2009). The anaerobic sequencing batch reactor (ASBR) has become the reactor chosen to overcome such process limitations to the production of H_2 using a wide variety of renewable substrates. Advantages of the ASBR over the CSTR include the ability to maintain higher concentrations of biomass in the reactor during operation, a high degree of process flexibility, and avoids the need for a separate clarifier (Dague et al., 1992).

Data collected by Environment Canada's wastewater technology centre (WTC) operating two 2.5 m^3 sequencing batch reactors (SBR) showed that the SBR process meets the specific needs of research better than the CSTR under several circumstances. The SBR can be operated by changing the process control parameters of the feed and react

sequences separately, making it more flexible than continuous flow systems, which require complex process modifications. Modifications to operational parameters can be made within minutes for a SBR with a control interface, whereas a CSTR requires more labour-intensive and time-consuming hardware modifications. An ASBR is best suited for treating the wastewater generated from brewery sludge (Zupancic et al., 2007).

Table 2.5 H_2 yields from various types of reactor configurations.

S.No.	Inoculum type	Substrate	Type of reactor	H_2 yield (mol mol^{-1} substrate)	Reference
1	Sewage sludge	Glucose	CSTR	1.81	Gavala et al. (2006)
2	Sewage sludge	Sucrose	CSTR	4.52	Chen and Lin (2003)
3	Sewage sludge	Fructose	CSTR	1.68	Lee et al. (2007)
4	Sewage sludge	Xylose	CSTR	1.63	Wu et al. (2008)
5	Sewage sludge	Sucrose	UASBR	1.5	Gavala et al. (2006)
6	Sewage sludge	Glucose	ABFBR	1.7	Zhang et al. (2008)
7	Sewage sludge	Sucrose	AGFBR	1.6	Zhang et al. (2008)
8	Sewage sludge	Xylose	PAC-GSBR	0.7	Wu et al. (2008)
9	Sewage sludge	Sucrose	PBR	3.9	Lee et al. (2003)
10	Sewage sludge	Glucose	MBR	1.72	Lee et al. (2007)
11	Sewage sludge	Xylose	IC-CSTR	0.8	Wu et al. (2008)
13	*Enterobacter cloacae* DM 11	Glucose	Packed bed	1.54	Koskinen et al. (2008)
14	Thermophilic mixed culture	Glucose	TBR	1.11	Oh et al. (2004)
15	Mesophilic mixed cultures	Sucrose	CIGSBR	3.03	Lee et al. (2004)
16	Sewage sludge	Glucose	CSTR	2.1	Fang et al. (2002)
17	Molasses wastewater	Sucrose	PAC-EGSBR	3.47	Guo et al. (2008)

Note: CSTR = Continuously stirred tank reactor; UASBR = Up-flow anaerobic sludge blanket reactor; PBR = Packed bed reactor; IC-CSTR = Immobilized cell-continuously stirred tank reactor; TBR = Trickling bed reactor; PAC-EGSBR = Powdered active carbon assisted-expanded granular sludge bed reactor; AGFBR = Anaerobic granular fluidized bed reactor; ABFBR = Anaerobic biofilm fluidized bed reactor; MBR = Membrane bio reactor; CIGSBR = Carrier induced granular sludge bed bioreactor.

Combined ASBR-UASBR treatment increases the biogas yield from all types of brewery wastewater compared to the yield from conventional UASBR. Combined ASBR-UASBR wastewater treatment also reduces the overall reactor volume by 25 % compared to conventional UASBR treatment (Zupancic et al., 2007). Recent developments in reactor design have increased the efficiency of ASBR to treat domestic sewage using higher loading rates than conventional digesters, and the development of systems capable of treating low to medium strength wastewaters.

The salient features of ASBR are relatively simple operation procedures combined with higher organic removal efficiency. The HRT is an important process parameter that determines the costs of the H_2 production process. Lower HRT is often attributed to the requirement for a smaller reactor volume, thereby reducing cost, but also enhancing the productivity of the H_2 production process (Kim et al., 2006).

Thus, optimizing the process parameters, such as HRT, organic loading rate (OLR) and SRT, as well as pH and temperature, will facilitate the scaling up of the H_2 production process. The H_2 yields from various types of reactor configurations are shown in Table 2.5.

2.6 Microbial pre-treatment methods

Biological H_2 production via dark fermentation can be carried out using pure cultures or mixed cultures, but H_2 production from pure cultures showed higher yields (2-4 mol H_2 mol^{-1} glucose) (Kumar et al., 2001; Schroder et al., 1994). Employing pure cultures for H_2 production is not economically feasible however, due to the maintenance costs associated with the process.

On the other hand, employing mixed cultures for H_2 fermentation can be problematic because of the loss of H_2 due to the presence of H_2 consuming bacteria (methanogens, homoacetogens and sulfur-reducing bacteria) as well as H_2 producing bacteria in mixed cultures. In order to selectively enrich the H_2 producing bacteria and inhibit the growth of H_2 consuming bacteria, several types of pretreatments (such as heat, acid, alkali, organic loading shock, BES, chloroform, and LCFAs) have been studied (Lalman and Bagley, 2002; Wang and Wan, 2008; Wang and Wan, 2009).

The main purpose of the pretreatments is to prevent the loss of H_2 to H_2 consumers and to enrich the spore-forming H_2 producing bacteria. Despite the wide range of pretreatment studies that have been conducted, the conclusions regarding the most effective method to enhance H_2 yield are contradictory. This may be due to differences in fermentation conditions, such as the temperatures, pH levels, sources of inoculum, and the nature of the substrates employed in these studies. Moreover, many of these studies were conducted as single batch cultivations, and studies describing the long term effects of pretreatment are scarce (Luo et al., 2011).

2.6.1 Heat treatment

Heat treatment is the most widely studied inoculum pretreatment. This type of pretreatment involves the application of moist heat (Lay, 2000) or dry heat (Van Ginkel et al., 2001) to disrupt the vegetative cells of the microorganisms. Heating the inoculum (microbial seed culture) at a temperature of about 100 °C for a few minutes results in the release of endospores (survival structures) by spore-forming microorganisms (e.g. *Clostridium* sp. and *Bacillus* sp.). Non-spore forming methanogens are destroyed by this treatment as they do not have the ability to form endospores. Endospores are released by organisms under unfavorable environmental conditions (such as high temperature, lack of a carbon source, desiccation and chemical toxicity). The heat treatment method was successfully employed for the pure culture isolation of spore-forming *Clostridium* species (Alexander, 1977; Gordon, 1990). Heat treatment is not successful against H_2 consuming homoacetogenic bacteria, however, since many of these bacteria can form spores. Oh et al (2003) studied the effectiveness of heat treatment for enhancing H_2 production in mixed culture, and found that heat treatment was inefficient because it could not prevent the H_2 loss due to homoacetogens (Savage et al., 1987).

2.6.2 Acid treatment

Pretreatment of the inoculum with acid is performed using dilute acids. An experiment was conducted to study the relative effectiveness of three dilute acids (HCl, HNO_3 and H_2SO_4) at enriching the HPB present in mixed anaerobic culture (Lee et al., 2009). Among them, HCl was effective at enriching the spore-forming *Clostridium* sp., which

accounted for 85 % of the total *Eubacteria* population after pretreatment. The volume of H_2 produced increased following HCl pretreatment to about 2.8 times that of the untreated controls. The other acids tested (HNO_3 and H_2SO_4) were not as effective as HCl because of the toxic nature of these acids, even when diluted. Nitrate (NO_3^-) acts as a potential inhibitor of HPB at concentrations above a specific threshold level. The acid pretreatment uses 2-3 N acid to adjust the pH of the inoculum to 3.0. This inoculum is then incubated for 24 hours following pH adjustment. After the incubation period, the pH of the inoculum is raised to the level specified in the experimental design using a base (NaOH or KOH). Kim et al. (2004) studied the effects of various concentrations of NO_3^-, and found that 1200 mg NO_3^- L^{-1} was inhibitory to HPB. Acid pretreatment was suitable for enriching acidophilic HPB present in the inoculum. This type of inoculum pretreatment is more suitable for carbohydrate rich wastewater.

2.6.3 Alkali treatment

Alkali treatment used sodium hydroxide (NaOH) or KOH to adjust the pH of the inoculum to alkaline levels (pH 12.0). This type of pretreatment selectively enriches mesophilic HPB, and is more suitable for protein rich wastewater. Alkaline pretreatment increases the solubilization of organic matter more than acid pretreatment. *Eubacterium* sp. and *Bacillus* sp. were the dominant bacteria found in alkaline pretreated sewage sludge (Cai et al., 2004).

2.6.4 BES treatment

2-bromoethane sulfonic acid (BES) is a specific inhibitor of methanogenesis (Balch and Wolfe, 1979), preventing the flow of carbon flux in methanogenic environments; the transfer of the methyl group during the final reductive step in the methanogenesis process is selectively inhibited by BES. BES is a structural analog of Co-enzyme M (2-mercaptoethanesulfonc acid), which catalyzes the methyl transfer reaction (Ma and Thauer, 1990). The growth of a wide variety of non-methanogenic *Archaebacteria* and *Eubacteria* is not affected by the addition of BES. For example, *Eubacterium limnosum* was uninhibited by the addition of 25 mM BES (Sparling and Daniels, 1987). This species reduced one carbon compound similarly to methanogenic A*rchaea*. BES has a

slight inhibitory effect (growth was 23 % less than control observations) on the growth of sulphurophiles (organisms that use SO_4^{2-} or S^- as their sole source of energy). Sparling and Daniels (1987) studied the inhibitory effects of the addition of BES (25 mM) on the growth of a wide variety of aerobic, facultative and strictly anaerobic bacteria. The study revealed that 25 mM BES is sufficient to inhibit the growth of methanogenic bacteria, whereas it has no significant effect on the growth of non-methanogens. In anaerobic environments, 50 mM BES is required to completely inhibit the H_2/CO_2 dependent methanogenesis (Zinder et al., 1984). The use of specific types of methanogenic inhibitors to examine the degradation products of organic substrate degradation in various anaerobic environments (e.g. lake sediments, rice paddies and anaerobic digesters) is useful in determining the importance and the role of methanogens in such environments.

2.6.5 Long chain fatty acid treatment

Long chain fatty acids (LCFAs) undergo β-oxidation and are degraded to short chain fatty acids, such as acetic acid (obtained by β-oxidation of even numbered saturated fatty acids) and propionic acid (obtained by β-oxidation of odd numbered saturated fatty acids). In an anaerobic digester, methanogens are unable to utilize LCFAs or lack the ability to degrade them. These methanogens rely on syntrophic organisms or acetogens to degrade LCFAs into acetic acid and H_2, which can then be used as substrates for methanogenesis. Unsaturated fatty acids must be saturated prior to undergoing β-oxidation. Thermodynamically, the oxidation of LCFAs is unfavorable under standard conditions. The P_{H2} must be kept low in order to drive the β-oxidation reaction forward. This can be achieved using syntrophic H_2 utilizing acetogenic bacteria or hydrogenotrophic methanogens that use H_2 as a substrate. Many edible oil industry and dairy wastewater effluents contain lipids and fats in their effluent stream. Fatty acids are abundant in many types of vegetable oils, such as safflower oil, corn oil, coconut oil, etc. They are also present in fish oil as polyunsaturated fatty acids (PUFAs). Fatty acids, such as caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), are inhibitory to both aceticlastic methanogenesis and hydrogenotrophic methanogenesis (Koster and Cramer, 1987; Lalman and Bagley, 2002).

2.6.5.1 Mechanism of action of LCFAs

The inhibitory actions of LCFAs can be explained in two possible ways. First, this type of inhibitory mechanism might involve direct chemical action of LCFAs on bacteria. However, there is no evidence of this mechanism. Moreover, this type of mechanism would be limited to unsaturated LCFAs. Birkinshaw and Raistrick (1943) concluded that H_2O_2 formed in the medium during the degradation of linolenic and linoleic acids was responsible for the inhibitory action of LCFAs. However, Kodicek and Worden (1944) found no evidence of H_2O_2 formation in media incubated with linoleic acid. The second possibility involves a physicochemical mode of action. Unsaturated fatty acids form a monolayer around bacteria and exert inhibitory effects by altering the permeability of adjacent surfaces, by exerting some type of chemical influence, and by altering the surface tension, thereby affecting bacterial division (Kodicek and Worden, 1944).

The nature of the monolayer formed by LCFAs varies for saturated and unsaturated fatty acids, with the latter having a larger inhibitory effect. The double bonds in unsaturated fatty acids will cause them to bend around the surface of bacteria, whereas saturated LCFAs tend to lie in parallel straight chains. The surface area of lactobacilli in 0.5 mL of inoculum is on the order of 3.3×10^{10} μ^2, with 400 millions/mL as the average concentration. The surface area covered by 160 µg of linoleic acid is on the order of 1.75×10^{11} μ^2, which is sufficient to form five monolayers around lactobacilli. Both linolenic and linoleic acid exert bacteriostatic action on gram positive microorganisms (Kodicek and Worden, 1944). The physicochemical mode of action of LCFAs was demonstrated using surface active agents, such as lecithin, cholesterol, calciferol and lumisterol, which form complexes with linoleic acid, and so LCFAs could be removed from the bacterial cell surface.

2.7 Lignocelluloses as renewable feedstocks for hydrogen fermentation:

The wastes from forestry, agricultural and agro-industrial wastes comprise lignocellulosic biomass (LB). They are abundant, renewable and inexpensive energy sources. These wastes are accumulated in large quantities every year. These LB residues rich in sugars

are utilized for the production of large number of value added products such as H_2, ethanol, food additives, organic acids, enzymes and others.

Despite the environmental problems caused by the accumulation of LB residues in nature, their potential for a variety of value added products make them a potentially valuable resource. The major building blocks of LB are cellulose, hemicellulose, and lignin polymers arranged closely with each other (Figure 2.4). Among them, cellulose is the dominant polysaccharide in LB accounting for (35- 50 %), followed by hemicellulose (20-35 %) and lignin (10-25 %) (Saha, 2003).

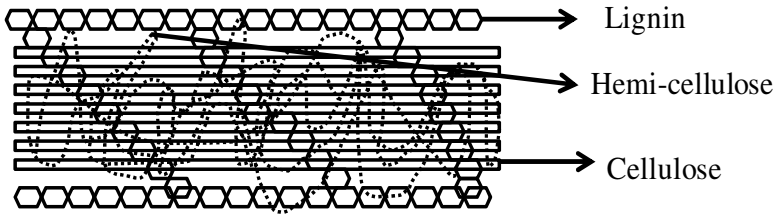

Figure 2.4 Typical lignocellulosic biomass structure showing arrangements of cellulose, hemicellulose and lignin.

Cellulose is a linear homopolymer with high molecular weight made up of repeated units of cellobiose. The cellobiose units are connected with each other via a B-1, 4 glycosidic linkages. The polymers are linked together by hydrogen and van-der walls bonds. This nature of the cellulose polymers cause them to be packed into micro fibrils (Ha et al., 1998). These long-chain polymers are arranged in parallel and form a crystalline structure. Around two-third of the total cellulose is made of compactly packed crystalline cellulose and the remaining portion by less-ordered amorphous regions. The crystalline portion of cellulose is less soluble and degradable (Taherzadeh and Karimi, 2008; Zhang and Lynd, 2004). Hemicellulose is typically made up of five different sugars namely L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose.

Table 2.6 Composition of cellulose, hemicellulose, and lignin in common agricultural residues and wastes - Source: Saratale et al. (2008).

S.No.	Type of Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
1	Hardwood stems	40-55	24-40	18-25
2	Softwood stems	45-50	25-35	25-35
3	Nut shells	25-30	25-30	30-40
4	Corn cobs	45	35	15
5	Grasses	25-40	35-50	10.0-30.0
6	Paper	85-99	0	0-15
7	Wheat straw	33-38	26-32	17-19
8	Sorted refuse	60	20	20
9	Leaves	15-20	80-85	0
10	Cotton seed hairs	80-95	20-5	0
11	Newspaper	40-55	24-40	18-30
12	Waste papers from chemical pulps	60-70	10.0-20.0	5.0-10.0
13	Primary wastewater solids	8.0-15.0	NA[a]	24-29
14	Swine waste	6	28	NA[a]
15	Solid cattle waste	1.6-4.7	1.4-3.3	2.7-5.7
16	Coastal Bermuda grass	25	35.7	6.4
17	Switch grass	45	31.4	12
18	Sorghum stalk	27	25	11
19	Rice bran	35	25	17
20	Rice straw	32-47	19-27	5.0-24.0
21	Coconut fiber	36-43	1.5-2.5	41-45
22	Wheat bran	30	50	15
23	Barley bran	23	32	21.4
24	Barley straw	31-45	27-38	14-19

Note: [a]Not available.

Hemicellulose polymers are both linear and heterogeneously branched in nature. The other components of hemicellulose include acetic, glucuronic and ferulic acids. The backbone of hemicellulose can be a homopolymer (made up of single type of sugar) or a heteropolymer (made of mixture of different sugars). Based on the major sugar residue in their backbone hemicellulose can be classified into different classifications e.g., xylans, mannans, glucuronoxylans, arabinoxylans, glucomannons, galactomannans, galactoglucomannons, β-glucans and xyloglucans. Hemicelluloses are amorphous in

nature, characterized by the presence of shorter polymer chains compared with glucose (Fengel and Wegener, 1984). This nature makes them easier to hydrolyze compared with cellulose.

Lignin on the other hand is a very complex molecule comprised of phenylpropane units linked with each other. The monomers of lignin are made of three phenyl propionic alcohols: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The major function of lignin is to provide rigidity and cohesion to the cell wall, to form a physico-chemical barrier against microbial attack. Lignin polymers are extremely resistant to chemical and enzymatic degradation. The composition of LB varies with type of plant species (Table 2.6). The constituents in a single plant species also found to vary with stage of growth and age of the plant.

2.7.1 Pretreatment of lignocellulosic biomass

The main aim of the pretreatment process is to remove lignin and hemicellulose and to a greater extent reduce the crystalline nature of cellulose resulting in increase in porosity of LB. The main requirements of the pre-treatment process are as follows:

(1). Greater release of sugars from LB or the ability to release sugars from LB upon hydrolysis.

(2). Prevent the degradation of carbohydrates.

(3). Prevent/limit the formation of inhibitory by-products (Phenols, furan derivates, etc.) resulting from sugar degradation which are otherwise inhibitory to the fermenting microorganisms.

(4). Must be cost effective in nature.

There are various type of pretreatments employed for different type of LB (Saratale et al., 2008). Despite the availability of a wide range of pretreatments, it is emphasized that pretreatment efficiency varies with the type of LB. The choice of pretreatment depends upon the composition of LB and the byproducts produced as a result of pretreatment. The costs associated with the pretreatment process are greatly affected by these factors. Many

researchers have conducted comparative analysis of various pretreatments to determine the most efficient pretreatment for a particular LB material (Rosgaard et al., 2007; Silverstein et al., 2007; Wyman et al., 2005a; Wyman et al., 2005b). The efficiency of acid/water impregnation followed by steam explosion and hot water extraction on barley and wheat straw was compared by Rosgaard et al (2007). These researchers concluded that acid/water impregnation followed by steam explosion was found to be the most effective pretreatment in terms of glucose recovery from barley straw. The pretreated LB residue was subjected to enzymatic hydrolysis by cellulase enzyme to compare their effectiveness of pretreatment.

Table 2.7 Effect of steam explosion pretreatment on enzymatic sachharification on different type of biomass materials (modified from (Kosaric and Vardar-Sukan, 2001; Orozco et al., 2007)).

S.No.	Biomass type		Steam explosion pretreatment	Total reducing sugars(g L^{-1})[a]
1	Hardwood	Poplar	No	2.4
			Yes	25.8
		Aspen	No	3
			Yes	24.8
2	Agricultural residues	Corn stover	No	7.8
			Yes	22.5
		Sugarcane Bagasse	No	2.5
			Yes	16.1
		Urban waste	No	18
			Yes	10.8
3	Softwoods	Eastern spruce	No	3.8
			Yes	6.4
		Douglas fir	No	3.2
			Yes	4.3

Note: [a] Sachharification of steam exploded biomass using enzyme from *Trichoderma reesei* (QM9414). The steam exploded biomass was washed before subjected to 19 IU of enzyme per g substrate (5 % substrate slurry) at pH 4.8 and 50°C.

Another study by Silverstein et al (2007) compared the effectiveness of sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozonation pretreatments on bioethanol production from corn stalk. These researchers observed that different pretreatments have proven efficient in various ways. The highest xylan conversion was observed with sulfuric acid pretreatment but with lower cellulose to glucose conversion. Maximum

lignin degradation and cellulose degradation occurred in sodium hydroxide pretreatment compared with hydrogen peroxide pretreatment. Ozone treatment was found to be the least efficient with corn stalk compared with other three types of pretreatment and does not affect lignin, glucan and xylan contents over time. Studies conducted on comparison of various pretreatments on corn residues also supported the theory that pretreatment efficiency varies with type of LB (Wyman et al., 2005a).

2.7.2 Steam explosion pretreatment

The most commonly used method for pretreatment of LB is steam explosion pretreatment (McMillan, 1994). Steam explosion pretreatment is more attractive compared with other types of pretreatment due to lower energy consumption and low use of chemicals. The LB is acted upon by the high-pressure saturated steam, followed by sudden release in pressure making the materials to undergo explosive decompression. The temperature during the steam explosion process ranges from about 160-260 °C and the corresponding pressure range is about 0.69-4.83 MPa. Such high temperatures and pressures are maintained from several seconds to few minutes before bringing the material down to the atmospheric pressure (Sun and Cheng, 2002). Explosive decompression of LB is necessary to facilitate hemicellulose hydrolysis. The high temperature used in the steam explosion process breaks down the lignin polymers and helps in hemicellulose degradation. This also increases the accessible surface area of LB for cellulose recovery by enzyme pretreatment. The hemicellulose polymers are hydrolyzed with the help of acetic and other type of acids released during steam explosion. Increase in enzymatic hydrolysis (about 90 %) was achieved in steam exploded poplar chips compared to 15 % efficiency in untreated chips (Grous et al., 1986). Incomplete melting and depolymerization/ repolymerization of lignin polymers occur as a result of explosive decompression. The turbulent flow of the material combined with rapid drop to atmospheric pressure result in fragmentation of material (Duff and Murray, 1996). This increases the accessible surface area of the LB. Compared with municipal solid waste (MSW) and soft wood chips, agricultural residues and hard wood chips are pretreated with higher efficiency using steam explosion pretreatment (Table 2.7). The efficiency of

the different types of material was compared on the basis of accessible surface area of LB after steam explosion for enzymatic hydrolysis.

The major factors affecting the steam explosion pretreatment are temperature, residence time, particle size, and moisture content of LB. Maximum hemicellulose recovery can be obtained by maintaining a shorter residence time of about one minute to few seconds at high temperature (240-270 °C) or maintaining a longer residence time about 5 to 10 minutes at low temperature (170-190 °C). The low energy requirement of steam explosion pretreatment makes it more commercially feasible method of sugar recovery and particle size reduction compared with other types of mechanical pretreatment. Steam explosion requires about 70 % less energy compared to other physical pretreatment methods to achieve the same particle size reduction.

Bio-ethanol production plant, Iogen, Canada employs steam explosion pretreatment on a variety of LB material. For larger scale application, steam explosion pretreatment is carried out with the addition of suitable catalyst such as dilute sulfuric acid (or SO_2) added in 0.3 – 3.0 % (wt/wt) to decrease the residence time and operating temperature of the pretreatment process. Water itself acts as an acid at high temperatures.

The steam generated in this process heats the cellulose polymers without excessive dilution of resulting sugars. Sudden change in process pressure causes the temperature to decrease in parallel. This resulting thermal expansion used to terminate the reaction increases the accessible surface area of the biomass for subsequent enzymatic hydrolysis. The constraints in steam explosion pretreatment are incomplete lignin removal, formation of fermentation inhibitors, and destruction of a portion of xylan. The steam pretreated biomass must be washed to remove the inhibitory by-products (generated during steam explosion) along with water soluble hemicellulose.

2.7.3 Inhibitors generated during steam explosion process

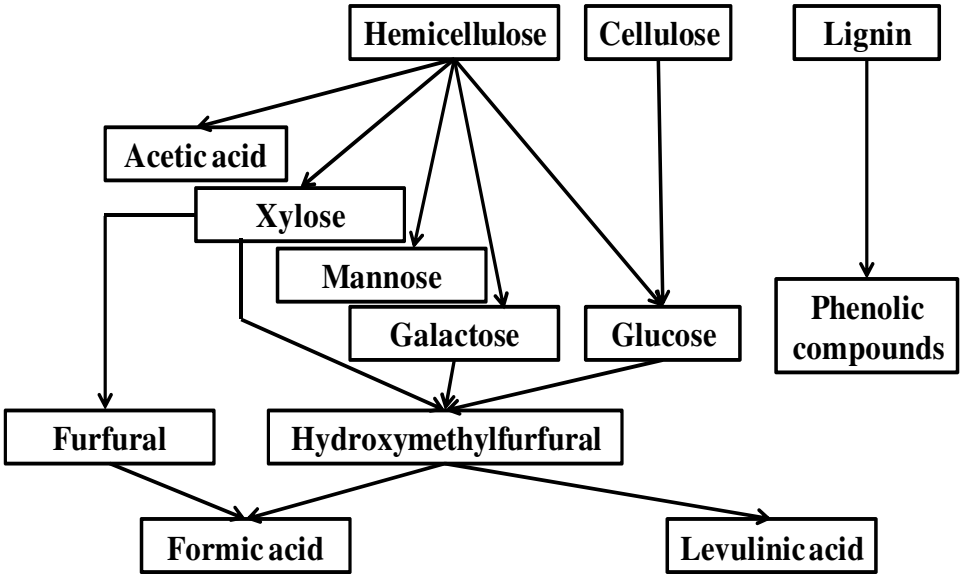

Figure 2.5 Major degradation compounds formed from polymer degradation of lignocellulosic biomass (Modified from Palmqvist and Hahn-Hägerdal (2000)).

The steam explosion process involves hydrolysis of LB under acidic conditions and at high temperatures. This results in the formation of wide range of compounds (Figure 2.5). Hemicellulose hydrolysis results in the formation of wide range of compounds such as xylose, mannose, acetic acid, galactose and glucose. On the other hand, cellulose polymers degradation results only on the formation of glucose. This xylose formed is further degraded into furfural at high temperature and pressure (Dunlop, 1948). Similarly, hexose degradation results in the formation of 5-hydroxy methyl furfural (HMF) (Ulbricht et al., 1984). HMF and furfural are further broken down to form formic acid (Dunlop, 1948; Ulbricht et al., 1984). The lignin polymer degradation mainly results in the formation of phenolic compounds (Bardet and Robert, 1985; Lapierre et al., 1983; Sears et al., 1971). HMF degradation can also result in the formation of levulinic acid (Ulbricht et al., 1984).

2.8 Microbial identification techniques

In past decades, the isolation of individual cultures for microbial analysis was carried out to understand their physiological, biochemical and morphological characteristics. For example, Lay (2000) detected *Clostridium* sp. as the dominant HPB present in mixed

culture fermentation (Lay, 2000). Analyses using such traditional methods, however, are often unreliable and time-consuming to perform. The major difficulty encountered is in distinguishing between the bacteria that are isolated from such anaerobic environments, as most of them share similar physiological, biochemical and morphological characteristics (Amann et al., 1995). Isolation of microbes using artificial growth medium is very difficult because different microorganisms require different growth conditions and some of their functions will vary depending upon the presence of other microorganisms present in closer association. Moreover, many of the microbes present in mixed culture environments are associated syntrophically and thus, cannot be cultured separately (Pike and Curds, 1971; Wagner et al., 1993).

Recent advancements in genomic analysis (such as fluorescence in-situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), and temperature gradient gel electrophoresis (TGGE)) allow traditional microbial analysis techniques (such as the most probable number (MPN) estimation technique) to be replaced (Ramette, 2009). The application of molecular finger printing techniques, such as T-RFLP, to generate microbial community profiles is useful for comparing samples grown under different fermentation conditions and for assessing similarities between them. The T-RFLP technique is relatively cost effective in comparison to other genomic analysis techniques.

T-RFLP is a robust and sensitive technique based on the analysis of the 16s rRNA gene (Clement et al., 1998). The results obtained with T-RFLP analysis are reproducible. The spatial and temporal changes in bacterial community structure can be studied with the help of this powerful tool (Lukow et al., 2000). This technique also has better resolution than the DGGE and TGGE techniques. The terminal restriction fragments can be differentiated at high resolutions (±1 bp) on gels according to the size of the restriction fragments (Tiedje et al., 1999). Phylogenetic characterization of mixed anaerobic communities can be performed using this technique (Kotsyurbenko et al., 2007; Rossetti et al., 2003). The terminal restriction site within the microbial community varies for different species of microorganisms due to variations in the sequences. This technique employs fluorescently-labeled terminal restriction fragments (T-RF) to detect the

existence of a particular DNA sequence in a PCR mixture. The accuracy of T-RFLP analysis relies on the proper selection of the optimal restriction enzyme for the purpose of the study.

2.9 Principal Component analysis (PCA)

Data reduction from correlated variables can be done using multivariate statistics tool known as PCA (Fievez et al., 2003). PCA technique can be used when very large numbers of multivariate datasets are analyzed to derive meaningful outcome. The dimensionality of the multivariate datasets can be reduced using PCA (Jolliffe, 2005). The PCA technique has been gained interest in biohydrogen production for relating the diversity of microbial community under different fermentation conditions. PCA provides good correlation of the fermentation metabolites profile with the microbial structure to provide more meaningful understanding of the fermentation process under different experimental conditions (Pendyala et al., 2012; Saady et al., 2012). Reduction of variables using PCA is done using the Eigen values. The principal components (PC) are arranged from the highest to the lowest values. Only PC with Eigen values higher than 1.0 is recommended for analysis using PCA because they reflect considerable amount of variation to the total variation (Kaiser, 1986). PC which has higher Eigen values greater than 1.0 are labeled as PC 1, PC 2, PC 3 from highest to lowest according to the percentage of variation compared with the total variation, i.e. PC 1 will have the highest percentage of variation compared to other components and PC 2 will have higher percentage variation compared with PC 3 and so on. The relationship between the original variable and the factor derived from PCA is known was factor loading. Value closer to -1 or +1 shows a strong relationship between the variable and the factor, whereas a value closer to 0 shows a weak correlation (Jayanegara et al., 2011). The relative score of each object in a principal component is known as factor score (Field, 2005).

2.10 References

Ahring, B.K., Ibrahim, A.A., Mladenovska, Z. (2001). Effect of temperature increase from 55 to 65 °C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. *Water Res*, **35**(10), 2446-52.

Ahring, B.K., Sandberg, M., Angelidaki, I. (1995). Volatile fatty acids as indicators of process imbalance in anaerobic digesters. *Appl Microbiol Biot*, **43**(3), 559-65.

Ahring, B.K., Westermann, P. (1988). Product inhibition of butyrate metabolism by acetate and hydrogen in a thermophilic coculture. *Appl Environ Microbiol*, **54**(10), 2393-7.

Alexander, M. (1977). *Introduction to Soil Microbiology* (2nd Ed.). John Wiley, New York pp.362-70.

Amann, R.I., Ludwig, W., Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev*, **59**(1), 143-69.

Andreesen, J.R., Gottschalk, G., Schlegel, H.G. (1970). *Clostridium formicoaceticum* nov. spec. Isolation, description and distinction from *C. aceticum* and *C. thermoaceticum*. *Arch Mikrobiol*, **72**(2), 154-74.

Atta, M., Meyer, J. (2000). Characterization of the gene encoding the [Fe]-hydrogenase from *Megasphaera elsdenii*. *Biochim Biophys Acta*, **1476**(2), 368-71.

Balch, W.E., Wolfe, R.S. (1979). Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). *J Bacteriol*, **137**(1), 256-63.

Bardet, M., Robert, D.R. (1985). On the reactions and degradation of the lignin during steam hydrolysis of aspen wood. *Sven Papperstidn*, **88**(6), R61-R67.

Barredo, M.S., Evison, L.M. (1991). Effect of propionate toxicity on methanogen enriched sludge, *Methanobrevibacter smithii*, and *Methanospirillum hungaetii* at different pH values. *Appl Environ Microbiol*, **57**(6), 1764-9.

Blaut, M., Gottschalk, G. (1985). Evidence for a chemiosmotic mechanism of ATP synthesis in methanogenic bacteria. *Trends Biochem Sci*, **10**(12), 486-9.

Brummeler, E.T., Pol, L.W.H., Dolfing, J., Lettinga, G., Zehnder, A.J.B. (1985). Methanogenesis in an Upflow Anaerobic Sludge Blanket Reactor at pH-6 on an acetate-propionate mixture. *Appl Environ Microbiol*, **49**(6), 1472-7.

Buhr, H.O., Andrews, J.F. (1977). The thermophilic anaerobic digestion process. *Water Res*, **11**(2), 129-43.

Cai, M., Liu, J., Wei, Y. (2004). Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. *Environ Sci Technol*, **38**(11), 3195-202.

Cammack, R., Frey, M., Robson, R. (2001). *Hydrogen as a Fuel: Learning from Nature*. Taylor & Francis, London 267.

Cha, G.C., Noike, T. (1997). Effect of rapid temperature change and HRT on anaerobic acidogenesis, Vol. 36, pp. 247-53.

Chen, C.C., Lin, C.Y. (2003). Using sucrose as a substrate in an anaerobic hydrogen producing reactor. *Adv Environ Res*, **7**(3), 695-9.

Chen, C.C., Lin, C.Y., Chang, J.S. (2001). Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. *Appl Microbiol Biot*, **57**(1-2), 56-64.

Chen, C.C., Lin, C.Y., Lin, M.C. (2002). Acid-base enrichment enhances anaerobic hydrogen production process. *Appl Microbiol Biot*, **58**(2), 224-8.

Chen, X., Sun, Y., Xiu, Z., Li, X., Zhang, D. (2006). Stoichiometric analysis of biological hydrogen production by fermentative bacteria. *Int J Hydrogen energy*, **31**(4), 539-49.

Chin, K.J., Lukow, T., Conrad, R. (1999). Effect of temperature on structure and function of the methanogenic archaeal community in an anoxic rice field soil. *Appl Environ Microbiol*, **65**(6), 2341-9.

Chu, C., Ebie, Y., Inamori, Y., Kong, H. (2009). Effect of hydraulic retention time on the hydrogen yield and population of *Clostridium* in hydrogen fermentation of glucose. *J Environ Sci*, **21**(4), 424-8.

Clement, B.G., Kehl, L.E., DeBord, K.L., Kitts, C.L. (1998). Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J Microbiol Meth*, **31**(3), 135-42.

Conrad, R. (1999). Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *Fems Microbiol Ecol*, **28**(3), 193-202.

Dabrock, B., Bahl, H., Gottschalk, G. (1992). Parameters affecting solvent production by *Clostridium pasteurianum*. *Appl Environ Microbiol*, **58**(4), 1233-9.

Dague, R.R., Habben, C.E., Pidaparti, S.R. (1992). Initial studies on the anaerobic sequencing batch reactor. *Water Sci Technol*, **26**(9-11), 2429-32.

Das, D., Veziroğlu, T.N. (2001). Hydrogen production by biological processes: A survey of literature. *Int J Hydrogen energy*, **26**(1), 13-28.

de Amorim, E.L.C., Barros, A.R., Damianouic, M.H.R.Z., Silva, E.L. (2009). Anaerobic fluidized bed reactor with expanded clay as support for hydrogen production through dark fermentation of glucose. *Int J Hydrogen Energy*, **34**(2), 783-90.

De Sá, L.R.V., De Oliveira, T.C., Dos Santos, T.F., Matos, A., Cammarota, M.C., Oliveira, E.M.M., Ferreira-Leitão, V.S. (2011). Hydrogenase activity monitoring in the fermentative hydrogen production using heat pretreated sludge: A useful approach to evaluate bacterial communities performance. *Int J Hydrogen energy*, **36**(13), 7543-9.

Demeyer, D., Fievez, V. (2000). Ruminants and environment: methanogenesis. *Ann Zootech*, **49**(2), 95-112.

Demirel, B., Scherer, P. (2008). The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. *Rev Environ Sci Biotechnol*, **7**(2), 173-90.

Doreau, M., Martin, C., Eugene, M., Popova, M., Morgavi, D.P. (2011). Tools for decreasing enteric methane production by ruminants. *Inra Prod Anim*, **24**(5), 461-74.

Doremus, M.G., Linden, J.C., Moreira, A.R. (1985). Agitation and pressure effects on acetone-butanol fermentation. *Biotechnol Bioeng*, **27**(6), 852-60.

Drake, H.L., Daniel, S.L., Matthies, C., Küsel, K. (1994). Acetogenesis, acetogenic bacteria, and the acetyl-CoA Wood/Ljungdahl Pathway: past and current perspectives. In: Drake HL, editor. *Acetogenesis*. New York: Chapman and Hall. pp.3-60.

Duff, S.J.B., Murray, W.D. (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. *Bioresour Technol*, **55**(1), 1-33.

Dunlop, A.P. (1948). Furfural formation and behavior. *Ind Eng Chem*, **40**(2), 204-9.

Duran, M., Speece, R.E. (1997). Temperature staged anaerobic processes. *Environ Technol*, **18**(7), 747-53.

Duval, B., Goodwin, S. (2000). Methane production and release from two New England peatlands. *Int Microbiol*, **3**(2), 89-95.

Enssle, M., Zirngibl, C., Linder, D., Thauer, R.K. (1991). Coenzyme F_{420} dependent N^5, N^{10} methylenetetrahydromethanopterin dehydrogenase in methanol grown *Methanosarcina barkeri*. *Arch Microbiol*, **155**(5), 483-90.

Ermler, U., Grabarse, W., Vaupel, M., Vorholt, J.A., Shima, S., Thauer, R.K., Wittershagen, A., Bourenkov, G., Bartunik, H.D. (1999). The crystal structure of methenyltetrahydromethanopterin cyclohydrolase from the hyperthermophilic archaeon *Methanopyrus kandleri*. *Struct Fold Des*, **7**(10), 1257-68.

Ermler, U., Grabarse, W.G., Mahlert, F., Shima, S., Thauer, R.K. (2000). Comparison of three methyl-coenzyme M reductases from phylogenetically distant organisms: Unusual amino acid modification, conservation and adaptation. *J Mol Biol*, **303**(2), 329-44.

Fan, K.S., Kan, N.R., Lay, J.J. (2006). Effect of hydraulic retention time on anaerobic hydrogenesis in CSTR. *Bioresour Technol*, **97**(1), 84-9.

Fang, H.H.P., Liu, H., Zhang, T. (2002). Characterization of a hydrogen producing granular sludge. *Biotechnol Bioeng*, **78**(1), 44-52.

Fengel, D., Wegener, G. (1984). *Wood: Chemistry, Ultrastructure, Reactions*. Wlater de Gruyter, Berlin.

Field, A. (2005). Discovering Statistics using SPSS.SAGE

Fievez, V., Vlaeminck, B., Dhanoa, M.S., Dewhurst, R.J. (2003). Use of principal component analysis to investigate the origin of heptadecenoic and conjugated linoleic acids in milk. *J Dairy Sci*, **86**(12), 4047-53.

Fox, J.D., Yiping, H.E., Shelver, D., Roberts, G.P., Ludden, P.W. (1996). Characterization of the region encoding the CO-induced hydrogenase of *Rhodospirillum rubrum*. *J Bacteriol*, **178**(21), 6200-8.

Fröstl, J.M., Seifritz, C., Drake, H.L. (1996). Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. *J Bacteriol*, **178**(15), 4597-603.

Fuchs, G. (1986). CO_2 fixation in acetogenic bacteria: Variations on a theme. *Fems Microbiol Lett*, **39**(3), 181-213.

Fuchs, G. (1994). Variations of the acetyl-CoA pathway in diversely related microorganisms that are not acetogens. *Acetogenesis*. Chapman and Hall Microbiology Series. pp.507-20.

Fukuzaki, S., Nishio, N., Shobayashi, M., Nagai, S. (1990). Inhibition of the fermentation of propionate to methane by hydrogen, acetate, and propionate. *Appl Environ Microbiol*, **56**(3), 719-23.

Gavala, H.N., Skiadas, I.V., Ahring, B.K. (2006). Biological hydrogen production in suspended and attached growth anaerobic reactor systems. *Int J Hydrogen Energy*, **31**(9), 1164-75.

Golueke, C.G. (1958). Temperature effects on anaerobic digestion of raw sewage sludge. *Sewage Ind Wastes*, **30**(10), 1225-32.

Goodwin, S., Zeikus, J.G. (1987). Ecophysiological adaptations of anaerobic bacteria to low pH - analysis of anaerobic digestion in acidic bog sediments. *Appl Environ Microbiol*, **53**(1), 57-64.

Gordon, S.A.B.S. (1990). Foodborne bacterial pathogens (Food science and technology series/31) Edited by Michael P. Doyle, Marcel Dekker, Inc, New York. *J Chem Technol Biot*, **47**(1), 88-88.

Graentzdoerffer, A., Rauh, D., Pich, A., Andreesen, J.R. (2003). Molecular and biochemical characterization of two tungsten and selenium containing formate dehydrogenases from *Eubacterium acidaminophilum* that are associated with components of an iron only hydrogenase. *Arch Microbiol*, **179**(2), 116-130.

Grous, W.R., Converse, A.O., Grethlein, H.E. (1986). Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. *Enzyme Microb Technol*, **8**(5), 274-80.

Guo, W.Q., Ren, N.Q., Wang, X.J., Xiang, W.S., Meng, Z.H., Ding, J., Qu, Y.Y., Zhang, L.S. (2008). Biohydrogen production from ethanol type fermentation of molasses in an expanded granular sludge bed (EGSB) reactor. *Int J Hydrogen Energy*, **33**(19), 4981-8.

Ha, M.A., Apperley, D.C., Evans, B.W., Huxham, M., Jardine, W.G., Vietor, R.J., Reis, D., Vian, B., Jarvis, M.C. (1998). Fine structure in cellulose microfibrils: NMR evidence from onion and quince. *Plant J*, **16**(2), 183-90.

Hallenbeck, P.C., Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? *Trends Biotechnol*, **27**(5), 287-97.

Happe, R.P., Roseboom, W., Egert, G., Friedrich, C.G., Massanz, C., Friedrich, B., Albracht, S.P.J. (2000). Unusual FTIR and EPR properties of the H_2 activating site of the cytoplasmic NAD-reducing hydrogenase from *Ralstonia eutropha*. *Febs Lett*, **466**(2-3), 259-63.

Happe, T., Hemschemeier, A., Winkler, M., Kaminski, A. (2002). Hydrogenases in green algae: do they save the algae's life and solve our energy problems? *Trends Plant Sci*, **7**(6), 246-50.

Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I. (2002). Sustainable fermentative hydrogen production: Challenges for process optimisation. *Int J Hydrogen energy*, **27**(11-12), 1339-47.

Henderson, G., Naylor, G.E., Leahy, S.C., Janssen, P.H. (2010). Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by analysis of formyltetrahydrofolate synthetase sequences from ruminants. *Appl Environ Microbiol*, **76**(7), 2058-66.

Horn, M.A., Matthies, C., Kusel, K., Schramm, A., Drake, H.L. (2003). Hydrogenotrophic methanogenesis by moderately acid-tolerant methanogens of a methane-emitting acidic peat. *Appl Environ Microbiol*, **69**(1), 74-83.

Hsu, T., Daniel, S.L., Lux, M.F., Drake, H.L. (1990). Biotransformations of carboxylated aromatic compounds by the acetogen *Clostridium thermoaceticum*: Generation of growth-supportive CO_2 equivalents under CO_2 limited conditions. *J Bacteriol*, **172**(1), 212-7.

Huser, B.A., Wuhrmann, K., Zehnder, A.J.B. (1982). *Methanothrix soehngenii* gen.nov.sp.nov, a new acetotrophic non hydrogen oxidizing methane bacterium. *Arch Microbiol*, **132**(1), 1-9.

Inatomi, K. (1986). Characterization and purification of the membrane bound ATPase of the Archaebacterium *Methanosarcina barkeri*. *J Bacteriol*, **167**(3), 837-41.

Jain, S.R., Mattiasson, B. (1998). Acclimatization of methanogenic consortia for low pH biomethanation process. *Biotechnol Lett*, **20**(8), 771-5.

Jayanegara, A., Wina, E., Soliva, C.R., Marquardt, S., Kreuzer, M., Leiber, F. (2011). Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. *Anim Feed Sci Technol*, **163**(2-4), 231-43.

Jolliffe, I. (2005). Principal Component Analysis. in: *Encyclopedia of Statistics in behavioral science*, John Wiley & Sons, Ltd.

Jung, J.Y., Lee, S.M., Shin, P.K., Chung, Y.C. (2000). Effect of pH on phase separated anaerobic digestion. *Biotechnol Bioprocess Eng*, **5**(6), 455-9.

Kaiser, H.F. (1986). Citation classic - the application of electronic computers to factor analysis. *Cc/Soc Behav Sci*(40), 1-18.

Kaosal, T., Sohgrathok, N. (2012). Influence of hydraulic retention time on biogas production from frozen seafood wastewater using decanter cake as anaerobic co-digestion material. *World Acad Sci Eng Technol*, **65**, 1017-21.

Kaparaju, P., Ellegaard, L., Angelidaki, I. (2009). Optimisation of biogas production from manure through serial digestion: Lab-scale and pilot-scale studies. *Bioresour Technol*, **100**(2), 701-9.

Kars, G., Gündüz, U., Rakhely, G., Yücel, M., Eroğlu, I., Kovacs, K.L. (2008). Improved hydrogen production by uptake hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* O.U.001. *Int J Hydrogen energy*, **33**(12), 3056-60.

Kaspar, H.F., Wuhrmann, K. (1978). Product inhibition in sludge digestion. *Microbial Ecol*, **4**(3), 241-8.

Khanal, S.K., Chen, W.H., Li, L., Sung, S. (2004). Biological hydrogen production: Effects of pH and intermediate products. *Int J Hydrogen energy*, **29**(11), 1123-31.

Kim, D.H., Kim, S.H., Ko, I.B., Lee, C.Y., Shin, H.S. (2008). Start-up strategy for continuous fermentative hydrogen production: Early switchover from batch to continuous operation. *Int J Hydrogen energy*, **33**(5), 1532-41.

Kim, J.O., Kim, Y.H., Ryu, J.Y., Song, B.K., Kim, I.H. (2004). Inhibition of methane formation and improvement of H_2 production by adding nitrate to anaerobic reactor. *Korean Soc Environ Eng*, **26**, 146-51.

Kim, S.H., Han, S.K., Shin, H.S. (2006). Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. *Process Biochem*, **41**(1), 199-207.

Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T., Friedrich, B. (2000). The H_2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases. *J Bacteriol*, **182**(10), 2716-24.

Kodicek, E., Worden, A.N. (1944). Effect of unsaturated fatty acids upon the growth of *Lactobacillus helveticus* and other gram-positive bacteria. *Nature*, **154**(3896), 17-8.

Kosaric, N., Vardar-Sukan, F. (2001). Substrates for industrial alcohol production. *The biotechnology of ethanol: Classic and Future Applications*. . 232.

Koskinen, P.E.P., Lay, C.H., Puhakka, J.A., Lin, P.J., Wu, S.Y., Orlygsson, J., Lin, C.Y. (2008). High efficiency hydrogen production by an anaerobic, thermophilic enrichment culture from an Icelandic hot spring. *Biotechnol Bioeng*, **101**(4), 665-78.

Koster, I.W., Cramer, A. (1987). Inhibition of methanogenesis from acetate in antigranulocytes sludge by long chain fatty acids. *Appl Environ Microbiol*, **53**(2), 403-9.

Kotsyurbenko, O.R., Friedrich, M.W., Simankova, M.V., Nozhevnikova, A.N., Golyshin, P.N., Timmis, K.N., Conrad, R. (2007). Shift from acetoclastic to H_2 dependent methanogenesis in a west Siberian peat bog at low pH values and isolation of an acidophilic *Methanobacterium* strain. *Appl Environ Microbiol*, **73**(7), 2344-8.

Kumar, N., Ghosh, A., Das, D. (2001). Redirection of biochemical pathways for the enhancement of H_2 production by *Enterobacter cloacae*. *Biotechnol Lett*, **23**(7), 537-41.

Künkel, A., Vorholt, J.A., Thauer, R.K., Hedderich, R. (1998). An Escherichia coli hydrogenase-3-type hydrogenase in methanogenic archaea. *Eur J Biochem*, **252**(3), 467-76.

Lalman, J., Bagley, D.M. (2002). Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. *Water Res*, **36**(13), 3307-13.

Lapierre, C., Rolando, C., Monties, B. (1983). Characterization of poplar lignins acidolysis products - capillary gas-liquid and liquid-liquid chromatography of monomeric compounds. *Holzforschung*, **37**(4), 189-98.

Larsen, H.E., Munch, B., Schlundt, J. (1994). Use of indicators for monitoring the reduction of pathogens in animal waste treated in biogas plants. *Int J Hygiene Environ Med*, **195**(5-6), 544-55.

Lay, J.J. (2000). Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol Bioeng*, **68**(3), 269-78.

Lee, K.S., Lin, P.J., Kai, F.C., Chang, J.S. (2007). Continuous hydrogen production by anaerobic mixed microflora using a hollow fiber microfiltration membrane bioreactor. *Int J Hydrogen Energy*, **32**(8), 950-7.

Lee, K.S., Lo, Y.S., Lo, Y.C., Lin, P.J., Chang, J.S. (2003). H_2 production with anaerobic sludge using activated carbon supported packed bed bioreactors. *Biotechnol Lett*, **25**(2), 133-8.

Lee, K.S., Wu, J.F., Lo, Y.S., Lo, Y.C., Lin, P.J., Chang, J.S. (2004). Anaerobic hydrogen production with an efficient carrier induced granular sludge bed bioreactor. *Biotechnol Bioeng*, **87**(5), 648-57.

Lee, M.J., Song, J.H., Hwang, S.J. (2009). Effects of acid pre-treatment on biohydrogen production and microbial communities during dark fermentation. *Bioresour Technol*, **100**(3), 1491-3.

Li, C., Fang, H.H.P. (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Crit Rev Environ Sci Technol*, **37**(1), 1-39.

Li, S.L., Kuo, S.C., Lin, J.S., Lee, Z.K., Wang, Y.H., Cheng, S.S. (2008). Process performance evaluation of intermittent continuous stirred tank reactor for anaerobic hydrogen fermentation with kitchen waste. *Int J Hydrogen Energy*, **33**(5), 1522-31.

Liang, T.M., Cheng, S.S., Wu, K.L. (2002). Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. *Int J Hydrogen energy*, **27**(11-12), 1157-65.

Lin, C.Y., Chang, R.C. (2004). Fermentative hydrogen production at ambient temperature. *Int J Hydrogen energy*, **29**(7), 715-20.

Lin, C.Y., Lee, C.Y., Tseng, I.C., Shiao, I.Z. (2006). Biohydrogen production from sucrose using base-enriched anaerobic mixed microflora. *Process Biochem*, **41**(4), 915-9.

Liu, Y., Yu, P., Song, X., Qu, Y. (2008). Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. *Process Biochem*, **33**(12), 2927-33.

Logan, B.E., Oh, S.E., Kim, I.S., Van Ginkel, S. (2002). Biological hydrogen production measured in batch anaerobic respirometers. *Environ Sci Technol*, **36**(11), 2530-5.

Lukow, T., Dunfield, P.F., Liesack, W. (2000). Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *Fems Microbiol Ecol*, **32**(3), 241-7.

Luo, G., Karakashev, D., Xie, L., Zhou, Q., Angelidaki, I. (2011). Long term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch

cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol Bioeng*, **108**(8), 1816-27.

Lusk, P. (1998). Methane recovery from animal manures: The current opportunities casebook.

Ma, K., Thauer, R.K. (1990). Single step purification of methylenetetrahydromethanopterin reductase from *Methanobacterium thermoautotrophicum* by specific binding to blue sepharose CL-6b. *Febs Lett*, **268**(1), 59-62.

Ma, K., Weiss, R., Adams, M.W.W. (2000). Characterization of hydrogenase II from the hyperthermophilic archaeon *Pyrococcus furiosus* and assessment of its role in sulfur reduction. *J Bacteriol*, **182**(7), 1864-71.

Mackie, R.I., Bryant, M.P. (1995). Anaerobic digestion of cattle waste at mesophilic and thermophilic temperatures. *Appl Microbiol Biot*, **43**(2), 346-50.

Maestrojuan, G.M., Boone, D.R. (1991). Characterization of *Methanosarcina barkeri* MS^T and 227, *Methanosarcina mazei* S-6^T, and *Methanosarcina vacuolata* Z-761^T. *Int J Syst Bacteriol*, **41**(2), 267-74.

Matthies, C., Freiberger, A., Drake, H.L. (1993). Fumarate dissimilation and differential reductant flow by *Clostridium formicoaceticum* and *Clostridium aceticum*. *Arch Microbiol*, **160**(4), 273-8.

McCabe, J., Eckenfelder, W. (1957). *Biological treatment of sewage and industrial wastes.* Reinbold Publishing. New York.

McKinlay, J.B., Harwood, C.S. (2010). Photobiological production of hydrogen gas as a biofuel. *Curr Opin Biotechnol*, **21**(3), 244-51.

McMillan, J.D. (1994). Pretreatment of lignocellulosic biomass. *Enzymatic Conversion of Biomass for Fuels Production.* American Chemical Society., **566**, pp.411-37.

Mertens, R., Greiner, L., Van Den Ban, E.C.D., Haaker, H.B.C.M., Liese, A. (2003). Practical applications of hydrogenase I from *Pyrococcus furiosus* for NADPH generation and regeneration. *J Mol Cat B Enzym*, **24-25**, 39-52.

Meyer, J. (2007). [FeFe] hydrogenases and their evolution: A genomic perspective. *Cell Mol Life Sci*, **64**(9), 1063-84.

Meynell, P.J. (1976). *Methane: Planning a Digester*. Schocken Books. New York., pp. 3.

Mikheeva, L.E., Schmitz, O., Shestakov, S.V., Bothe, H. (1995). Mutants of the cyanobacterium *Anabaena variabilis* altered in hydrogenase activities. *Z Naturforsch C*, **50**(7-8), 505-10.

Mizuno, O., Dinsdale, R., Hawkes, F.R., Hawkes, D.L., Noike, T. (2000). Enhancement of hydrogen production from glucose by nitrogen gas sparging. *Bioresour Technol*, **73**(1), 59-65.

Morimoto, K., Kimura, T., Sakka, K., Ohmiya, K. (2005). Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production. *Fems Microbiol Lett*, **246**(2), 229-34.

Mu, Y., Zheng, X.J., Yu, H.Q., Zhu, R.F. (2006). Biological hydrogen production by anaerobic sludge at various temperatures. *Int J Hydrogen energy*, **31**(6), 780-5.

Nandi, R., Sengupta, S. (1998). Microbial production of hydrogen: An overview. *Crit Rev Microbiol*, **24**(1), 61-84.

Oh, Y.K., Kim, S.H., Kim, M.S., Park, S. (2004). Thermophilic biohydrogen production from glucose with trickling biofilter. *Biotechnol Bioeng*, **88**(6), 690-8.

Orozco, A., Ahmad, M., Rooney, D., Walker, G. (2007). Dilute acid hydrolysis of cellulose and cellulosic bio-waste using a microwave reactor system. *Process Saf Environ Prot*, **85**(B5), 446-9.

Oxelfelt, F., Tamagnini, P., Lindblad, P. (1998). Hydrogen uptake in Nostoc sp. strain PCC 73102. Cloning and characterization of a hupSL homologue. *Arch Microbiol*, **169**(4), 267-74.

Öztürk, Y., Yücel, M., Daldal, F., Mandaci, S., Gündüz, U., Türker, L., Eroğlu, I. (2006). Hydrogen production by using *Rhodobacter capsulatus* mutants with genetically modified electron transfer chains. *Int J Hydrogen energy*, **31**(11), 1545-52.

Palmqvist, E., Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. *Bioresour Technol*, **74**(1), 25-33.

Patel, G.B., Sprott, G.D., Fein, J.E. (1990). Isolation and characterization of *Methanobacterium espanolae* sp.nov, a mesophilic, moderately acidiphilic methanogen. *Int J Syst Bacteriol*, **40**(1), 12-8.

Pendyala, B., Chaganti, S.R., Lalman, J.A., Shanmugam, S.R., Heath, D.D., Lau, P.C.K. (2012). Pretreating mixed anaerobic communities from different sources: Correlating the hydrogen yield with hydrogenase activity and microbial diversity. *Int J Hydrogen Energy*, **37**(17), 12175-86.

Pierce, E., Xie, G., Barabote, R.D., Saunders, E., Han, C.S., Detter, J.C., Richardson, P., Brettin, T.S., Das, A., Ljungdahl, L.G., Ragsdale, S.W. (2008). The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). *Environ Microbiol*, **10**(10), 2550-2573.

Pike, E.B., Curds, C.R. (1971). The microbial ecology of the activated sludge process. *Sym Ser Soc Appl Bacteriol*, **1**, 123-47.

Pohorelic, B.K.J., Voordouw, J.K., Lojou, E., Dolla, A., Harder, J., Voordouw, G. (2002). Effects of deletion of genes encoding Fe-only hydrogenase of *Desulfovibrio vulgaris* hildenborough on hydrogen and lactate metabolism. *J Bacteriol*, **184**(3), 679-86.

Ragsdale, S.W., Pierce, E. (2008). Acetogenesis and the Wood-Ljungdahl pathway of CO_2 fixation. *BBA-Proteins Proteom*, **1784**(12), 1873-98.

Ramette, A. (2009). Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl Environ Microbiol*, **75**(8), 2495-505.

Reeve, J.N., Nolling, J., Morgan, R.M., Smith, D.R. (1997). Methanogenesis: genes, genomes, and who's on first? *J Bacteriol*, **179**(19), 5975-86.

Rosgaard, L., Pedersen, S., Meyer, A.S. (2007). Comparison of different pretreatment strategies for enzymatic hydrolysis of wheat and barley straw. *Appl Biochem Biotechnol*, **143**(3), 284-96.

Rossetti, S., Blackall, L.L., Majone, M., Hugenholtz, P., Plumb, J.J., Tandoi, V. (2003). Kinetic and phylogenetic characterization of an anaerobic dechlorinating microbial community. *Microbiology-Sgm*, **149**, 459-69.

Saady, N.M.C., Chaganti, S.R., Lalman, J.A., Heath, D. (2012). Impact of culture source and linoleic acid (C18:2) on biohydrogen production from glucose under mesophilic conditions. *Int J Hydrogen energy*, **37**(5), 4036-45.

Saha, B.C. (2003). Hemicellulose bioconversion. *J Ind Microbiol Biot*, **30**(5), 279-91.

Saratale, G.D., Chen, S.D., Lo, Y.C., Saratale, R.G., Chang, J.S. (2008). Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation - A review. *J Sci Ind Res*, **67**(11), 962-79.

Sargent, F., Berks, B.C., Palmer, T. (2006). Pathfinders and trailblazers: A prokaryotic targeting system for transport of folded proteins. *Fems Microbiol Lett*, **254**(2), 198-207.

Sauter, M., Böhm, R., Böck, A. (1992). Mutational analysis of the operon (hyc) determining hydrogenase 3 formation in *Escherichia coli*. *Mol Microbiol*, **6**(11), pp.1523-32.

Savage, M.D., Wu, Z.G., Daniel, S.L., Lundie Jr, L.L., Drake, H.L. (1987). Carbon monoxide dependent chemolithotrophic growth of *Clostridium thermoautotrophicum*. *Appl Environ Microbiol*, **53**(8), 1902-6.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev*, **61**(2), 262-80.

Schlegel, H.G., Bowien, B. (1989). *Autotrophic bacteria*. Scinece Tech Publishers; Springer- Verlag, Madison (Wiscosin); Berlin.

Schroder, C., Selig, M., Schonheit, P. (1994). Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium thermotoga maritima: Involvement of the Embden-Meyerhof pathway. *Arch Microbiol*, **161**(6), 460-70.

Schut, G.J., Adams, M.W.W. (2009). The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: A new perspective on anaerobic hydrogen production. *J Bacteriol*, **191**(13), 4451-7.

Sears, K.D., Hamilton, J.K., Engen, R.J., Casebier, R.L., Beelik, A., Hergert, H.L. (1971). Southern pine prehydrolyzates - characterization of polysaccharides and lignin fragments. *J Polym Sci Pol Symp*(36), 425-43.

Seifritz, C., Daniel, S.L., Gossner, A., Drake, H.L. (1993). Nitrate as a preferred electron sink for the acetogen *Clostridium thermoaceticum*. *J Bacteriol*, **175**(24), 8008-13.

Shen, L.H., Bagley, D.M., Liss, S.N. (2009). Effect of organic loading rate on fermentative hydrogen production from continuous stirred tank and membrane bioreactors. *Int J Hydrogen Energy*, **34**(9), 3689-96.

Shima, S., Trauer, R.K. (2007). A third type of hydrogenase catalyzing H_2 activation. *Chem Rec*, **7**(1), 37-46.

Shin, H.S., Youn, J.H., Kim, S.H. (2004). Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *Int J Hydrogen Energy*, **29**(13), 1355-63.

Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J. (2007). A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresour Technol*, **98**(16), 3000-11.

Slobodkin, A., Reysenbach, A.L., Mayer, F., Wiegel, J. (1997). Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. *Int J Syst Bacteriol*, **47**(4), 969-74.

Snoeyink, V.L., Jenkins, D. (1980). *Water Chemistry*. John Wiley and Sons, New York., pp.177–90.

Sparling, R., Daniels, L. (1987). The specificity of growth inhibition of methanogenic bacteria by bromoethanesulfonate. *Can J Microbiol*, **33**(12), 1132-6.

Speece, R.E. (1983). Anaerobic biotechnology for industrial wastewater treatment. *Environ Sci Technol*, **17**(9), 416A-427A.

Speece, R.E. (1996). *Anaerobic Biotechnology for Industrial Wastewaters*. Archae Press, Nashville, TN. . pp.29-58.

Staley, B.F., de los Reyes, F.L., Barlaz, M.A. (2011). Effect of spatial differences in microbial activity, pH, and substrate levels on methanogenesis initiation in refuse. *Appl Environ Microbiol*, **77**(7), 2381-91.

Sun, Y., Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour Technol*, **83**(1), 1-11.

Taherzadeh, M.J., Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *Int J Mol Sci*, **9**(9), 1621-51.

Tebrommelstroet, B.W., Hensgens, C.M.H., Keltjens, J.T., Vanderdrift, C., Vogels, G.D. (1990). Purification and properties of 5,10-methylenetetrahydromethanopterin reductase, a coenzyme F_{420} dependent enzyme, from *Methanobacterium thermoautotrophicum* strain ΔH. *J Biol Chem*, **265**(4), 1852-7.

Tebrommelstroet, B.W.J., Geerts, W.J., Keltjens, J.T., Vanderdrift, C., Vogels, G.D. (1991). Purification and properties of 5,10-methylenetetrahydromethanopterin dehydrogenase and 5,10-methylenetetrahydromethanopterin reductase, coenzyme F_{420} dependent enzymes, from *Methanosarcina barkeri*. *Biochim Biophys Acta*, **1079**(3), 293-302.

Tiedje, J.M., Asuming-Brempong, S., Nüsslein, K., Marsh, T.L., Flynn, S.J. (1999). Opening the black box of soil microbial diversity. *Appl Soil Ecol*, **13**(2), 109-22.

Tijhuis, L., Van Loosdrecht, M.C.M., Heijnen, J.J. (1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. *Biotechnol Bioeng*, **44**(5), 595-608.

Ulbricht, R.J., Northup, S.J., Thomas, J.A. (1984). A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. *Fundam Appl Toxicol*, **4**(5), 843-53.

Valdez-Vazquez, I., Ríos-Leal, E., Esparza-García, F., Cecchi, F., Poggi-Varaldo, H.M. (2005). Semi-continuous solid substrate anaerobic reactors for H_2 production from organic waste: Mesophilic versus thermophilic regime. *Int J Hydrogen energy*, **30**(13–14), 1383-91.

Valentine, D.L., Blanton, D.C., Reeburgh, W.S. (2000). Hydrogen production by methanogens under low hydrogen conditions. *Arch Microbiol*, **174**(6), 415-21.

Van Ginkel, S., Logan, B.E. (2005). Inhibition of biohydrogen production by undissociated acetic and butyric acids. *Environ Sci Technol*, **39**(23), 9351-6.

Van Ginkel, S., Sung, S.W., Lay, J.J. (2001). Biohydrogen production as a function of pH and substrate concentration. *Environ Sci Technol*, **35**(24), 4726-30.

Van Lier, J.B. (1995). Thermophilic anaerobic wastewater treatment; Temperature aspects and process stability. Ph.D. Thesis, Wageningen Agricultural University, Wangeningen, The Netherlands.

van Lier, J.B., Tilche, A., Ahring, B.K., Macarie, H., Moletta, R., Dohanyos, M., Pol, L.W.H., Lens, P., Verstraete, W. (2001). New perspectives in anaerobic digestion. *Water Sci Technol*, **43**(1), 1-18.

Vandenberg, L., Patel, G.B., Clark, D.S., Lentz, C.P. (1976). Factors affecting rate of methane formation from acetic acid by enriched methanogenic cultures. *Can J Microbiol*, **22**(9), 1312-9.

VanLier, J.B., Martin, J.L.S., Lettinga, G. (1996). Effect of temperature on the anaerobic thermophilic conversion of volatile fatty acids by dispersed and granular sludge. *Water Res*, **30**(1), 199-207.

Vardar-Schara, G., Maeda, T., Wood, T.K. (2008). Metabolically engineered bacteria for producing hydrogen via fermentation. *Microb Biotechnol*, **1**(2), 107-25.

Vaupel, M., Dietz, H., Linder, D., Thauer, R.K. (1996). Primary structure of cyclohydrolase (Mch) from *Methanobacterium thermoautotrophicum* (strain Marburg) and functional expression of the mch gene in Escherichia coli. *Eur J Biochem*, **236**(1), 294-300.

Vignais, P.M., Billoud, B. (2007). Occurrence, classification, and biological function of hydrogenases: An overview. *Chem Rev*, **107**(10), 4206-72.

Vignais, P.M., Colbeau, A. (2004). Molecular biology of microbial hydrogenases. *Curr Iss Mol Biol*, **6**(2), 159-88.

Wagner, M., Amann, R., Lemmer, H., Schleifer, K.H. (1993). Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. *Appl Environ Microbiol*, **59**(5), 1520-5.

Wang, C.H., Lu, W.B., Chang, J.S. (2007). Feasibility study on fermentative conversion of raw and hydrolyzed starch to hydrogen using anaerobic mixed microflora. *Int J Hydrogen energy*, **32**(16), 3849-59.

Wang, J.L., Wan, W. (2008). Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. *Int J Hydrogen Energy*, **33**(12), 2934-41.

Wang, J.L., Wan, W. (2009). Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy*, **34**(2), 799-811.

Wang, M.Y., Tsai, Y.L., Olson, B.H., Chang, J.S. (2008). Monitoring dark hydrogen fermentation performance of indigenous *Clostridium butyricum* by hydrogenase gene expression using RT-PCR and qPCR. *Int J Hydrogen energy*, **33**(18), 4730-8.

Wang, Q., Noguchi, C.K., Kuninobu, M., Hara, Y., Kakimoto, K., Ogawa, H.I., Kato, Y. (1997). Influence of hydraulic retention time on anaerobic digestion of pretreated sludge. *Biotechnol Tech*, **11**(2), 105-8.

Westermann, P., Ahring, B.K., Mah, R.A. (1989). Temperature compensation in *Methanosarcina barkeri* by modulation of hydrogen and acetate affinity. *Appl Environ Microbiol*, **55**(5), 1262-6.

Wheatley, A. (1990). *Anaerobic Digestion: A waste treatment technology*. Elseiver Applied Science., 229.

Wiegant, W.M., Zeeman, G. (1986). The mechanism of ammonia inhibition in the thermophilic digestion of livestock wastes. *Agr Wastes*, **16**(4), 243-53.

Wieringa, K.T. (1936). About the disappearance of hydrogen and carbon dioxide under anaerobic conditions. *Antonie Van Leeuwenhoek*, **3**(4), 263-73.

Wieringa, K.T. (1939). The formation of acetic acid from carbon dioxide and hydrogen by anaerobic spore-forming bacteria. *Antonie Van Leeuwenhoek*, **6**(1), 251-62.

Wieringa, K.T. (1941). Überdie bildung von essigsäure aus kohlensäure und wasserstoff durch anaerobe bazillen. *Brennstoff-Chem*, **22**, 161-4.

Wijffels, R.H., Englund, G., Hunik, J.H., Leenen, E.J.T.M., Bakketun, A., Gunther, A., Obon de Castro, J.M., Tramper, J. (1995). Effects of diffusion limitation on immobilized nitrifying microorganisms at low temperatures. *Biotechnol Bioeng*, **45**(1), 1-9.

Wu, S.Y., Lin, C.Y., Lee, K.S., Hung, C.H., Chang, J.S., Lin, P.J., Chang, F.Y. (2008). Dark fermentative hydrogen production from xylose in different bioreactors using sewage sludge microflora. *Energy Fuels*, **22**(1), 113-9.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y. (2005a). Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresour Technol*, **96**(18), 2026-32.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y. (2005b). Coordinated development of leading biomass pretreatment technologies. *Bioresour Technol*, **96**(18), 1959-66.

Xu, J.F., Ren, N.Q., Su, D.X., Qiu, J. (2010). Bio-hydrogen production from acetic acid steam-exploded corn straws by simultaneous saccharification and fermentation with *Ethanoligenens harbinense* B49. *Int J Energy Res*, **34**(5), 381-6.

Xu, K.W., Liu, H., Du, G.C., Chen, J. (2009). Real-time PCR assays targeting formyltetrahydrofolate synthetase gene to enumerate acetogens in natural and engineered environments. *Anaerobe*, **15**(5), 204-13.

Yang, S.T., Okos, M.R. (1987). Kinetic study and mathematical modeling of methanogenesis of acetate using pure cultures of methanogens. *Biotechnol Bioeng*, **30**(5), 661-7.

Yu, H., Hu, Z., Hong, T. (2003). Hydrogen production from rice winery wastewater by using a continuously-stirred reactor. *J Chem Eng Jpn*, **36**(10), 1147-51.

Yuan, Z., Yang, H., Zhi, X., Shen, J. (2010). Increased performance of continuous stirred tank reactor with calcium supplementation. *Int J Hydrogen Energy*, **35**(7), 2622-6.

Yusoff, M.Z.M., Abdul Rahman, N., Abd-Aziz, S., Ling, C.M., Hassan, M.A., Shirai, Y. (2010). The effect of hydraulic retention time and volatile fatty acids on biohydrogen production from palm oil mill effluent under non-sterile condition. *Aust J Basic Appl Sci*, **4**(4), 577-87.

Yusoff, M.Z.M., Hassan, M.A., Abd-Aziz, S., Rahman, N.A.A. (2009). Start-Up of biohydrogen production from palm oil mill effluent under non-sterile condition in 50 L continuous stirred tank reactor. *Int J Agr Res*, **4**(4), 163-8.

Zhang, Y.H.P., Lynd, L.R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnol Bioeng*, **88**(7), 797-824.

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T., Lee, D.J. (2008). Biohydrogen production with anaerobic fluidized bed reactors - A comparison of biofilm-based and granule-based systems. *Int J Hydrogen Energy*, **33**(5), 1559-64.

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T., Lee, D.J., Jiang, W.J. (2006). Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. *Process Biochem*, **41**(10), 2118-23.

Zheng, X.J., Yu, H.Q. (2005). Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. *J Environ Manage*, **74**(1), 65-70.

Zinder, S.H., Anguish, T., Cardwell, S.C. (1984). Selective inhibition by 2-bromoethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor. *Appl Environ Microbiol*, **47**(6), 1343-5.

Zupancic, G.D., Straziscar, M., Ros, M. (2007). Treatment of brewery slurry in thermophilic anaerobic sequencing batch reactor. *Bioresour Technol*, **98**(14), 2714-22.

CHAPTER 3: MATERIALS AND METHODS

3.1 Lignocellulosic biomass procurement

Lignocellulosic biomass (LB) (corn stalks) was procured from a local Ontario farmer (Leamington, ON). The corn stalks were dried in a hot air oven at 105 °C for 24 h after delivery to the University of Windsor. The oven dried corn stalks (final moisture content < 3 %) were then milled using a hammer mill (Restech, USA) into uniformly sized pellets smaller than 5 mm x 2 mm. The shredded corn stalks were then collected in airtight bags and stored in a cold room at 4 °C.

3.1.1 Steam explosion

Pretreatment of the lignocellulosic biomass (raw corn stalk) was carried out using a 4-L pilot-scale batch steam explosion reactor (Autoclave Engineers, Inc., USA). The reactor was designed to withstand high temperatures and pressures. Corn stalk residues were loaded at an initial concentration of 10 % wt/wt of dry corn stalk/water and soaked overnight in 1 % (wt/wt) H_2SO_4. The mixing speed of the reactor was 815 rpm. Heating the contents of the reactor was performed with the assistance of a temperature controlled heating jacket. The contents of the reactor were heated to a temperature of 190 °C for 10 min. The pressure within the reactor was then released rapidly in order to provide the conditions necessary for exploding the raw material. Next, the contents of the reactor were cooled until ambient temperature was attained. This liquor was then filtered using a filter cloth in order to remove suspended solids and the pH of the filtered liquor was adjusted to pH 5.0 with 10 M NaOH. The hydrolyzate was filtered using a 0.45 µm glass microfiber filter and stored at 4 ^{o}C in a 10 L reactor vessel for further analysis. The composition of monomeric sugars present in the hydrolyzate was analyzed using chemical methods (see Section 3.6.2.1), and the individual sugar composition (xylose, glucose, arabinose, mannose and galactose) was estimated using chromatographical methods (see Section 3.7.1).

3.2 Inoculum Source

The cultures used as microbial inocula were obtained were collected from an up-flow anaerobic sludge blanket reactor (UASBR) at a brewery wastewater treatment facility (Guelph, ON). The cultures were stored at 4 °C upon arrival at the University of Windsor. The volatile suspended solids (VSS) and total suspended solids (TSS) content of these granular cultures were measured according to *Standard Methods* (APHA, 2010).

3.3 Serum bottle preparation

The experimental methods used for conducting batch reactor studies were adapted from previous reports (Lalman et al., 2003; Lalman and Bagley, 2000; Lalman and Bagley, 2002). A schematic of the batch reactor is shown in Plate 3.1. The composition of the basal media used in both the H_2 production and H_2 consumption experiments is described in Table 3.1. The basal media composition was adapted from Wiegant and Lettinga (1985). All chemical reagents were analytical grade (Spectrum Chemicals, CA).

Serum bottle batch reactors (160 mL) was filled with a specific quantity of basal media and microbial culture (2000 mg L^{-1} VSS) in an anaerobic glove box (COY Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 76 % N_2, 20 % CO_2 and 4 % H_2 (all 99.99 % purity Praxair Inc., ON). After allowing the biosolids to settle for 5 minutes, a specific amount of liquid equivalent to the amount of substrate to be added was removed from each reactor. The volumes of substrate and inhibitor added were variable and dependent on the experimental condition. Each reactor had a final working volume of 50 mL (or 75 mL for the long-term study). The initial pH levels within the batch reactors were adjusted using 3N HCl and 3M KOH. After adjusting the pH, the reactors were then sealed using Teflon®-lined silicone rubber septa and aluminum caps. The reactors were then over-pressurized using 20 mL of the gas mixture from the anaerobic glove box atmosphere to avoid the formation of negative pressure in the headspace during sampling.

Table 3.1 Composition of the basal media used for H_2 production and consumption studies.

Chemical Ingredient	Concentration (mg L^{-1})
$NaHCO_3$	6000
NH_4HCO_3	70
KCl	25
K_2HPO_4	14
$(NH_4)_2SO_4$	10
Yeast Extract	10
$MgCl_2.4H_2O$	9
$FeCl_2.4H_2O$	2
Resazurin	1
Ethylenediamine tetra acetic acid (EDTA)	1
$MnCl_2.4H_2O$	0.5
$CoCl_2.6H_2O$	0.15
$NaSeO_3$	0.1
$(NH_4)_6MoO_7.4H_2O$	0.09
$ZnCl_2$	0.05
H_3BO_3	0.05
$NiCl_2.6H_2O$	0.05
$CuCl_2.2H_2O$	0.03

After pressurization, the batch reactors were transferred to an orbital shaker and left to agitate at 200 rpm for 24 hours at the temperature (21 °C, 37 °C, or 53 °C) corresponding to a particular experimental condition. Liquid samples were removed and checked for VFAs before the start of each experiment.

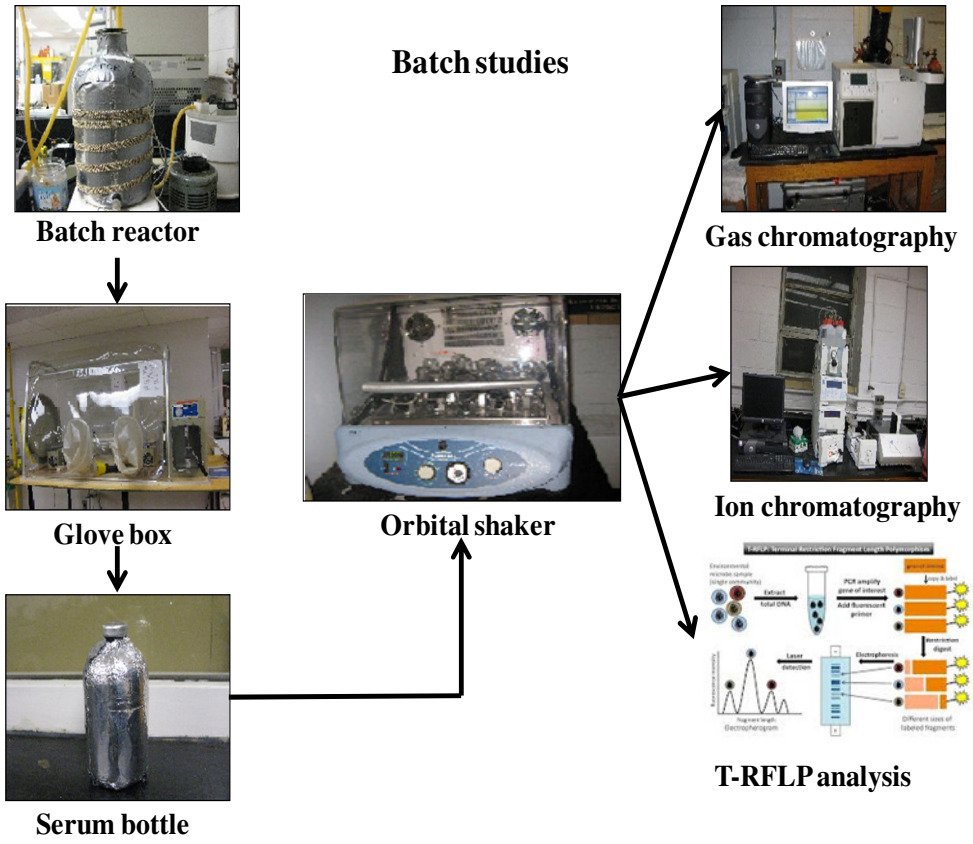

Plate 3.1 Schematic of batch reactor (serum bottle) studies.

3.4 Sequencing batch reactor operation

A schematic illustration of the anaerobic sequencing batch reactor (ASBR) operation used in these studies is shown in Plate 3.2. Two 6-L (total volume) continuous stirred-tank reactors with a 5-L working volume were operated in sequencing batch mode under anaerobic conditions. These ASBRs were inoculated with flocculated culture obtained a wastewater treatment facility (Guelph, ON). Flocculated cultures were prepared by crushing the granular sludge. Flocculated or suspended cultures were transferred from the anaerobic glove box and into three 10-L mother reactors (designated as Reactor A, B and C) with an 8-L working volume. The contents of the reactors were diluted with the basal media (described in Section 3.2) to a final VSS concentration of 20 g L^{-1}.

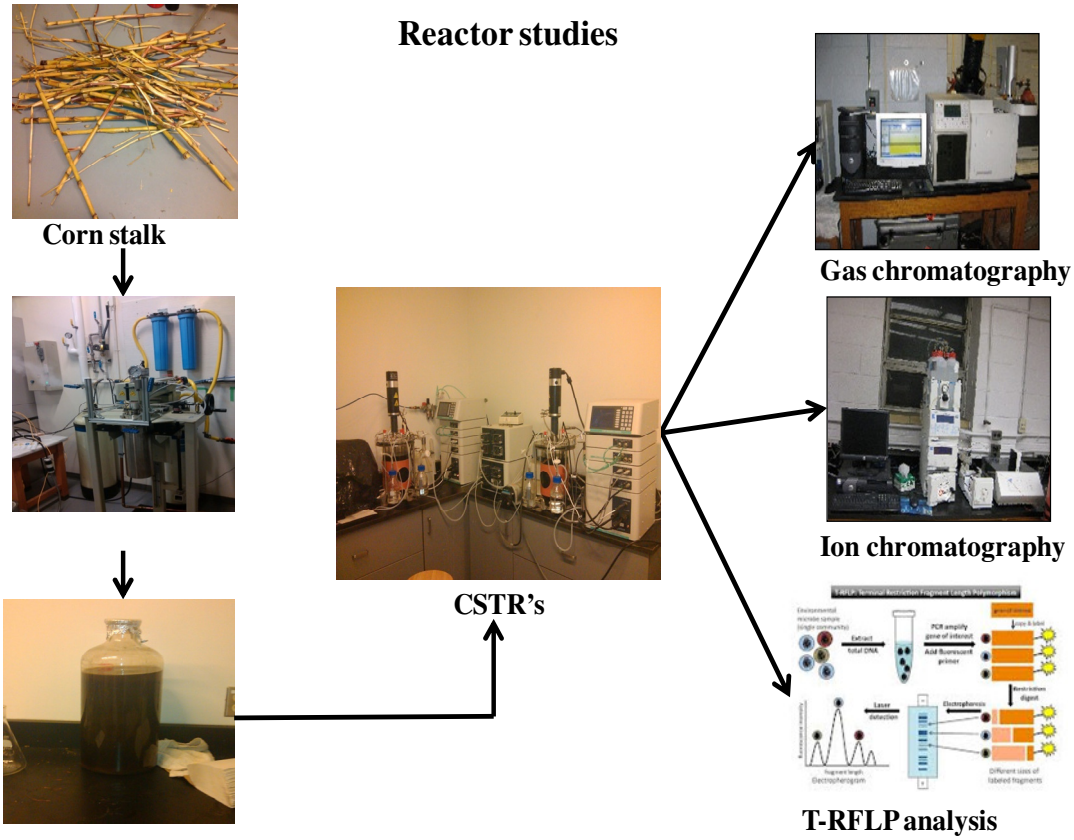

Plate 3.2 Schematic of sequencing batch reactor studies.

The feeding periods were determined based on gas production and the level of residual VFAs in the reactor. The cultures in reactors A, B and C were fed regularly (on a weekly basis) with either pure substrate glucose (5 g L^{-1}) or glucose: xylose (1:1 ratio; 5 g L^{-1}).

3.5 Experimental plan

The detailed experimental plan for each study comprising this thesis is presented at the beginning of the chapter that describes the findings of a particular experiment. A brief summary of the experimental design is presented in Table 3.2.

Table 3.2 Brief summary of the studies performed in this thesis.

Chapter No.	Reactor type	Focus of the experiment	Fermentation parameters	Stressors used
4	Batch	H_2 consumption	Temperature - 21°C,37°C and 55°C; pH - 4.5, 6.0 and 7.5	LA
5	Batch	H_2 consumption	Temperature - 37°C; pH - 5.5	Furfural, BES, LUA, LA, Fish oil
6	Batch	H_2 production (0 - 25 days)	Temperature - 37°C and 55°C; pH - 5.5	Chemical (BES and LA), Physiochemical (Acid and alkali), Physical (Loading and Heat shock)
		H_2 consumption (26^{th} - 30^{th} day)		Stress treated culture from H_2 production study (25^{th} day
7	Sequencing batch	H_2 production	Temperature - 37°C; pH - 5.5; HRT - 37.5, 17.5 h, 10.8 h, 7.5 h and 3.3 h	LA - 0, 2 g L^{-1}
			Temperature - 37°C; pH - 5.5; HRT - 37.5, 17.5 h and 7.5 h	
8	Sequencing batch	H_2 production	Temperature - 21°C, 37°C and 55°C; pH - 4.5, 5.5 and 6.5; HRT - 6, 12 h and 18 h	Furfural, HMF present in corn stalk liquor
	Batch	H_2 consumption	Temperature - 21°C, 37°C and 55°C; pH - 4.5, 5.5 and 6.5	Stress treated cultures obtained at the end of each fermentation condition

3.6 Chemical methods

3.6.1 Biomass characterization

Characterizing the agricultural LB for cellulose, hemicellulose, lignin, moisture and ash content is described in the following sections (3.6.1.1 to 3.6.1.4).

3.6.1.1 Estimation of moisture and ash content

The moisture content of the LB (corn stalk) sample was estimated by measuring the oven dry weight of the LB sample after oven-drying at 105 °C for 24 h. The moisture content was calculated as a percentage (%) using the difference in weight of the initial biomass and the oven-dried biomass. The ash content of the LB sample was calculated by incinerating the oven-dried LB sample in a muffle furnace at 550 °C for 4 h. The difference in the weight of the original LB sample and the ash weight of the incinerated LB sample was then used to estimate the ash content as a percentage (%).

3.6.1.2 Estimation of cellulose content

The protocol developed by Updegraf (1969) was followed to estimate the amount of cellulose in the lignocellulosic materials (corn stalks) used in this study. Briefly, 0.5 g of dry biomass sample was mixed with 3 mL acetic/nitric reagent (a mixture of 80 % acetic acid and concentrated nitric acid in the ratio of 10:1). This reaction mixture was then heated to 100 °C for 30 minutes. The cellulose present in the biomass was acetylated to form acetylated cellodextrin. This mixture was cooled at room temperature, and then centrifuged (Beckman Coulter, Inc., USA) at 3000 rpm for 20 minutes. The supernatant was discarded, and the residue was washed three to four times with Milli-Q water (Millipore, Barnstead, USA). The residues were then hydrolyzed with 10 mL of 67 % H_2SO_4 for 1 h to extract simple sugars (e.g. glucose), and then 1 mL of the above solution was diluted (100X) for further analysis. The diluted sample (1 mL) was added to 10 mL of cold anthrone reagent (prepared by dissolving 200 mg anthrone in 100 mL conc. H_2SO_4). The resulting mixture was boiled at 100 °C for 10 minutes using a water bath to dehydrate the sugars to form hydroxymethyl furfural (HMF). Calibration standards were prepared using pure cellulose in various known concentrations ranging from 20 µg to 200

µg. Similarly, to the preparation of samples, 1mL of the standard stock solution was mixed with 10 mL of cold anthrone reagent, and then heated for 10 minutes in a water bath along with the samples. The intensity of the colour formed in the samples and calibration standards was then measured at 630 nm using a UV Spectrophotometer (CARY 50 Scan, Varian, CA). The steps followed for the estimation of cellulose levels in the biomass samples were repeated for a blank or control sample using Milli-Q water. The calibration graph generated from the calibration standards containing a known amount of cellulose was then used to determine the concentration of cellulose present in the experimental biomass samples.

3.6.1.3 Estimation of hemicellulose content

The protocol developed by Goering and Van Soest (1970) was used to calculate the hemicellulose content present in the sample by determining the amount of neutral detergent fiber (NDF) and acid detergent fiber (ADF). The hemicellulose content (%) was estimated using the difference between NDF and ADF. The procedure for estimating the amount of NDF in the sample was as follows: to 1 g of dry biomass sample, 10 mL of cold neutral detergent solution (prepared according to Goering and Van Soest, 1970), 2 mL of decahydronaphthalene and 0.5 g sodium sulfite were added to a 250 mL flat bottomed round necked flask, and heated with a reflux column for 1 h. This removes the water content and non-fibrous portions of the biomass. The contents were removed from the reflux apparatus and cooled at room temperature for 10 minutes. The contents were filtered and washed with hot water using a sintered glass crucible connected to a vacuum suction port. The biomass sample was then washed with acetone to remove unwanted particles and chemicals. The residue was then oven-dried at 105 °C for 8 h, and the oven dry weight was measured. The NDF content of the biomass sample was then calculated using the weight of the remaining residue as a percentage of the original 1 g sample.

ADF was estimated using 100 mL acid detergent solution (ADS) (2 % wt/vol Cetyl trimethyl ammonium bromide (CTAB) in 1 N H_2SO_4) and 2 mL of decahydronaphthalene; 1 g of dry biomass sample was added to the mixture of ADS and decahydronaphthalene to dissolve the acid soluble contents present in the biomass. This

reaction mixture was then heated with a reflux for 1 h. The contents were cooled and washed with hot water and acetone in a sintered glass crucible using vacuum suction. The residue was dried in a hot air oven at 105 °C for 8 h. The weight of the remaining residue was used to estimate the percentage of ADF content in the original 1 g sample of dry biomass. The hemicellulose content was then calculated as a percentage of the 1 g dry biomass sample using the difference between NDF and ADF.

3.6.1.4 Estimation of lignin content

The protocol for estimation of the amount of lignin content present in the biomass was modified from Hubbell and Ragauskas (2010). The total lignin content in the biomass sample was estimated using delignification of the biomass by acid-chlorite treatment. To 5 g of dry biomass sample, 100 mL of 2 % sodium chlorite was added and adjusted to a final pH of 3 using glacial acetic acid, then heated in a water bath at 70 °C for 2 to 3 h while occasionally stirring the contents with a glass rod. The contents were then cooled and filtered through a sintered glass crucible using vacuum suction to remove dissolved lignin from the filtrate. The residual biomass was then diluted and adjusted to neutral pH using a sodium sulfate solution (1 g of sodium sulfate in 1 L of Milli-Q water). The sodium sulfate solution containing the residual biomass was filtered again using the glass crucible, and the remaining residue was then oven-dried at 105 °C for 2-3 h. The oven dry weight of the residual biomass was recorded, and the lignin content of the biomass sample was calculated using the difference in weight of the initial biomass (5 g) and the oven dry weight of the residual biomass (in g). The weight loss represents the holocellulose (cellulose + hemicellulose) content of the biomass. This procedure will estimate the total lignin (Acid soluble lignin + acid insoluble lignin) content of the biomass sample.

The acid insoluble lignin (Klason lignin) was estimated according to the protocol developed by Goering and Van Soest (1970). Briefly, 0.5 g of the ADF residue (Section 3.6.1.3) was mixed with 100 mL ice cold 72 % H_2SO_4 in a 250 mL conical flask. The mouth of the flask was covered with paraffin and the contents were stirred occasionally. This mixture was then filtered through a sintered glass crucible using a vacuum. The

filtered residue was washed thoroughly with Milli-Q water to remove the acid residues from the biomass. The residual biomass was then oven-dried at 105 °C for 8 h, and weighed. The difference in biomass weight before and after oven-drying to the original weight of the biomass taken for ADF analysis gives the amount of Klason lignin. The VSS (ash) content of this residual oven-dried biomass was calculated by incinerating the biomass sample to ash in a muffle furnace at 550 °C. The weight of the sample after ashing was recorded. The difference in weight between the initial biomass and incinerated biomass was also used to calculate the ash content.

3.6.2 Characterization of liquor

3.6.2.1 Estimation of reducing sugars and total sugar content

The total sugar content in the steam exploded liquor was determined using the dinitrosalicylic acid (DNS) method (Miller (1959). The steam exploded liquor was adjusted to pH 5.0 using 3 M NaOH. The residue present in the liquor was removed by passing it through a 0.45 µm glass fiber filter. The filtered liquor was stored in a glass container and stored at 4 °C. Ten mL of the filtered sample was passed through a syringe filter fitted with a 0.45 µm polypropylene membrane (GE Osmonics, MN) to remove any suspended particles present in the liquor. This liquor sample was then diluted 50X for estimation of the levels of furan aldehydes (furfural and HMF), phenol, COD and sugars. The reagents for the DNS method were prepared according to the protocol followed by Miller (1959). Briefly, 2 mL of the diluted liquor sample was mixed with 2 mL of the DNS reagent and heated in a water bath (100 °C) for 5-8 minutes. The calibration curve (Appendix A) for the standards was prepared by varying the amounts of the sugars (glucose, xylose and a glucose/xylose mixture) added to the solution (2 mL of DNS reagent) from 100 to 500µg. In addition, known concentrations of the standards were added to a liquor sample to estimate the concentration of unknown sugars present in the liquor sample.

Actual sugar concentration measured = Concentration of sugar in the sample – known concentration of sugar added in the sample.

The blank was prepared by mixing 2 mL of Milli-Q water with 2 mL of DNS reagent. The contents were cooled using cold water and the optical density if the samples was measured at 540 nm using a UV Spectrophotometer (CARY 50 Scan, Varian, CA). The total sugar content was estimated using the anthrone method described by Hedge and Hofreiter (1962). Briefly, 1 mL of diluted liquor was mixed with 0.1 mL of 2.5 N HCl and the samples were heated in a hot water bath (100 °C). The oligomers (disaccharide sugars) present in the liquor samples were hydrolyzed into monomers (monosaccharide sugars) by the addition of the hot acid. The samples were neutralized using 3 M NaOH and subjected to centrifugation at 3000 rpm for 20 minutes. 1mL of the supernatant was mixed with 4 mL of freshly prepared anthrone reagent and heated in a hot water bath (100 °C). The calibration curve for total sugar estimation was prepared by varying the amounts (100 to 500 µg) of pure glucose added to standard samples (Dutch baby, CA). Blank standards were prepared according to the same procedure, but pure Milli-Q water was mixed with the reagent instead of liquor. Internal standards with known amounts of added sugar were prepared with diluted liquor and these internal standards were run in parallel with pure standards and the test samples of liquor. The intensity of the colour formation in the samples was assessed at 630 nm using a UV Spectrophotometer (CARY 50 Scan, Varian, CA).

3.6.2.2 Estimation of chemical oxygen demand (COD)

The chemical oxygen demand (COD) of diluted liquor samples was measured using closed reflux calorimetric methods according to standard methods (Greenberg et al., 2010). The COD was measured for raw unfiltered samples and also for filtered samples to determine the COD of the residual biomass present in the steam exploded liquor. Briefly, 2.5 mL of both filtered and unfiltered liquor samples were mixed with 1.5 mL of 0.25 N potassium dichromate ($K_2Cr_2O_7$) solution and 3.5 mL of sulfuric acid reagent (prepared by dissolving 0.55 g $AgSO_4$ in 100 g conc.H_2SO_4). The standards for COD estimation were prepared within the range of 50 – 500 µg COD equivalent using potassium hydrogen phthalate (KHP) (0.425 g KHP = 0.5 g COD). The blank was prepared using the same procedure, but with Milli-Q water replacing the liquor. The

COD tubes were heated at 150 °C for 2 h. The digested contents were then cooled, and the intensity of colour formation was assessed at 600 nm using a UV Spectrophotometer (CARY 50 Scan, Varian, CA).

3.6.2.3 Estimation of phenol content

The total content of phenols present in the diluted liquor samples was estimated calorimetrically using Folin-Ciocalteau (FC) reagent (Singleton and Rossi, 1965). Briefly, 5 mL of liquor were added to 1 mL of FC reagent and diluted to a volume of 11 mL with Milli-q water. The sample was mixed with 10 mL of 20 % sodium carbonate (Na_2CO_3) solution, and heated to 30 °C for 1.5 h followed by measurement of OD using a UV Spectrophotometer. Standard calibration curves were prepared with standards containing various amounts of catechol (20 – 100 µg). These standards were prepared using the same procedures as were used for the preparation of the test samples. The blanks were prepared similarly, but with Milli-Q water replacing the liquor. In addition, several internal standards were also prepared (adding known amounts of catechol to liquor samples). The intensity of colour formation in the standard and test samples was measured at 730 nm using a UV Spectrophotometer (CARY 50 Scan, Varian, CA).

3.7 Analytical methods:

3.7.1 Liquid metabolite analysis

The volatile fatty acids (VFAs) and alcohols produced during the course of the experiment were analyzed using high performance liquid chromatography (HPLC) and ion chromatography (IC) (Dionex, Oakville, ON) systems equipped with an auto sampler. The composition of the sugars (glucose, xylose, arabinose, mannose and galactose) in the samples were analyzed with a CarboPac^{TM} PA20 (15 cm x 3 mm) analytical column (Dionex Sunnyvale, CA) using an ion-exchange chromatography (IC) (Dionex, DX-600) system equipped with a GP 50 gradient pump, AS 40 auto sampler, ED 50 electrochemical detector and a 25 µL sample loop. The sample was passed through a CarboPac^{TM} PA 20 guard column (Dionex Sunnyvale, CA) before entering the PA 20 analytical column. An isocratic elution method was used at a flow rate of 0.2 L min^{-1} for

40 mM NaOH (50 %) and H_2O (50 %). The calibration curves were prepared using standards containing mixtures of all 5 monomeric sugars in varying concentrations (ranging from 100 to 500 mg L^{-1}). The sugar standards were prepared using diluted (10X) basal medium. All of the sugars used in this analysis were of analytical grade (> 99 % purity). Standards containing an individual sugar of a known concentration were prepared and analyzed with the test samples in order to identify the peak retention times of the corresponding sugars by the IC instrument. The detection limit for monomeric sugars was 1 mg L^{-1} with a 25 µL injection volume. Another set of calibration curves was constructed using a CarboPac MA 1 column (Dionex, CA) equipped with a CarboPac MA1 guard column (Dionex, CA) for the estimation of the levels of alcohols and sugars present in the fermented samples. The alcohols (iso-propanol, ethanol, propanol, iso-butanol and butanol) were mixed with the five monomeric sugars in varying concentrations (100 – 500 mg L^{-1}) and then analyzed with the MA 1 column.

3.7.1.1 Liquid sample processing

The following steps were followed to process the liquid samples for HPLC and IC analysis. The samples were diluted to obtain comparable peak resolutions from IC and HPLC. The samples for alcohol analysis using IC were diluted 5X since the electrochemical detector was most accurate (ED50) for concentrations in this range. Similarly, the samples for VFA analysis using HPLC were diluted 5X to allow the accurate detection of VFAs in the amounts produced during this experiment.

Initially, 1 ml of sample was withdrawn from each serum bottle reactor, and was then added to 4 ml of Milli-Q® grade water (Millipore) that had been previously added to an 8 ml glass vial. The vials were capped, and then centrifuged (Beckman Coulter, Inc, USA) at 3000 rpm for 10 minutes. Following centrifugation, the supernatant was left undisturbed on plastic racks. Using a 10 ml plastic syringe, the supernatant was carefully withdrawn and then filtered using a dual stage filtering process into a 5 ml plastic IC vial. In the first stage, the samples were filtered through a 25-mm diameter, 0.45µm polypropylene membrane. This filtered sample was then passed through a 1 ml polypropylene cartridge with a 20 µm PE frit. The 1 ml cartridge was filled with Chelex®

100 to 200 mesh silicon resin (Bio-Rad, California). After the dual stage filtering process was completed, 1 ml of the filtered sample was transferred from the 5 ml plastic vial into a 1.5 ml HPLC vial for VFA analysis, and capped with rubber septa. After the samples for VFA analysis were collected and prepared, the remaining samples within the 5 ml vials were capped and loaded into IC cartridges for the alcohol analysis.

After the samples were run through the IC or HPLC systems, the output data generated from the analysis of the test samples were converted into concentration values using the calibration curves corresponding to the appropriate standards. Finally, these concentration values were multiplied by the dilution factor in order to obtain the actual concentrations of the metabolites in the original samples.

3.7.2 Gas metabolite analysis

The headspace gases (H_2, CH_4 and CO_2) were measured using a gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a 2-mm x 1-mm diameter packed Shincarbon ST (Restek) column. The temperature set points for the TCD, injector and column oven were 200 °C, 100 °C and 200 °C, respectively. N_2 was used as the carrier gas (99.99 % purity, Praxair) at a flow rate of 15 mL / min with a total analysis time of 2.2 minutes. The standards used for gas calibration were H_2 (99.99 %, Praxair), CH_4 (99.99 %, Praxair) and CO_2 (99.99 %, Praxair). The standards were prepared in 160 mL serum bottles, which were initially purged with pure N_2 (99.99 %, Praxair), and then sealed with Teflon®-lined silicon rubber septas and aluminium caps. The standard concentrations of the H_2, CH_4 and CO_2 used for calibration were: 0.25, 0.5, 1.0, 5, 10, 20, 30 and 50 ml gas / 160 ml. The detection limits of the three gases were 0.1900 KPa (2.5 µL / bottle), 0.1226 KPa (2.5 µL / bottle) and 0.0737 KPa (2.5 µL / bottle) for H_2, CH_4 and CO_2, respectively. Known volumes of each gas were prepared as standards within the calibration range and analyzed for each individual gas in order to calibrate the experimental headspace gas analysis. The gas calibration curves are shown in Appendix A.

3.7.2.1 Reactor gas analysis

Headspace gas samples (25 µL) were extracted from batch reactors using a Teflon lined Hamilton gas tight syringe (100 µL) (VWR, ON). A pressure meter was used to measure the pressure in each bottle at this time. Gas samples from all of the reactors were withdrawn using the same 100 µL syringe. The syringe was rapidly inserted through the rubber septa in order to prevent leakage of gas from the reactor. The syringe was removed from the reactor, and then 25 µL of the gas in the syringe was injected into the GC. Using the calibration curves, the composition (percentage of each gas) in the headspace (H_2, CO_2 and CH_4) was determined in order to find the volume of each gas produced within the reactor. Then, using the ideal gas law, the volume of each gas produced was converted into moles of gas.

3.7.3 Volatile suspended solids (VSS) and pH measurement

The concentration of VSS in the biomass was measured before the experiment started and again at the end of the experiment. The assessment of VSS was conducted using standard methods (Greenberg et al., 2010). The initial VSS concentration was measured in samples of the biomass to ensure that the initial baseline for VSS was 500 mg L^{-1} in all of the serum bottle reactors. The measurements of VSS in multiple reactors were conducted in triplicates using a liquid biomass sample from each reactor of 5 ml. This biomass sample was filtered using glass fiber filters with a 0.45 µm pore size (VWR, ON). The pH levels were measured with a VWR SR40C Symphony pH meter (Orion) during sample preparation. The intial and final pH of the fermentation media were measured using the pH meter. The pH meter was calibrated using pH 4.0 and pH 7.0 standard buffer solutions.

3.7.4 Furan aldehydes estimation

The quantity of furan aldehydes (furfural and 5-hydroxymethyl furfural (HMF)) present in the steam exploded liquor was estimated using high performance liquid chromatography (HPLC) (Dionex Ultimate 3000, CA). The liquor samples were filtered using a 0.45 µm polypropylene membrane before HPLC analysis. The HPLC system was

configured with a photo-diode array (PDA) detector. The composition of furfural, HMF, furoic acid and furyl alcohol in the liquor was analyzed using a Dionex, Eclipse plus C18 column (3 mm x 100 mm ID) (Dionex, CA). The temperature of the column oven was set at 50 °C, and an isocratic elution method was used with a solution composed of 0.5 % H_3PO_4 (80 %) and methanol (20 %) at a flow rate of 0.2 mL min^{-1}. The instrument was programmed to scan each sample at three different wavelengths: 280 nm (for furfural and HMF), 215 nm (for furoic acid) and 250 nm (for furyl alcohol). Individual standards were prepared using mixtures of these furan compounds (furfural, HMF, furyl alcohol and furoic acid) with concentrations ranging from 10 - 100 mg L^{-1} in 10X diluted basal medium. The liquor samples from each reactor were filtered using a 0.45 µm polypropylene membrane and the samples (10 µL) were prepared in capped 1.5 mL HPLC vials for HPLC analysis. The detection limits for furan aldehydes (furfural and HMF) were 0.5 mg L^{-1} and were 1 mg L^{-1} for furan derivatives (furyl alcohol and furoic acid).

3.8 Long chain fatty acid (LCFA) addition to reactors

The fatty acids used in my experiments, lauric acid (C 12, LAU) and linoleic acid (C 18:2, LA), are relatively insoluble in water . In order to properly distribute the fatty acids in the fermentation medium, they must be dissolved in water prior to the start of the experiment. The fatty acids were saponified using lye (NaOH) according to the method developed by (Rinzema et al., 1994). Stock solutions (50,000 mg L^{-1}) were prepared in 160 mL glass vials and stored in a refrigerator at 4 °C prior to use. The fatty acids were melted *au bain-marie* and dissolved in vigorously stirred NaOH solution maintained at 50 °C. The amount of NaOH added to saponify each fatty acid is expressed as g NaOH added per g fatty acid (Table 3.3).

Table 3.3 Amount of NaOH used in LCFA stock solution preparation.

Fatty acid	Amount of lye used (g NaOH g^{-1} fatty acid)
Lauric acid (LUA); C 12	0.20
Linoleic acid (LA); C 18:2	0.14

3.9 Enzymatic methods

3.9.1 Uptake Hydrogenase Assay (UHA)

The procedure for the UHA was carried out in a similar manner to that described by Pendyala et al. (2012). The UHA protocols include a cell mixture, an electron mixture and a reaction mixture. In the case of this *in vitro* H_2 uptake assay, oxidized benzyl viologen (BV) (TCI America, OR) was used as an artificial electron acceptor during H_2 oxidation by uptake [Ni-Fe] hydrogenase. The UHA was carried out in 5 mL glass vials with continuous N_2 purging to ensure anaerobic conditions. The electron mixture contained 0.3 mL of 40 mM BV, 2.4 mL of anaerobic milli-Q water and 0.3 mL of 100 mM phosphate buffer. The electron mixture was purged with pure H_2 for 3 minutes, and then this mixture was placed in a 37 °C water bath.

A cell mixture containing 0.3 mL of the inoculum (obtained from the batch reactors), 1.8 mL of anaerobic milli-Q water, 0.3 mL of 100 mM phosphate buffer (pH-6.0), and 0.3 mL of dithiothreitol (DTT) (TCI America, OR) was prepared anaerobically. The cell mixture was then purged with H_2 for 2 minutes, then 1% (v/v) Triton X-100 (Sigma Aldrich, ON) was added, and the mixture was purged again for 1 minute. The cell mixture was then placed in the water bath.

The reaction mixture was prepared by adding 2.1 mL of the electron mixture and 0.7 mL of the cell mixture into a 4 mL anaerobic cuvette. Previously, the cuvette was purged with H_2 and sealed under anaerobic conditions. The time course of benzyl viologen reduction was monitored with a spectrophotometer (Cary 50 scan, Varian, CA) at 546 nm for 10 minutes at 0.1 sec intervals. The initial slope obtained from the plot of the optical density (OD) versus time (min) was used to compute the total activity (µmol H_2 min^{-1}). The specific activity was reported as U_u mg^{-1} VSS where 1 U_u represents 1 µL of H_2 consumed h^{-1}.

3.9.2 Evolution Hydrogenase Assay (EHA)

The EHA was carried out using a similar procedure to that described by Pendyala et al. (2012). *In vitro* H_2 evolution assays were performed using methyl viologen (MV) (99 %

purity) (Sigma, ON) as an artificial electron donor. This system mimics H_2 evolution by Pyruvate: ferredoxin oxido-reductase (PFOR) in the anaerobic glucose degradation. Pyruvate decarboxylation to acetyl-CoA reduces ferredoxin, thereby resulting in H_2 evolution. [Fe]-hydrogenase catalyzes H_2 production using electrons from the reduced ferredoxin. The HEA that measures this enzymatic activity is carried out under anaerobic conditions using 0.3 mL of inoculum obtained from the incubated batch reactors.

A cell mixture containing 0.3 mL of inoculum, 0.3 mL of 0.5 M phosphate buffer (pH 7.0), and 0.3 mL of a 0.1 % Triton X-100 solution (ACP chemicals Inc., QC) was mixed with 2.1 mL of anaerobic milli-Q water (Milli-Q water purged with N_2 for 10 min) for a final working volume of 3 mL.

During preparation of the reaction mixture, the solution was continuously purged with nitrogen gas (Praxair, ON; purity > 99 %) during the addition of these ingredients, so as to maintain strict anaerobic conditions. The EHA was performed in its entirety in a 5 mL glass vial sealed tightly with a Teflon®-lined cap with a rubber septa and aluminum crimp. The glass vial was purged with N_2 gas through a sterile needle inserted through the septa, and another needle was also inserted into the vial for gas to exit. The reaction mixture (approximately 2.0 mL) contained 0.1 mL of the cell mixture (purged with N_2 for 3 mins), 1.6 mL of anaerobic milli-Q water, 0.2 mL of 0.5 M phosphate buffer (pH 7.0), and 40 µL of oxidized MV (20 mM). 100 µL of sodium dithionite (ACP chemicals Inc., QC) was added from a previously prepared stock solution (2.5 mg mL^{-1}) to initiate the reaction. After the reaction mixtures in the glass vials were incubated for 1 h (at 37 °C for inocula from mesophilic batch reactors or at 55 °C for inocula from thermophilic batch reactors), the reaction was terminated by the addition of 0.1 mL of trichloro acetic acid (TCA) (MP Biochemicals, OH). Headspace gas analysis was then conducted for each of the incubated 5 mL glass vials to measure H_2 evolution (the amount of H_2 produced by the various reaction mixtures). The specific activity of hydrogenase was reported as U_e, mg VSS^{-1} where 1 U_e represents 1 µL H_2 produced h^{-1}.

3.10 Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

3.10.1 Genomic DNA extraction

Genomic analyses of the microbial consortia in the batch reactors were performed using terminal restriction polymorphism analysis (T-RFLP) according to the protocol described by Chaganti et al. (2012). Microbial samples were collected from the anaerobic sequencing batch reactors (AnSBR) at the start and the end of the experiment, and at the end of each batch for the batch reactor studies. The samples were collected from the reactors with sterile 10 mL plastic syringes, and were immediately transferred into 15 mL plastic centrifuge tubes. The centrifuge tubes were labeled (to identify the study, experimental conditions, and the time when the sample was collected), and the samples were capped and stored in a freezer at -20 °C until analysis. Samples were prepared for analysis by withdrawing 0.4 mL of the thawed contents from the 15 mL conical centrifuge tube, and then adding it to a 2 mL tube containing approximately 250 mg of zirconia/silica beads, 0.4 mL cetyl trimethylammonium bromide (CTAB) extraction buffer (20 % (wt/vol) (Sigma, Toronto, ON) in 1.4 mM NaCl with 480 mM of potassium phosphate buffer at pH 8.0, and 0.4 mL of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0). These ingredients were then subjected to three freeze-thaw cycles (-80 °C). The microbial cells were then lysed with a Thermo Savant Bio 101 Fast Prep FP 120 homogenizer (Savant Instruments, NY) for 45 s at the 6.5 speed setting. The homogenized samples were centrifuged at 16,000 x g for 10 min at 4 °C to achieve clear phase separation. The clear supernatant was then transferred into 1.5 mL micro-centrifuge tubes, and re-extracted using an equal volume of chloroform-isoamyl alcohol (24:1), followed by centrifugation at 10,000 x g for 10 min. The nucleic acids were precipitated by the addition of isopropanol solution (2/3 vol) to the extract. The sample was incubated at room temperature (21 °C) for 10 min, and then centrifuged at 10,000 x g for 20 min at 4 °C. The supernatant was discarded, and the pellet (precipitate) was washed using 70 % (vol/vol) ice-cold ethanol, and then air-dried. The precipitates from each sample were then re-suspended in 50 µL of sterile Milli-Q water for PCR and T-RFLP profiling.

3.10.2 Polymerase chain reaction (PCR) and T-RFLP profiling

Polymerase chain reaction (PCR) of the 16S rRNA gene present in the microbial samples was performed using dye-labeled forward primer B8F (5' – AGAGTTTGATCCTGGCTCAG - 3') (Edwards et al., 1989) and with reverse primer Eub-539R (5' – ATCGTATTACCGCGCTGCTGGC – 3'). The archeal 16S rRNA genes were amplified similarly using forward primer Arc- 112F (5' – GCTCAGTAACACGTGG – 3') and with reverse primer Arc-533R (5' – TTACCGCGGCGGCTGGCA – 3'). The PCR mixtures (25 µL) containing 10.2 mM Tris buffer, 2.3 mM $MgCl_2$, 50 mM KCl, 2 % DMSO, 5 µg BSA, 0.2 mM of individual dNTP, 0.2 mM of each primer, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Streetsville, ON) were cycled as follows: 95 °C for 5 min, 35 cycles of 94 °C for 1 min, 42 °C for 15 s, 72 °C for 15 s, followed by a final elongation step of 72 °C for 1 min. PCR products were purified using QIAquickspin columns (Invitrogen, Carlsbad, CA). The PCR products were then digested using 2.5 U of various restriction enzymes (*Hae* III, *Hha* I, *MSP* I and *Hinf* I) in a total volume of 20 µL for 2 h at 37 °C. The restriction enzymes, *Hae* III and *Hha* I, were selected in order to produce short terminal restriction fragments (T-RFs < 300 bases) from most bacteria and archea. The restriction digest products (1µL) were mixed with 3 µL of stop solution (LI-COR, Inc., Lincoln, NE), and these samples and size markers (50-700 base pairs, LI-COR, Inc., Lincoln, NE) were denatured at 95 °C for 2 min, followed by rapid chilling on ice. The denatured samples were then loaded onto a 6.5 % polyacrylamide gel (KBPlus™, LI-COR, Inc., Lincoln, NE). The T-RFs were then separated according to their size by electrophoresis (2.5 h at 1500 V, 35 mA, 35 W, 45 °C) using a DNA analyzer (Model 4300L, LI-COR, Inc., Lincoln, NE).

3.10.3 Analysis of T-RFLP patterns

The fragment sizes and relative abundances (band intensity) of the separated T-RFs were estimated using the software application, Gene ImagIR 4.05 (Scanalytics, Inc., Rockville, MD). The T-RFs that were found in different profiles, but differed by only ±1 bp were considered to be identical in order to facilitate the comparison of T-RFLP profiles

between samples. The relative abundance (based on band intensity) of each T-RF within a given T-RFLP pattern was calculated by dividing the peak area for a particular T-RF by the total peak area of all the T-RFs detected within a fragment (ranging in length from 50 to 500 bp for archea and bacteria). Short fragments (< 50 bp) were excluded from the T-RFLP analysis. The pros of this technique are it is rapid and highly automated (Lau et al., 2005). The cons of this technique are as follows. This method doesn't allow for the bacterial species identification in the samples. Results from T-RFLP could only be viewed as "semi-quantitative" because the same peak may represent many species of bacteria that share the same restriction enzyme digest sites (Zhang et al., 2008). Moreover, minority bacterial populations may not be detected by T-RFLP analysis due to the fact that the template DNA from these populations represents a small fraction of the total extracted DNA and may not be amplified by PCR due to the kinetic bias (Liu et al., 1997). T-RFLP analysis cannot differentiate closely related DNA sequences which are likely to have the same terminal restriction site, and thus may reduce the number of detectable OUT's (Muyzer et al., 1993). T-RFLP also over estimates the bacterial diversity due to the formation of pseudo-TRF formation (i.e., single stranded amplicon that are recalcitrant to enzyme digestion) (Egert and Friedrich, 2003). Due to the above mentioned disadvantages bands generated using T-RFLP analysis should always be used with a caution. This will also affect the microbial cluster analysis when using abundance based index.

3.10.4 Phylogenetic assignment of T-RFs

The T-RFLP data generated by the digestion of PCR-amplified genetic material from microbial samples were formatted according to the requirements of the Phylogenetic Assignment Tool (PAT), and analyzed online (https://secure.limnology.wisc.edu/trflp/) using default fragment bin tolerance window settings. Phylogenetic assignment was performed using a database modified from the default database generated by the microbial community analysis (MiCA) site (http://mica.ibest.uidaho.edu/).

3.11 Flux Balance Analysis (FBA)

Flux balance analysis (FBA) of the flow of electrons during metabolic reactions was performed using MetaFluxNet software Version.1.8.6.2. Flux analysis was applied to only the experimental data for the H_2 production studies using pure glucose as the substrate using the metabolic reactions network and the stoichiometry reported for glucose.

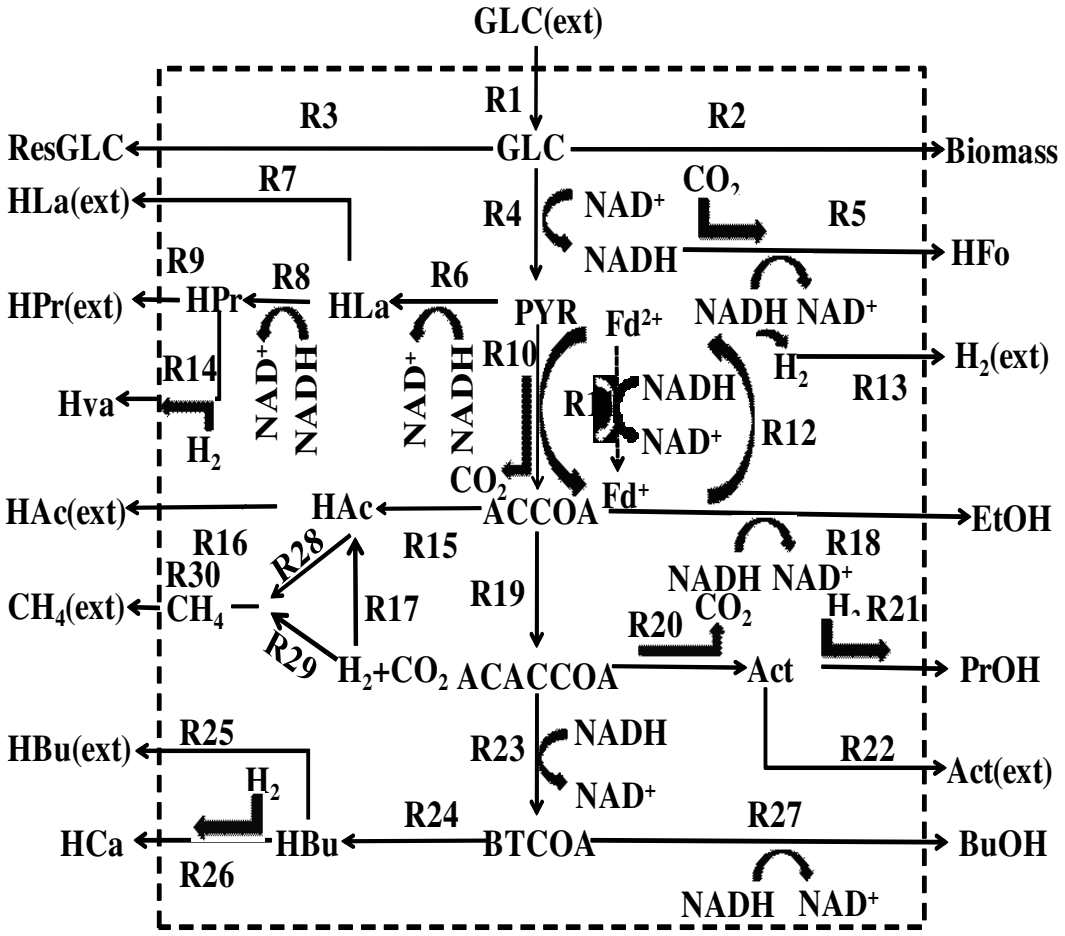

Figure 3.1 Metabolic reaction network adapted from the model developed for glucose by Chaganti *et al*., 2011.
Note: The flux model network was adapted from Chaganti et al. (2011).

The reaction stoichiometries are listed in Table 3.4. In order to illustrate the actual cell environment, dashed lines in Figure 3.1 represent the cell wall, which separates the intracellular and extracellular metabolites. Moles of electrons derived from substrate

(glucose) and the by-products (VFAs, alcohols and gases in moles) that were measured during testing provided the inputs for the FBA. H_2 production was selected as the objective function for determining the metabolic flux distribution.

Table 3.4 Stoichiometries of the biological reactions (R1 to R30) in the metabolic reaction network model used in the flux balance analysis.

Reaction No.	Reaction Stoichiometry
R1	**Glucose (ext) → Glucose**
R2	**Glucose → Biomass**
R3	**Glucose → Residual glucose**
R4	**Glucose → 2 Pyruvate + 2 NADH**
R5	**NADH → HFo**
R6	**NADH + Pyruvate → HLa**
R7	**HLa → HLa (ext)**
R8	**HLa + NADH → HPr**
R9	**HPr → HPr (ext)**
R10	**Pyruvate → Acetyl-CoA + 2 Fd_{red}**
R11	**NADH → 2 Fd_{red}**
R12	**2 Fd_{red} → H_2**
R13	**H_2 → H_2 (ext)**
R14	**HPr + 6 H_2 → HVa**
R15	**Acetyl-CoA → HAc**
R16	**HAc → HAc (ext)**
R17	**4 H_2 → HAc**
R18	**Acetyl-CoA + 2 NADH → EtOH**
R19	**2 Acetyl-CoA → Acetoacetyl CoA**
R20	**Acetoacetyl CoA → Acetone**
R21	**Acetone + H_2 → PrOH**
R22	**Acetone → Acetone (ext)**
R23	**Acetoacetyl CoA + 2 NADH → ButyrylCoA**
R24	**ButyrylCoA → HBu**
R25	**HBu → HBu (ext)**
R26	**HBu + 6 H_2 → HCa**
R27	**ButyrylCoA + NADH → BuOH**
R28	**HAc → CH_4**
R29	**4 H_2 → CH_4**
R30	**CH_4 → CH_4 (ext)**

3.12 Principal component analysis (PCA)

A multivariate statistical technique, Principal Component Analysis (PCA), was used to reveal underlying patterns within the microbial populations examined under various

operating conditions during these studies. A PCA bi-plot of scores and loadings was generated. A cluster analysis of the microbial communities was also carried out using the Jaccard, Kulczynski and Bray-Curtis similarity index. In addition, rarefaction curves were generated to study species richness under each experimental condition (in Chapter 5). These analyses were conducted using the PAST: paleontological statistics software package for education and data analysis (Version 2.13; http://folk.uio.no/ohammer/past/). Tukey's test for multiple comparisons was used to compare the degree of similarity between the results obtained for different experimental conditions ($p < 0.05$); Tukey's test was performed using Microsoft Excel 2007.

3.13 References

APHA. (2010). Standard methods for the examination of water and wastewater. 20th ed. Washington D.C: American publishers health association.

Chaganti, S.R., Kim, D.H., Lalman, J.A. (2011). Flux balance analysis of mixed anaerobic microbial communities: Effects of linoleic acid (LA) and pH on biohydrogen production. *Int J Hydrogen Energy*, **36**(21), 14141-52.

Chaganti, S.R., Lalman, J.A., Heath, D.D. (2012). 16S rRNA gene based analysis of the microbial diversity and hydrogen production in three mixed anaerobic cultures. *Int J Hydrogen Energy*, **37**(11), 9002-17.

Egert, M., Friedrich, M.W. (2003). Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl Environ Microbiol*, **69**(5), 2555-62.

Goering, H.K., Van Soest, P.J. (1970). Forage fiber analysis. *Agricultural Handbook*, **379**.

Greenberg, A.E., Clesceri, L.S., Eaton, A.D. (2010). American Public Health Association (APHA); American Water Works Association (AWWA); and Water Environment Federation (WEF). *Standard Methods for the Examination of Water and Wastewater.*

Hedge, J.E., Hofreiter, B.T. (1962). Estimation of carbohydrate. *Carbohydr Chem*, 17-22.

Hubbell, C.A., Ragauskas, A.J. (2010). Effect of acid-chlorite delignification on cellulose degree of polymerization. *Bioresour Technol*, **101**(19), 7410-5.

Lalman, J.A., Alosta, H., Bejankiwar, R., Bellmer, D. (2003). Kinetics of glucose fermentation by a mixed culture in the presence of linoleic, oleic, and stearic acid. *Environ Technol*, **24**(12), 1471-8.

Lalman, J.A., Bagley, D.M. (2000). Anaerobic degradation and inhibitory effects of linoleic acid. *Water Res*, **34**(17), 4220-8.

Lalman, J.A., Bagley, D.M. (2002). Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. *Water Res*, **36**(13), 3307-13.

Lau, S.C.K., Thiyagarajan, V., Cheung, S.C.K., Qian, P.Y. (2005). Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates. *Aquat Microb Ecol*, **38**(1), 41-51.

Liu, W.T., Marsh, T.L., Cheng, H., Forney, L.J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol*, **63**(11), 4516-22.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*, **31**(3), 426-8.

Muyzer, G., Dewaal, E.C., Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16s ribosomal RNA. *Appl Environ Microbiol*, **59**(3), 695-700.

Pendyala, B., Chaganti, S.R., Lalman, J.A., Shanmugam, S.R., Heath, D.D., Lau, P.C.K. (2012). Pretreating mixed anaerobic communities from different sources: Correlating the hydrogen yield with hydrogenase activity and microbial diversity. *Int J Hydrogen Energy*, **37**(17), 12175-86.

Rinzema, A., Boone, M., Van Knippenberg, K., Lettinga, G. (1994). Bactericidal effect of long chain fatty acids in anaerobic digestion. *Water Environ Res*, **66**(1), 40-9.

Singleton, V.L., Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Amer J Enol Viticult*, **16**, 144-58.

Updegraf, D.M. (1969). Semimicro determination of cellulose in biological materials. *Anal Biochem*, **32**(3), 420-4.

Wiegant, W.M., Lettinga, G. (1985). Thermophilic anaerobic digestion of sugars in upflow anaerobic sludge blanket reactors. *Biotechnol Bioeng*, **27**(11), 1603-7.

Zhang, R., Thiyagarajan, V., Qian, P.Y. (2008). Evaluation of terminal-restriction fragment length polymorphism analysis in contrasting marine environments. *FEMS Microbiol Ecol*, **65**(1), 169-78.

CHAPTER 4: STATISTICAL OPTIMIZATION OF HYDROGEN CONSUMPTION IN MIXED ANAEROBIC CULTURE USING THE BOX-BEHNKEN DESIGN

4.1 Introduction

Effluents from many food processing industries contain neutral lipids, such as fats and oils, along with carbohydrates (Cirne et al., 2007). Under favourable thermodynamic conditions, lipids and carbohydrates are degraded into methane via a series of biochemical reactions. Fats and oils contain long chain fatty acids (LCFAs) connected to a glycerol molecule. During anaerobic digestion of these organic substrates, H_2 is produced as an intermediate, which is then consumed by the H_2 consumers to produce methane.

Maintaining low H_2 partial pressures is important for the efficient operation of anaerobic bioreactors. According to (Cord-Ruwisch et al., 1997), the threshold H_2 limit for the stable operation of anaerobic digesters is 6.5 Pa. However, in practice, typical H_2 levels are in the range of 1.5-19.9 Pa (Collins and Paskins, 1987). The threshold H_2 value is defined as the minimum amount of energy (Critical Gibb's free energy) that is required for hydrogenotrophic growth or that allows H_2 utilization (Seitz et al., 1990). Hydrogen is utilized by anaerobic chemolithotrophic bacteria, such as methanogens, sulfate reducers and homoacetogens, in reactions that have exergonic Gibbs free energy under standard conditions (25 °C; 1M/1 bar (100 kPa) reactants or products). Hydrogen is also utilized under -standard conditions, as long as the partial pressure of H_2 is greater than a threshold level (Cord-Ruwisch et al., 1988; Lovley, 1985). The threshold values for H_2 utilization by different H_2 consumers are dependent on the redox potential of the terminal electron acceptor (Table 4.1). Note the H_2 threshold values decrease with increasing redox potential of the terminal electron acceptor.

Temperature is an important factor influencing microbial habitats. Homoacetogens, methanogens and sulfate reducers generally tolerate change in temperature only within

specific limits, but quickly adapt to a particular optimum temperature. Homoacetogens out-compete methanogens in anaerobic environments at low temperatures because their growth rates are much higher than those of methanogens at these temperatures (Kotsyurbenko et al., 2001). The competition for H_2 depends upon variation in the uptake kinetics of different bacterial strains. The H_2 threshold values are also strain specific and vary within a particular group. In a study carried out by Kotsyurbenko et al., (2001), homoacetogenic *A.bakii*, *A.tundrae* and a methanogenic strain MSB exhibited the largest temperature range (4 °C – 30 °C) for optimum H_2 consumption. On the other hand, *A. fimetarium*, *A. faludosam* and the methanogenic strain MSP, exhibited lower H_2 consumption rates when the temperature decreased to below 10 °C. Temperature also plays a key role in determining the Gibbs free energy. The free energy of a particular reaction ($\Delta G^{o'}$) is calculated using the standard Gibbs free energy (ΔG^{o}) and the concentration-dependent logarithmic term, which increases linearly with increasing temperature.

The H_2 threshold is inversely related to the changes in Gibb's free energy ($\Delta G^{o'}$) and the electrochemical potential of the H_2-consuming reaction (Loffler et al., 1999). The steady state H_2 threshold concentrations are independent of the kinetics of H_2 producing or H_2 consuming reactions and dependent on the physiological characteristics of the H_2 consuming organisms (Lovley and Goodwin, 1988). The H_2 threshold values are ranked in the following order from the least to most energetically favourable terminal electron acceptor process: acetogenesis (430-4660 ppm_v) > methanogenesis (6-120 ppm_v) > sulfate reduction (sulfidogenesis) ($SO_4^{2-} \rightarrow HS^-$) (1.3-19 ppm_v) > Fe (III) reduction (0.3-1 ppm_v) > denitrification (< 0.06 ppm_v) (Loffler et al., 1999).

The inhibition of hydrogen consumers by LCFAs leads to the accumulation of volatile fatty acids, which in turn creates an imbalance between H_2 producers and consumers and subsequent reactor failure. LCFAs inhibit microorganisms by adsorbing to external and internal cell membranes. In solution, they can be precipitated by divalent ions and resulting in biomass floatation (Dereli et al., 2014). Adsorption is the most widely accepted mode of inhibition by LCFAs because the physical interaction of the LCFA

with the cell membranes affects both transport and protective functions (Galbraith and Miller, 1973). The metabolic activities of several microorganisms, such as acidogens, acetogens and methanogens, are affected by the inhibition caused by LCFAs at their threshold levels (Hanaki et al., 1981; Hwu and Lettinga, 1997; Koster and Cramer, 1987; Lalman et al., 2003; Mykhaylovin et al., 2005).

Table 4.1 Selected H_2 threshold values of major H_2 consumers present in mixed anaerobic communities.

Name of microorganism	H_2 threshold (Pa)	Role in anaerobic digestion	Reference
Acetobacterium woodii	52.689	Acetogen	Cord-Ruwisch *et al*. 1988
Acetobacterium carbinolicum	95.000	Acetogen	
Spormusa acidovorans	43.570	Acetogen	
Spormusa termitida	84.100	Acetogen	
Desulfovibrio desulfuricans Essex	0.912	Sulfate Reducer	
Desulfovibrio desulfuricans	0.003	Nitrate reducer	
Wolinella succinogens	0.002	Fumarate reducer	
Methanospirillum hungatei	3.040	Methanogen	
Methanobrevibacter smithii	10.133	Methanogen	
Methanobrevibacter arboriphilus	9.119	Methanogen	
Methanobrevibacter formicum	2.837	Methanogen	
Methanococcus vannielii	7.599	Methanogen	
Methanothermobacter thermoautotrophicus ΔH	12-14	Methanogen	Lee *et al*. 1988
Methanothermobacter thermoautotrophicus THF	12-14	Methanogen	
Methanobacterium formicicum JF-1	6.5 ± 0.6	Methanogen	Lovely, 1985
Methanobacterium bryantii M.o.H	6.5 ± 1.5	Methanogen	
Methanospirillum hungatei JF-1	9.5 ± 1.3	Methanogen	
Methanogenic strain MSB	0.8-1	Methanogen	Kotsyurbenko *et al*. 2001
Methanogenic strain MSP	3-4	Methanogen	
Methanobacterium bryantii strain Bab.1	0.04	Methanogen	Conrad and wetter, 1990
Methanobacterium thermoautotrophicum	0.19	Methanogen	
Methanogenium fridgium Ace-2	0.57	Methanogen	Chong *et al*. 2002

Only a few studies have described the effects of LCFAs on fermentative H_2 production (Demeyer and Henderickx, 1967; Hanaki et al., 1981; Lalman and Bagley, 2000; Lalman

and Bagley, 2001; Templer et al., 2006). Hanaki et al. (1981) examined with effects of LCFAs with levels varying up to 2 g L^{-1} and concluded a LCFAs mixture containing C10:0-C18:0 fatty acids was effective on H_2 metabolism at 37 °C when compared to control (without LCFA added) cultures. Templer et al. (2006) also conducted a study of H_2 metabolism using mixtures of three different LCFAs, namely stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (LA) (C18:2), at 21 °C and concluded that with increasing C=C bond unsaturation, the greater the degree of inhibition. The nature of the monolayer formed by unsaturated fatty acids is not the same as monolayers formed by saturated fatty acids. The unsaturated molecules tend to arrange themselves with bends at the double bonds while saturated fatty acids tend to be positioned in parallel straight chains (Galbraith and Miller, 1973). The bacteriostatic effect of LA (C18:2) is of particular interest since it is regarded as an unsaturated fatty acid that is essential to the nutrition of higher animals. The fact that LA inhibits gram positive bacteria more than gram negative bacteria is interesting because most chemotheraupetic agents are similarly more effective on gram positive bacteria (Demeyer and Henderickx, 1967). The extent of the inhibition by these fatty acids depend upon the concentration of bacteria, the length of incubation, the amount and nature of the fatty acid added, and the presence of other lipids in the medium (Kodicek and Worden, 1945).

Many past studies have examined using a one-factor-at-a-time approach to examine the impact of LCFAs on microbial cultures (Hanaki et al., 1981; Lalman and Bagley, 2000; Lalman and Bagley, 2001; Templer et al., 2006). These studies examined the impact of LCFA by varying LCFA concentration, pH or the type of fatty acid. In addition to their inhibitory effects, LCFAs such as linoleic acid (LA) also plays a role in diverting the flow of electrons derived during the degradation of reduced carbon substrates towards proton reduction (Ray et al., 2010b). The electron flow in mixed anaerobic systems is affected by the temperature, pH and the presence of chemical additives (e.g. LCFAs, 2-Bromoethane sulfonic acid (BES), or acetylene) (Chowdhury et al., 2007; Ray et al., 2008; Ray et al., 2009). pH is also a major factor controlling the flow of electrons in metabolic pathways and also in suppressing the activity of methanogenic archea. pH affects the hydrogenases which are involved in either proton oxidation or reduction.

Hydrogen fermentation by different microbial populations is optimum under different pH ranges. For example, methanogens are more active in the pH range of 6.5 - 8.0. Decreasing the pH to 5.0 without adjusting other parameters can impair the activity of methanogens.

The effect of multiple parameters on a single response can be studied effectively and efficiently using statistical modeling. Statistical modeling offers several advantages, such as the analysis of potential interactions between variables, determination of the optimum response under design conditions, assessment of the validity of the model and the reliability and significance of test results, and optimization of test parameters to reduce experimental variation. Response surface methodology (RSM) is a statistical tool used to explore the relationship between the explanatory variable (independent variable) and one or more response variables. RSM is used to optimize a response variable (i.e., to minimize, maximize or attain a specific goal). Response surface designs are advantageous over a full factorial design (FFD) for predicting the positions of the factor space, which are equidistant from the center space. Response surface designs, such as central composite design (CCD) and Box-Behnken design (BBD) are widely used for performing optimization studies. The BBD is a three-level design using a combination of two-level factorial and incomplete block designs. The BBD requires fewer experiments than FFD and CCD.

The effects of multiple variables (temperature, pH and varying LA concentration) on H_2 consumption have not been examined. Understanding the combined effect of these variables will assist in optimizing the conditions required for minimizing H_2 consumption. Hence, the objectives of this study are to optimize the conditions for minimum H_2 consumption using a statistical Box-Behnken design (BBD); and to examine the diversity of homoacetogens, hydrogenotrophic methanogens and aceticlastic methanogens in the presence of LA using T-RFLP.

4.2. Materials and methods

The materials and methods utilized in this research study are described in Chapter 3. The source of the inocula and the maintenance protocol are detailed in Section 3.2. The protocol followed in the preparation of the batch reactors (160 mL) is described in Section 3.3. The experimental design matrix is shown in Table 4.2. The analytical methods for analyzing gas by-products (H_2, CH_4 and CO_2), and liquid by-products (VFAs) accord with the protocols described in Sections 3.7.1 and 3.7.2.

A three-factor three-level Box-Behnken design (BBD) was used optimizing the variables affecting H_2 consumption. All experimental conditions were conducted in triplicate. Preliminary studies were conducted to determine which design factors influence H_2 consumption, and the range of values to apply during testing (data not shown). Based on these pilot studies, the design factors examined in this study were as follows: temperature (21, 37, 53 °C), pH (4.5, 6.0, 7.5) and LA concentration (0, 1, 2 g L^{-1}). The variable X_i was coded as x_i according to the following equation (4.1) where X_o corresponded to the central value.

$$\boldsymbol{x_i = \frac{X_i - X_o}{\Delta X_i}} \tag{4.1}$$

Where, x_i and X_i are the coded and actual values of the independent variable, respectively. X_o is the actual value of the independent variable at the centre point, and ΔX_i is the step change of Xi.

The design factors (independent variables) used in this study and their levels are tabulated in Table 4.2. The response variable was % H_2 consumption, and the mean response was calculated for further analysis. The following quadratic polynomial (Eq. 4.2) was used to model the relationship between the response and the design variables.

$$\begin{aligned}\boldsymbol{H_2\ consumption}\ (\%) = \boldsymbol{a_0} + \boldsymbol{a_1} * (\boldsymbol{Temperature}) + \boldsymbol{a_2} * (\boldsymbol{pH}) + \boldsymbol{a_3} * \\ (\boldsymbol{LA\ Concentration}) + \boldsymbol{a_4} * (\boldsymbol{Temperature})^2 + \boldsymbol{a_5} * (\boldsymbol{pH})^2 + \boldsymbol{a_6} * \\ (\boldsymbol{LA\ Concentration})^2 + \boldsymbol{a_7} * (\boldsymbol{Temperature}) * (\boldsymbol{pH}) + \boldsymbol{a_8} * \\ (\boldsymbol{Temperature}) * (\boldsymbol{LA\ Concentration}) + \boldsymbol{a_9} * (\boldsymbol{pH}) * (\boldsymbol{LA\ Concentration}) + \boldsymbol{\varepsilon}\end{aligned} \tag{4.2}$$

Table 4.2 Experimental design matrix for determining the optimum condition to minimize H_2 consumption.

Exp #	Temperature (°C)		pH		LA (g L^{-1})		Metabolite analysis	
	X_1	Code	X_2	Code	X_3	Code	Gas (H_2, CH_4, CO_2) every 24 hrs till 96 hrs	Liquid (VFAs) at 96 hr
1	21	-1	4.5	-1	1	0		
2	53	1	4.5	-1	1	0		
3	21	-1	7.5	1	1	0		
4	53	1	7.5	1	1	0		
5	21	-1	6.0	0	0	-1		
6	53	1	6.0	0	0	-1		
7	21	-1	6.0	0	2	1		
8	53	1	6.0	0	2	1		
9	37	0	4.5	-1	0	-1		
10	37	0	7.5	1	0	-1		
11	37	0	4.5	-1	2	1		
12	37	0	7.5	1	2	1		
13[a]	37	0	6.0	0	1	0		
14[a]	37	0	6.0	0	1	0		
15[a]	37	0	6.0	0	1	0		

Note: [a]denotes centre point repeated three times.

Different amounts of LA (0, 1 and 2 g L^{-1}) were injected into the serum bottles by adding the appropriate volume of a 50 g L^{-1} saponified LA stock solution. LA was fed to the cultures 24 h before the addition of substrate, so as to provide enough time for LA to impose its inhibitory effect. After 24 h, 60 mL of substrate (3000 µmol H_2) was injected into each bottle. The optimum conditions were predicted according to the quadratic equation above (Eq. 4.2). The software application, MINITAB (version 16, Minitab Inc., State College, PA), was used to analyze the model predicted outcomes with the experimental outcomes, and to generate two- and three-dimensional contour plots. An analysis of variance (ANOVA) was conducted to further evaluate the validity of the model.

Samples for microbial analysis were collected at the end of the experiment (51 h). The microbial (T-RFLP) analysis was carried out according to the protocol described in section 3.10. A principal component analysis (PCA) was performed using PAST software

to assess the effects of different process parameters (temperature, initial pH and LA concentration) on the distribution of fermentation metabolites. By-products from H_2 consumption, which included acetic acid, formic acid, propionic acid and the remaining residual H_2 were used as inputs for the PCA analysis.

4.3. Results and Discussion

The optimum levels for selected key factors and the effect of their interactions on H_2 consumption were determined using a 3-factor 3-level BBD. The effect of temperature on H_2 consumption by methanogens, homoacetogens, and to some extent sulfate reducers, was studied previously (Conrad and Wetter, 1990; Kotsyurbenko et al., 2001; Sonne-Hansen et al., 1999); however, these studies were conducted in pure cultures of homoacetogens, methanogens or sulfate reducers. The effects of multiple process variables (pH, LA) in addition to temperature on H_2 consumption in mixed microbial communities comprised of these populations have not been examined.

4.3.1 Experimental design analysis

The % H_2 consumption for each experimental condition at various levels of selected key factors is shown in Table 4.3. The experimental response for each condition over the three factors and levels tested was used for the response surface optimization study. The % H_2 consumption obtained under each of the experimental condition varied from 0 % to 100 % 51 hours after adding the substrate. The variation in the response (% H_2 consumption) observed under similar fermentation conditions, but with different temperature, pH and LA concentration, indicates the importance of these experimental parameters on H_2 metabolism. Similar changes in the H_2 consumption rates of LA-treated culture have been reported previously in a study conducted at pH 7.6 and at temperature 21 °C (Templer et al., 2006). In comparison, the current study provides better understanding of H_2 consumption under conditions characterized by different combinations of pH, temperature and LA concentration.

Table 4.3 Hydrogen consumption (%) and metabolites produced under different experimental conditions.

Exp #	% H_2 consumption		CH_4 (mol mol^{-1} H_2)	HAc (meq^-)	HPr (meq^-)
	Experimental[b]	Predicted			
1	2±2	8	0.00±0.00	0.00±0.00	0.00±0.00
2	0±0	23	0.00±0.00	0.00±0.00	0.00±0.00
3	96±1	75	0.12±0.02	1.18±0.16	0.83±0.08
4	35±6	28	0.05±0.01	0.51±0.16	0.00±0.00
5	100±0	107	0.22±0.01	0.37±0.08	0.00±0.00
6	100±0	92	0.21±0.01	0.35±0.06	0.00±0.00
7	9±1	17	0.01±0.00	0.24±0.01	0.00±0.00
8	6±2	-1	0.00±0.00	0.25±0.01	0.00±0.00
9	96±0	84	0.22±0.00	0.39±0.02	0.00±0.00
10	100±0	114	0.21±0.01	0.67±0.14	0.00±0.00
11	0±0	-14	0.00±0.00	0.00±0.00	0.00±0.00
12	16±6	29	0.02±0.01	0.11±0.01	0.29±0.03
13[a]	21±3	21	0.04±0.01	0.00±0.00	0.18±0.03

Notes: [a]The centre point run was repeated three times; [b]average of the triplicate data sets is presented along with standard deviation. HAc = acetic acid; HPr = propionic acid.

The regression coefficients obtained for the response variables were used to develop a model regression equation (Eq. 4.3).

$$\%\ H_2\ consumption = 21.10 - 8.07 * (Temperature) + 18.20 * (pH) - 45.63 * (LA) + 6.70 * (Temperature)^2 + 5.85 * (pH)^2 + 26.00 * (LA)^2 - 15.57 * (Temperature) * (pH) - 0.87 * (Temperature) * (LA) + 3.19 * (pH) * (LA) \quad (4.3)$$

4.3.2 Accuracy of Model (Analysis of variance (ANOVA))

ANOVA was conducted with the data for the observed experimental response (% H_2 consumption) in order to evaluate the accuracy of the quadratic model (i.e., to determine the fit of the model to actual observations) (Table 4.4). The *p*-value indicates the likelihood that the variation in the experimental data is due to coincidence (random noise), which is not explained by the model. The ANOVA result is significant if the F-value is large (> F critical) together with corresponding *p-value* < 0.05. In this study, the overall fit or accuracy of the model was statistically significant ($F_{9, 34}$=2.16, $p < 0.0001$). In general, large values for the F-statistic (which exceed the critical value for F, and

correspond to *P*-values lower than 5 % indicate that > 95 % of the variation in the response data is explained by the quadratic model equation, and is not random. The ANOVA also examined the significance associated with the model's parameters (main effects) as well as the interactions between the terms (Table 4.4). The results from the ANOVA (Table 4.4) analysis showed that the linear terms (temperature (X_1), pH(X_2) and LA(X_3)), the square term (X_3^2) and the interaction term (X_1*X_2) was significant (p < 0.05) and account for a significant variation in the response data.

Table 4.4 ANOVA results for the model's fit with the experimental response (% H_2 consumption) including the significance of different factors and interactions.

Factors	Statistics				
	Sequential Sum of square (SS)	**Degrees of freedom**	**Mean square (MS)**	***F-value***	***p-value (F > $F_{0.05}$)***
Model	67547.2	9	7495.2	38.39	0.000
Linear	59471.2	3	19823.7	101.54	0.000
Temperature (X_1)	1561.5	1	1561.5	8.00	0.008
pH (X_2)	7949.6	1	7949.6	40.72	0.000
LA (X_3)	49960.1	1	49960.1	255.90	0.000
Square	4947.4	3	1649.1	8.45	0.000
X_1^2	75.2	1	308	1.58	0.219*
X_2^2	235.5	1	234.6	1.20	0.282*
X_3^2	4636.7	1	4636.7	23.75	0.000
Interaction	3038.6	3	1012.9	5.19	0.005
X_1*X_2	2907.6	1	2907.6	14.89	0.001
X_1*X_3	9.2	1	9.2	0.05	0.830*
X_2*X_3	121.9	1	121.9	0.62	0.436*
Residual Error	5661.7	29	195.2		
Lack-of-Fit	5442.9	3	1814.3	215.55	0.000
Pure Error	218.8	26	8.4		
Total	73119.0	38			

Notes: 1. $F_{(critical)\ 0.05,9,38} = 2.14$. 2.* denotes *p-values* which are insignificant at $\alpha = 0.05$

4.3.3 Effects of experimental variables on the response variable

4.3.3.1 Main effects plot

The linear effects of the independent variables on the response variable were significant components of the quadratic model assessed in the previous section (ANOVA; Table 4.4). The main effects can also be examined using response surface methodology. The impact of experimental parameters (temperature, pH and LA concentration) on % H_2 consumption is shown in a three factor main effects plot (Figure 4.1). These plots are generated by considering only the main effects of the individual factors and without considering the effects of (or interactions with) other experimental factors. Hence, the trends apparent in the main effects plot must be viewed with appropriate caution. Variation of the response (% H_2 consumption) values with different experimental conditions is shown in Figure 4.1. In general, the main effects of increasing the temperature or LA concentration were associated with reducing % H_2 consumption. However, increasing the % H_2 consumed was linked with increasing pH condition (Figure 4.1). The mean average response was approximately 68 % H_2 consumption.

4.3.3.1.1 Temperature

As evident in the main effects plot (Figure 4.1a), temperature had a significant impact on the response variable (Table 4.4; *p-value* = 0.000). Higher response values (51.8 % H_2 consumption) were observed at low temperature (21 °C). However, the consumption decreased (46.6 % H_2 consumption) at mesophilic temperature (37 °C). Further increasing the temperature to 53 °C was accompanied by a decrease in the mean % H_2 consumption (35.7 % H_2 consumption).

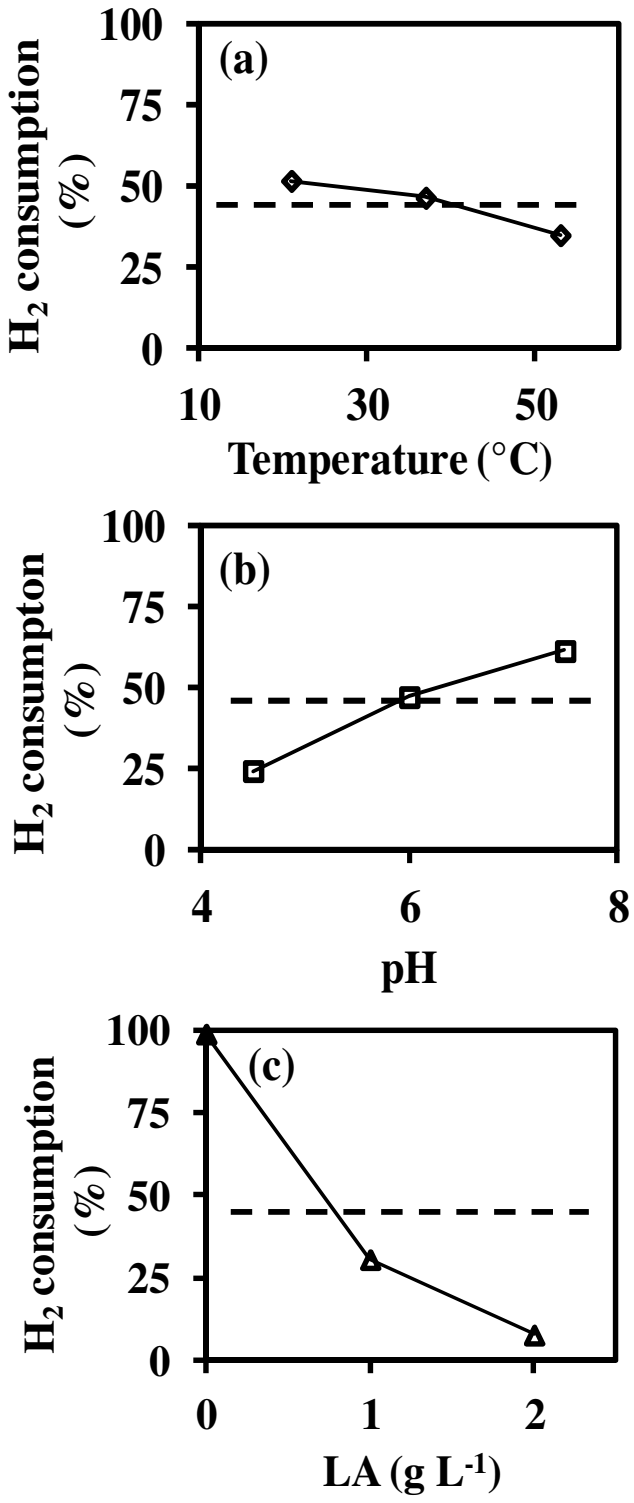

Figure 4.1 Main effects plot showing effects of experimental factors on H_2 consumption: (a) Temperature; (b) pH; (c) LA concentration.

4.3.3.1.2 pH

The pH term also had a significant impact on the response variable (see Table 4.4; *p-value* = 0.000). The impact is evident in the main effects plot (Figure 4.1). The % H_2 consumed was greater at pH 7.5 (approximately 61.5 %) when compared to pH 4.5 (approximately 25.1 %) (Figure 4.1b). These results indicate that pH strongly affects the activity of H_2 consumers in the mixed anaerobic culture. In the absence of sulfate reducing bacteria, methanogens and homoacetogens actively compete for H_2. The change in Gibbs free energy values associated with H_2 consuming reactions in methanogens is higher than in homoacetogens (Table 4.5) indicating that the activity of methanogenic bacteria is strongly affected by pH.

4.3.3.1.3 LA Concentration

The LA concentration had a significant impact on the response variable (see Table 4.4; *p-value* = 0.000). Notice the influence of LA is evident in the main effects plot (Figure 4.1c). The main effects plot shows that as the LA concentration increases, the H_2 consumption decreases. Minimum H_2 consumption was observed at 2 g L^{-1} LA and maximum consumption was obtained in control culture (without the addition of LA). These results indicate that the inhibitory impact of LA was more pronounced on H_2 consumers at higher LA concentrations.

4.3.4 Factor interaction plots

4.3.4.1 Two factor interaction plot

The interaction term (Temperature * pH [X_1 * X_2]) was significant (Table 4.4; *p-value* < 0.05) whereas the pH * LA concentration [X_2 * X_3] term and Temperature * LA concentration [X_1 * X_3] term were not significant (*p-value* > 0.05). The two-factor interaction plots (Figure 4.2) also show evidence of interactions between the experimental variables. The interaction plot profiles shown in Figure 4.2 will lie parallel

to each other if there is no interaction between the experimental variables shown in that particular panel of the figure.

Table 4.5 Gibbs free energies for selected H_2 consumption reactions under standard (1M; 1 bar) conditions.

Eq. No.	Reaction	ΔG^{o} (kJ mol^{-1})	$\Delta G^{o'}$ (kJ mol^{-1})
4.4	$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	-175.49	-135.50
4.5	$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	-144.44	-104.48
4.6	$7\ H_2 + 3\ HCO_3^- + 2H^+ \rightarrow CH_3CH_2COO^- + 7\ H_2O$	-260.81	-180.88
4.7	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-130.70	-130.70
4.8	$4\ H_2 + 2\ CO_2 \rightarrow CH_3COO^- + H^+ + 2\ H_2O$	-55.00	-94.96
4.9	$7\ H_2 + 3\ CO_2 \rightarrow CH_3CH_2COO^- + H^+ + 4\ H_2O$	-126.72	-86.74
4.10	$CH_3COO^- + H^+ + 3\ H_2 + CO_2 \rightarrow CH_3CH_2COO^- + H^+ + 2\ H_2O$	-71.67	-31.64
4.11	$CH_3COO^- + H+ \rightarrow CH_4 + CO_2$	-75.75	-81.46
4.12	$CH_3COO^- + HCO_3^- + H^+ + 3\ H_2 \rightarrow CH_3CH_2COO^- + 7\ H_2O$	-116.36	-76.40

4.3.4.2 Contour plots

The response surfaces and the corresponding contour plots for the model were constructed using MINITAB 16 software (Minitab Inc., State College, PA). A contour plot is a graphical representation of a 3-dimensional surface in a 2-dimensional format using constant z-slices. In simple terms, if there is a given value for z, then lines are drawn connecting the (x, y) coordinates at the point of occurrence of the z-value. Three dimensional contour plot is a useful tool for understanding the complex data generated by the model. The lines in the contour plot are known as the iso-response values. These values are response values for independent variable 2 on the vertical axis and independent variable 1 on the x-axis. The shape of the contour plot represents the effect of two independent variables at the optimum point for keeping the third variable at a constant value. The interactions between the independent variables are clearly illustrated

by the shape of the contour plot. Circular contour plots indicate that the interactions between the independent variables are insignificant while elliptical plots indicate that there are significant differences or interactions between the influences of the independent variables depicted in the contour plot.

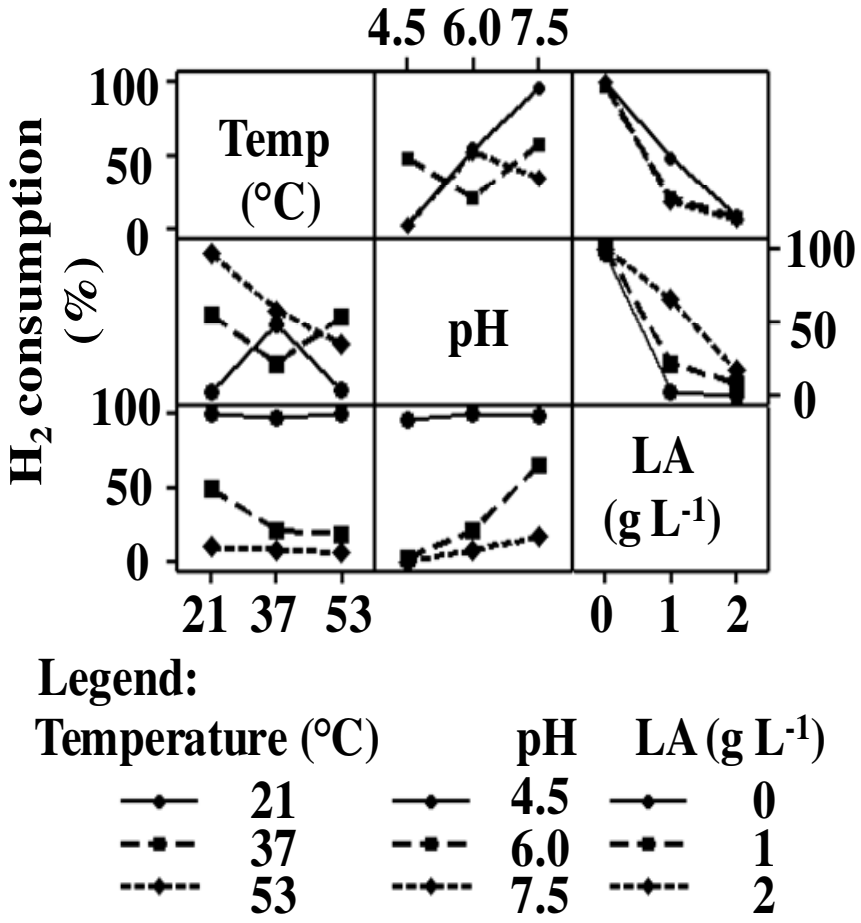

Figure 4.2 Two factor interaction plots showing effects of interactions between experimental factors (Temperature, pH, and LA concentration) on H_2 consumption.

The contour plots for the response variable (% H_2 consumption) (Figure 4.3a-c) show that the optimum conditions are positioned between the design boundaries. The contour plots were generated at the midrange of each independent variable. The percent H_2 consumed is below 30 % for temperatures from 21 °C to 53 °C and at a pH below 5.7 (Figure 4.3a). This plot was generated at a LA concentration of 1 g L^{-1}, whereas increasing the pH clearly favors H_2 consumption. Lin et al. (2008) reported that H_2 production is favored at

a pH range of 4.5-5.5. If the pH level falls below 6.3 to 7.8, then conditions are not optimum rate for methane production. The contour plot for the independent variables, temperature and LA concentration (Figure 4.3b) also clearly shows elevated LA concentrations (approximately 2 g L^{-1}) is important in reducing H_2 consumption at temperatures greater than 37 °C when pH is maintained at 6.0. The response (H_2 consumed) decreased to less than 20 % (Figure 4.3c) when the LA concentration was between 1.3-2.0 g L^{-1}, the pH is less than 5.5 and the temperature was constant at 37 °C. Methane production was not observed at a pH value of 4.5 and 2 g L^{-1} LA. Note at a lower LA concentration of 1 g L^{-1} and at a temperature of 37 °C, methane production was observed. This observation confirms data from previous studies showing 2 g L^{-1} LA as a effective methanogenic inhibitor (Brahmaiah et al., 2012; Lalman and Bagley, 2001; Saady et al., 2012). Under standard conditions (i.e., at 25 °C and 1 atm or 1M concentration of reactants and products), methanogens are able to outcompete homoacetogens for H_2 in the absence of other electron acceptors, such as sulfate, nitrate, fumarate, etc. This is evident from the Gibbs free energy values (Table 4.5) and the H_2 threshold values (Table 4.1) associated with the relevant reactions. Conditions for minimizing H_2 consumption and maximizing H_2 production in fermentative H_2 producing cultures have not been identified. Statistical modeling of H_2 production studies using glucose as the substrate combined cultures treated with LA have shown maximum H_2 production at 37 °C under low pH conditions (< 5.0) and at high LA concentrations of 2 g L^{-1} (Ray et al., 2010a). In comparison, using the conditions for maximum H_2 production to assess the % H_2 consumed indicate the level was approximately 0 % (Figure 4.3c). Hydrogen production coupled with VFAs is accompanied with a reduction in pH. Low pH is unfavourable for H_2 consumers and higher H_2 yields are observed.

4.3.4.3 Surface plots

Interaction between the experimental factors and their impact on the response variable is illustrated in 3D surface plots (Figure 4.3d-e).

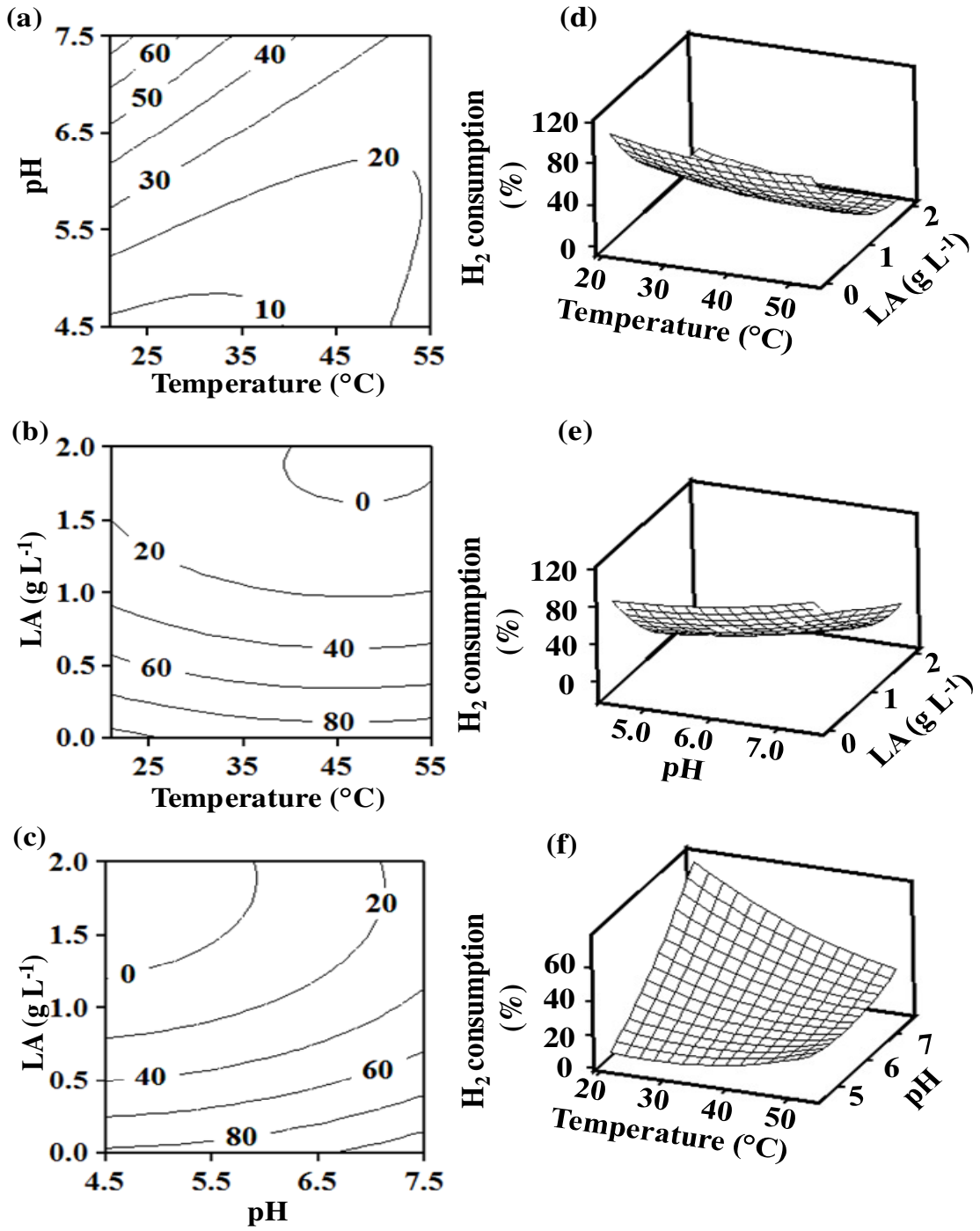

Figure 4.3 Contour (a, b and c) and 3D-surface plots (d, e and f) showing the experimental response (percent H_2 consumption) under different experimental conditions shown in Table 4.3.

Notes: 1. LA = linoleic acid.

The surface plot (Figure 4.3f) shows minimum H_2 consumption occurs at a pH below 5 and at all temperatures studied. The effects of temperature and LA are clearly demonstrated in Figure 4.3d. Less than 10 % of the available H_2 was consumed at all temperatures conditions and with increasing LA concentration (> 2 g L^{-1}). However, by inspecting the data in Figure 4.3e, very little interaction between pH and LA concentration on H_2 metabolism is observed. The data in Table 4.4 confirms interaction between these two factors was insignificant. Methane production at higher temperatures was almost 30 % less than production at 21 °C, at the same pH and LA concentration. At higher temperatures, growths of aceticlastic methanogens are not favorable (Noll et al., 2010) while higher temperatures are favorable to the growth of hydrogenotrophic methanogens. Hence, methane production at higher temperatures is likely due to hydrogenotrophic methanogens. Hydrogenotrophic methanogens generally have shorter doubling times than aceticlastic methanogens, and their growth is a major factor contributing to major loss of the electron equivalents from substrate oxidation. Lalman and Bagley (2001) investigated the effect of LA on hydrogenotrophic and aceticlastic methanogens and concluded 0.3 g L^{-1} LA were not sufficient inhibit both methanogens. In contrast, 2.0 g L^{-1} LA used in this study inhibited both these methanogens which is clearly evident from the low CH_4 yields (Table 4.3).

Apart from methanogens, homoacetogens are the primary H_2 consumers. When methanogens are suppressed, homoacetogens become more active. The enhanced activity of acetogens under conditions suppressing methanogens has been observed in BES treated mixed cultures. Saady et al. (2012) noted that the electron equivalents derived from substrate oxidation was diverted towards homoacetogens, and concluded that acetogenic H_2 consumption was primarily responsible for the loss of electron equivalents when methanogens were inhibited by LCFA.

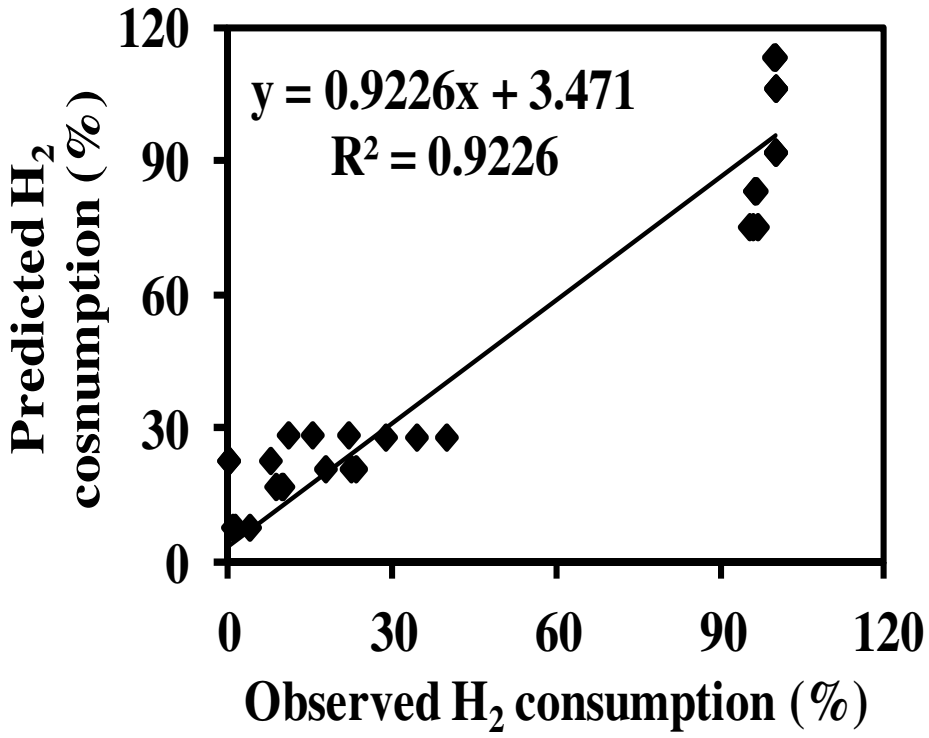

Figure 4.4 Model predicted Vs observed experimental response values.

4.3.5 Response surface model verification

The predicted response (% H_2 consumption) computed using the model correlated reasonably well with the experimental data (R^2 = 0.9266) (Figure 4.4). The residuals (model predicted value – experimental value) for the response are useful in judging the normal distribution. The Anderson-Darling (A-D) probability plot (Figure 4.5) confirmed that the residuals are normally distributed. In this study, the model A-D statistic which was less than the critical A-D value of 0.752 for the sample size of 39 at 5 % level of significance (A-D statistic = 0.538; p = 0.157) confirmed a normal distribution of the residuals. This verifies that the model is reliable and that the response values predicted by the model are correlated to the experimental measures of the response over the factor space under consideration.

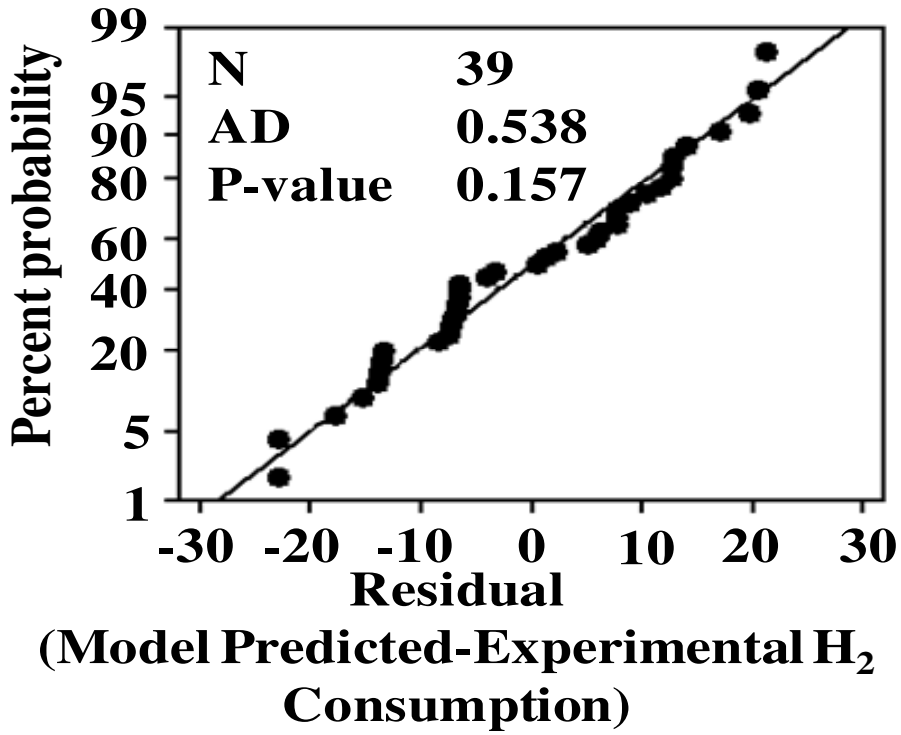

Figure 4.5 Anderson-Darling (A-D) normality plot of residuals.

4.3.6 Model Validation

A popular optimization procedure, D-optimality, was used to optimize the response variable (i.e., minimize H_2 consumption by determining the optimal operating conditions). In general, D-optimality is used to either minimize or maximize the determinant of the information matrix of the design. In this study, the goal was to minimize H_2 consumption in mixed anaerobic culture by varying the process parameters (temperature, pH, and LA concentration). The numerical optimization function in the MINITAB software was used to evaluate the minimum response (% H_2 consumption) within the factor space under the design conditions. The optimization was performed using the D-optimality index which range is between zero and one for all factors. The MINITAB application computed the entire domain of factor settings, which results in the largest D-optimality value possible. A D-optimality value of 0.9997 was obtained with the minimum response (0.97 % H_2 consumption) predicted for operation at pH 5.5, temperature of 39.7 °C, and LA concentration of 1.4 g L^{-1} (Figure 4.6). In comparison to

the operating conditions applied during testing, 0±0 % of the injected H_2 was consumed at a pH of 4.5, temperature of 37 °C and LA concentration of 2.0 g L^{-1}. This result is close to the response predicted by the model of 0.97 %.

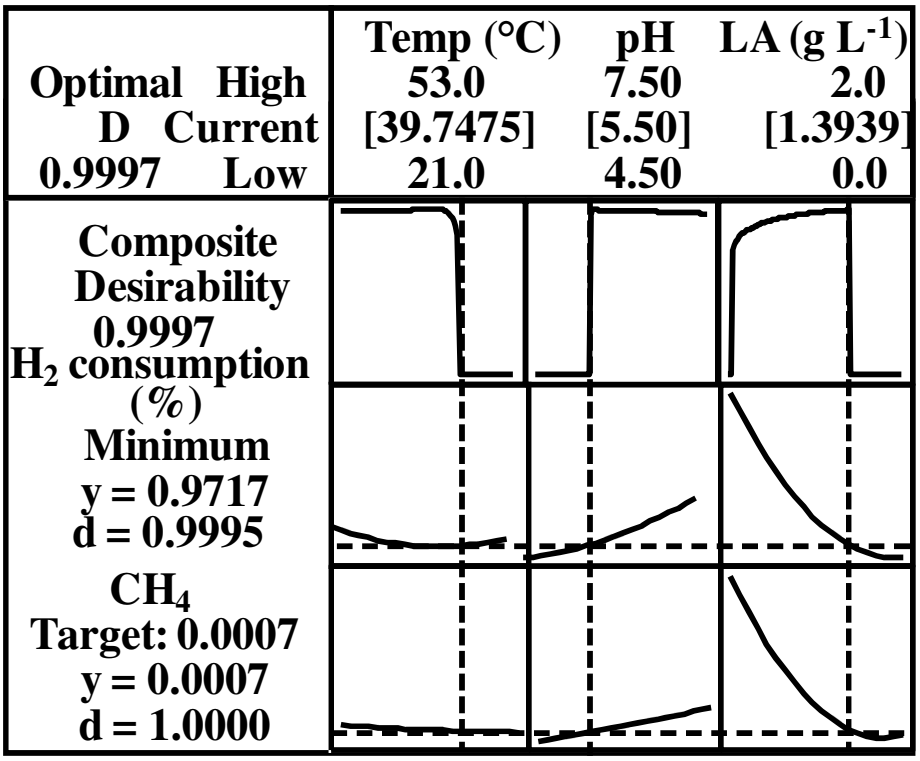

Figure 4.6 Composite desirability and optimization (D-optimality) plot.

In order to validate the optimum condition predicted by the model, a validation study was performed for the three independent variables in the model under evaluation. In the validation study, the model's predicted response values were compared with the experimental response data obtained using the same operational parameters. The substrate (3000 µmol H_2) was injected into the serum bottles after 24 h following the addition of 1.4 g L^{-1} of LA. The % H_2 consumption was calculated by performing the headspace gas analysis at regular time intervals. A response of 0.0±0.0 % hydrogen consumption was observed during testing under the optimum conditions, which was similar to the model's prediction of 0.97 % (data not shown).

4.3.7 Principal Component Analysis

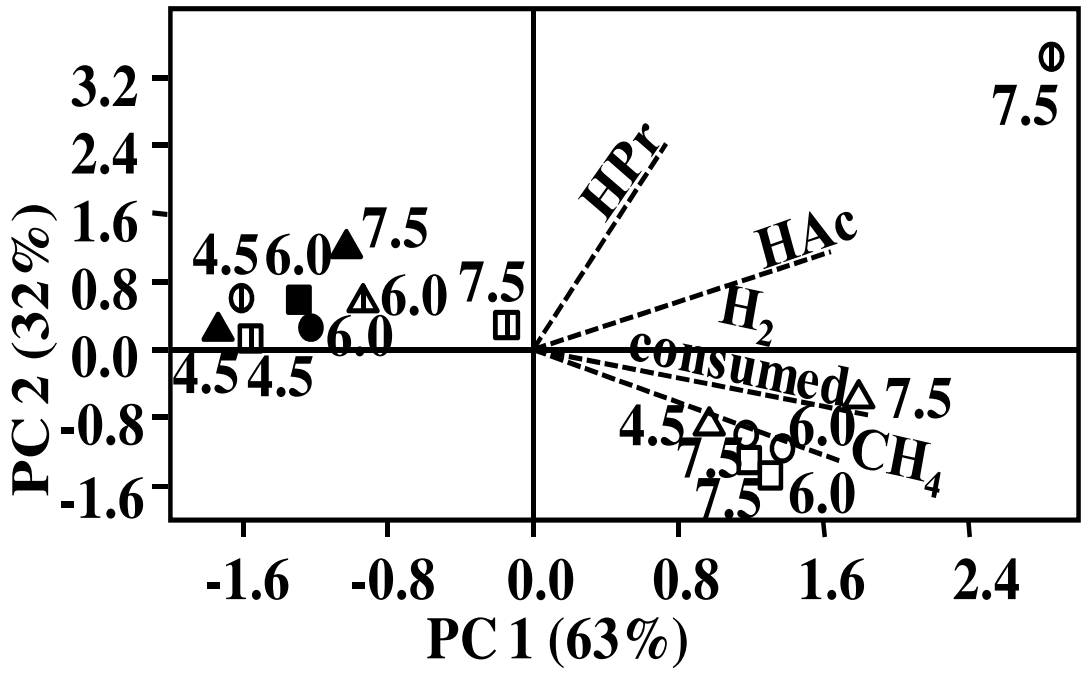

Figure 4.7 PCA of fermentation metabolites under different experimental conditions. Notes:

1. Shapes- 'Circle', 'square' and 'triangle' indicate incubation temperatures 21°C, 37°C and 53°C.
2. Closed circle (●), square (■) and triangle (▲) indicates control cultures (1 g L^{-1} LA)
3. open circle (○), square (□) and triangle (△) indicates 0 g L^{-1} LA treated culture
4. dashed circle (◍), square (▣) and triangle (◬) indicates 2 g L^{-1} LA treated culture
5. HAc = acetic acid; HPr = propionic acid; and CH_4 = methane.

Similarities and differences in the outcomes under different experimental conditions (Table 4.2) were assessed using principal component analysis (PCA). A PCA bi-plot (Figure 4.7) shows the loading and score values obtained for the principal components, PC 1 and PC 2. The fermentation metabolites acetic acid (HAc), propionic acid (HPr), methane (CH_4), along with the response variable (H_2 consumed) were provided as inputs to the PCA. The first two principal components, PC 1 and PC 2, accounted for 95 % of the total variation in the data set. Based on the loading values, PC 1 was associated with H_2 consumed (0.60), CH_4 (0.55), HPr (0.23) and HAc (0.53), whereas PC 2 was

associated with HAc (0.37) and HPr (0.78). The PCA bi-plot indicated that the H_2 consumed was positively correlated with both CH_4 and HAc production. The PCA bi-plot also shows that cultures formed a cluster based on the addition of LA. Cultures incubated at different temperatures with LA addition were clustered together and not associated with the controls (no LA added) cultures. The PCA results are consistent with the inhibitory effect of LA on H_2 consumers. Similar grouping of cultures under different operational pH conditions was reported by Pendyala et al. (2013). The clustering for LA fed cultures indicated that they exhibited a different fermentation pattern compared to the control cultures.

4.3.8 Microbial analysis

Cluster analysis is a grouping technique, which partitions the data elements into groups denoted as clusters based on a distance or dissimilarity function. The similarities of the T-RFLP patterns between cultures subjected to the tested parameters (temperature, pH and LA concentration) were assessed with the Jaccard coefficient. This index is selected based on a higher Cophenetic correlation value (Figure 4.8a). The Cophenetic correlation coefficient is a non-linear regression coefficient, which provides an assessment of the reliability of the dendrogram. The Jaccard similarity measure has a Cophenetic correlation of approximately 0.87. The samples were grouped according to the Jaccard coefficient of the T-RFs which were present in all samples (Kaufman and Rousseeuw, 2005). The groups appear to be related to the experimental conditions (Table 4.2), and three clusters of similar microbial organisms are shown in the dendrogram (Figure 4.8a). In clade I, cultures associated with lower temperature conditions in the methanogenic pH range (pH $\geq$ 6.0) are grouped together with a 40 % similarity index. In clade II, cultures from both lower and higher temperature conditions were grouped together and showed a 22 % similarity with clade I cultures.

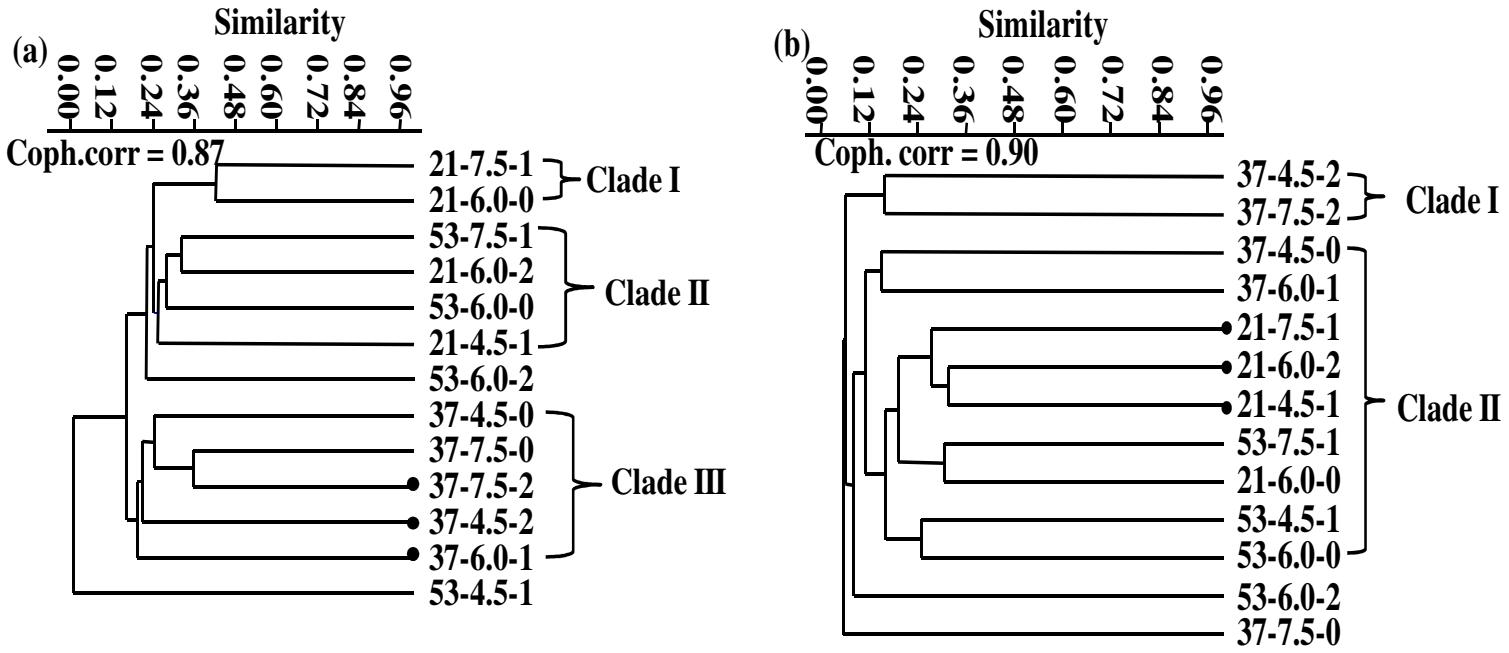

Figure 4.8 Cluster analysis using Jaccard index of 16S rRNA gene T-RFLP profiles. **(a) Archaeal *Hae* III enzyme digest; (b) Bacterial *Hae* III enzyme digest.**

Notes: 1. (●) represents LA-treated cultures with lower H_2 consumption within the same clade in Figure 4.8a.

2. The 1st, 2nd and 3rd numbers are the temperature (C), the pH and the LA concentration (g L^{-1}).

3. Cophenetic correlation = Coph. Corr.

In clade III, all of the cultures from the mesophilic temperature (37 °C) conditions were grouped together. In this clade, the LA-treated cultures showed less H_2 consumption regardless of the initial pH (Fig. 4.8a). Cultures from Exp #2 were grouped separately from all of the other treatment conditions and showed no detectable levels of H_2 consumption (Table 4.2). This might be due to exposure to the combination of lower fermentation pH (4.5), higher temperature (53 °C) along with a LA concentration of 1 g L^{-1}. This particular combination of conditions resulted in a different microbial community composition and hence, shared lower similarity (< 5 %) with the other clades represented in the dendrogram. The cluster analysis using the Jaccard similarity index for the bacterial *Hae* III enzyme digested T-RFs showed a Cophenetic correlation score of 0.90. Cultures incubated at 37 °C and fed 2 g L^{-1} were clustered together in clade I (Fig. 9b). Similar clustering of microbial cultures incubated at 37 °C was observed with Archaeal *Hae* III enzyme digested T-RFs profile (Fig. 9a). In general, bacterial enzyme digests were not clustered based on incubation temperature.

Genomic analysis of the microbial diversity under different incubation conditions using terminal restriction fragment length polymorphism (T-RFLP) revealed that at 37 °C *Clostridium* sp., *Kosmotoga olearia*, *Thermoanaerobacter* sp. were present in all of the cultures. However abundance of *Clostridium novyi* was greater in the LA-treated cultures at pH 4.5 compared to other cultures. *C. novyi* is a known propionate producer (Kim et al., 2010).

Methanogens utilizing H_2/CO_2 (Methanomicrobiales (hydrogenotrophic methanogen)) or acetate (Methanosarcinales (aceticlastic methanogen)) were present in the control cultures at pH 7.5. The abundance of *M. halotolerans* was higher in LA-treated cultures compared to the control cultures at pH 7.5. At pH 4.5, in both control and LA-treated cultures, inhibition of these microorganisms indicate that *M. halotolerans* are likely LCFA degrading organisms which are active at neutral pH. The presence of *Methanococcoides alaskense* only in the control cultures at pH 7.5 indicate that either the

addition of LA or reducing the pH to 4.5 are inhibitory to these organisms. Hydrogenotrophic methanogens was abundant at pH 4.5 in the control cultures and for this reason, the quantity of H_2 consumed reached 96±0 %. However, reducing the relative abundance of these methanogens by adding LA resulted in no detectable levels of H_2 consumed. *M. alaskense*, *Methanocalculus taiwanensis*, *M. halotolerans* were observed under all experimental conditions except Exp # 2 (Table 4.3).

The bacterial *Hae* III enzyme digested T-RFs showed the presence of organisms closely related to *Clostridium sporogenes* and *Clostridium beijerinckii* in cultures incubated at 21 °C. These organisms were abundant in LA treated cultures compared to the control cultures under the same temperature conditions. *Syntrophus aciditrophus*, a syntrophic fatty acid degrading microorganism (McInerney et al., 2007) was detected in cultures incubated at 21 °C with LA. This organism can utilize a wide range of organic compounds such as fatty acids, alcohols aromatic acids, sugars using syntrophic association under anaerobic conditions.

Clostridium algidicarnis, *Clostridium innocuum*, *Desulfotomaculum thermosapovorans* and *Eubacterium cylindroides* were detected in LA treated cultures at 53 °C. Lower levels of these organisms were detected in control cultures at 53 °C. In 2 g L^{-1} LA treated cultures incubated at pH 6.0 and 53 °C microorganisms related to *Clostridium ljungdahlii* were detected. Lower level of acetate (0.25±0.01 meq^-) detected under these conditions (Table 4.3) indicate LA might have exerted an inhibitory effect on homoacetogenic bacteria. Cluster analysis showed 10 % similarity in 2 g L^{-1} LA treated cultures compared to cultures incubated at the same temperature (Figure 4.8b). *Clostridium ljungdahlii*, a homoacetogenic bacterium, is capable of growing on H_2/CO_2 and producing acetate (Kopke et al., 2010). Microorganisms related to *Desulfovibrio magneticus* RS-1, *Bacillus marismortui* were observed under all the experimental conditions except in Exp # 3 (Table 4.3). Control cultures incubated at 37 °C were dominated by *Eubacterium* sp., a homoacetogen (Mechichi et al., 1998). Note this organism was not present cultures treated with 2 g L^{-1} LA (Exp # 11, 12). This might be the reason for lower H_2 consumption in LA treated cultures at 37 °C. Propionic acid production under different

experimental conditions might also be due to the presence of organisms related to *Propionobacterium acnes* (Bruggemann et al., 2004).

The formation of propionic acid in cultures containing H_2, CH_3COO^- and HCO_3^- indicate reaction 4.12 in Table 4.5 is energetically favourable. The formation of propionic acid from acetate and HCO_3^- has been previously reported (Conrad and Klose, 1999). Microorganisms mediating equation 4.10 include *Desulfobulbus propionicus* (Laanbroek et al., 1982).

4.4 Conclusions

1. A response surface model of H_2 metabolism based on a BBD was developed to include temperature, pH and LA concentration.
2. The response (% H_2 consumption) obtained under different experimental conditions (various combinations of temperature, pH, and LA concentration) were modeled.
3. The combination of 2.0 g L^{-1} LA together with a lower pH value of 4.5 exerts strong inhibitory effects on H_2 consuming microorganisms.
4. Maintaining a low pH during fermentation without adding LA has no effect on H_2 consumers. This was evident from higher H_2 consumption values (approximately 100 %) under low pH conditions.
5. Contour plots indicate that there were significant interactions between the three experimental factors.
6. The A-D statistic was used to confirm the normal distribution of the residuals.
7. Calculation of Gibbs free energy values indicated that the experimental conditions were favourable for both hydrogenotrophic and aceticlastic methanogenesis. Even though the conditions for CH_4 formation were favourable under all of the experimental conditions studied, the amount of CH_4 generated varied from 0.00±0.00 to 0.22±0.01 mol mol^{-1} H_2 depending on the pH and the LA concentration.

8. Negligible/no CH_4 was detected in cultures incubated at 37 °C with 2 g L^{-1} LA at either pH 7.5 or 4.5 with favourable thermodynamic condition. These results show that the addition of LA is an effective methanogenic inhibitor.
9. Both acetogenesis and propionogenesis were the alternative electron sinks for H_2 in the absence of methanogens. Since there is a possibility of propionic acid formation using acetic acid, H_2, CO_2 or HCO_3^- no clear conclusion should be made on the effect of LA on homoacetogenic bacteria.
10. The PCA found that PC 1 and PC 2 accounted for 95 % of the variation in the original data set. Cultures treated with 1 g L^{-1} were clustered together in a separate quadrant of the PCA bi-plot regardless of fermentation pH and temperature, which also correlated with lower H_2 consumption.
11. Cluster analysis using the Jaccard similarity index for the archaeal *Hae* III digested T-RFs showed that cultures incubated at 37 °C were clustered together in clade III, separately from cultures incubated at 21 °C and 53 °C. Cultures maintained at high pH during fermentation (6.0 and 7.5), and incubated at 21 °C showed 45 % similarity and were clustered together in clade I. However, decreasing the pH (to 6.0 or 4.5) in combination with LA treatment may have resulted in a different composition of the microbial population. This was shown by separate grouping of cultures exposed to these conditions in clade II (apart from clade I).
12. The dendrogram constructed using bacterial *Hae* III digested T-RFs showed grouping of 2 g L^{-1} LA treated cultures incubated at 37 °C similar to archaeal *Hae* III digested T-RFs.
13. Cultures treated with LA were abundant with *Clostridium* sp. Control cultures showed the presence of both aceticlastic (methanosarcinales) and hydrogenotrophic (Methanomicrobiales) methanogens. LCFA degrading microorganisms, such as *Methanocalculus halotolerans*, were abundant only in LA-treated cultures at neutral pH 7.5 (37 °C). Decreasing the pH together with LA addition had a synergistic effect on the methanogens.

14. *Methanococcoides alaskense*, *Methanocalculus taiwanensis* and *Methanocalculus halotolerans* were present under all experimental conditions except in cultures incubated with initial pH 4.5, 53 °C together with 1 g L^{-1} LA addition.
15. *Clostridium sporogenes* and *Clostridium beijerinckii* were abundant in cultures incubated at 21 °C and treated with LA when compared to the control cultures. These results indicate that LA addition selectively enriched *Clostridium* sp. In addition organism related to *Syntrophus aciditrophus* was detected in LA treated cultures.
16. *Eubacterium cylindroides*, *Desulfotomaculum thermosapovorans* in addition to *Clostridium algidicarnis*, *Clostridium ljungdahlii* and *Clostridium innocuum* were detected in cultures incubated at 53 °C. In comparison, *Eubacterium* sp. was abundant in cultures incubated at 37 °C under all experimental conditions.
17. Propionic aicd formation under different experimental conditions might be due to the presence of organisms related to *Propionobacterium acnes* and *Desulfobulbus propionicus*.

4.5 References:

Brahmaiah, P., Chaganti, S.R., Lalman, J.A., Saravanan, R.S., Heath, D., Lau, C.K. (2012). Pretreating mixed anaerobic communities from different sources: Correlating the hydrogen yield with hydrogenase activity and microbial diversity. *Int J Hydrogen Energy*, **37**(17), 12175-86.

Bruggemann, H., Henne, A., Hoster, F., Liesegang, H., Wiezer, A., Strittmatter, A., Hujer, S., Durre, P., Gottschalk, G. (2004). The complete genome sequence of *Propionibacterium acnes*, a commensal of human skin. *Science*, **305**(5684), 671-3.

Chowdhury, N., Lalman, J.A., Seth, R., Ndegwa, P. (2007). Biohydrogen production by mesophilic anaerobic fermentation of glucose in the presence of linoleic acid. *J Environ Eng*, **133**(12), 1145-52.

Cirne, D.G., Paloumet, X., Björnsson, L., Alves, M.M., Mattiasson, B. (2007). Anaerobic digestion of lipid-rich waste-Effects of lipid concentration. *Renew Energy*, **32**(6), 965-75.

Collins, L.J., Paskins, A.R. (1987). Measurement of trace concentrations of hydrogen in biogas from anaerobic digesters using an exhaled hydrogen monitor. *Water Res*, **21**(12), 1567-72.

Conrad, R., Klose, M. (1999). Anaerobic conversion of carbon dioxide to methane, acetate and propionate on washed rice roots. *Fems Microbiol Ecol*, **30**(2), 147-55.

Conrad, R., Wetter, B. (1990). Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. *Archiv Microbiol*, **155**(1), 94-8.

Cord-Ruwisch, R., Mercz, T.I., Hoh, C., Strong, G.E. (1997). Dissolved hydrogen concentration as an on-line control parameter for the auomated operation and optimization of anaerobic digesters. *Biotechnol Bioeng*, **56**, 626-34.

Cord-Ruwisch, R., Seitz, H.J., Conrad, R. (1988). The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Archiv Microbiol*, **149**, 350-7.

Demeyer, D.I., Henderickx, H.K. (1967). The effect of C_{18} unsaturated fatty acids of methane production *in vitro* by mixed rumen bacteria. *BBA-Lipid Lipid Met*, **137**(3), 484-97.

Dereli, R.K., van der Zee, F.P., Heffernan, B., Grelot, A., van Lier, J.B. (2014). Effect of sludge retention time on the biological performance of anaerobic membrane bioreactors treating corn-to-ethanol thin stillage with high lipid content. *Water Res*, **49**, 453-64.

Galbraith, H., Miller, T.B. (1973). Physicochemical effects of long chain fatty acids on bacterial cells and their protoplasts. *J Appl Bacteriol*, **36**(4), 647-58.

Hanaki, K., Nagase, M., Matsuo, T. (1981). Mechanism of inhibition caused by long chain fatty acids in anaerobic digestion process. *Biotechnol Bioeng*, **23**(7), 1591-610.

Hwu, C.S., Lettinga, G. (1997). Acute toxicity of oleate to acetate utilizing methanogens in mesophilic and thermophilic anaerobic sludges. *Enzyme Microb Technol*, **21**(4), 297-301.

Kaufman, L., Rousseeuw, P. (2005). *Finding Groups in Data: An Introduction to Cluster Analysis (Wiley Series in Probability and Statistics)*. Wiley-Interscience; March 2005.

Kim, M.D., Song, M., Jo, M., Shin, S.G., Khim, J.H., Hwang, S. (2010). Growth condition and bacterial community for maximum hydrolysis of suspended organic materials in anaerobic digestion of food waste recycling wastewater. *Appl Microbiol Biotechnol*, **85**(5), 1611-8.

Kodicek, E., Worden, A.N. (1945). The effect of unsaturated fatty acids on *Lactobacillus helveticus* and other gram positive microorganisms. *Biochem J*, **39**(1), 78-85.

Kopke, M., Held, C., Hujer, S., Liesegang, H., Wiezer, A., Wollherr, A., Ehrenreich, A., Liebl, W., Gottschalk, G., Durre, P. (2010). *Clostridium ljungdahlii* represents a microbial production platform based on syngas. *PNAS USA*, **107**(29), 13087-92.

Koster, I.W., Cramer, A. (1987). Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. *Appl Environ Microbiol*, **53**(2), 403-9.

Kotsyurbenko, O.R., Glagolev, M.V., Nozhevnikova, A.N., Conrad, R. (2001). Competition between homoacetogenic bacteria and methanogenic archaea for hydrogen at low temperature. *Fems Microbiol Ecol*, **38**(2-3), 153-9.

Laanbroek, H.J., Abee, T., Voogd, I.L. (1982). Alcohol Conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. *Archiv Microbiol*, **133**(3), 178-84.

Lalman, J.A., Alosta, H., Bejankiwar, R., Bellmer, D. (2003). Kinetics of glucose fermentation by a mixed culture in the presence of linoleic, oleic, and stearic acid. *Environ Technol*, **24**(12), 1471-8.

Lalman, J.A., Bagley, D.M. (2000). Anaerobic degradation and inhibitory effects of linoleic acid. *Water Res*, **34**(17), 4220-8.

Lalman, J.A., Bagley, D.M. (2001). Anaerobic degradation and methanogenic inhibitory effects of oleic and stearic acids. *Water Res*, **35**(12), 2975-83.

Loffler, F.E., Tiedje, J.M., Sanford, R.A. (1999). Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. *Appl Environ Microbiol*, **65**(9), 4049-56.

Lovley, D.R. (1985). Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl Environ Microbiol*, **49**(6), 1530-1.

Lovley, D.R., Goodwin, S. (1988). Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochimica Et Cosmochimica Acta*, **52**(12), 2993-3003.

McInerney, M.J., Rohlin, L., Mouttaki, H., Kim, U., Krupp, R.S., Rios-Hernandez, L., Sieber, J., Struchtemeyer, C.G., Bhattacharyya, A., Campbell, J.W., Gunsalus, R.P. (2007). The genome of *Syntrophus aciditrophicus*: Life at the thermodynamic limit of microbial growth. *PNAS USA*, **104**(18), 7600-5.

Mechichi, T., Labat, M., Woo, T.H.S., Thomas, P., Garcia, J.L., Patel, B.K.C. (1998). *Eubacterium aggregans* sp. nov., a new homoacetogenic bacterium from olive mill wastewater treatment digestor. *Anaerobe*, **4**(6), 283-91.

Mykhaylovin, O., Roy, J.M., Jing, N., Lalman, J.A. (2005). Influence of C18 long chain fatty acids on butyrate degradation by a mixed culture. *J Chem Technol Biotechnol*, **80**(2), 169-75.

Noll, M., Klose, M., Conrad, R. (2010). Effect of temperature change on the composition of the bacterial and archaeal community potentially involved in the turnover of acetate and propionate in methanogenic rice field soil. *Fems Microbiology Ecology*, **73**(2), 215-25.

Pendyala, B., Chaganti, S.R., Lalman, J.A., Heath, D.D., Shanmugam, S.R., Veeravalli, S.S. (2013). Using a food and paper-cardboard waste blend as a novel feedstock for hydrogen production: Influence of key process parameters on microbial diversity. *Int J Hydrogen Energy*, **38**(15), 6357-67.

Ray, S., Chowdhury, N., Lalman, J.A., Seth, R., Biswas, N. (2008). Impact of initial pH and linoleic acid (C18:2) on hydrogen production by a mesophilic anaerobic mixed culture. *J Environ Eng*, **134**(2), 110-7.

Ray, S., Lalman, J.A., Biswas, N. (2009). Using the Box-Benkhen technique to statistically model phenol photocatalytic degradation by titanium dioxide nanoparticles. *Chem Eng J*, **150**(1), 15-24.

Ray, S., Reaume, S.J., Lalman, J.A. (2010a). Developing a statistical model to predict hydrogen production by a mixed anaerobic mesophilic culture. *Int J Hydrogen Energy*, **35**(11), 5332-42.

Ray, S., Saady, N.M.C., Lalman, J.A. (2010b). Diverting electron fluxes to hydrogen in mixed anaerobic communities fed with glucose and unsaturated C:18 long chain fatty acids. *J Environ Eng*, **136**(6), 568-75.

Saady, N.M.C., Chaganti, S.R., Lalman, J.A., Heath, D. (2012). Impact of culture source and linoleic acid (C18:2) on biohydrogen production from glucose under mesophilic conditions. *Int J Hydrogen Energy*, **37**(5), 4036-45.

Seitz, H.J., Schink, B., Pfennig, N., Conrad, R. (1990). Energetics of syntrophic ethanol oxidation in defined chemostat cocultures. *Archiv Microbiol*, **155**(1), 89-93.

Sonne-Hansen, J., Westermann, P., Ahring, B.K. (1999). Kinetics of sulfate and hydrogen uptake by the thermophilic sulfate-reducing bacteria *Thermodesulfobacterium* sp. strain JSP and *Thermodesulfovibrio* sp. strain R1Ha3. *Appl Environ Microbiol*, **65**(3), 1304-7.

Templer, J., Lalman, J.A., Jing, N., Ndegwa, P.M. (2006). Influence of C18 long chain fatty acids on hydrogen metabolism. *Biotechnol Prog*, **22**(1), 199-207.

CHAPTER 5: COMPARISON OF SPECIFIC AND NON-SPECIFIC CHEMICAL INHIBITORS ON HYDROGEN METABOLISM

5.1 Introduction

Hydrogen (H_2) production by dark fermentation is a promising method for sustainable H_2 production using various types of organic materials (e.g. lignocellulosic biomass, municipal solid wastes, etc.) can be used as feed-stocks (Han and Shin, 2004; Tanisho et al., 1987). Higher H_2 yields can be obtained by employing pure *Clostridium* cultures or mixed cultures containing predominantly *Clostridium* sp. At current prices, H_2 fermentation employing pure cultures is not economically feasible because of high maintenance and operational costs. On the other hand, using mixed cultures for H_2 fermentation also poses some disadvantages. The major problem in employing mixed cultures for dark fermentation is H_2 losses caused by the consumption of H_2 by homoacetogenic bacteria, hydrogenotrophic methanogens and sulfate-reducing bacteria which are present in close association with H_2-producers in mixed culture. Successful elimination or inhibition of these H_2-consuming bacteria improves the H_2 yield of dark fermentation. Selective enrichment of H_2-producing seed inocula can be obtained using a variety of inoculum pretreatment methods such as heat (Baghchehsaraee et al., 2008; Zhu and Beland, 2006), acid (Ren et al., 2008), alkali (Cai et al., 2004), aeration (Zhu and Beland, 2006), loading shock, 2-bromoethanesulfonate (BES) (Zhu and Beland, 2006) and long chain fatty acids (LCFAs) (Lalman and Bagley, 2002). Among these methods, heat treatment is most widely used to enrich spore-forming *Clostridium* sp. from mixed cultures. However, many H_2-consuming acetogenic bacteria such as *Clostridium aceticum* are spore-forming and can resist heat treatment (Lin and Chang, 2004).

Hydrogen losses due to homoacetogenesis has been a major problem with using heat-treated inoculum (Oh et al., 2003). Moreover, heat pretreatment cannot be used for large-scale operations due to high heating costs that limit commercial application. The use of different types of chemical stressing agents such as BES, furfural, LCFAs (saturated and

unsaturated) to selectively suppress the growth of H_2-consuming microorganisms are alternatives to heat treatment. Fatty acids are relatively cheap and are readily available from fried foods, slaughterhouse wastes, dairy industries, and edible oil industries. LCFAs are produced upon the hydrolysis of fats and oils by hydrolytic bacteria and employing them as stressing agents for H_2 production offers an economic advantage over other types of chemical stressors.

Microbial chemical inhibitors can be divided into the following two major categories: 1. specific and 2. non-specific inhibitors (Liu et al., 2011). For example, BES imposes a specific inhibitory effect on methanogens while LCFA can affect numerous cellular structures and enzymes. BES is a popular chemical stressor which is widely used in many ecological studies (Sparling and Daniels, 1987). BES is a structural analog of coenzyme M (2-mercaptoethanesulfonic acid), which plays an important role in the final step of methane formation. In this reaction, Coenzyme M (CoM) functions as the methyl (CH_3) carrier, which then reduces protons (H^+) to form methane (CH_4). Coenzyme M reductase is unique to methanogens, and many inhibitors have been synthesized are structural analogs of CoM. BES is capable of effectively inhibiting Coenzyme M reductase at low concentrations (Gunsalus et al., 1978). BES is synthesized from non-renewable chemicals and in large-scale bio-refinery application, availability relative to similar inhibitory renewable chemicals and environmental concerns related to discharge into receiving water bodies could become factors deterring the use of BES.

The BES concentration required to inhibit methanogens is variable and depends on the anaerobic environmental conditions. For example, 10 mM BES is sufficient to inhibit the growth of methanogens in ecological studies (Balch and Wolfe, 1979), whereas 50 mM is required to completely inhibit hydrogenotrophic methanogens in an anaerobic digester (Zinder et al., 1984). Inhibitors such as BES, 2-chloroethanesulfonate (CES) and chloroform are known as specific inhibitors because of their functional role in specifically inhibiting Coenzyme M reductase. In addition some of chlorinated hydrocarbons such as carbon tetrachloride and methylene chloride were also effective in

inhibition of methanogens. However, no data is available on their biochemical mechanism of action (Sanz et al., 1997).

These classic methanogenic inhibitors do not affect the activity of non-methanogens which operate in close association with H_2 consumers such as methanogens. Sparling and Daniels (1987) reported that 25 mM BES inhibited only methanogens but no effect was observed on gram positive and gram negative microorganisms. According to Chiu and Lee (2001), BES and chloroform are able to alter the activity of archea communities over long periods. They also influence the activities of Gram-negative bacteria and sulfate reducers. In additions, BES can impair the syntrophic activity of acetogens such as *Syntrophomonas* sp. and *Syntrophobacter* sp. and can disturb the syntrophic association between fatty acid degraders and reductive homoacetogens such as *Eubacterium* sp. and *Moorella* sp. (Xu et al., 2010a; Xu et al., 2009; Xu et al., 2010b).

Methanogens are inhibited by saturated as well as unsaturated fatty acids. Saturated medium chain fatty acids, such as lauric acid (C12 (LUA)), have been shown to exhibit methane-suppressing effects in ruminants (Faciola et al., 2013). In comparison, monounsaturated fatty acids, such as oleic acid (C18:1 (OA)), and polyunsaturated long chain fatty acids, such as linoleic acid (C18:2 (LA)), are able to inhibit gram positive bacteria and methanogens (Lalman and Bagley, 1999; Ray et al., 2010). Synergistic inhibition by two LCFAs acting together on anaerobic microorganisms has been reported by (Koster and Cramer, 1987). Studies conducted by Koster and Cramer (1987) have shown evidence for synergistic inhibition by LUA and myristic acid (C14 (MA)) on aceticlastic methanogens when compared to the effect caused by the individual LCFA.

To date, the exact mechanism of LCFA inhibition remains unclear. However, two types of hypotheses have been postulated by Galbraith and Miller (1973). The first is direct chemical action upon the metabolism of bacteria, and the second is the formation of lipid monolayers around the bacterial cell wall, thereby increasing the surface tension of the cell and interfering with cell division (Galbraith and Miller, 1973). The extent of inhibition is assumed to depend upon the degree of unsaturation (i.e., number of C=C double bonds) within the length of the fatty acid chain. Polyunsaturated fatty acids, such

as docosahexanoic acid (DHA, C22:6) and eicosapentanoic acid (EPA, C20:5), which are present in many types of fish oils, are also capable of inhibiting methanogens in ruminants (Patra and Yu, 2013). To the best of our knowledge, studies concerning the use of fish oil as H_2 consumption inhibitor in mixed anaerobic cultures have not been carried out. Hence, fish oils were chosen as one of the methanogenic inhibitor to compare the effect of fish oils having higher chain fatty acids (with increased number of C=C unsaturation) with individual fatty acids (both saturated and unsaturated).

Similarly, furfural generated during the dehydration of pentose sugars during acid hydrolysis might also offer an economic advantage like that of fatty acids in inhibiting methanogenesis. At low threshold levels, furan aldehyde stimulates fermentation by regenerating the nicotinamide adenine dinucleotide (NAD^+) which is necessary for hexose fermentation (glycolysis) (Boopathy et al., 1993). At levels beyond threshold levels, furans can impair the function of anaerobic microorganisms particularly methanogens (Belay et al., 1997; Boopathy, 2009). Furfural concentrations 20 mM and 25 mM are found to be growth inhibitory to methanogens such as *Methanococcus deltae* (Delta) LH (Belay et al., 1997). In another study conducted by Boopathy (2009), furfural concentrations greater than 15 mM were found inhibitory to *Methanococcus* sp. Strain B. Hydrogen fermentation via dark fermentation is promising if it generated using renewable lignocellulosic waste materials. Since furfural is produced in the process of steam exploding (pressure cooking) lignocellulosic biomass, the role of furfural on the H_2 consumers present in mixed anaerobic culture need to be investigated

The chemical stressing agents such as BES, acetylene, ethylene, chloroacetate, and fluoroacetate used to study the methanogenic inhibition in natural environments are also helpful in identifying the mechanism of methane formation and to study the microbial relationships between methanogens and various carbon mineralizing groups (Liu et al., 2011).

Using LCFAs, byproducts from vegetable oil hydrolysis, to control electron fluxes in anaerobic cultures is documented in many studies (Lalman and Bagley, 2001; Lalman and Bagley, 1999; Ray et al., 2010; Raychowdhury et al., 1985). Threshold levels of

vegetable oils and other constituents in olive mill effluents (OME) have been shown to affect the activities of various anaerobic populations in mixed cultures (Goncalves et al., 2012; Li et al., 2005). In OME, polyphenols, tannins, and lipids can negatively impact methanogenic growth at threshold levels (Goncalves et al., 2012). According to (Bertin et al., 2004), *Methanobacterium formicicum*, a hydrogenotrophic methanogen, was the dominant archaeal community in anaerobic microbial communities treating OME. In a culture treating oleate (C18:1) rich feed, H_2 scavengers including both *Methanococcus* sp. (strictly hydrogenotrophic) and *Methanosarcina* sp. (some members of *Methanosarcina* sp. can utilize acetic acid and H_2) was associated with the syntrophic consortium required for LCFA degradation (Baserba et al., 2012). According to (Baserba et al., 2012), the continuous addition of oleate (1500-2000 mg L^{-1}) enhanced the growth hydrogenotrophic methanogens. Since contrasting results with respect to different type of LCFAs have been reported in literature with respect to methanogen inhibition and also no information was provided about the other major H_2 consumer (homoacetogens). Hence, this study was aimed in addressing these issues.

The motivation for conducting this study was to evaluate the effectiveness of several readily available low cost non-specific renewable chemical inhibitors on H_2 metabolism because of the unavailability of data for H_2 fermentative microbial cultures. The objectives of this study were as follows: 1. Compare the relative effectiveness of BES, furfural, LUA, LA and fish oil (containing the omega-3 fatty acids, EPA (C22:5) and DHA (C20:6)) on H_2 consumption in a mixed anaerobic culture; 2. Evaluate the impact of LCFA carbon chain length and degree of unsaturation; 3. Examine the flux of electrons and carbon during methanogen inhibition and 4. Compare the microbial community structure after subjecting the inocula to different chemical inhibitors.

5.2. Materials and methods

The materials and methods utilized in this research study are described in detail in Chapter 3. The source of the inocula and the maintenance protocol are detailed in section 3.2. The protocol followed in the preparation of the batch reactors (160 mL) is described in Section 3.3. The experimental design matrix is shown in Table 5.1. The analytical

methods for analyzing gas by-products (H_2, CH_4 and CO_2), and liquid by-products (VFAs) were in accordance with the protocols described in Sections 3.7.1 and 3.7.2.

The experiments were designed to examine the effects of different types of chemical inhibitors (BES, furfural, lauric acid (LUA), linoleic acid (LA), and fish oil containing EPA and DHA) on mesophilic (37±2°C) H_2 consumption. Except for the fish oil purchased from Jamieson™, the remaining chemicals were of reagent grade with > 98 % purity and purchased from Sigma Aldrich. All experiments were performed in 160 mL serum bottle batch reactors with a final working volume of 50 mL. Each experimental condition was conducted in triplicate. The control condition (without the addition of a chemical inhibitor) was run in parallel with the experimental conditions to compare the effects on H_2 consumption over the 4.3 day (104 h) period. Batch reactors were mixed using an orbital shaker (Lab line instruments, Model 3520) set at 200 rpm. The initial pH in the batch reactors was adjusted to 5.5 using 1 N HCl and the initial volatile suspended solids (VSS) concentration was 2000 mg L^{-1}.

Table 5.1: Experimental design matrix to study the effects of different types of chemical stressors on H_2 consumption.

Experiment #	Chemical inhibitor[1,2]	Substrate[7] (H_2/CO_2) addition after 24 hours	Metabolite[8] analysis
1	Control[3]	√	√
2	BES (50[2,4,5])	√	√
3	Furfural (2000[1,4,5])	√	√
4	Fish oil(2000[1,4,6])	√	√
5	Lauric acid (2000[1,4,5])	√	√
6	Linoleic acid (2000[1,4,5])	√	√

Notes:
1. Denotes inhibitor concentration in mg L^{-1}
2. Denotes inhibitor concentration in mM
3. No inhibitor addition
4. LA = linoleic acid; LUA = lauric acid; BES = 2-bromoethanesulfonic acid
5. 50 mM BES = 10,550 mg L^{-1}; 2000 mg L^{-1} furfural = 20.83 mM; 2000 mg L^{-1} LA = 6.69 mM; 2000 mg L^{-1} LUA = 7.13 mM
6. Fish oil 2000 mg L^{-1} contains 360 mg L^{-1} eicosapentanoic acid (EPA) and 240 mg L^{-1} docosahexanoic acid (DHA)
7. Cultures fed inhibitor at 37 °C for 24 hours before adding substrates

8. Gas (H_2, CH_4, and CO_2) and liquid (VFAs) metabolites analyzed. All experimental analysis was conducted in triplicate.

After adding the chemical inhibitor for 24 hours, each batch reactor was fed with a H_2/CO_2 gas mix (Praxair, ON, 99.99% purity; 4:1 mol basis). The concentration of H_2/CO_2 was selected based on the K_m values obtained using the substrate (H_2) consumption kinetics at 37 °C (Appendix Figure C.1). The substrate concentration (greater than K_m) was selected so that the reaction to reached a maximum velocity. The BES concentration selected was based on the optimum reported value of 50 mM for inhibiting methanogenesis in an anaerobic digester (Zinder et al., 1984). The fatty acids concentration was 2000 mg L^{-1} for both saturated (LUA) and unsaturated (LA) fatty acids was based on studies by Saady et al. (2012). The stock solutions of the fatty acids (100,000 mg L^{-1}) were prepared according to the procedure described by (Rinzema et al., 1994). Wild fish oil rich in the omega-3 fatty acids, EPA (180 mg g^{-1} oil) and DHA (120 mg g^{-1} oil), was used to study the effects of higher polyunsaturated fatty acids on H_2 consumption. Fish oil at a final concentration of 2000 mg L^{-1} was used after saponification with NaOH (0.18 g g^{-1}NaOH). The stock solution of fish oil (100,000 mg L^{-1}) was prepared using 1 g of fish oil saponified with hot NaOH (50 °C). The stock solutions were cooled and stored at 0^oC. The furfural concentration of 2000 mg L^{-1} was selected based on the average furfural concentration obtained using steam explosion of corn stalk liquor at 190 °C with 10 min residence time (data not shown).

The degree of diversity in the microbial communities found in mixed anaerobic cultures subjected to different experimental conditions was determined according to the protocol described in section 3.10. The terminal restriction fragments (T-RFs) obtained with the restriction enzyme *Hae III* were used to obtain the T-RFLP profiles for the inhibitor treated and control cultures. The data obtained from T-RFLP analysis is comprised of peaks reflecting the size of the terminal restriction fragments (T-RFs) in terms of the number of base pairs (bp) together with the area of each peak measured in fluorescence units. Calculating the area of each peak as a percentage of the total area provides the relative abundance of each band within a particular dataset. The diversity or mean richness of the microbial species was estimated from the plot of the relative abundance

(as a percentage) versus the fragment size (in bp). The T-RFs assessment of species diversity using the Shannon-Weiner diversity index (H') and rare fraction index was estimated using PAST software (Hammer et al., 2001).

The statistical differences observed between different sets of metabolite data obtained under various experimental conditions was evaluated using Tukey's paired comparison procedure for multiple means at the 95 % confidence level (Box et al., 1978). The half-life values based on the substrate consumption profiles obtained for each condition were used to determine the effects of different types of chemical inhibitors on H_2 metabolism. The half-life is the time required for the microorganisms to consume 50 % of the initial substrate concentration. Faster microbial reactions have a shorter half-life. Hence, half-life is a useful parameter for determining and comparing the inhibitory effects under different conditions. A PCA was performed using PAST software (Hammer et al., 2001) to compare the variations in the dataset obtained using different types of chemical stressors along with the untreated control samples. The metabolite data (H_2, CH_4, and VFAs) were used as inputs for the metabolite PCA. The terminal restriction fragment length polymorphism (T-RFLP) data obtained under different experimental conditions were used as the inputs for the microbial PCA. The terminal restriction fragments (T-RFs) were used to obtain the T-RFs profiles for the stressor-treated and control cultures. These T-RFs profiles were also used in a non-parametric multivariate cluster analysis (MCA) that uses Kulczynski similarity index to form clusters. The MCA was performed using the PAST software.

5.3. Results and discussion

5.3.1 Hydrogen consumption profiles

In the untreated control cultures, CH_4 production reached 1390 ± 40 µmol CH_4 after 104 h of incubation (Figure 5.1b). No CH_4 was detected in cultures treated with BES, furfural, LUA or fish oil. In LA-treated cultures, a negligible amount of CH_4 (112 ± 8 µmol) was produced during the 104 h of incubation.

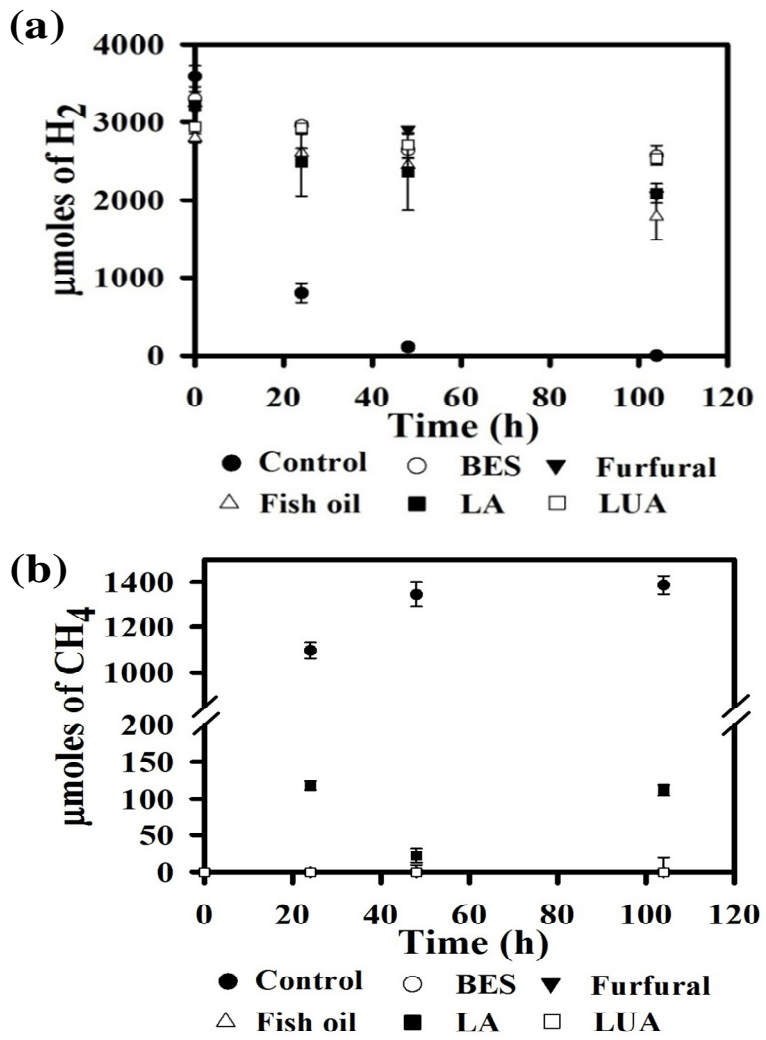

Figure 5.1. Gas metabolites profiles for different chemical stressors (a). Hydrogen consumption; (b). Methane formation.

Note: The error bars in this figure represents the standard deviation for n = 3

Approximately 100 % of the H_2 injected was consumed within 48 h by the untreated control cultures (Figure 5.1a). Of the different types of chemical inhibitors that were compared, LUA exhibited the greatest inhibitory effect on H_2 consumption with 85±1 % of the injected H_2 remained in the batch reactor after 104 h (Table. 5.2). BES was the second most effective inhibitor of H_2 consumption with only 22±1 % of the H_2 consumed after 104 h. Fish oil, LA and furfural exhibited similar levels of inhibition with approximately 35 % of the H_2 consumed after 104 h. Similar results for H_2 consumption with a 50-60 % residual were reported by Templer et al. (2006) for mixed cultures fed 2 g

L^{-1} LA. Anaerobic degradation of fats and lipid-containing wastewaters is not successful because of the inhibitory effects of fatty acids on the microorganisms involved in the anaerobic digestion. Hydrolysis of fats and oils is the rate-limiting step during anaerobic digestion (Cirne et al., 2007). After hydrolysis, the liberated LCFAs are available to impose their effects on different microbial populations.

The relative inhibitory, based on Tukey's pair-wise comparison of the quantity of H_2 consumed, is as follows: LUA > BES > furfural = LA = fish oil > control. The data trend indicates the variable inhibitory effect on H_2 consumers is dependent on the chemical inhibitor. Compared to BES (a specific methanogenic inhibitor), LUA exerted a stronger inhibitory effect on H_2 consumers. Notice, a saturated shorter carbon chain acid, LUA, had a stronger inhibitory effect on H_2 consumption when compared to longer LA and fish oil. Fish oil has similar inhibitory effects as LA, although the concentration of polyunsaturated fatty acids (EPA and DHA) in the fish oil was much lower (about 300 mg L^{-1} of EPA and DHA in 2000 mg L^{-1} oil). Supporting work by Fievez et al. (2003) has shown evidence that CH_4 inhibition was proportional to the relative amount of polyunsaturated fatty acids fish oil. Studies by Song et al. (2011) have shown the impact of fish oil on mRNA synthesis. They reported reduced mRNA expression by archaeal 16S rDNA related to methanogens. The mechanism of inhibition is likely different for LCFAs and furfural. However, at the levels examined, the impact of furfural on H_2 consumption was similar to fish oil and LA.

The impact of saturated and unsaturated LCFAs on methane production has been described extensively in rumen studies. Fievez et al. (2003) and Lourenço et al. (2010) examined the effects of fish oil and a feed rich in LUA on the inhibition of methanogenesis and hypothesized that the effects of a feed rich in LUA and fish oil on archaeal and bacterial community composition is not the same because of the differences in fatty acid composition. Based on this work, the influence of LCFAs on methanogenic inhibition and fermentation byproducts profile could vary in rumen mixed anaerobic communities. A greater understanding of the effects of these oils on rumen microbiome

potentially could assist in developing effective strategies to decrease H_2 consumption and hence, increase the H_2 yield in fermentative H_2 producing cultures.

5.3.2 H_2 consumption half-life values

The half-life values for cultures treated with different chemical inhibitors suggest that both LUA and BES exerted the greatest inhibitory effects followed by LA, fish oil, and furfural when compared to control (untreated) cultures. The half-life value for the control (untreated) cultures was 9.69±3 h (Table 5.2). This indicates that the injected H_2 was consumed within 24 h by the control cultures while for LUA treated cultures, the H_2 degradation half-life reached 480±5 h.

Table 5.2: Hydrogen consumption half-life values under various experimental conditions.

Exp. Set	% H_2 consumption	H_2 degradation Half life values (h)
Control	100 ± 4^{a}	9.69 ± 3^{e}
BES	22 ± 1^{d}	287.52 ± 9^{b}
Furfural	35 ± 1^{c}	169 ± 2^{c}
Fish oil	36 ± 7^{b}	166 ± 18^{d}
LA	$35\pm2^{b, c}$	$162\pm10^{c, d}$
LUA	14 ± 1^{e}	480 ± 5^{a}

Notes: 1. $^{a, b, c}$ and ^{d} indicate statistically different means in the same column. No comparison should be made between columns.
2. ND= Not detected.
3. BES = 2-bromoethane sulfonic acid; LA = linoleic acid; LUA = lauric acid
4. The error bars in this table represents the standard deviation for n = 3.

Since the control cultures exhibited maximum H_2 consumption, the half-life value of the control culture was therefore considered as 100 % and the half-life values of all of the other stressor-treated cultures were calculated as relative percent with respect to the control cultures (Table 5.2). When comparing the relative effectiveness of different types of chemical stressing agents on H_2 consumption using half-life values, LUA had the highest H_2 degradation half-life value of 480±5 h followed by BES 287.5±9 h (Table 5.2). These results clearly show that of the chemical inhibitors examined, LUA has the greatest inhibitory effect on H_2 consumers. LUA can replace BES as an effective methanogenic inhibitor in many anaerobic habitats. Previous studies (Lalman and Bagley,

2000; Lalman and Bagley, 2001; Templer et al., 2006) have showed that LA is more inhibitory to H_2 consumers in mixed culture when compared to OA. The percent H_2 degradation based on half-life values showed a five-fold increase in LA treated cultures (2 g L^{-1}) in comparison to control cultures (Templer et al., 2006). Templer et al. (2006) concluded that the extent of inhibition by fatty acids is dependent on the length of the carbon chain, and that longer chain fatty acids with a higher degree of unsaturation exerts a greater inhibitory effect on H_2 metabolism. However, the work reported by Templer et al. (2006) did not compare the effect of lauric acid (LUA) with longer chain fatty acids. LUA is more inhibitory H_2 consumers -when compared to LA or fish oil. Saturated medium chain fatty acids (MCFAs) have been shown to exhibit great potential for suppressing methanogenesis. Lauric acid (C 12, LUA) is more potent at suppressing methane production than myristic acid (C 14, MA) (68 % compared to 49 % inhibition, respectively) (Blaxter and Czerkawski, 1966). Dohme et al. (2001) claim that adding LUA depressed CH_4 production more than MA in vitro Batch studies with granular sludge using mixtures of LUA and MA showed that the mixture was highly effective compared to LUA (Koster and Cramer, 1987). *In vitro* methane production decreased by 88 % and *in vivo* methane production by 73 % when ruminal diets were supplemented with coconut oil, which contains high amounts of esterified LUA and MA (Dong et al., 1997; Machmüller and Kreuzer, 1999).

Table 5.3 Gibbs free energies for selected H_2 consumption reactions under standard (1M; 1 bar) conditions.

Product	Stoichiometric reaction	$\Delta G^{o'}$ (kJ mol^{-1})	Eq. No.
HPr	$7\ H_2 + 3\ CO_2 \rightarrow CH_3CH_2COO^- + H^+ + 4\ H_2O$	-166.68	5.1
HPr	$7\ H_2 + 3\ HCO_3^- + 2H^+ \rightarrow CH_3CH_2COO^- + 7\ H_2O$	-180.88	5.2
HPr	$CH_3COO^- + H^+ + 3\ H_2 + CO_2 \rightarrow CH_3CH_2COO^- + H^+ + 2\ H_2O$	-71.67	5.3
HPr	$CH_3COO^- + HCO_3^- + H^+ + 3\ H_2 \rightarrow CH_3CH_2COO^- + 7\ H_2O$	-76.40	5.4
HAc	$4\ H_2 + 2\ CO_2 \rightarrow CH_3COO^- + H^+ + 2\ H_2O$	-94.96	5.5
CH_4	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-130.70	5.6

Notes: 1. HPr = propionic acid; HAc = acetic acid; CH_4 = methane.
2. Gibbs free energy values were calculated using the free energy of formation values for the substrates and products.

5.3.3 Soluble metabolites profile

Volatile fatty acids (VFAs), such as acetic acid (HAc), propionic acid (HPr) and formic acid (HFr), were produced during H_2 degradation following different chemical stress treatments. Higher levels of acetic acid detected in the control cultures when compared to the inhibitor treated cultures clearly indicated a hydrogenotrophic methanogenesis (Figure 5.2). HPr production arising in cultures treated with LA or LUA is possible by two pathways. The first pathway via reduction can proceed by Eq. 5.1 and 5.2 in Table 5.3. The second pathway is via reduction of acetic acid/hydrogen/carbondioxide ($CH_3COOH/H_2/CO_2$) or acetic acid/hydrogen/carbonic acid ($CH_3COOH/H_2/HCO_3^-$) to HPr (Eq. 5.3 and 5.4). The Gibbs free energy for formation of HPr under standard conditions (1M, 1 bar) indicates that all of these reactions were possible during this study. Conrad and Klose (1999) have indicated that HPr was the second most abundant fermentation product after HAc for cultures fed H_2/CO_2. The amount of HAc (30-40 %) reported by Conrad and Klose (1999) was very similar to the amount of acetic acid produced (32 %) in the control cultures in this study, Conrad and Klose (1999) concluded that a substantial proportion of the HPr was formed from both H_2/CO_2 and HAc. Using radio-labeled acetic acid and HCO_3^-, they estimated 40-60 % of the HPr was produced from $H^{14}CO_3^-$ (Eq. 5.4 in Table 5.3).

Fixation of atmospheric CO_2 into propionic acid in rice field soil has been reported (Rothfuss and Conrad, 1992). These studies indicate that propionogenesis might become more favorable for the diversion of electron and carbon fluxes from H_2/CO_2 under conditions suppressing homoacetogenesis. These researchers noted that although propionic acid formation is favorable under standard conditions, no such results were observed previously in a natural environment. In cultures treated with LA and LUA, more than 15 % of the electron flux was diverted towards HPr formation. The results obtained from this study also demonstrate that HPr formation is possible in effluents containing mixed anaerobic cultures metabolizing complex wastes rich in fatty acids. Similar findings of enhancing HPr production were obtained with wastewater containing chloroform-treated mesophilic mixed anaerobic culture fed with synthetic sugar. On

studies conducted with chloroform, a methanogenic and homoacetogenic inhibitor, Abreu et al. (2011) reported HPr production from H_2/CO_2. Since no HAc and only a negligible amount of CH_4 was produced in LUA- and LA-treated cultures, no conclusions could be made regarding the effects of these fatty acids on homoacetogens. This is due to the fact that the produced HAc could have been converted to HPr. Unlike, LA and LUA treated cultures, in fish oil-treated cultures containing long chain poly unsaturated fatty acids, HPr formation was not observed indicating that higher chain fatty acids might inhibit HPr formation.

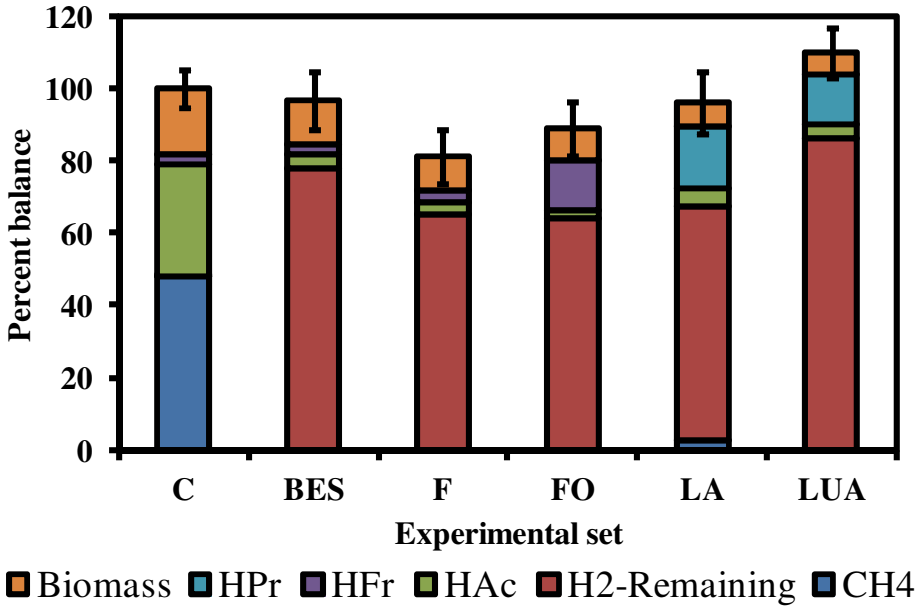

Figure 5.2. Percent distribution of electron fluxes from H_2 under different stress treatments.

Notes:1. It was assumed that 10% of the initial electrons goes to biomass. The error bars in this figure represents the standard deviation for n = 3; 2. Hpr = propionic acid; HAc = acetic acid; HFr = formic acid; CH_4 = methane.3. Abbrevations: BES = 2-bromoethane sulfonic acid; LA = linoleic acid; LUA = lauric acid; C = control; F = furfural; FO = fish oil.

Formate (HFr) was the major liquid metabolite produced in the fish oil-treated cultures (Figure 5.2). Under these conditions, HFr was likely produced via the Wood-Ljungdahl pathway. In the methyl branch of this pathway, the first step in the reduction of CO_2 by H_2 leads to the formation of HFr. The HFr produced serves as the precursor for the

formation of formyl tetrahydrofolate in a subsequent step. This intermediate byproduct then undergoes a sequence of reactions to form the methyl (CH_3) group of acetyl-CoA. The main reason for the higher HFr concentrations observed in the fish oil-treated cultures might the inhibition of subsequent steps in the Wood-Ljungdahl pathway during the conversion of HFr. Another possible reason might be the selective enrichment of HFr producing population and a decrease in the hydrogenotrophic methanogens in response to the fish oil stress treatment. In the current study, the accumulation of H_2 and/or formate is contradictory and indicates that specific and nonspecific chemical inhibitors are able to decouple the syntrophic relationship between H_2 consumers and H_2 producers. In the untreated control cultures, approximately 48 % of the electron equivalents (of the 100 % H_2 that was consumed) were diverted to CH_4 formation (Eqn. 5.6, Table 5.3) and 31 % to HAc formation (Eqn. 5.5, Table 5.3) (Figure 5.2).

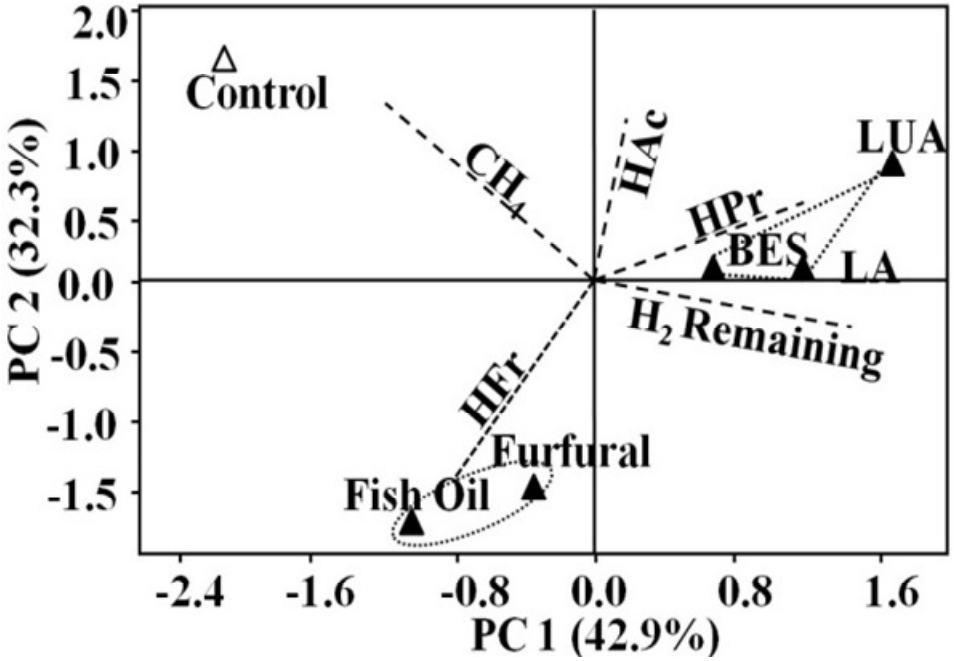

Figure 5.3. Principal component analysis of the fermentation metabolites obtained under different stress treatments. Note: HPr = propionic acid; HFr = formic acid; HAc = acetic acid; Rem H_2 = H_2 remaining.

In the BES-, furfural-, LUA- and fish oil-treated cultures, no detectable levels of CH_4 were produced. Notice in the LA-treated culture, less than 2.5 % of the electron

equivalents were diverted towards CH_4 formation. In contrast to the untreated control culture, less than 5 % of the electrons derived from H_2 were diverted towards HAc formation in any of the cultures treated with chemical inhibitors.

5.3.4 Principal component analysis

Analysis of the fermentation metabolites by PCA revealed similarities and differences between different types of chemical stress treatments. The loadings and the score values of the first two principal components (PC 1 and PC 2) are shown in the biplot (Figure 5.3). The volatile fatty acids (acetic acid (HAc), propionic acid (HPr) and formic acid (HFr)) produced during the experimental phase were used as the inputs for the PCA together with the gas data (H_2-Remaining and CH_4). The first two principal components, PC 1 and PC 2, accounted for 75% of the total variance in the original dataset. The loading values for Rem H_2 (0.90), HAc (0.11) and HPr (0.74) correlated positively with the first principal component (PC 1) whereas the loading values for CH_4 (0.54), HAc (0.50), and HPr (0.24) correlated positively with PC 2. The bi-plot shows that H_2-remaining is negatively correlated with CH_4. The lengths of the lines (loading vectors) showed in the bi-plot approximate the variance of these response variables. The longer the length of the line, the higher is the variance of the associated variable. Among the variables investigated CH_4, HFr, and H_2 remaining, had higher variances compared to HAc and HPr under the conditions examined. In addition, the untreated control culture is located differently, in a separate quadrant and at a distance from the other stressor-treated cultures. A similar pattern showing separation of the control cultures from chemically treated cultures was reported by Chaganti et al. (2013). In the control culture, the formation of CH_4 is evident in the bi-plot from its placement in the quadrant dominated by CH_4.

Similarly, LUA- and LA-treated cultures are grouped closely with the BES-treated culture (upper-right quadrant of Figure 5.3). In this case, propionate formation was characteristic of these types of chemical inhibitors. Fish oil- and furfural-treated cultures were grouped together in a different quadrant (lower-left) of the bi-plot with the HFr levels greater in both of these cultures. The results from the PCA showing the association

between the chemical inhibitors to the cultures with the metabolite data indicate that all of the inhibitors tested were very effective against CH_4 formation.

5.3.5 Microbial analysis

Genomic analyses of the microbial communities subjected to different types of chemical stressors were investigated using T-RFLP. For the analyses, only T-RFs with bands greater than 50 arbitrary fluorescence units were considered. Microbial communities showing variation in dominant microbial species were related to the types of chemical stressors applied and the resulting fermentation patterns. A Kulczynski similarity index was used to construct a cluster tree in order to quantify the relationships based on the variation in the T-RFs. Cluster analysis is a tool used to form hierarchical groupings of the experimental sets based on the multivariate data obtained from T-RFLP analysis. LA- and fish oil-treated cultures were grouped together in clade I while BES- and furfural-treated cultures were grouped together in clade II (Figure 5.4).

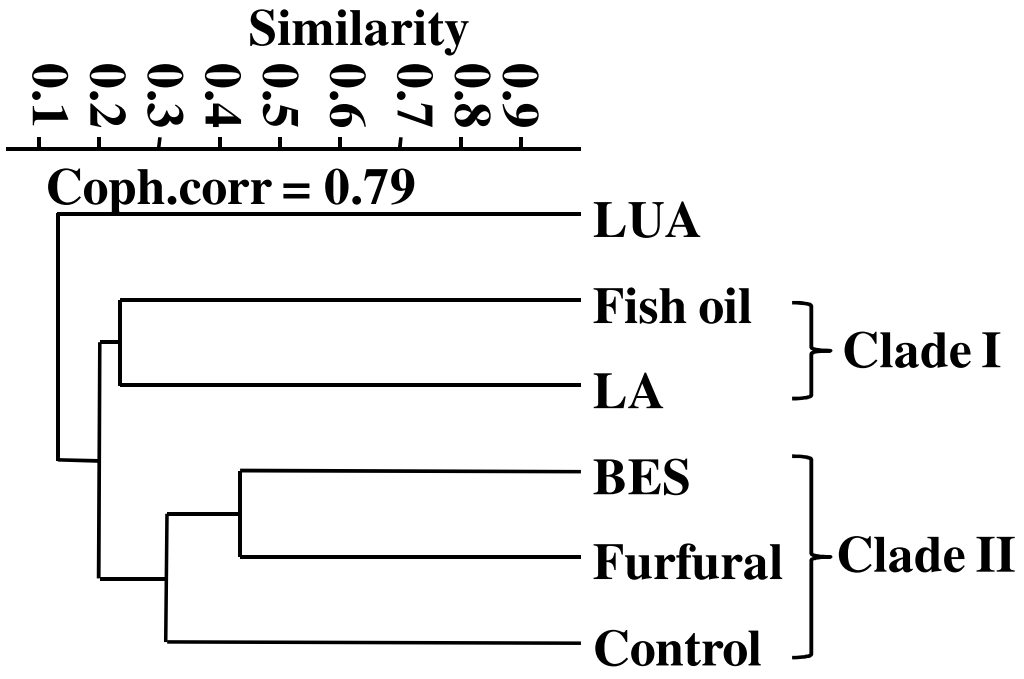

Figure 5.4. Dendrogram of cultures treated with different chemical stressors constructed using the Kulczynski similarity index for 16S rRNA T-RFs.
Note: Cophenetic correlation coefficient = Coph. Corr.

A 25 % similarity between cultures treated with LA and fish oil might be due similar inhibitory effects by fish oil and LA and moreover both are long chain fatty acids. Note both fish oil and LA are unsaturated LCFAs with more than 16 carbon atoms in which inhibition is based on the degree of unsaturation. According to Demeyer and Henderickx (1967), fatty acids with two unsaturated C=C double bonds have a greater inhibitory effect on methanogens than saturated fatty acids.

LUA-treated culture appears to be different from both LA- and fish oil-treated cultures in its structure and its inhibitory actions. Notice the dendrogram of the T-RFLPs separates the LUA-treated samples from the other conditions. LUA is more soluble than longer chain LCFAs and has no *cis* double bonds. Hence, its inhibitory effects will differ from unsaturated fatty acids such as LA and fish oil. The nature of the monolayer formed by unsaturated fatty acids is different from the monolayer staked structures formed by saturated fatty acids. Unsaturated molecules tend to arrange themselves with bends at double bonds, while saturated fatty acids tend to lie in parallel straight chains. The bacteriostatic effect of LA is also of interest since LA is regarded as an unsaturated fatty acid that is essential in the nutrition of higher animals. The fact that LA is inhibitory to gram positive bacteria more than gram negative bacteria is interesting because most chemotherapeutic agents are similarly more effective against gram positive bacteria (Nieman, 1954). The extent of inhibition imposed by bacteriostatic fatty acids, such as LA, depends not only on the concentration of bacteria but also on the duration of incubation, the amount and nature of the fatty acid added to the culture, as well as the presence of other lipids in the medium.

Both BES and furfural-treated cultures were grouped together in clade II. Note, the untreated control culture is also grouped in clade II. The similarity index indicates that the microbial composition of cultures grouped in clade II showed 32-45 % similarity while cultures grouped in clade I showed a 25 % similarity. LUA-treated culture showed about 15 % similarity compared to the other stressor-treated cultures, and is placed separately in the dendrogram. A Cophenetic correlation (C) co-efficient (non-linear regression co-efficient) was used to evaluate the quality of the dendrogram generated

using the Kulczynski similarity measure. A C-value of 0.79 indicates a high quality output for this analysis.

Table 5.4 Richness and diversity estimates of OTUs

Library	OTU richness[a]	OTU diversity[b]
Control	28	3.067
BES	31	3.126
Furfural	29	3.130
Fish oil	35	3.331
LUA	17	2.694
LA	20	2.694

Note: [a]Non-parametric estimators of species (OTU) richness (total number of different OTUs in a given sample). (OTU = operational taxonomic units)
[b]Shannon diversity index (considers both species richness and evenness (relative abundance)).

Rarefaction curves are used to assess species richness (number of taxa) based on the number of specimens (samples) examined. The construction of rarefaction curves to estimate the richness of species within the experimental samples is illustrated in Figure 5.5. The curve shown describes the plot of the number of species observed in the current study as a function of the number of samples or specimens tested. If the rarefaction curve has a steep slope, then this indicates that a large fraction of the species present under that experimental condition need to be explored. The rarefaction curves for most of the experimental (both control and stress treated) cultures in the current study reached an asymptote, which indicates that a reasonable number of samples were considered in the microbial analysis. More intensive sampling would likely yield few additional species (Gotelli and Colwell, 2001). Fish oil treatment showed a high level of species diversity with 35 operational taxonomic units (OTUs) (Table 5.4). Both LA (20 OTUs) and LUA (17 OTUs) treatment produced the lowest levels of species diversity compared to the other chemical stressor treatments. Sample richness increased in parallel with the sample diversity (H'). Fish oil-treated cultures showed the highest H' index (3.331) followed by both furfural and BES-treated cultures (3.13) (Table 5.4). LA and LUA-treated cultures showed the least amount of microbial diversity (H' = 2.694). One possible reason for this

low level of microbial diversity might be attributed to the selective enrichment of specific groups of bacteria by both of these chemical inhibitors. This enrichment is clearly evident in their terminal restriction fragments (T-RFs) profiles (Table 5.6). Both LA and LUA treatment showed higher levels of *Clostridium* sp. diversity compared to the other types of chemical stressors. Similar work has provided evidence showing bacterial community changes in response to oleate addition resulted in a less diverse bacterial consortium related to functional specialization of the species towards oleate degradation (Baserba et al., 2012).

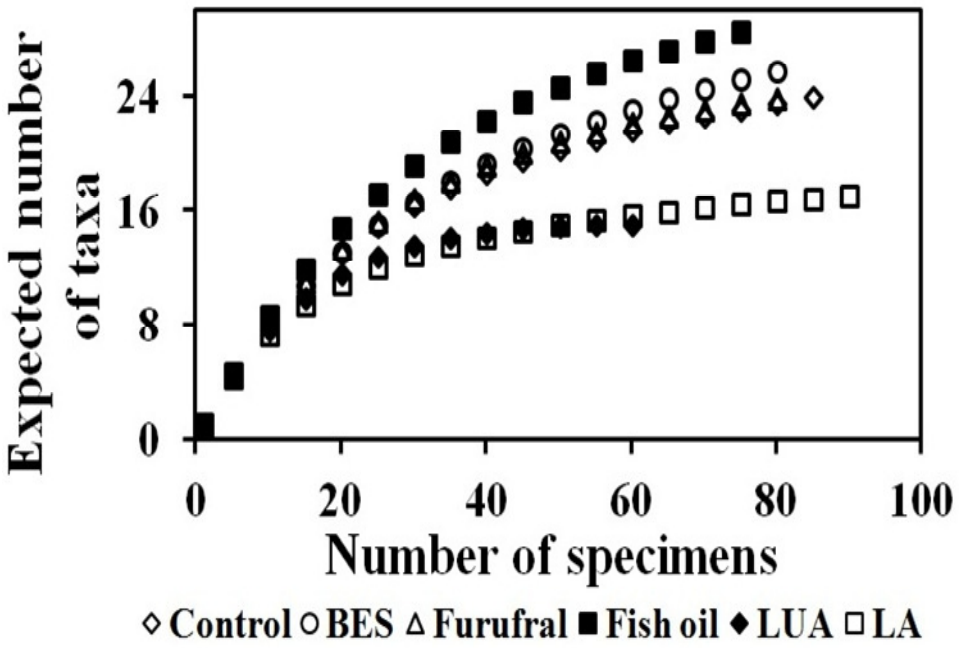

Figure 5.5. Rarefaction curves for different stress treated cultures.
Note: LA - Linoleic acid; LUA - lauric acid; BES - 2-bromoethane sulfonate

The T-RFs from untreated control cultures confirmed the presence of both aceticlastic methanogens, such as *Methanosarcina* sp. (Mori et al., 2012) and *Methanosaeta* sp. (Patel and Sprott, 1990) as well as hydrogenotrophic methanogens, such as *Methanococcus* sp. (Hendrickson et al., 2004) (Table 5.5). The presence of both microbial populations in the control cultures resulted in higher CH_4 production. Methanogens were undetectable in the chemical treated cultures. Among the *Clostridium* sp. detected, organisms closely related to *Clostridium ljungdahlii* were present in all of

the cultures except for the untreated control cultures. *C.ljungdahlii* is a homoacetogenic bacterium capable of performing syngas (synthesis gas) fermentation (Kopke et al., 2010). *Clostridium* sp. was dominant in both LA and LUA treatment conditions. Some of the *Clostridia* closely associated with *Clostridium propionicum* and *Propionibacterium acnes* are capable of HPr production, so may be responsible for the higher levels of HPr production observed under both of these treatment conditions.

Table 5.5 Microbial communities identified after treatment with different stressors.

Sets	Microbial diversity
Control (C)	*Propionibacterium acnes, Methanococcus* sp.*, Methanosarcina* sp.*, Methanosaeta* sp.*, Clostridium formicaceticum, Methylomicrobium buryaticum, Meiothermus ruber, Desulfovibrio magneticus, Lactobacillus acidophilus*
BES (B)	*Desulfitobacterium dehalogenans, Eubacterium saphenum , Clostridium ljungdahlii , Desulfovibrio magneticus, Lactobacillus acidophilus*
Furfural (F)	*Eubacterium timidum, E.infirmum, Clostridium ljungdahlii, C. gasigenes, Bacteroides* sp.
Fish oil (O)	*Bacteroides distasonis, Flavobacterium ferrugineum, Clostridium estertheticum, Methylomicrobium buryaticum, Meiothermus ruber, Bifidobacterium inopinatum, Eubacterium saphenum, Thermanaerovibrio acidaminovorans, C. ljungdahlii*
Lauric acid (LUA)	*Bacteroides thetaiotaomicron , Clostridium propionicum, Propionibacterium acnes, uncultured Clostridium* sp.*, C. ljungdahlii*
Linoleic acid (LA)	*Clostridium Frigidicarnis, C. aceticum C. gasigenes, C. Scatologenes, C. paraputrificum, C. propionicum, Propionibacterium acnes, C. ljungdahlii, Lactobacillus* sp.

Bands related to *Bacteroides* sp. were not dominant in the T-RFLP profiles for the LA and LUA treatment conditions. However, *Eubacteria* sp. in cultures subjected to BES, furfural or fish oil treatments were responsible for producing HFr (Holdeman et al., 1980). High HFr levels were observed in the fish oil-treated cultures (Table 5.2). Many *Eubacteria sp.* capable of producing HAc use the acetyl-CoA pathway (Wood-Ljungdahl pathway) (Drake et al., 2008).

Table 5.6 Minimum inhibitory concentration (MIC) values for chemical inhibitors on different microorganisms.

Organism	Temp (°C)	pH	Substrate	Chemical inhibitor	Inhibitory conc. (mM)	Reference
Methanococcus deltae ΔLH	37	NR	H_2/CO_2	Furfural	> 20	Belay et al. (1997)
Methanococcus thermolithotrophicus *Methanosarcina barkeri* 227 *Methanobrevibacter ruminatum*					> 15	
Clostridium acetobutylicum ATCC 824 *Clostridium beijerinckii* BA101	35±1	6.7	Glucose	Furfural	> 30	Zhang et al. (2012)
Methanococcus sp. strain B	20±2	NR	H_2/CO_2	Furfural	> 15	Boopathy (2009)
Escherichia coli ATCC 1175 *Enterobacter aerogenes* *Proteus vulgaris*	37	7	Glucose	Furfural	> 35 > 39 > 17	Boopathy et al. (1993)
Desulfovibrio sp. Strain F-1	37	7	Furfural	Furfural	> 10	Brune et al. (1983)
Methanothrix sp.	30±1	7±0.1	Acetate	LUA	1.6	Koster and Cramer (1987)
Bacillus megaterium	37	7.4	Glucose	LUA, LA	0.15, 0.02	Galbraith et al. (1971)
Methanosarcina acetivorans C2A	35	NR	Sodium acetate	BES	0.4	Zhang et al. (2000)

In the methyl branch of this pathway, the first step is the reduction of H_2 with CO_2 to form HFr, which then undergoes a series of reductive steps to form the CH_3 group of the HAc molecule. Fish oil treatment could have likely resulted in inhibiting the degradation of HFr. A weak band of *Lactobacillus* sp. was detected in the control, BES- and LA-treated cultures, which indicated that both the BES and LA treatments were not able to completely inhibit lactic acid bacteria (LAB). The minimum concentrations of the various chemical inhibitors required for inhibition are shown in Table 5.6. The concentration of furfural required to inhibit H_2-consuming methanogenic archaea is quite low compared to that required for H_2-producing *Clostridia* sp. For example, 15 mM of furfural is sufficient to inhibit strains of methane-producing methanogenic archaea, such as *Methanococcus thermolithotrophicus* and *Methanosarcina barkeri* 227, whereas more than 30 mM of furfural is required to inhibit H_2 producers, such as *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* BA101 (Zhang et al., 2012). The results obtained from this study (i.e., no methane formation in furfural-treated cultures) (Table. 5.2) are consistent with evidence that methanogens are inhibited by the addition of 2 g L^{-1} (20.83 mM) furfural. However, H_2-producing *Clostridium* sp. are not inhibited at these concentrations (Table 5.6).

The minimum inhibitory concentration (MIC) values required for gram positive bacteria are less than the MIC for gram negative bacteria. The inhibitory mechanisms for polyunsaturated fatty acids on bacterial growth are not clear. Only a few studies have examined the mechanism of LA action (Butcher et al., 1976; Greenway and Dyke, 1979). The results revealed that free LA inhibits bacterial growth by altering the permeability of the cell membrane. According to Greenway and Dyke (1979), the surfactant properties of various LCFAs are responsible for cell leakage. LCFAs can also act by altering oxidative phosphorylation (Schonfeld et al., 1989).

5.4. Conclusions

The effects of different types of chemical inhibitors on H_2 consumption by a flocculated mixed anaerobic culture were investigated under anaerobic conditions at 37 °C at pH 5.5. The conclusions from this study are as follows:

1. Under the conditions examined, the chemical inhibitors were effective in inhibiting methanogenesis when compared to control cultures. The H_2 consumption (%) for the controls and stress treated cultures revealed the following trend: Control > Fish oil = LA = Furfural > BES > LUA respectively. (Based on using the Tukey's the test at α = 0.05).
2. Of the chemical inhibitors studied, LUA showed a stronger inhibitory effect on H_2 consumption when compared to BES. In addition, similarly to BES treatment, no methane was detected in LUA-treated cultures. These results indicate that LUA can be used as an effective methanogenic inhibitor comparable to BES.

3. Among the fatty acids studied, LUA has a greater inhibitory effect on H_2 consumption than LA and fish oil. Cultures treated with either LA or fish oil showed similar levels of H_2 consumption (about 35 %). This suggests that unsaturated fatty acids have similar mechanisms of actions on H_2 consumption.

4. A statistical analysis of the half-life values clearly showed an inhibitory trend influencing the threshold concentration levels required for different types of chemical inhibitors. LUA treated cultures showed higher H_2 degradation half-life value compared to other stress treatments.

5. Since no HAc was formed in cultures treated with LA or LUA, no conclusions could be made about the impact of these chemical agents on homoacetogenesis as it was possible that any HAc produced was consumed by the formation of HPr using HAc, H_2 and CO_2.

6. An examination of the PCA biplot (i.e., analysis of the first two principal components associated with the fermentation metabolites) shows that untreated control culture was located separately from all of the stressor-treated cultures, and was associated with CH_4 production. HPr production was associated with both the LA- and LUA-treated cultures. Since no HAc was observed in both LA and LUA treated cultures no conclusion should be made on the effect of these stressing agents on homoacetogens since HPr could be formed using HAc, H_2 and CO_2. Higher HFr formation associated

with fish oil-treated cultures might be due to selective inhibition of subsequent steps of the metabolic pathway (inhibition of the conversion of formate in the acetyl-CoA pathway).

7. A cluster analysis using the Kulczynski similarity index showed both LA and fish oil treated cultures are clustered together indicating higher chain fatty acids are grouped together include I. A Cophenetic correlation value of 0.79 indicates a very good level of correspondence of the index value with the cluster grouping.

8. The Shannon-Wiener diversity index indicates that the fish oil-treated cultures exhibited the highest degree of species diversity and both LA and LUA treated cultures showed the lowest levels of species diversity. In addition, both the LA and the LUA treatments resulted in greater abundance of *Clostridium* sp. when compared to cultures treated with other chemical inhibitors. Since *Clostridium* sp. is mainly involved in H_2 production, treatments with both LA and LUA could be used for selectively enhancing the H_2 producers present in mixed culture.

9. An analysis of microbial 16S rRNA profiles indicated that untreated control cultures consisted of both aceticlastic (*Methanosaeta* sp. and *Methanosarcina* sp.) and hydrogenotrophic (*Methanococcus* sp.) methanogens. These microorganisms were absent in all cultures treated with chemical inhibitors. *Clostridium propionicum* and *Propionobacterium acnes* were present in LA and LUA treated cultures which might be responsible for HPr formation. However, both these organisms were absent in fish oil treated cultures. *Eubacteria* sp. (homoacetogenic bacteria), a microorganism responsible for HFr production, was detected in fish oil treated cultures.

5.5 References:

Abreu, A.A., Alves, J.I., Pereira, M.A., Sousa, D.Z., Alves, M.M. (2011). Strategies to suppress hydrogen consuming microorganisms affect macro and micro scale structure and microbiology of granular sludge. *Biotechnol Bioeng*, **108**(8), 1766-75.

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G. (2008). The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. *Int J Hydrogen Energy*, **33**(15), 4064-73.

Balch, W.E., Wolfe, R.S. (1979). Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). *J Bacteriol*, **137**(1), 256-63.

Baserba, M.G., Angelidaki, I., Karakashev, D. (2012). Effect of continuous oleate addition on microbial communities involved in anaerobic digestion process. *Bioresource Technol*, **106**, 74-81.

Belay, N., Boopathy, R., Voskuilen, G. (1997). Anaerobic transformation of furfural by *Methanococcus deltae* ΔLH. *Appl Environ Microbiol*, **63**(5), 2092-4.

Bertin, L., Colao, M.C., Ruzzi, M., Fava, F. (2004). Performances and microbial features of a granular activated carbon packed-bed biofilm reactor capable of an efficient anaerobic digestion of olive mill wastewaters. *FEMS Microbiol Ecol*, **48**(3), 413-23.

Blaxter, K.L., Czerkawski, J. (1966). Modifications of the methane production of the sheep by supplementation of its diet. *J Sci Food Agric*, **17**(9), 417-21.

Boopathy, R. (2009). Anaerobic biotransformation of furfural to furfuryl alcohol by a methanogenic archaebacterium. *Int Biodeter Biodegr*, **63**(8), 1070-2.

Boopathy, R., Bokang, H., Daniels, L. (1993). Biotransformation of furfural and 5-hydroxymethyl furfural by enteric bacteria. *J Ind Microbiol*, **11**(3), 147-50.

Box, G.E.P., Hunter, W.G., Hunter, J.S. (1978). Statistics for experimenters: An introduction to design, data analysis, and model building. New York: John Wiley and Sons. p.672.

Brune, G., Schoberth, S.M., Sahm, H. (1983). Growth of a strictly anaerobic bacterium on furfural (2-furaldehyde). *Appl Environ Microbiol*, **46**(5), 1187-92.

Butcher, G.W., King, G., Dyke, K.G.H. (1976). Sensitivity of *Staphylococcus aureus* to unsaturated fatty acids. *J Gen Microbiol*, **94**(2), 290-6.

Cai, M., Liu, J., Wei, Y. (2004). Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. *Environ Sci Technol*, **38**(11), 3195-202.

Chaganti, S.R., Pendyala, B., Lalman, J.A., Veeravalli, S.S., Heath, D.D. (2013). Influence of linoleic acid, pH and HRT on anaerobic microbial populations and metabolic shifts in ASBRs during dark hydrogen fermentation of lignocellulosic sugars. *Int J Hydrogen Energy*, **38**(5), 2212-20.

Chiu, P.C., Lee, M. (2001). 2-bromoethanesulfonate affects bacteria in a trichloroethene-dechlorinating culture. *Appl Environ Microbiol*, **67**(5), 2371-4.

Cirne, D.G., Paloumet, X., Björnsson, L., Alves, M.M., Mattiasson, B. (2007). Anaerobic digestion of lipid-rich waste-Effects of lipid concentration. *Renew Energy*, **32**(6), 965-75.

Conrad, R., Klose, M. (1999). Anaerobic conversion of carbon dioxide to methane, acetate and propionate on washed rice roots. *FEMS Microbiol Ecol*, **30**(2), 147-55.

Demeyer, D.I., Henderickx, H.K. (1967). The effect of C_{18} unsaturated fatty acids of methane production *in vitro* by mixed rumen bacteria. *BBA Lipid Lipid Met*, **137**(3), 484-97.

Dohme, F., Machmuller, A., Wasserfallen, A., Kreuzer, M. (2001). Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. *Lett Appl Microbiol*, **32**(1), 47-51.

Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W., Cheng, K.J. (1997). Lipid-induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). *Can J Anim Sci*, **77**(2), 269-78.

Drake, H.L., Gossner, A.S., Daniel, S.L. (2008). Old acetogens, new light. *Ann NY Acad Sci*, **1125**, 100-28.

Faciola, A.P., Broderick, G.A., Hristov, A., Leao, M.I. (2013). Effects of lauric acid on ruminal protozoal numbers and fermentation pattern and milk production in lactating dairy cows. *J Anim Sci*, **91**(1), 363-73.

Fievez, V., Dohme, F., Danneels, M., Raes, K., Demeyer, D. (2003). Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*. *Anim Feed Sci Technol*, **104**(1-4), 41-58.

Galbraith, H., Miller, T.B. (1973). Physicochemical effects of long chain fatty acids on bacterial cells and their protoplasts. *J Appl Bacteriol*, **36**(4), 647-58.

Galbraith, H., Miller, T.B., Paton, A.M., Thompson, J.K. (1971). Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol. *J Appl Bacteriol*, **34**(4), 803-13.

Goncalves, M.R., Costa, J.C., Marques, I.P., Alves, M.M. (2012). Strategies for lipids and phenolics degradation in the anaerobic treatment of olive mill wastewater. *Water Res*, **46**(6), 1684-92.

Gotelli, N.J., Colwell, R.K. (2001). Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecol Lett*, **4**(4), 379-91.

Greenway, D.L.A., Dyke, K.G.H. (1979). Mechanism of the inhibitory action of linoleic-acid on the Growth of *Staphylococcus aureus*. *J Gen Microbiol*, **115**(1), 233-45.

Gunsalus, R.P., Romesser, J.A., Wolfe, R.S. (1978). Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum. Biochemistry*, **17**, 2374-77.

Hammer, O., Harper, D.A.T., Ryan, P.D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, **4**, 1-9.

Han, S.K., Shin, H.S. (2004). Biohydrogen production by anaerobic fermentation of food waste. *Int J Hydrogen Energy*, **29**(6), 569-77.

Hendrickson, E.L., Kaul, R., Zhou, Y., Bovee, D., Chapman, P., Chung, J., de Macario, E.C., Dodsworth, J.A., Gillett, W., Graham, D.E., Hackett, M., Haydock, A.K., Kang, A., Land, M.L., Levy, R., Lie, T.J., Major, T.A., Moore, B.C., Porat, I., Palmeiri, A., Rouse, G., Saenphimmachak, C., Soll, D., Van Dien, S., Wang, T., Whitman, W.B., Xia, Q., Zhang, Y., Larimer, F.W., Olson, M.V., Leigh, J.A. (2004). Complete genome sequence of the genetically tractable hydrogenotrophic methanogen *Methanococcus maripaludis*. *J Bacteriol*, **186**(20), 6956-69.

Holdeman, L.V., Cato, E.P., Burmeister, J.A., Moore, W.E.C. (1980). Descriptions of *Eubacterium timidum* sp. nov., *Eubacterium brachy* sp. nov., and *Eubacterium nodatum* sp.nov. Isolated from human periodontitis. *Int J Syst Bacteriol*, **30**(1), 163-9.

Kopke, M., Held, C., Hujer, S., Liesegang, H., Wiezer, A., Wollherr, A., Ehrenreich, A., Liebl, W., Gottschalk, G., Durre, P. (2010). *Clostridium ljungdahlii* represents a microbial production platform based on syngas. *P Natl Acad Sci USA*, **107**(29), 13087-92.

Koster, I.W., Cramer, A. (1987). Inhibition of methanogenesis from acetate in granular sludge by long-chain fatty acids. *Appl Environ Microbiol*, **53**(2), 403-409.

Lalman, J., Bagley, D.M. (2002). Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. *Water Res*, **36**(13), 3307-13.

Lalman, J.A., Bagley, D.M. (2000). Anaerobic degradation and inhibitory effects of linoleic acid. *Water Res*, **34**(17), 4220-8.

Lalman, J.A., Bagley, D.M. (2001). Anaerobic degradation and methanogenic inhibitory effects of oleic and stearic acids. *Water Res*, **35**(12), 2975-83.

Lalman, J.A., Bagley, D.M. (1999). Anaerobic degradation of linoleic and oleic acids. *Environ Eng*, 786-95.

Li, Z.K., Wrenn, B.A., Venosa, A.D. (2005). Anaerobic biodegradation of vegetable oil and its metabolic intermediates in oil-enriched freshwater sediments. *Biodegradation*, **16**(4), 341-52.

Lin, C.Y., Chang, R.C. (2004). Fermentative hydrogen production at ambient temperature. *Int J Hydrogen Energy*, **29**(7), 715-20.

Liu, H., Wang, J., Wang, A.J., Chen, J.A. (2011). Chemical inhibitors of methanogenesis and putative applications. *Appl Microbiol Biot*, **89**(5), 1333-40.

Lourenço, M., Ramos-Morales, E., Wallace, R.J. (2010). The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal*, **4**(7), 1008-23.

Machmüller, A., Kreuzer, M. (1999). Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. *Can J Anim Sci*, **79**(1), 65-72.

Mori, K., Iino, T., Suzuki, K.I., Yamaguchi, K., Kamagata, Y. (2012). Aceticlastic and NaCl-requiring methanogen "*Methanosaeta pelagica*" sp. nov., isolated from marine tidal flat sediment. *Appl Environ Microbiol*, **78**(9), 3416-23.

Nieman, C. (1954). Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol Rev*, **18**(2), 147-63.

Oh, S.E., Van Ginkel, S., Logan, B.E. (2003). The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ Sci Technol*, **37**(22), 5186-90.

Patel, G.B., Sprott, G.D. (1990). *Methanosaeta concilii* gen.nov, sp.nov (*Methanothrix concilii*) and *Methanosaeta thermoacetophila* nom.rev, comb.nov. *Int J Syst Bacteriol*, **40**(1), 79-82.

Patra, A.K., Yu, Z. (2013). Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations *in vitro*. *J Dairy Sci*, **96**(3), 1782-92.

Ray, S., Saady, N.M.C., Lalman, J.A. (2010). Diverting electron fluxes to hydrogen in mixed anaerobic communities fed with glucose and unsaturated C18 long chain fatty acids. *J Environ Eng-Asce*, **136**(6), 568-75.

Raychowdhury, M.K., Goswami, R., Chakrabarti, P. (1985). Effect of unsaturated fatty acids in growth inhibition of some penicillin resistant and sensitive bacteria. *J Appl Bacteriol*, **59**(2), 183-8.

Ren, N.Q., Guo, W.Q., Wang, X.J., Xiang, W.S., Liu, B.F., Wang, X.Z., Ding, J., Chen, Z.B. (2008). Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. *Int J Hydrogen Energy*, **33**(16), 4318-24.

Rinzema, A., Boone, M., Vanknippenberg, K., Lettinga, G. (1994). Bactericidal effect of long chain fatty acids in anaerobic digestion. *Water Environ Res*, **66**(1), 40-9.

Rothfuss, F., Conrad, R. (1992). Vertical profiles of CH_4 concentrations, dissolved substrates and processes involved in CH_4 production in a flooded Italian rice field. *Biogeochemistry*, **18**(3), 137-52.

Saady, N.M.C., Chaganti, S.R., Lalman, J.A., Heath, D. (2012). Impact of culture source and linoleic acid (C18:2) on biohydrogen production from glucose under mesophilic conditions. *Int J Hydrogen Energy*, **37**(5), 4036-45.

Sanz, J.L., Rodríguez, N., Amils, R. (1997). Effect of chlorinated aliphatic hydrocarbons on the acetoclastic methanogenic activity of granular sludge. *Appl Microbiol Biot*, **47**(3), 324-8.

Schonfeld, P., Schild, L., Kunz, W. (1989). Long-Chain fatty-acids act as protonophoric uncouplers of oxidative-phosphorylation in rat-liver mitochondria. *Biochim Biophys Acta*, **977**(3), 266-72.

Song, M.K., Li, X.Z., Oh, Y.K., Lee, C.K., Hyun, Y. (2011). Control of methane emission in ruminants and industrial application of biogas from livestock manure in korea. *Asian Austral J Anim Sci*, **24**(1), 130-6.

Sparling, R., Daniels, L. (1987). The specificity of growth inhibition of methanogenic bacteria by bromoethanesulfonate. *Can J Microbiol*, **33**(12), 1132-6.

Tanisho, S., Suzuki, Y., Wakao, N. (1987). Fermentative hydrogen evolution by *Enterobacter aerogenes* Strain E-82005. *Int J Hydrogen Energy*, **12**(9), 623-7.

Templer, J., Lalman, J.A., Jing, N., Ndegwa, P.M. (2006). Influence of C18 long chain fatty acids on hydrogen metabolism. *Biotechnol Progr*, **22**(1), 199-207.

Xu, K.W., Liu, H., Chen, J. (2010a). Effect of classic methanogenic inhibitors on the quantity and diversity of archaeal community and the reductive homoacetogenic activity during the process of anaerobic sludge digestion. *Bioresource Technol*, **101**(8), 2600-7.

Xu, K.W., Liu, H., Du, G.C., Chen, J. (2009). Real-time PCR assays targeting formyltetrahydrofolate synthetase gene to enumerate acetogens in natural and engineered environments. *Anaerobe*, **15**(5), 204-13.

Xu, K.W., Liu, H., Li, X.F., Chen, J.A., Wang, A.J. (2010b). Typical methanogenic inhibitors can considerably alter bacterial populations and affect the interaction between fatty acid degraders and homoacetogens. *Appl Microbiol Biot*, **87**(6), 2267-79.

Zhang, J.K., Pritchett, M.A., Lampe, D.J., Robertson, H.M., Metcalf, W.W. (2000). *In vivo* transposon mutagenesis of the methanogenic archaeon *Methanosarcina acetivorans* C2A using a modified version of the insect mariner family transposable element Himar1. *P Natl Acad Sci USA*, **97**(17), 9665-70.

Zhang, Y., Han, B., Ezeji, T.C. (2012). Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by *Clostridium acetobutylicum* ATCC 824 during butanol fermentation. *New Biotechnol*, **29**(3), 345-51.

Zhu, H.G., Beland, M. (2006). Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *Int J Hydrogen Energy*, **31**(14), 1980-8.

Zinder, S.H., Anguish, T., Cardwell, S.C. (1984). Selective inhibition by 2-bromoethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor. *Appl Environ Microbiol*, **47**(6), 1343-5.

CHAPTER 6: LONG TERM IMPACT OF STRESSING AGENTS ON FERMENTATIVE HYDROGEN PRODUCTION AT MESOPHILIC (37 °C) AND THERMOPHILIC (55 °C) TEMPERATURES

6.1 Introduction

Increasing energy demands along with the inability to meet future needs have caused a rise in research and development for alternative energy sources. Hydrogen (H_2) is a promising sustainable alternative to fossil fuels because it is carbon neutral, releasing water as its sole by-product. Its calorific value of 142 kJ g^{-1}, is about 2.8 times higher than other fossil fuel (Fan et al., 2006). At present, the need for more practical and efficient ways to produce H_2 is a growing concern among the research community (Hawkes et al., 2002; Logan, 2004). Considering the abundance of municipal and industrial effluents containing useable organics together with the availability of renewable agriculture residues, microbial H_2 production is thought to be an ideal approach for generating renewable energy supplies..

Hydrogen (H_2) can be produced by direct bio-photolysis, indirect bio-photolysis, photo fermentation and dark fermentation (Kovacs et al., 2006; Levin and Chahine, 2010; Manish and Banerjee, 2008). Fermentative H_2 production has garnered considerable interest over the last couple of decades because yields are generally greater than other biological methods (Hallenbeck and Benemann, 2002; Levin, 2004; Mathews and Wang, 2009). Moreover, it has the advantage of using a wide range of feed stocks as substrate, such as lignocellulosic or starch materials, glycerol, food and dairy wastes, crop residues, palm oil mill effluent, among others (Guo et al., 2010; Prasertsan et al., 2009; Wang and Wan, 2009). In dark fermentation, complex organic matter is converted into simpler substances, such as volatile fatty acids (VFAs) (e.g. acetic acid, propionic acid, formic acid, lactic acid and butyric acid), alcohols (e.g. ethanol, iso-propanol, and butanol), H_2, methane and carbon dioxide (Hallenbeck and Ghosh, 2009; Li et al., 2007; Sterling Jr. MC., 2001; Valdez-Vazquez and Poggi-Varaldo, 2009). The proper identification of the VFAs and alcohols produced during the fermentation process provides pertinent

information regarding the metabolic pathways followed by the microorganisms. From an engineering perspective, employing mixed cultures to generate H_2 is preferred because using organic waste as feedstock is cost effective and also ease of process control (Das and Veziroglu, 2001; Fan et al., 2008; Fang et al., 2006; Lay et al., 1999; Liu and Shen, 2004).

The H_2 yield obtained by dark fermentation is less than maximal because the H_2 intermediate product is converted into acetic acid, methane, propionic acid and ethanol by H_2-consuming bacteria (HCB). To increase the H_2 yield, decoupling the syntrophic relationship between H_2 consumers and H_2 producers will enrich the H_2-producing bacteria (HPB). Earlier published reports showed that microorganisms of the genus *Clostridium* are responsible for H_2 generation during dark fermentation (Chang et al., 2008; Hung et al., 2008; Skonieczny and Yargeau, 2009).

Hydrogenases are the principal enzymes responsible for molecular H_2 metabolism (Das et al., 2006; Vignais et al., 2001; Voordouw, 1992). Hydrogenases are classified as follows based on the type of metal ion present at their active site: [Ni-Fe] hydrogenases, which contain a nickel (Ni) ion at their active site, [Fe-Fe] hydrogenases, which contain an iron (Fe) ion at their active site and [Fe] hydrogenases or metal-free hydrogenases. [Fe-Fe] hydrogenases which are mainly present in HPB, such as *Clostridium*, are responsible for H_2 evolution. [Ni-Fe] hydrogenases which are mainly present in HCB, such as homoacetogens and methanogens are responsible for much of the H_2 consumption (Meyer, 2007; Peters, 1999; Vignais and Colbeau, 2004). Recently, the level of hydrogenase gene (*hyd*) expression, and its correlation with H_2 production, has been used as an assessment tool (Wang et al., 2008; Wang et al., 2009).

In order to inhibit HCB and enrich HPB, various inoculum pre-treatment methods such as heat, acid, alkali, organic loading shock, 2-bromoethane sulfonate (BES), long chain fatty acids (LCFAs), ultrasonication, and chloroform have been studied (Ray et al., 2010; Wang and Wan, 2008; Wang and Wan, 2009). Many HPB have the ability to generate spores under unfavorable conditions in contrast to many HCBs. All of the pre-treatment methods mentioned above have been shown to enrich HPB, although there are controversies regarding which method is most effective. The lack of consensus is likely

due to the wide variety of operational conditions (e.g. temperature and pH), substrate concentrations and types of inocula employed. Many studies of inoculum pre-treatment have reported using single batch methods (Mu et al., 2007; Wang and Wan, 2008). Over the course of long-term or continuous operation, the pre-treatment effects on a H_2 producing culture will likely differ from those observed in single batch cultivations. According to Luo et al. (2010), the impact of different pre-treatment methods, based on cultures fermenting cassava stillage to H_2, was different in batch and continuous reactors. These researchers observed that the differences in the H_2 yields were reduced after an initial operational period in continuous experiments using cultures treated with chloroform, base, acid, heat and loading-shock. The data reported by Luo et al. (2010) indicate that after pretreatment, differences in H_2 production were minimized over an initial short-term period. Their claim that similar performances in the continuous fermentative H_2 production studies could be attributed to similar dominant microbial community was not supported by microbial characterization studies.

Table 6.1 Role of homoacetogenesis in dark fermentative H_2 production.

Inoculum type	pH	Temp (°C)	Substrate	Type of pre-treatment	H_2 loss due to homoacetogenesis[3]	Reference
Municipal wastewater	6.2	25±1	Glucose	Heat	33%	Oh et al. (2003)
Municipal wastewater	6.2	24	Glucose	Heat	43%	Park et al. (2005)
Municipal wastewater	7.5[1]	35	Glucose	Heat	24.3%	Dinamarca et al. (2011)
Activated sludge	5.5	37	Glucose	Acid Heat Loading shock	56% 43% 36%	Luo et al. (2011)
Municipal wastewater	5.3	35	Starch	Heat	57%	Arooj et al. (2008)
Digested sewage sludge	5.2	35	Wheat starch	Heat	32%	Hussy et al. (2003)

Notes:
1. Average pH value
2. Average % electron equivalents from H_2 diverted to acetate
3. Percent based on total H_2 produced

Inoculum pre-treatment is mainly used to inhibit methanogenic archea (non-spore-forming bacteria) (Mu et al., 2007; O-Thong et al., 2009). However, very little is known about the effects of these methods on other major HCB such as homoacetogenic bacteria.

Under conditions suppressing methanogenesis, H_2 oxidation proceeds via homoacetogenesis (Siriwongrungson et al., 2007) or sulfidogenesis if sulfate is present in the feed (Mueller and Nielsen, 1996). The impact of homoacetogenesis (% H_2 loss) on mixed anaerobic cultures (in pre-treated and untreated cultures) is shown in Table 6.1. According to Oh et al. (2003), a large fraction of H_2 produced from glucose was consumed by the homoacetogens after 30 h and none was undetected after 80 h. Very few published reports have described the effects of pre-treatment of the inocula on homoacetogens. Hence, the objectives of this study are as follows:

1. Investigate the long-term effects of various pre-treatments (i.e., acid, alkali, heat, BES, linoleic acid (LA) and loading shock) on H_2 production under mesophilic (37 °C) and thermophilic (55 °C) temperatures;

2. Evaluate the impact of various pre-treatments and successive glucose feedings on H_2 consumers under mesophilic (37 °C) and thermophilic (55 °C) temperatures;

3. Assess H_2 metabolism under different pre-treatment conditions using hydrogenase activity assays;

4. Perform flux balance analyses (FBA) to elucidate the internal metabolic fluxes over long-term H_2 production (25 days); and

5. Use T-RFLP to examine the microbial community structure under different pretreatment and growth conditions.

6.2. Materials and Methods

6.2.1 Inoculum Source

Experiments were conducted using a mixed anaerobic inoculum (the effluent from a brewery's wastewater facility as described in section 3.2). The culture was refrigerated at 4 °C in a cold room. The inoculum had a volatile suspended solids (VSS) concentration

of approximately 40,000 mg L^{-1}. Two mother reactors were maintained at 37 °C (Reactor A) and 55 °C (Reactor B) with a working VSS concentration of 10,000 mg L^{-1}. The reactors were fed glucose (5 g L^{-1}) every 7 days and the gas production was monitored daily. The cultures served as the inoculum source for the work described in this chapter. Before pretreatment was administered, the VSS concentration of the inoculum was adjusted to 2,000 mg L^{-1}. The following stressors (heat shock, acid, alkali, loading shock, 2-bromo ethane sulfonate (BES) (Sigma Aldrich, ON), and linoleic acid (C18:2 (LA)) (TCI America, OR)) were used to pre-treat the inoculum according to the procedures outlined in Table 6.2.

Table 6.2 Methodology for administration of different pretreatment.

Pretreatment type	Methodology
Heat	Heat the inoculum at 100 °C for 30 minutes
Acid	Adjust the pH value to 3.0 using 3 N HCl and incubate for 24 hours
Alkali	Adjust the pH value to 12.0 using 3 M KOH and incubate for 24 hours
BES	Add 50 mM BES and incubate for 24 hours
LA	Add 2 g L^{-1} linoleic acid and incubate for 24 hours
LS	Add 20 g L^{-1} glucose and incubate for 24 hours

Note: BES = 2-bromoethanesulfonic acid; LA = linoleic acid; LS = loading shock

6.2.2 Experimental Procedures

The long-term effects of pretreatments involving different stressing agents on H_2 production and consumption were assessed in 160 mL batch reactors with a working volume of 75 mL. The final VSS concentration in the batch reactors was 2000 mg L^{-1}. All the experimental runs were conducted in triplicate. The protocol followed in the preparation of the batch reactors (160 mL) is described in Section 3.3. The experimental design matrix is shown in Table 6.3. The analytical methods for analyzing gas by-products (H_2, CH_4 and CO_2) and liquid by-products (VFAs) accord with the protocols described in Sections 3.7.1 and 3.7.2. All experiments were conducted at an initial pH of 5.5.

Untreated inoculum (without the application of a chemical inhibitor) was used as the positive control. Glucose (5 g L^{-1}) was added to the mesophilic (37 °C) and thermophilic (55 °C) cultures 24 h (batch 1 after applying the pretreatment condition to enrich the H_2 producing population. Hydrogen production was monitored for 4 days (96 h) after feeding glucose. At the end of the 1st glucose addition, the pH in the batch reactor was measured. The VSS concentration at the end each glucose feeding (at day 5, 10, 15, 20 and 25) was measured using the optical density (OD) versus VSS curve (Appendix A).

Table 6.3 Experimental design matrix to study the long-term effects of various pretreatments on H_2 production and consumption.

Expt. No.[4]	Exp. Set	Pre-treatment	Incubation at 37 °C and 55 °C for 24 hours	Substrate (glucose) addition after 24 hours[3]	Metabolite analysis	Hydrogenase assay
1	Control (C)	X	√	√	Gas (H_2, CH_4, CO_2) every 24 hrs until 96 hrs Liquid (VFAs, Alcohols, Glucose) at 96 hrs	Evolution and uptake assay (during H_2 consumption study) after 24 h
2	Heat (H)	√	√	√		
3	Acid (A)	√	√	√		
4	Alkali (K)	√	√	√		
5	BES[1]	√	√	√		
6	LA[2]	√	√	√		
7	Loading shock (LS)	√	√	√		

Note: 1. BES = 2-bromoethanesulfonic acid; 2. LA = linoleic acid; 3. Repeated glucose feedings. Fed glucose 5 times every 5 days. Culture washed; 4. All experiments were conducted in triplicate.

Before beginning the second and subsequent glucose feedings, the microbial culture was washed to remove VFAs which were produced during the previous glucose addition. The culture-washing procedure was as follows: 1. De-pressurize the batch reactors by inserting a needle into the rubber septa and place them in an anaerobic glove box (COY Laboratory Products Inc., Grass Lake, MI). 2. Open the batch reactors and transfer the contents from each triplicate set into a 300 mL glass beaker. 3. Decant the supernatant and add fresh basal medium to a final volume of 40 mL. 4. Transfer the liquid into a 50 mL centrifuge tube with a screw cap. 5. Centrifuge (Beckman Coulter, Inc, USA) at 3000

rpm for 10 minutes and decant the liquid. Resuspend the pellet in fresh basal medium by vortexing. After repeating the washing, centrifugation and resuspension (designated as C1, C2, C3, C4 and C5) procedure for cultures fed glucose, the pH and VSS level was determined. After step C5, the control cultures were fed H_2 (3000 µmol) (designated as C6).The pretreated cultures were fed glucose and washed repetitively in the same manner as the controls after pretreatment with various stressing agents. After the 5^{th} glucose feeding period, washing and resuspending the culture in fresh basal media, all the pretreated cultures were fed 3000 µmol H_2. The H_2 levels were monitored periodically for 96 h. The VSS at the end of each batch was measured using OD versus VSS concentration curve (Appendix Figure A.10). Byproducts and pH were also determined at the end of all the experiments. The nomenclature used to designate the control and pretreated cultures over the different glucose and H_2 feeding period were based on the acronyms used in Table 6.3 for the various pretreatment methods (C, H, A, K, LA and LS) and the number of glucose and H_2 feeding periods (1-5 (glucose feed) and 6 (H_2 feed)).

6.2.3 Statistical analyses

A principal component analysis (PCA) was performed using PAST software (Hammer et al., 2001) to assess the long-term effects of pretreatment with different stressors (i.e., heat, acid, alkali, loading shock, BES and LA) on the fermentation metabolite patterns compared with untreated controls. The quantities of acetic acid, propionic acid, butyric acid, ethanol, H_2 and CH_4 produced during glucose degradation were used as the input data for the analysis (PCA). The Tukey's pair-wise comparisons ($\alpha = 0.05$) was used to compare the relative effectiveness of the pretreatments. Flux balance analysis (FBA) was used to study the electron flux diverted to homoacetogens and methanogens. FBA was conducted according to the methods described in section 3.11.

6.2.4 Hydrogenase assay

The hydrogenase uptake and evolution assays for the cultures pretreated with different stressing agents were performed according to the protocols described in sections 3.9.1

and 3.9.2. The evolution hydrogenase assay and uptake hydrogenase assay was performed for the long term stress treated cultures (after feeding H_2).

6.2.5 Molecular biology techniques

The composition of the microbial community following pretreatment of mixed culture with different stressors was analyzed using the protocols previously described in section 3.10. The microbial samples were collected at the end of day 4 for each batch. The cultures collected after feeding period 5 (C5, H5, A5, K5, LA5 and LS5) and period feed 6 (C6, H6, A6, K6, LA6 and LS6) were considered in the microbial analysis. The Bray-Curtis similarity index was used to construct the dendrogram based on the similarities in the microbial community composition for the bacterial and archaeal *Hha* I enzyme digests. This similarity matrix compares the percent similarity of each T-RF pattern compared to the other T-RF patterns obtained under different pretreatment conditions.

6.3. Results and discussion

6.3.1 Repeated glucose feedings after pretreatment

After pretreating the cultures, 5 successive glucose feedings over 25 days was implemented to selectively increase the H_2 producing populations. Repeated culture cultivation has been utilized to increase the H_2 yield by enriching the H_2 producing population. This method has been used to increase microbial productivity by changing the fermentation medium at regular intervals (Duangmanee et al., 2007). Similar methodology of repeated batch cultivation has been reported in previous studies (Liu et al., 2008; Luo et al., 2011; Radmann et al., 2007; Yokoi et al., 2002; Yokoyama et al., 2007).

6.3.2 Hydrogen (H_2) and methane (CH_4) production

At mesophilic (37 °C) temperatures, stressor pretreatment showed an average H_2 yield of 1.89±0.19 mol mol^{-1}glucose after adding the 5^{th} glucose feed. In comparison, control (untreated) cultures exhibited lower H_2 yields than the pretreated cultures at the end of the 5^{th} glucose feeding period (C5). Control cultures after period C5 showed an initial H_2 yield of 1.35±0.27 mol mol^{-1} glucose (24 h after adding glucose). However, the H_2

produced during the first 24 h of at the C5 period was depleted immediately. After 48 h, H_2 levels in the untreated control cultures were undetectable. In comparison, the impact of different types of pretreatment methods revealed that cultures treated with linoleic acid (LA), loading shock (LS), acid (A), or heat (H) exhibited similar H_2 yields which were significantly higher than the yield from untreated control culture. For pretreated cultures operating at 37 °C and fed glucose during the 5th period, the H_2 yields were statistically the same (Table 6.4; Tukey's test, $\alpha = 0.05$). In all the cultures fed with glucose during the 1st period (controls and those pretreated), the H_2 yields at 37 °C were statistically different (Table 6.4; Tukey's test, $\alpha = 0.05$).

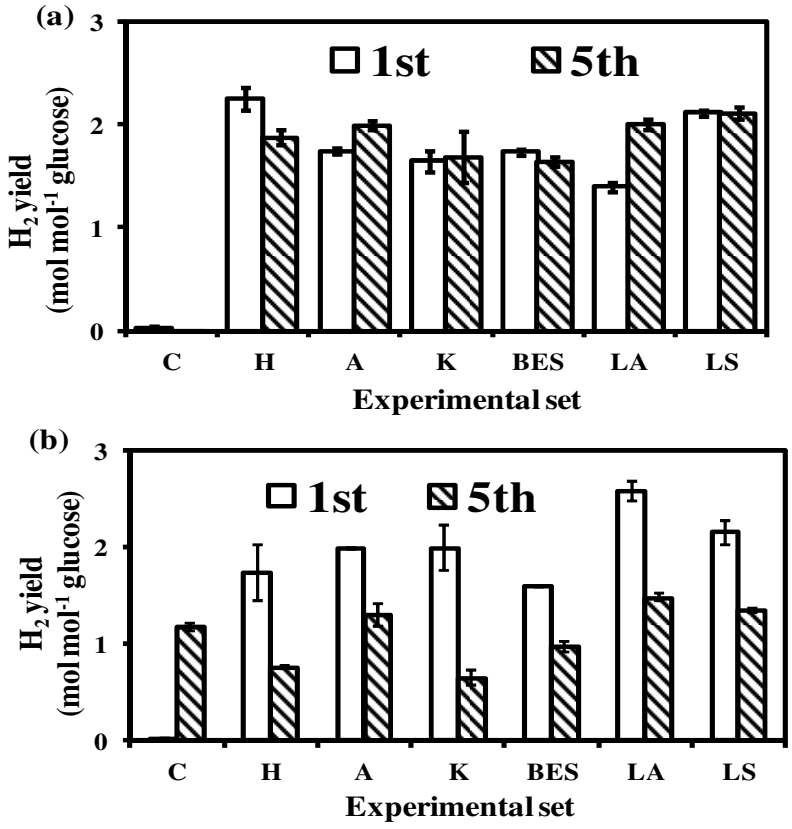

Figure 6.1 H_2 yields for pretreated cultures after 1st and 5th glucose feedings (a) mesophilic (M) (37 °C); and (b) thermophilic (T) (55 °C) temperatures.
Note: C = control; H = heat; A = acid; K = alkali; BES = 2-bromoethanesulfonate; LA = linoleic acid and LS = loading shock; the error bars shown in this figure represents the standard deviation for n = 3.

No methane (CH_4) was observed in the pre-treated cultures in all five glucose feed periods except for the LS treatment which showed lower CH_4 yields < 0.04 mol mol^{-1} glucose at both mesophilic (feeding period 1 to feeding period 4) (B1-B4) and at thermophilic (glucose feed period 1) temperatures, respectively (Appendix Table C.3 and C.4). These results indicate that these pre-treatments were very effective at selectively enriching spore-forming H_2 producers, such as *Clostridium* sp., at mesophilic temperatures and were capable of inhibiting the methanogens in these cultures over the course of the longer time periods examined in this study. The average H_2 yields obtained from cultures pretreated with different stressors and incubated at thermophilic temperatures (55 °C) were lower than those obtained from maintained at mesophilic temperatures (Table 6.4). The H_2 yields observed after the 5^{th} glucose feeding period for the controls and pre-treated cultures were statistically the same (Tukey's test, $\alpha = 0.05$). The largest H_2 yield observed after the 1^{st} glucose feeding under thermophilic conditions was 2.58±0.1 mol mol^{-1} glucose (Figure 6.1b) in the LA pre-treated culture. Methane production of 0.72±0.1 mol mol^{-1} glucose (Appendix Table C.4) was detected after the 1^{st} glucose feed in control cultures at thermophilic temperatures. Over successive glucose feeds, the H_2 yields decreased below the levels observed after the 1^{st} feed. In addition, no CH_4 was detected in the untreated thermophilic control cultures after the 1^{st} glucose feed. These observations indicate that the pre-treatments administered were not effective at thermophilic operational temperatures by repeated batch cultivations. The results obtained from this study are similar to those of Valdez-Vazquez and Poggi-Varaldo (2009), who obtained higher levels of H_2 production from cultures pretreated with heat shock and incubated at mesophilic temperatures (37 °C) compared to those maintained at thermophilic temperatures (55 °C). In general, the H_2 yields obtained over the course of this study are comparable to the yields reported previously by other researchers (Table 6.5). The lower hydrogen yields observed in pre-treated cultures maintained at thermophilic temperatures shows the temperature is a major factor in maximizing H_2 production. According to Gibbs (1967), heat shock pretreatment is able to selectively enrich the spore-forming *Clostridium* sp. Incubation temperature is the major factor affecting the growth rate of *Clostridium* sp. (Stringer et al., 1997).

Table 6.4: Comparison and ranking of pre-treatment effects on H_2 yields.

Expt.	Pre-treatment methods [a]	Substrate	pH	Temp. (°C)	H_2 yield (mol mol^{-1} glucose)[1, 3]	Statistical analysis of treatment methods [2]
1	A, K, H, BESA, LA, LS	Glucose	5.5	(37 °C)	2.25±0.11	H^a, LS^a, A^b, $BES^{b, c}$, K^c, LA^d, C^e
2					2.49±0.10	LA^a, H^b, $K^{b, c}$, A^c, $LS^{c, d}$, BES^d, C^e
3					2.26±0.15	LA^a, H^a, LS^b, $A^{b, c}$, K^c, C^d, $BES^{d, e}$
4					1.93±0.01	A^a, LS^a, H^a, LA^b, $BES^{b, c}$, C^d
5					2.12±0.06	LS^a, A^a, H^a, LA^a, K^a, BES^a, C^b
1	A, K, H, BESA, LA, LS	Glucose	5.5	(55 °C)	2.58±0.10	LA^a, LS^b, $K^{a, b}$, A^b, $H^{b, c}$, BES^c, C^d
2					2.23±0.12	BES^a, K^a, C^b, A^c, $H^{c, d}$, LA^e, LS^f
3					1.79±0.15	K^a, BES^b, $LS^{b, c}$, A^c, H^c, C^c, LA^d
4					1.65±0.12	A^a, LA^a, H^a, $LS^{a, b}$, K^b, $C^{b, c}$, BES^d
5					1.48±0.04	LA^a, LS^a, A^a, C^a, BES^a, H^a, K^a

Notes: [a]denotes: A = Acid; K = Alkali; BES = 2-Bromo ethane Sulfonic acid; LA = Linoleic acid; LS = Loading Shock; H = Heat; C = control. [1]Average of triplicate values is shown. [2]Statistically analyzed using Tukey's test at α value = 0.05, superscripts for treatments in the same cell with the same letters are statistically the same. [3]Maximum H_2 yield obtained at the end of each batch is shown.

Table 6.5 Comparison of reported H_2 yields obtained after repeated batch cultivations.

S.No.	Inoculum type	Temp. (°C)	pH	No. of repeated batches	Type of inoculum pretreatment	Maximum H_2 yield	Reference
1	Household solid waste	70	7.0	7	No	2.38 mol mol^{-1} glucose	Lu et al. (2012)
2	Sewage sludge	35±1	5.0-9.0	3	heat	2.00- 2.30 mol mol^{-1} glucose	Han et al. (2003)
3	Municipal sludge	37	5.5	7	Repeated heat	1.52 mol mol^{-1} sucrose	Duangmanee et al. (2007)
4	Cow manure	75	7.3	7	No	2.65 mol mol^{-1} glucose	Yokoyama et al. (2007)
5	Household solid waste	70	NA	15	No	101.7±9.1 mL g^{-1} VS_{added}	Liu et al. (2008)
6	Mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* HO-39	37	5.3	13	No	2.70 mol mol^{-1} glucose	Yokoi et al. (2002)
7	Digested sludge	37, 55	5.5	5	heat, acid, alkali, BES, linoleic acid, loading shock	1.68 - 2.12 mol mol^{-1} glucose, 0.65 - 1.48 mol mol^{-1} glucose	*This study*

6.3.3 Hydrogen consumption

In order to assess methanogenic and homoacetogenic activity after long-term operation following pretreatment, a H_2 consumption study was conducted using mesophilic or thermophilic cultures after 5 glucose feeding periods. In general, all the cultures incubated at thermophilic temperatures showed lower H_2 consumption rates than cultures incubated at mesophilic temperatures with the consumption level varying greatly for cultures subjected to different pretreatments and incubated at mesophilic temperatures. The results indicate that pre-treatment with LS was the most effective method for inhibiting H_2 consumers. Cultures pretreated with LS and incubated at mesophilic temperature showed 39±1 % H_2 consumption (Figure 6.2). LA treatment was the second most effective pretreatment (62±3 % H_2 consumed) method against H_2 consumers. In contrast, in the untreated controls cultures at mesophilic temperature, all the H_2 was consumed (100% H_2 consumption). Overall, the minimum H_2 consumption (19±2 %) was observed in cultures pre-treated with heat and incubated at thermophilic temperatures. For all of the other types of pre-treatment, approximately 35 % of the injected H_2 was consumed after 96 h.

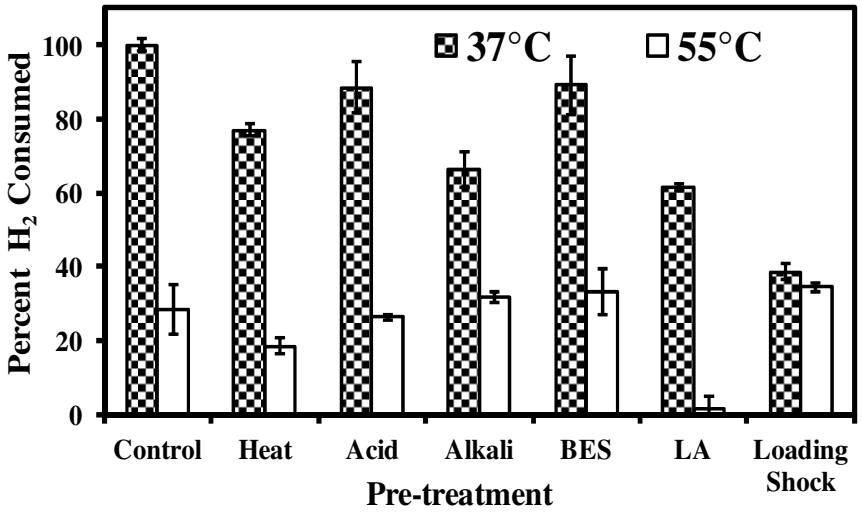

Figure 6.2 Percent H_2 consumed by pretreated cultures subjected to 5 glucose feedings (25 days). Note: BES = 2-bromoethanesulfonic acid; LA = linoleic acid**;** the error bars shown in this figure represents the standard deviation for n = 3.

Methane was produced in the mesophilic cultures (Appendix C; Table C.5). In contrast, no CH_4 was produced in the thermophilic cultures. Acetate production was observed in cultures fed H_2 plus CO_2 and incubated at either mesophilic or thermophilic temperatures (Appendix C; Table C.5). Acetate production from H_2 and CO_2 is a strong indication of homoacetogenic activity.

6.3.4 Effect of unionized volatile fatty acids on hydrogen production

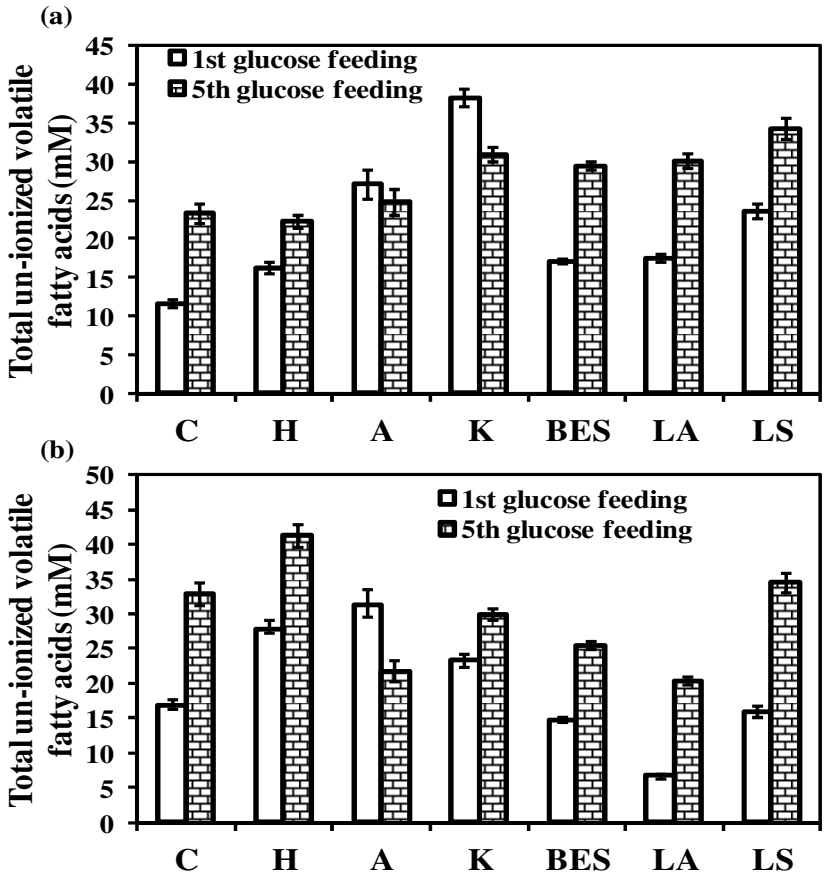

Figure 6.3 Comparison of unionized volatile fatty acids concentration for the 1st and 5th glucose feeding. (a) Cultures maintained at 37 °C; (b) Cultures maintained at 55 °C.

Note: C = control; H = heat; A = acid; K = alkali; BES = 2-bromoethanesulfonic acid; LA = linoleic acid; LS = loading shock; the error bars shown in this figure represents the standard deviation for n = 3.

The concentration of unionized (undissociated or free) acids present in a fermentation medium will exert a greater inhibitory effect on microorganisms in the inocula over successive batches. Undissociated acids traverse the cell membrane at low pH and dissociate after entering the cell, thus altering the internal pH. The influx of undissociated acids results in acidification, and subsequent dissociation inside the cytoplasmic membrane decreases the internal pH. This increase in H^+ concentration is effluxed out using ATP. In general, the internal pH gradient (ΔpH) collapses as soon as the energy for H^+ extrusion decreases (Gatje and Gottschalk, 1991). The increase in the H^+ ion concentration inside the cell disrupts the proton pump resulting in alteration of the membrane potential and intracellular functions (Jones and Woods, 1986).

The undissociated acid concentration was calculated using the following relationships:

$$Ka = [H^+]*[A^-]/ [HA] \quad \text{Eq. 6.1}$$

$$Ka = 10^{-pKa} \quad \text{Eq. 6.2}$$

$$[H+] = 10^{-pH} \quad \text{Eq. 6.3}$$

Upon rearrangement,

$[HA]/ [A^-]$ = unionized acid/ ionized acid = $10^{-pH}/10^{-pKa}$.

The VFAs concentration is a good indicator of the operational stability of anaerobic digesters. The extent of inhibition imposed by VFAs on specific microbial populations is variable and depends on the type and concentration for individual VFAs (Stafford, 1982). The undissociated VFAs level at threshold levels are inhibitory to acidogens and methanogens (Moletta et al., 1986). The undissociated acetic acid accounted for nearly 50 % of the total undissociated VFA concentration in cultures fed once with glucose and the same culture fed after the 5^{th} glucose feeding period and maintained at 37 °C and 55 °C. When considering the number of glucose feeds (1 to 5) to the same mesophilic culture, elevated H_2 yields in the pretreated cultures (except heat treatment) was coupled with an increase in the total undissociated acid concentration after the 5^{th} glucose feeding period when compared to the 1^{st} feeding.

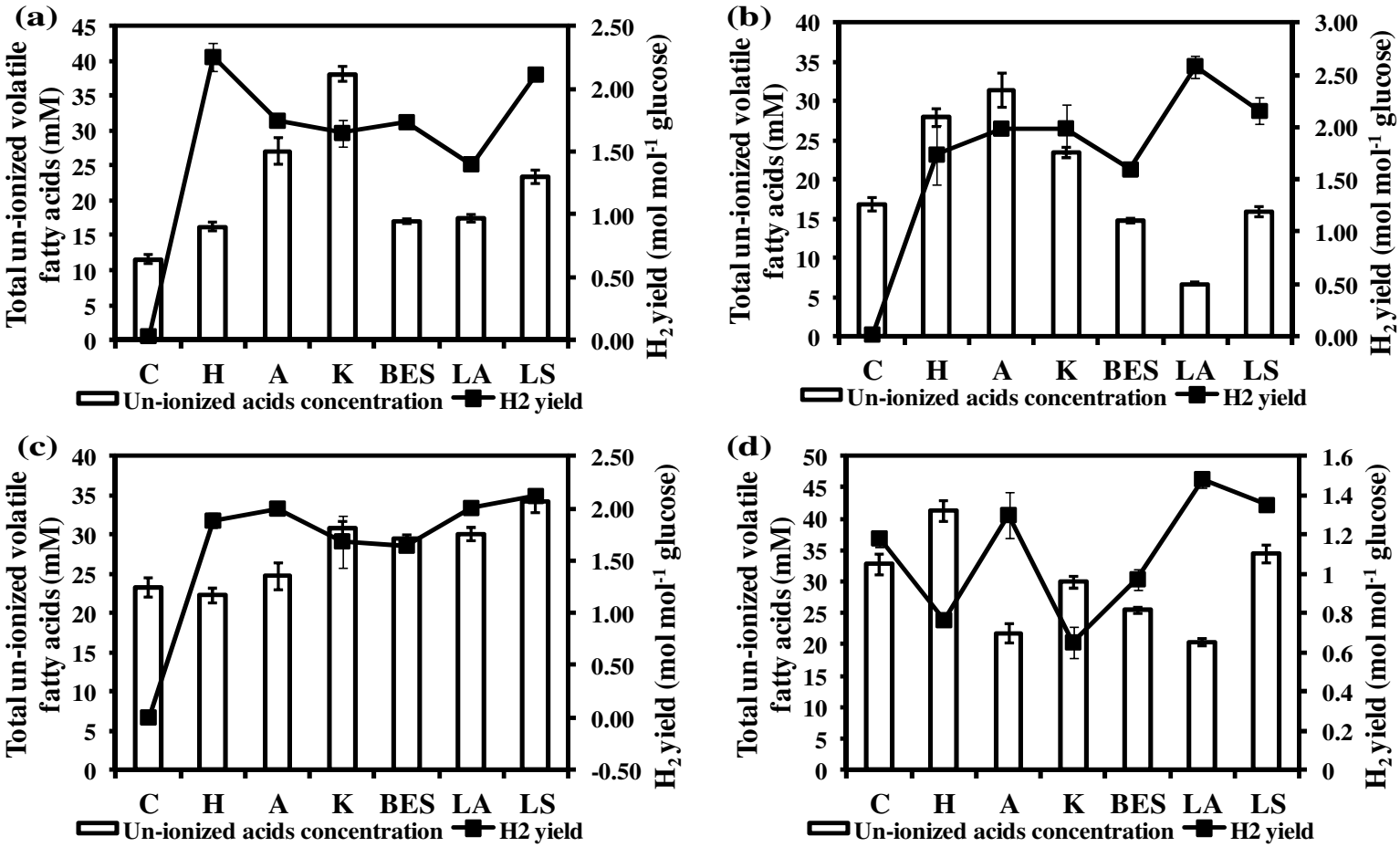

Figure 6.4 Comparison of unionized volatile fatty acids concentrations and H_2 yields for the 1st and 5th glucose feeding. **(a) Cultures at 37 °C and receiving the 1st glucose feeding; (b) Cultures at 55 °C and receiving the 1st glucose feeding; (c) Cultures at 37 °C and receiving the 5th glucose feeding; (d) Cultures at 55 °C and receiving the 5th glucose feeding.** Note: the error bars shown in this figure represents the standard deviation for n = 3.

In contrast to these findings, decreasing H_2 yields were detected in the pretreated thermophilic cultures after the 5th glucose cultivation period. These results (Figure 6.4 (b and d)) indicate that incubation at thermophilic temperatures, along with an increase in undissociated acid concentration, likely affected H_2 production in cultures pretreated with different stressing agents. In all control cultures (untreated) fed 5 times with glucose, the increase in the H_2 yield might be due to increasing levels of total undissociated VFA levels from 16±0.8 mM to 32±1.6 mM. The inhibitory effect exerted by these acids on H_2 consumers likely resulted in higher H_2 production. According to Van Ginkel and Logan (2005) an undissociated acid concentration of 60 mM is inhibitory to H_2 producers. van den Heuvel et al. (1992) conducted a study to determine the inhibitory concentrations of self-produced acids in repeated batch cultivations using glucose as substrate, and concluded that 48 mM undissociated butyric acid concentration was inhibitory to acidogenesis at mesophilic temperatures. In the current study the undissociated acid concentrations resulting from repeated batch cultivation were less than the threshold inhibitory values reported in the literature and H_2 production was unaffected under mesophilic conditions. The threshold inhibitory undissociated VFA concentrations reported for specific acids in mixed anaerobic cultures are 50-70 mM lactic acid (Gatje and Gottschalk, 1991), 40-50 mM acetic acid and 40-50 mM butyric acids (Monot et al., 1984; Terracciano and Kashket, 1986; Wang and Wang, 1984).

A metabolic shift to solventogenesis did not occur even though the concentration of undissociated VFAs was above 20 mM. However, as excess glucose is required to initiate solventogenesis, this expected shift to solvent production would not have occurred. Studies conducted by Van Ginkel and Logan (2005) have shown that at lower pH levels of 4.5 with an undissociated VFA concentration above 15 mM, solvent production was not observed. Moreover, the undissociated butyric acid concentration was much less than the concentration of acetic acid. Butyric acid is able to exert a stronger inhibitory effect on acetone-butanol-ethanol (ABE) fermentation when compared to acetic acid (Ezeji et al., 2004). Husemann and Papoutsakis (1988) concluded that undissociated butyric acid is correlated with solvent production more than acetic acid. The reason for the influence of undissociated butyric acid on solvent production initiation is not clear. However, Husemann and Papoutsakis (1988) hypothesized that acetic acid

production regenerates NAD^+ via H_2 production, whereas butyric acid production regenerates NAD^+ by H_2 production and also by butyryl-phosphate reduction. If the butyric acid concentration increases in the medium subsequent inhibition of H_2 production and excess dissolved H_2 can only be disposed of by increasing solvent production. Although there are many contradicting reports in the literature, the H_2 yield for the cultures incubated at thermophilic temperature were affected by increasing unionized VFA levels in cultures repeatedly fed with glucose 5 times when compared to those receiving 1 glucose fed. Also, the inhibitory effect varies with the incubation temperature and the type of stress treatment employed.

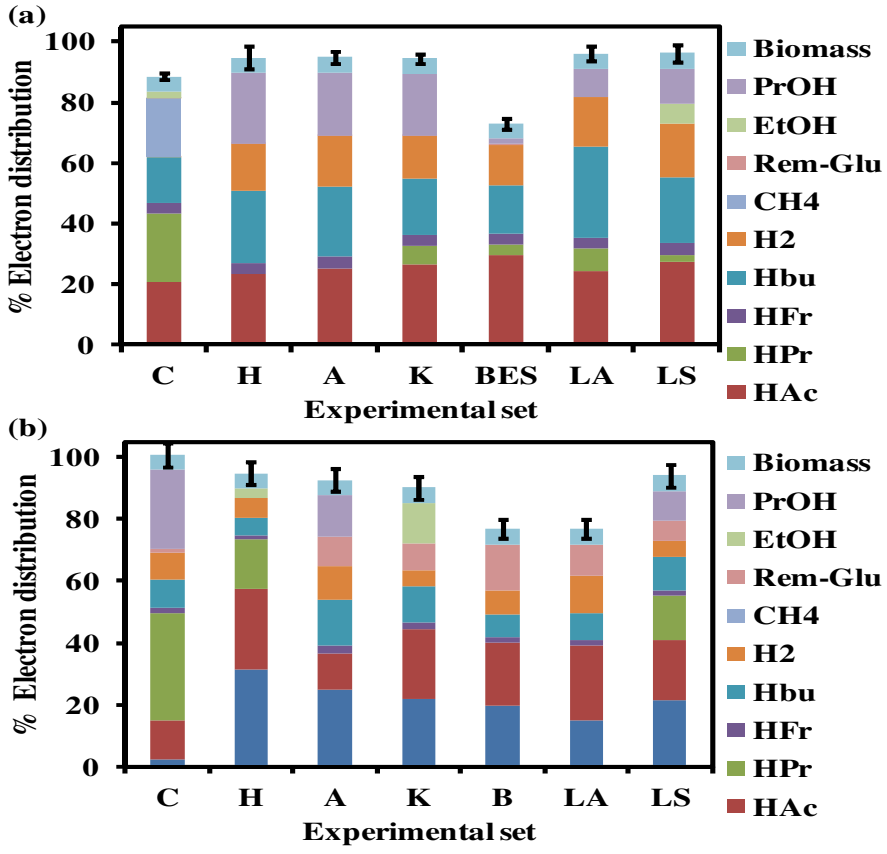

Figure 6.5 Percent electron equivalent distributed among the fermentation metabolites for pretreated cultures repeatedly fed with glucose 5 times. (a) 37 °C; (b) 55 °C. Notes: 1. C = control; H = heat; A = acid; K = alkali; B = 2-bromoethanesulfonate; LA = linoleic acid; LS = loading shock. 2. PrOH = propanol; EtOH = ethanol; CH_4 = methane; H_2 = hydrogen; HBu = butyric acid; HFr = formic acid; HPr = propionic acid; HAc = acetic acid. 3. The error bars shown in this figure represents the standard deviation for n = 3.

6.3.5 Electron balance

An analysis of liquid metabolites for long term stress treated (repeated glucose feeding 5 times) cultures showed different metabolic profiles for cultures incubated at mesophilic or thermophilic temperatures (Figure 6.5a and b). No lactic acid (HLa) was produced in cultures incubated at mesophilic temperatures (Figure 6.5a), whereas greater than 15 % of the derived electrons from glucose were diverted towards lactic acid production in all of the pre-treated cultures, except for the untreated control cultures incubated at thermophilic temperatures (Figure 6.5b). Greater than 40 % of the electrons were diverted towards acetic acid and butyric acid production in the cultures incubated at mesophilic temperatures. Ethanol production was observed only in the untreated control and the LS-treated cultures incubated at mesophilic temperatures. Control cultures incubated at thermophilic temperatures showed higher levels of propionic acid (HPr) production (45 % of electrons from glucose were diverted to HPr).

6.3.6 Hydrogenase activity

Evolution hydrogenase activity (EHA) for all of the long term pre-treated cultures, except for the culture stressed by alkali treatment, showed lower activities at thermophilic temperature compared to the cultures incubated at mesophilic temperatures (Figure 6.6). Alkali pre-treated cultures, however, showed an increase of approximately 120 % in their EHA at 55 °C when compared to 37 °C. The alkali pre-treated cultures exhibited the highest level of EHA of 96 ± 1 μL H_2 evolved mg^{-1} VSS h^{-1} (Figure 6.6). Decreasing EHA activity at 55 °C was detected in all the pretreated cultures including the untreated control cultures. Control cultures incubated at thermophilic temperatures showed 50 % relative decrease in EHA compared to the control cultures at 37 °C. A possible reason for the reduction observed in EHA at 55 °C is likely due to the VFA concentration produced during fermentation. Because the final pH in the cultures was below 4.0, a shift in fermentation pathway could take place.

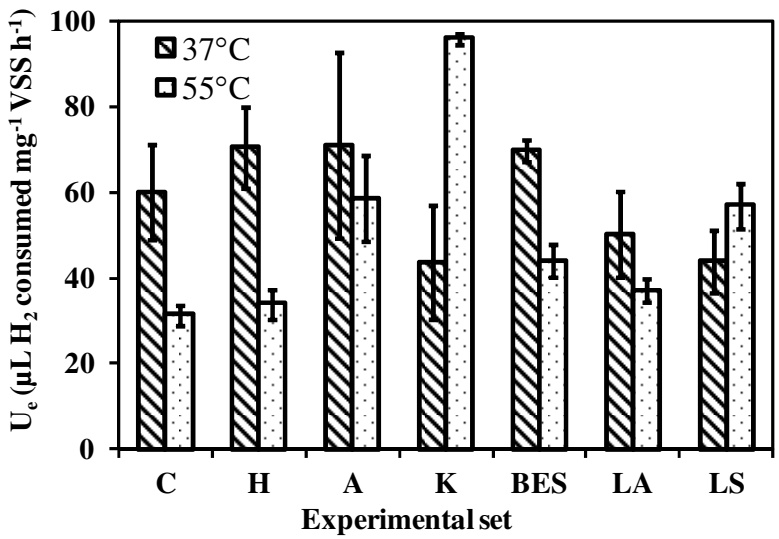

Figure 6.6 Hydrogenase evolution activity levels for long term stress treated cultures pretreated with different stressors and maintained at 37 °C and 55 °C.
Note: C = control; H = heat; A = acid; K = alkali; BES = 2-bromoethanesulfonate; LA = linoleic acid; LS = loading shock; the error bars shown in this figure represents the standard deviation for n = 3.

The hydrogenase activity of whole cells obtained from acid-producing cultures (pH 5.8) was approximately 2.2 times greater than that of solvent-producing cultures maintained at pH 4.5 (Andersch et al., 1983; Kim and Zeikus, 1985). Furthermore, solvent-producing cultures exhibited hydrogenase activity only after a lag in the growth phase of approximately 10 to 15 minutes. However, a study conducted to examine the influence of pH and VFA concentration on *in vivo* hydrogenase activity revealed that neither of these factors affected hydrogenase activity (Kim and Zeikus, 1985). The study concluded that the decreasing H_2 production observed at low pH values was due to the regulation of hydrogenase enzyme's activation rather than to its inhibition. Studies conducted by Andersch et al. (1983) revealed that both acid and solvent-producing cultures exhibited the same levels of hydrogenase enzyme activity under similar assay conditions.

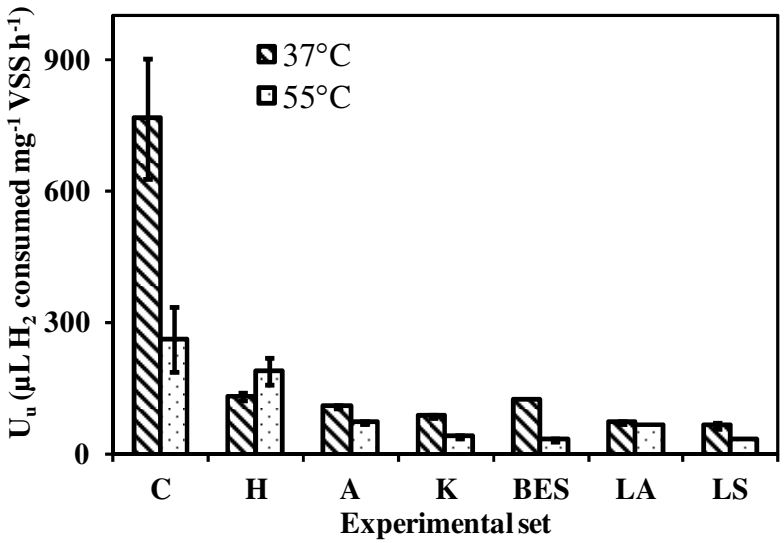

Figure 6.7 Hydrogenase uptake activity of long term stress treated cultures.
Notes: 1. U_u = uptake hydrogenase activity (UHA)
2. BES = 2-bromoethanesulfonic acid; LA = linoleic acid.
3. The error bars shown in this figure represents the standard deviation for n = 3.

The optimum level of hydrogenase enzyme activity was observed at a pH of 8.5, whereas a negligible level of activity was detected in cultures assayed below pH 6. The solvent-producing cultures assayed in the studies conducted by Andersch et al. (1983) were maintained at a pH of 4.5 at which the hydrogenase enzyme was inactive, but was then activated after a lag period, the EHA at pH 5.0 was 10 % lower than that of cells maintained at pH 7.0. Moreira et al. (1982) compared NADPH and NADH for their relative effectiveness at regulating hydrogenase activity (EHA), and the results obtained revealed that NADPH was responsible for only 12 % of the activity produced by NADH. Furthermore, FADH and reduced ferredoxin were unable to replace NADH in its role in regulating EHA. The researchers reported the shift from acid to solvent production coincided with the evolution of molecular H_2 (Moreira et al., 1982).

The uptake hydrogenase activity (UHA) was measured in order to better understand the activities of H_2 consumers (methanogens and homoacetogens) over successive batch glucose feeding. Higher UHAs in the untreated control cultures, incubated at either mesophilic or thermophilic temperatures, was observed when compared to the pre-treated cultures (Figure 6.7). Higher UHA in mesophilic cultures when compared to thermophilic

cultures correlated with increasing H_2 consumption by mesophilic cultures relative to thermophilic cultures (Figure 6.2). Cultures pre-treated with K, BES, LA and LS, and maintained thermophilic temperatures had lower UHA compared to mesophilic cultures. This decrease in UHA may be the reason for lower H_2 consumption under thermophilic conditions (Figure 6.2).

6.3.7 Flux balance analysis

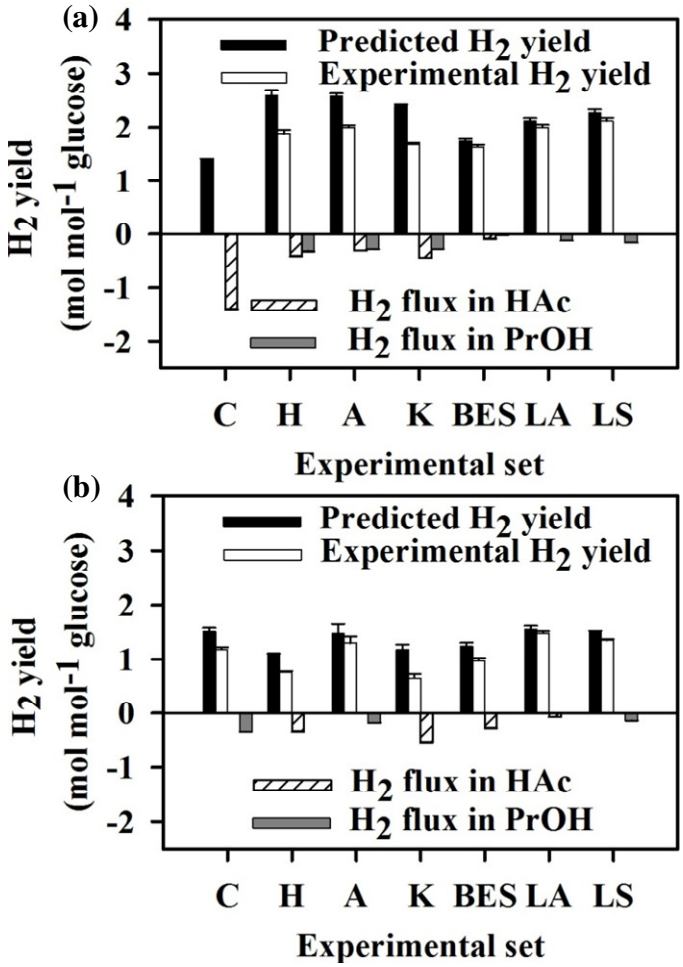

Figure 6.8 Flux balance analysis of fermentation metabolites in long term stress treated cultures (a) 37 °C; (b) 55 °C.

Notes: 1. Predicted H_2 yield was obtained from the flux model based on R12 flux (Table 3.4; Figure 3.3).

(2) C= Control; H= Heat; A= Acid; K= Alkali; BES = 2-Bromoethane sulfonic acid; LA = linoleic acid; LS= loading shock.

(3) H_2 flux in HAc indicates the amount of H_2 consumed via homoacetogenesis (R17 flux) and H_2 flux in PrOH indicates the amount of H_2 consumed for propanol formation (R21 flux).
(4) HAc = acetic acid; PrOH = propanol.
(5) The error bars shown in this figure represents the standard deviation for n= 3**.**

FBA is a tool used to predict the internal electron fluxes based on the assessment of the external fluxes. Fermentation metabolites (both gas and liquid) from 5 times glucose fed cultures were given as the inputs for the FBA. The model predicted H_2 yield (hydrogenase activity) was based on R12 ($2Fd^+ + 2H^+ \rightarrow 2\ Fd^{2+} + H_2$) flux (Table 3.4). The experimental H_2 yield was compared against this model predicted H_2 yield to measure the H_2 consumption activity. The experimental H_2 yield was lower than the model predicted H_2 yield under all experimental conditions. This loss in H_2 yield may due to the formation of acetate (R17) by the activity of homoacetogenic bacteria, valeric acid (R14); caproic acid (R26); Iso-propanol (R21) and CH_4 (R29) by the activity of hydrogenotrophic methanogens. Both valerate and caproate was not detected under the experimental conditions. In addition, CH_4 was not detected in the stress treated cultures. So, the possibility of H_2 loss could be due to acetate and iso-propanol formation. The Gibb's free energy values of both these reactions (Eq. 6.4 and 6.5) indicate that both of these reactions are thermodynamically feasible.

$$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O \quad \Delta G^{o'} = -104.6\ kJ\ mol^{-1} \quad \text{(Eq. 6.4)}$$

$$CH_3COCH_3 + H_2 \rightarrow CH_3CHOHCH_3 \quad \Delta G^{o'} = -24.7\ kJ\ mol^{-1} \quad \text{(Eq. 6.5)}$$

The output from the flux balance analysis indicated that untreated control cultures exhibited both aceticlastic methanogenic activity (data not shown) and homoacetogenic activity for mesophilic cultures (Figure 6.8). Aceticlastic methanogenic activity indicates the amount of CH_4 that was formed using acetate as the substrate. Homoacetogenic activity indicates the amount of acetate that was formed using H_2/CO_2 as the substrate. Interestingly, no hydrogenotrophic methanogenic activity was detected in cultures at 37 °C and 55 °C and fed with glucose 5 times (data not shown). Homoacetogenic activity (R17 flux; Table 3.4) was observed in heat, acid, alkali and BES treated mesophilic cultures. The CH_4 formed in the untreated control cultures is mainly due to the activity of aceticlastic methanogens (R28 flux) in both mesophilic and thermophilic incubated

control cultures. Similarly, homoacetogenic activity was undetectable in both LA and LS treated cultures maintained at mesophilic temperature. This activity may be lost by successive batch cultivations. The decrease in experimental H_2 yield in cultures treated LA and LS and fed with glucose 5 times is due to iso-propanol formation by R21 flux. No homoacetogenic activity was observed in the controls, acid (A) and LS treated thermophilic cultures but a R21 flux (Eq. 6.5) was observed.

6.3.8 Principal component analysis

PCA was conducted using data for fermentation metabolites (both gas and liquid) as input. The metabolites levels determined after conducting experiments after 1st glucose addition and with 5th glucose addition were considered in the analysis. The PCA bi-plots derived using metabolite data for the two glucose feeding conditions and cultures at mesophilic and thermophilic temperatures are shown in Figure 6.9a and 6.9b. The PCA constructed using metabolite data gathered from mesophilic cultures fed glucose 5 times indicates that PC 1 accounted for 44 % of the total variation in the data set while PC 2 accounted for 64 % of the total variation. Cultures repeatedly fed with glucose 5 times are grouped in a separate quadrant away from cultures receiving 1 glucose feed (Figure 6.9a). The stress treated cultures grouped together away from the control cultures indicate difference in the metabolite pattern and the H_2 yield. Similar grouping of the control cultures away from the stress treated cultures were observed in cultures incubated at thermophilic temperature (Figure 6.9b). The loading values for the mesophilic cultures, HLa (0.11), HAc (0.39), HBu (0.27), HFr (0.08), PrOH (0.29) and H_2 (0.46) correlate positively with PC 1 while HLa (0.44), HBu (0.44), CH_4 (0.03), Rem Glu (0.18) and Et-OH (0.59) correlate positively with PC 2. The pattern of the loading values for the thermophilic cultures are different from mesophilic cultures with HBu (0.36), H_2 (0.49), Rem Glu (0.34), Et-OH (0.24) and PrOH (0.35) correlating positively with PC 1 while HPr (0.56), HBu (0.32), CH_4 (0.29), PrOH (0.33) correlating positively with PC 2. These differences in the loading values in the PCA biplots clearly indicate that the fermentation pattern of the microorganisms cultivated at different incubation temperatures showed a clear variation.

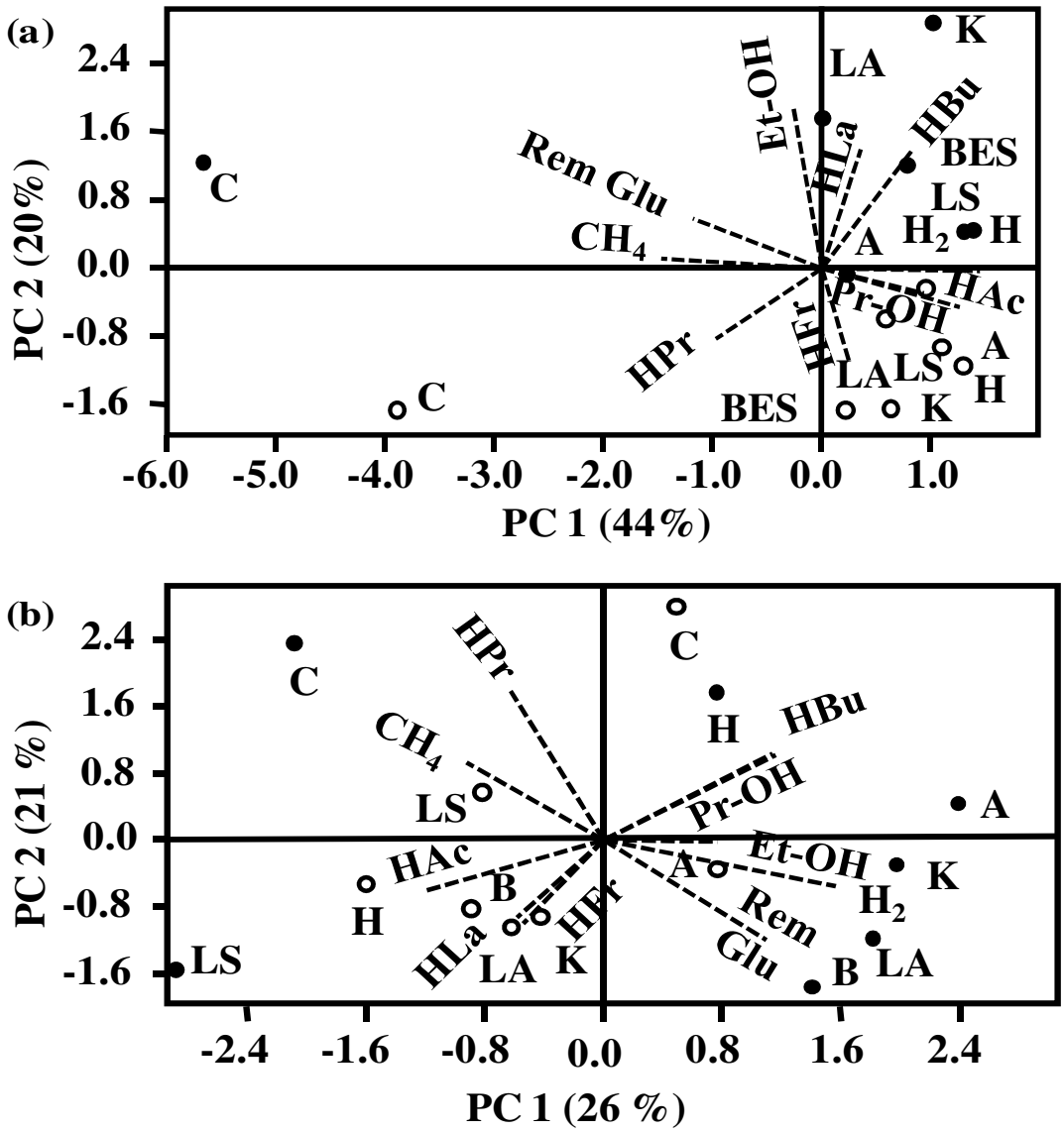

Figure 6.9 Biplot of PCA of fermentation metabolites for cultures incubated at (a) 37 °C and (b) 55 °C.

Notes: 1. C = control; H = heat; A = acid; K = alkali; B = 2-bromoethanesulfonic acid; LA = linoleic acid; LS = loading shock.

2. Closed circle (●) indicates 1st time glucose fed cultures and open circle (◯) indicates 5th time glucose fed cultures.

3. HAc = acetic acid; HPr = propionic acid; HFr = formic acid; HBu = butyric acid; HLa = lactic acid; EtOH = ethanol; CH_4 = methane; PrOH = propanol; H_2 = hydrogen; Rem Glu = remaining glucose.

This is evident from the groupings of the microbial cultures subjected to different pretreatments with stressors.

6.3.9 Microbial cluster analysis of control and stress treated cultures

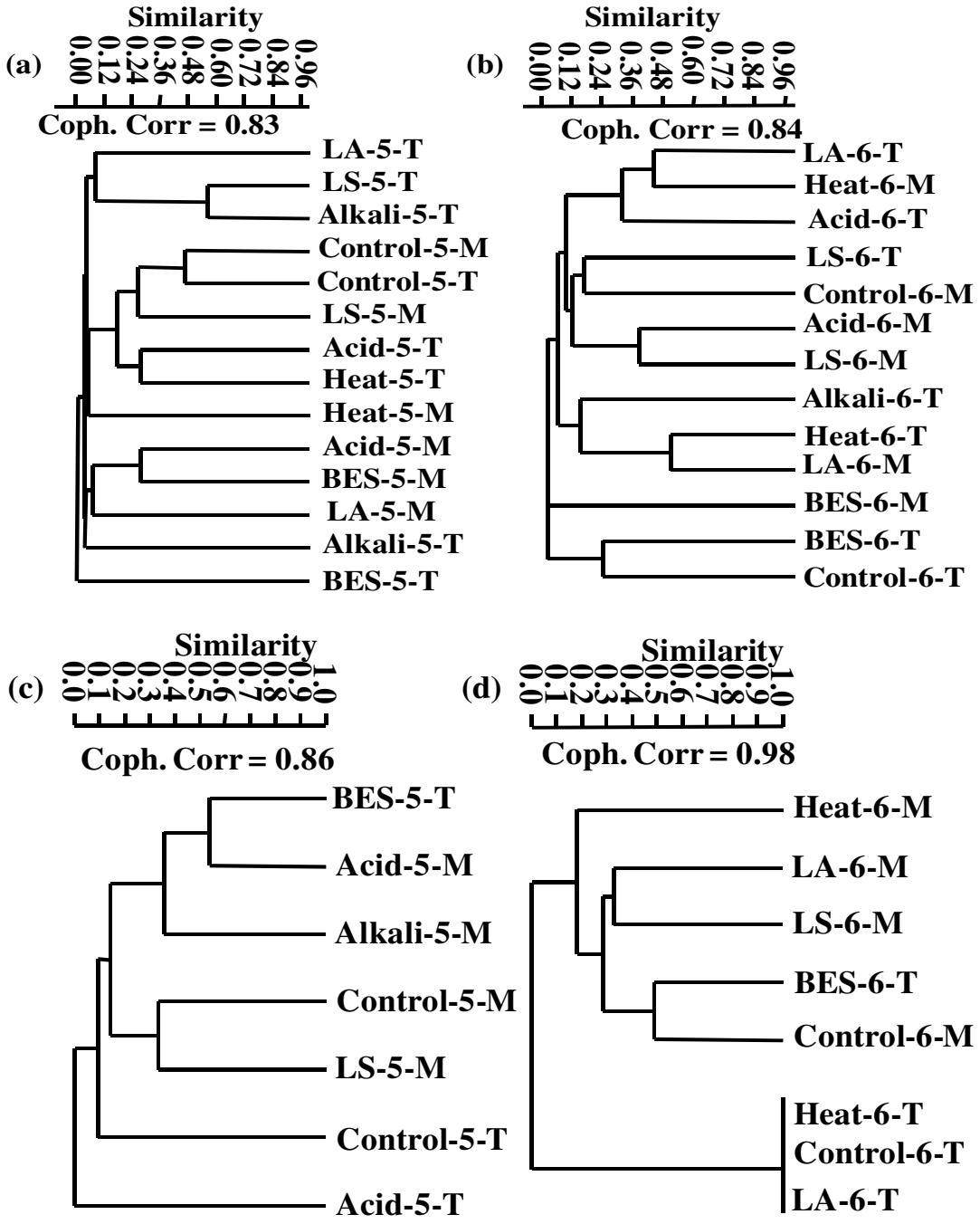

Figure 6.10 Microbial cluster analyses using the Bray-Curtis similarity index of 16S rRNA gene-based T-RFLP profiles. (a) Bacterial *Hha I* digest - cultures fed glucose repeatedly 5 times; (b) Bacterial *Hha I* digest - cultures fed H_2/CO_2; (c) Archaeal *Hha I* digest – cultures fed glucose; (d) Archaeal *Hha I* digest – cultures fed H_2/CO_2.
Notes: LS = loading shock; LA = linoleic acid; BES = 2-bromoethanesulfonic acid; T = cultures incubated at 55 °C; and M = cultures incubated at 37 °C.

The microbial cluster analysis (MCA) was performed using the Bray-Curtis similarity index for the bacterial and archaeal *Hha* I enzyme digests from 16S rRNA gene-based T-RFLP profiles (Figure 6.10 a-d). The Cophenetic correlation coefficients for both the archaeal and the bacterial populations show strong correlations with values above 0.80. Cultures repeatedly fed glucose 5 times were chosen to study the variation in microbial populations fed glucose and incubated at either mesophilic or thermophilic temperatures. Cultures fed with H_2/CO_2 were also analyzed using T-RFLP to study the long-term variations that developed in cultures pretreated with stressors, and which were fed H_2 (consumption study).

An analysis of Archaeal T-RFLP profiles of cultures receiving 5 glucose feedings indicates that bands related to methanogens were present only in the control, acid-, alkali, BES- and LS-treated cultures incubated at mesophilic temperatures, and only in the untreated control and acid-treated cultures incubated at thermophilic temperatures (Figure 6.10c). The results indicate that the bands related to Archaeal 16S rRNA genes were absent in other stress treated cultures successively fed glucose 5 times and incubated at 37 ^{o}C and 55 ^{o}C. Similarly, in the case of cultures fed H_2 (Figure 6.10d), the Archaeal genes were present only in the control, heat-, LA- and LS-treated cultures for the mesophilic-incubation temperature condition and the control, heat- and LA-treated cultures for the thermophilic-incubation temperature condition.

Both the LA- and heat-treated cultures incubated at mesophilic temperatures showed the presence of Archaeal genes in cultures receiving H_2/CO_2, but not in cultures fed repeatedly with glucose 5 times. In comparison, Archaeal bands were not observed following acid- and alkali-treatment for cultures receiving H_2/CO_2 incubated at the same temperature. These results suggest that some archaeal populations present in the mixed anaerobic culture become active depending upon the substrate available and the fermentation media pH. A decrease in pH to levels below 5.0 was not observed in cultures fed H_2. This might account for the patterns of bands in the T-RFLP profiles observed under different fermentation conditions. Microorganisms present within the mixed culture have different pH optima and preferred substrates (Hwang et al., 2004; Nielsen et al., 2004).

6.3.10 Microbial community analysis

Microbial community analysis, performed using T-RFLP, revealed the presence of *Moorella thermoacetica* (14% of the total bacterial population), *Methanosarcina mazei* and *Methanosaeta thermophila* in control cultures (after 5 glucose feedings). *Moorella thermoacetica* is a homoacetogenic bacterium (Henderson et al., 2010) capable of proton reduction, whereas *Methanosarcina mazei* and *Methanosaeta thermophila* are aceticlastic methanogens capable of growth with acetate (Murray and Zinder, 1985; Welte and Deppenmeier, 2011). Homoacetogenic activity was detected in control culture (37 °C) (Figure 6.8a). Analysis of the archaeal community structure for cultures fed repeatedly with glucose 5 times did not detect the presence of hydrogenotrophic methanogens under all of the experimental conditions. Flux balance analysis using the metabolite data also revealed no evidence of hydrogenotrophic methanogen activity. Microbial analysis of thermophilic cultures fed repeatedly with glucose showed abundant levels lactic acid (Figure 6.5b) as well as *Lactobacillus ruminis* and *Lactobacillus salivarius*. These species were particularly abundant in control (27 %), BES- (26 %), LA- (25 %) and LS- (74 %) treated cultures. In comparison, *Lactobacillus* sp. was not detected in mesophilic cultures repeatedly fed glucose. Lactic acid producing bacteria such as *Lactobacillus* sp. excrete bacteriocins which have an adverse effect on HPB thereby decreasing H_2 production (Noike et al., 2002). This might be the reason for the lower H_2 yields observed in B5 thermophilic cultures (Figure 6.4d). In similar studies conducted by Boboescu et al. (2014), elevated lactic acid levels were associated with lower H_2 yields. However, these researchers did not report the presence of *Lactobacillus* sp.

Higher levels of *Clostridium* sp. were detected in mesophilic cultures treated with various stressors and fed repeatedly with glucose (5 times) when compared to the thermophilic cultures. Note variation in *Clostridium* sp. was a function of the pretreatment method. *C. sporogenes* (60 %) and *C. bifermentans* (10.3 %) were abundant in cultures pre-treated with alkali and repeatedly fed with glucose under mesophilic conditions while in acid-treated cultures *C. acidiuric* (30 %), *C. beijerinckii* (11 %), *C. argentinense* (9 %) and *C. bifermentans* (11.5 %) were abundant. In the mesophilic LA treated cultures; *C. sporogenes* (> 95 % relative abundance) was detected after 5 repeated glucose feedings.

Microorganisms related to *Eubacterium* sp. were abundant in cultures treated with BES (13.6 %) and loading shock (16 %). These observations are consistent with those of previous studies (Freier et al., 1994; Zindel et al., 1988). In the repeatedly fed thermophilic cultures, *Flavobacterium* sp., *Bacillus* sp., *Thermoanaerobacter* sp., *Bacteroides* sp., *Thioalkalivibrio* sp., and several species of *Clostridium*, such as *C. septicum*, *C. carnis* and *C. botulinum* were detected in both control and stress-treated cultures with low abundance of *Clostridium* sp. Microorganisms such as *Bacillus* sp., *Thermoanaerobacter* sp. are often found closely associated with *Clostridium* sp. which in turn plays a crucial role in H_2 production (Hung et al., 2011). Organisms related to *Bacteroides* sp. and *Propionibacterium acnes* were detected in control- (15 %), acid- (6 %) and LA- (6 %) treated cultures. Song et al. (2012) conducted a study to quantify the dominant populations in cow dung compost using different pre-treatments such as infrared drying, boiling, forced aeration and aeration with sugar addition. These researchers identify that *Bacteroides* sp. was found to be dominant microorganism after *Clostridium* sp. and *Enterobacter* sp. in stress-treated H_2 producing cultures.

Similar type of microbial populations was observed in a similar study with different stress treated cultures (acid, base, loading shock, BES and heat shock) incubated at 60 °C (O-Thong et al., 2009). *Thioalkalivibrio* sp. was found to be the dominant population (87 %) in alkali treated thermophilic cultures whereas *M. thermoacetica* was found to be dominant (50 %) in BES treated thermophilic cultures. This result confirms the fact that homoacetogenic bacteria become the alternative electron sink from H_2 under suppressed methanogenesis (Wang et al., 2013). The presence of *M. thermoacetica* in BES- treated cultures also supports the detection of R17 flux shown in FBA (Figure 6.8b). *Thermoanaerobacter* sp. has the ability to use lignocellulosic biomass and produce ethanol and H_2 as the by-products (Jessen and Orlygsson, 2012). The lower relative abundance of *Clostridium* sp., together with the presence of *Lactobacillus* sp., accounts for the lower H_2 yields observed at 55 °C. *Thioalkalivibrio* sp., a salt tolerant microorganism, was detected in thermophilic alkali-treated cultures which were repeatedly enriched by feeding glucose.

Methanogens similar to those isolated from marine sediments (Shlimon et al., 2004) mesophilic anaerobic butyric acid degrading reactors (Vanlier et al., 1993) and a Canadian oil reservoir (Mayumi et al., 2013) were detected in mesophilic control cultures fed H_2/CO_2,. In comparison, methanogens were not detected in thermophilic control cultures fed H_2/CO_2. This might be the reason for the lower H_2 consumption (28±7%) by thermophilic control cultures compared to mesophilic control cultures (100±2% H_2 consumption) (Figure 6.2).

Clostridium sp. was detected in mesophilic cultures repeatedly fed glucose and H_2/CO_2 cultures. After pretreatment, repeated feeding with glucose and adding H_2/CO_2, methane or acetate production indicate hydrogenotrophic methanogens or homoacetogens were still active. Many species of *Clostridium* such as *C. ljungdahlii* are homoacetogens have the ability to produce acetate from H_2/CO_2 (Leang et al., 2013).

The survival and subsequent growth of H_2 consumers is likely the reason for greater H_2 consumption observed pretreated mesophilic cultures compared the pretreated thermophilic cultures (Figure 6.2). The data indicate repeated batch cultivation of the pre-treated cultures at 37 °C with glucose selectively enhanced the growth of *Clostridium* sp. In comparison, cultures at 55 °C lower quantities of *Clostridium* sp. were detected. The results also demonstrate that H_2 production using cultures produced using different stress treatments at 37 °C and 55 °C by successive batch cultivations with glucose resulted in different microbial communities.

Mixed anaerobic culture pretreatment is used to inactivate H_2 consumers; however, many non-spore H_2 producers such as *Enterobacter* are also destroyed. This major limitation of could result in reducing the H_2 production efficiency. Many studies have claimed the effectiveness of heat-shock; acid and base on increasing the H_2 yield (Mu et al., 2007; Wang and Wan, 2008). However, the efficacy of culture pretreatment on H_2 yields over the long-term operation is not clearly documented in the literature. Many pretreatment methods inactivate microbial growth using a variety of mechanisms. In case of utilizing a chemical, physical and engineering pretreatment control method, the stress is temporarily applied and any surviving H_2 consumers are able to proliferate under favourable growth conditions. A sustainable H_2 producing culture utilizing non-sterilized

feedstocks must utilize a stressing agent or a combination of stressing agents to permanently inactivate H_2 consumers. Although several methods have been used to inactive H_2 consumers, a suitable culture pretreatment technology has not been fully developed. Temporarily applying stresses such as heating, adding acid and adding alkali have been effective in suppressing H_2 consumers and achieving high H_2 yields; however, all reported data are based on short-term studies (Mu et al., 2007). Heating and utilizing BES are impractical from an economical point-of-view in full-scale operations. In case of using BES, discharging effluents containing this chemical could damage natural habitats. In comparison, addition of LCFAs to continually suppress H_2 consumers (compared to control cultures) during glucose fermentation was found to be effective from the results obtained in this study. These results indicate that in addition to the stress treatment, proper maintenance of operational parameter such as pH is necessary to inhibit/suppress the activity of H_2 consumers during growth on glucose.

6.4. Conclusions

The pretreatment methods used during the enrichment step showed different effects on the H_2 yield. Generally, the pretreated inocula produced higher amount of H_2 in comparison with the controls.

1. When compared to thermophilic cultures, on average higher H_2 yields (1.89±0.19 mol mol^{-1} glucose) were observed in mesophilic cultures receiving repeated glucose feedings.

2. Methane production was only observed in control mesophilic cultures receiving repeated 5 glucose additions. No CH_4 was detected in the thermophilic cultures.

3. The effect of lower fermentation pH (below 4.5 obtained at the end of each batch cultivation) in combination with higher incubation temperatures (55 °C) likely inhibited methanogenic activity in control cultures receiving repeated glucose feeds.

4. The low H_2 yield observed for the thermophilic cultures is likely due to the large amounts of unionized VFAs produced during repeated glucose feedings.

5. Lower H_2 consumption and no detectable quantities of methane were observed in the thermophilic cultures.

6. Lower EHA activity observed in the thermophilic cultures (except alkali treated cultures) compared to mesophilic cultures might be due to the effects of low fermentation pH. The fermentation pH was in the range of 4.0-4.5 at the end of each batch for both mesophilic and thermophilic-incubated cultures. This low pH level might have affected the EHA of the hydrogenase enzyme, which has a pH optima (8.5) above the neutral pH.

7. For both mesophilic and thermophilic cultures, the UHA of the untreated control cultures exhibited higher levels of activity than the pretreated cultures.

8. The flux balance analysis showed no detectable hydrogenotrophic methanogenic activity in cultures receiving repetitive glucose feedings. Mesophilic control cultures producing methane was due to the activity of aceticlastic methanogens. Homoacetogenic activity (R17 flux) accounted for more than 98% loss in H_2 yield in untreated mesophilic control cultures. No detectable homoacetogenic activity was observed in both LA and LS treated mesophilic cultures. In cultures, showing undetectable homoacetogenic activity, H_2 loss occurs via propanol formation (R21 flux).

9. The PCA bi-plot of the fermentation metabolites showed a cluster of pre-treated cultures away from the control cultures irrespective of the incubation temperature. This indicates that stress treated cultures followed different fermentation pattern compared to the control cultures. The stress-treated cultures maintained at mesophilic temperature and fed 5 times with glucose were clustered separately from cultures receiving only 1 glucose feed. Together, the principal components, PC 1 and PC 2, accounted for more than 64 % and 47 % of the variation in the dataset for the cultures incubated at mesophilic and thermophilic temperatures, respectively.

10. In pre-treated cultures fed successively glucose additions and cultures receiving H_2/CO_2, the microbial cluster analysis showed lack of bands related to Archaea.

Fewer T-RFs were detected for Archaeal enzyme digestion compared to bacterial *Hha* I enzyme digestion. The Bray-Curtis similarity index showed a high correlation co-efficient (above 0.83) for both types of enzyme digestion. Control cultures receiving successive glucose feedings and incubated at different temperatures were clustered together with a 40 % similarity (approximate value)**.**

11. Analysis of 16S rRNA gene based T-RFs showed the presence of homoacetogen (*Moorella thermoacetica*), aceticlastic methanogens (*Methanosarcina mazei* and *Methanosaeta thermophila*) in control cultures (receiving 5 glucose feedings). Thermophilic cultures receiving successive 5 glucose feedings were dominated by the presence of *Lactobacillus* sp. such as *Lactobacillus ruminis* and *Lactobacillus salivarius*.

12. In general, all the pre-treatments resulted in selective enrichment of *Clostridium* sp. in mesophilic cultures. Microorganisms related to *Eubacterium* sp. were abundant in both BES and LS pre-treated cultures.

13. Although *Clostridium* sp was detected in pre-treated thermophilic cultures, the dominant microorganisms were *Flavobacterium* sp., *Bacillus* sp., *Thermoanaerobacter* sp., *Bacteroides* sp. and *Thioalkalivibrio* sp

6.5 References

Andersch, W., Bahl, H., Gottschalk, G. (1983). Levels of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. *Eur J Appl Microbiol Biotechnol*, **18**(6), 327-32.

Arooj, M.F., Han, S.K., Kim, S.H., Kim, D.H., Shin, H.S. (2008). Continuous biohydrogen production in a CSTR using starch as a substrate. *Int J Hydrogen Energy*, **33**(13), 3289-94.

Boboescu, I.Z., Gherman, V.D., Mirel, I., Pap, B., Tengölics, R., Rákhely, G., Kovács, K.L., Kondorosi, É., Maróti, G. (2014). Simultaneous biohydrogen production and wastewater treatment based on the selective enrichment of the fermentation ecosystem. *Int J Hydrogen Energy*, **39**(3), 1502-10.

Chang, J.J., Wu, J.H., Wen, F.S., Hung, K.Y., Chen, Y.T., Hsiao, C.L., Lin, C.Y., Huang, C.C. (2008). Molecular, monitoring of microbes in a continuous hydrogen-producing system with different hydraulic retention time. *Int J Hydrogen Energy*, **33**(5), 1579-85.

Das, D., Dutta, T., Nath, K., Kotay, S.M., Das, A.K., Veziroglu, T.N. (2006). Role of Fe-hydrogenase in biological hydrogen production. *Curr Sci*, **90**(12), 1627-37.

Das, D., Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. *Int J Hydrogen Energy*, **26**(1), 13-28.

Dinamarca, C., Gañán, M., Liu, J., Bakke, R. (2011). H_2 consumption by anaerobic non-methanogenic mixed cultures. *Water Sci Technol*, **63**(8), 1582-9.

Duangmanee, T., Padmasiri, S.I., Simmons, J.J., Raskin, L., Sung, S. (2007). Hydrogen production by anaerobic microbial communities exposed to repeated heat treatments. *Water Environ Res*, **79**(9), 975-83.

Ezeji, T.C., Qureshi, N., Blaschek, H.P. (2004). Acetone butanol ethanol (ABE) production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. *Appl Microbiol Biotechnol*, **63**(6), 653-8.

Fan, Y.T., Xing, Y., Ma, H.C., Pan, C.M., Hou, H.W. (2008). Enhanced cellulose hydrogen production from corn stalk by lesser panda manure. *Int J Hydrogen Energy*, **33**(21), 6058-65.

Fan, Y.T., Zhang, Y.H., Zhang, S.F., Hou, H.W., Ren, B.Z. (2006). Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. *Bioresour Technol*, **97**(3), 500-5.

Fang, H.H.P., Li, C.L., Zhang, T. (2006). Acidophilic biohydrogen production from rice slurry. *Int J Hydrogen Energy*, **31**(6), 683-92.

Freier, T.A., Beitz, D.C., Li, L., Hartman, P.A. (1994). Characterization of *Eubacterium coprostanoligenes* sp.nov., a cholesterol reducing anaerobe. *Int J Syst Bacteriol*, **44**(1), 137-42.

Gatje, G., Gottschalk, G. (1991). Limitation of growth and lactic acid production in batch and continuous cultures of *Lactobacillus helveticus*. *Appl Microbiol Biotechnol*, **34**(4), 446-9.

Gibbs, P.A. (1967). The activation of spores of *Clostridium bifermentans*. *J Gen Microbiol*, **46**(2), 285-91.

Guo, X.M., Trably, E., Latrille, E., Carrere, H., Steyer, J.P. (2010). Hydrogen production from agricultural waste by dark fermentation: A review. *Int J Hydrogen Energy*, **35**(19), 10660-73.

Hallenbeck, P.C., Benemann, J.R. (2002). Biological hydrogen production; fundamentals and limiting processes. *Int J Hydrogen Energy*, **27**(11-12), 1185-93.

Hallenbeck, P.C., Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? *Trends Biotechnol*, **27**(5), 287-97.

Hammer, O., Harper, D.A.T., Ryan, P.D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, **4**, 1-9.

Han, S.K., Kim, S.H., Sung, S., Shin, H.S. (2003). Effects of pH and repeated heat-shock treatment on hydrogen fermentation of sucrose by mixed anaerobic culture. *Environ Eng Res*, **8**(4), 202-11.

Hawkes, F.R., Dinsdale, R., Hawkes, D.L., Hussy, I. (2002). Sustainable fermentative hydrogen production: challenges for process optimisation. *Int J Hydrogen Energy*, **27**(11-12), 1339-47.

Henderson, G., Naylor, G.E., Leahy, S.C., Janssen, P.H. (2010). Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by analysis of formyltetrahydrofolate synthetase sequences from ruminants. *Appl Environ Microbiol*, **76**(7), 2058-66.

Hung, C.H., Chang, Y.T., Chang, Y.J. (2011). Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems - A review. *Bioresour Technol*, **102**(18), 8437-8444.

Hung, C.H., Cheng, C.H., Cheng, L.H., Liang, C.M., Lin, C.Y. (2008). Application of *Clostridium*-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. *Int J Hydrogen Energy*, **33**(5), 1586-92.

Husemann, M.H.W., Papoutsakis, E.T. (1988). Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. *Biotechnol Bioeng*, **32**(7), 843-52.

Hussy, I., Hawkes, F.R., Dinsdale, R., Hawkes, D.L. (2003). Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. *Biotechnol Bioeng*, **84**(6), 619-26.

Hwang, M.H., Jang, N.J., Hyun, S.H., Kim, I.S. (2004). Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH. *J Biotechnol*, **111**(3), 297-309.

Jessen, J.E., Orlygsson, J. (2012). Production of ethanol from sugars and lignocellulosic biomass by *Thermoanaerobacter* J1 isolated from a hot spring in Iceland. *J Biomed Biotechnol*, **2012**, 1-7.

Jones, D.T., Woods, D.R. (1986). Acetone-butanol fermentation revisited. *Microbiol Rev*, **50**(4), 484-524.

Kim, B.H., Zeikus, J.G. (1985). Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum*. *Dev Ind Microbiol*, **26**, 1-14.

Kovacs, K.L., Maroti, G., Rakhely, G. (2006). A novel approach for biohydrogen production. *Int J Hydrogen Energy*, **31**(11), 1460-8.

Lay, J.J., Lee, Y.J., Noike, T. (1999). Feasibility of biological hydrogen production from organic fraction of municipal solid waste. *Water Res*, **33**(11), 2579-86.

Leang, C., Ueki, T., Nevin, K.P., Lovley, D.R. (2013). A genetic system for *Clostridium ljungdahlii*: a chassis for autotrophic production of biocommodities and a model homoacetogen. *Appl Environ Microbiol*, **79**(4), 1102-9.

Levin, D.B. (2004). Re: Biohydrogen production: Prospects and limitations to practical application - Erratum. *Int J Hydrogen Energy*, **29**(13), 1425-6.

Levin, D.B., Chahine, R. (2010). Challenges for renewable hydrogen production from biomass. *Int J Hydrogen Energy*, **35**(10), 4962-4969.

Li, Y.F., Ren, N.Q., Chen, Y., Zheng, G.X. (2007). Ecological mechanism of fermentative hydrogen production by bacteria. *Int J Hydrogen Energy*, **32**(6), 755-60.

Liu, D.W., Zeng, R.J., Angelidaki, I. (2008). Enrichment and adaptation of extreme thermophilic (70 °C) hydrogen producing bacteria to organic household solid waste by repeated batch cultivation. *Int J Hydrogen Energy*, **33**(22), 6492-7.

Liu, G.Z., Shen, J.Q. (2004). Effects of culture and medium conditions on hydrogen production from starch using anaerobic bacteria. *J Biosci Bioeng*, **98**(4), 251-6.

Logan, B.E. (2004). Extracting hydrogen electricity from renewable resources. *Environ Sci Technol*, **38**(9), 160-7.

Lu, W.J., Fan, G.Y., Zhao, C.X., Wang, H.T., Chi, Z.F. (2012). Enhancement of fermentative hydrogen production in an extreme thermophilic (70 °C) mixed culture environment by repeated batch cultivation. *Curr Microbiol*, **64**(5), 427-32.

Luo, G., Karakashev, D., Xie, L., Zhou, Q., Angelidaki, I. (2011). Long term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol Bioeng*, **108**(8), 1816-27.

Luo, G., Xie, L., Zou, Z.H., Wang, W., Zhou, Q. (2010). Evaluation of pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. *Bioresour Technol*, **101**(3), 959-64.

Manish, S., Banerjee, R. (2008). Comparison of biohydrogen production processes. *Int J Hydrogen Energy*, **33**(1), 279-86.

Mathews, J., Wang, G.Y. (2009). Metabolic pathway engineering for enhanced biohydrogen production. *Int J Hydrogen Energy*, **34**(17), 7404-16.

Mayumi, D., Dolfing, J., Sakata, S., Maeda, H., Miyagawa, Y., Ikarashi, M., Tamaki, H., Takeuchi, M., Nakatsu, C.H., Kamagata, Y. (2013). Carbon dioxide concentration dictates alternative methanogenic pathways in oil reservoirs. *Nat Commun*, **4**.

Meyer, J. (2007). [Fe-Fe] hydrogenases and their evolution: a genomic perspective. *Cell Mol Life Sci*, **64**(9), 1063-84.

Moletta, R., Verrier, D., Albagnac, G. (1986). Dynamic modeling of anaerobic digestion. *Water Res*, **20**(4), 427-34.

Monot, F., Engasser, J.M., Petitdemange, H. (1984). Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol*, **19**(6), 422-6.

Moreira, A.R., Dale, B.E., Doremus, M.G. (1982). Utilization of the fermentor off-gases from an acetone-butanol fermentation. pp. 263-77.

Mu, Y., Yu, H.Q., Wang, G. (2007). Evaluation of three methods for enriching H_2 producing cultures from anaerobic sludge. *Enzyme Microb Technol*, **40**(4), 947-53.

Mueller, R.F., Nielsen, P.H. (1996). Characterization of thermophilic consortia from two souring oil reservoirs. *Appl Environ Microbiol*, **62**(9), 3083-7.

Murray, P.A., Zinder, S.H. (1985). Nutritional requirements of *Methanosarcina sp.* Strain-TM-1. *Appl Environ Microbiol*, **50**(1), 49-55.

Nielsen, H.B., Mladenovska, Z., Westermann, P., Ahring, B.K. (2004). Comparison of two-stage thermophilic (68 °C/55 °C) anaerobic digestion with one-stage thermophilic (55 °C) digestion of cattle manure. *Biotechnol Bioeng*, **86**(3), 291-300.

Noike, T., Takabatake, H., Mizuno, O., Ohba, M. (2002). Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. *Int J Hydrogen Energy*, **27**(11-12), 1367-71.

O-Thong, S., Prasertsan, P., Birkeland, N.K. (2009). Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis. *Bioresour Technol*, **100**(2), 909-18.

Oh, S.E., Van Ginkel, S., Logan, B.E. (2003). The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ Sci Technol*, **37**(22), 5186-90.

Park, W., Jang, N.J., Hyun, S.H., Kim, I.S. (2005). Suppression of hydrogen consuming bacteria in anaerobic hydrogen fermentation. *Environ Eng Res*, **10**(4), 181-90.

Peters, J.W. (1999). Structure and mechanism of iron-only hydrogenases. *Curr Opin Struct Biol*, **9**(6), 670-6.

Prasertsan, P., O-Thong, S., Birkeland, N.K. (2009). Optimization and microbial community analysis for production of biohydrogen from palm oil mill effluent by thermophilic fermentative process. *Int J Hydrogen Energy*, **34**(17), 7448-59.

Radmann, E.M., Reinehr, C.O., Costa, J.A.V. (2007). Optimization of the repeated batch cultivation of microalga *Spirulina platensis* in open raceway ponds. *Aquaculture*, **265**(1-4), 118-26.

Ray, S., Saady, N.M.C., Lalman, J.A. (2010). Diverting electron fluxes to hydrogen in mixed anaerobic communities fed with glucose and unsaturated C:18 long chain fatty acids. *J Environ Eng*, **136**(6), 568-75.

Siriwongrungson, V., Zeny, R.J., Angelidaki, I. (2007). Homoacetogenesis as the alternative pathway for H_2 sink during thermophilic anaerobic degradation of butyrate under suppressed methanogenesis. *Water Res*, **41**(18), 4204-10.

Skonieczny, M.T., Yargeau, V. (2009). Biohydrogen production from wastewater by *Clostridium beijerinckii*: Effect of pH and substrate concentration. *Int J Hydrogen Energy*, **34**(8), 3288-94.

Song, Z.X., Dai, Y., Fan, Q.L., Li, X.H., Fan, Y.T., Hou, H.W. (2012). Effects of pretreatment method of natural bacteria source on microbial community and bio-hydrogen production by dark fermentation. *Int J Hydrogen Energy*, **37**(7), 5631-6.

Stafford, D.A. (1982). The effects of mixing and volatile fatty acid concentrations on anaerobic digester performance. *Biomass*, **2**(1), 43-55.

Sterling Jr. MC., L.R.E., Engler C.R., Ricke S.C. (2001). Effects of ammonia nitrogen on H_2 and CH_4 production during anaerobic digestion of dairy cattle manure. *Bioresour Technol*, **77**, 9-18.

Stringer, S.C., Fairbairn, D.A., Peck, M.W. (1997). Combining heat treatment and subsequent incubation temperature to prevent growth from spores of non-proteolytic *Clostridium boltulinum*. *J Appl Microbiol*, **82**(1), 128-36.

Terracciano, J.S., Kashket, E.R. (1986). Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum*. *Appl Environ Microbiol*, **52**(1), 86-91.

Valdez-Vazquez, I., Poggi-Varaldo, H.M. (2009). Hydrogen production by fermentative consortia. *Renew Sust Energ Rev*, **13**(5), 1000-13.

van den Heuvel, J.C., Verschuren, P.G., Beeftink, H.H., de Beer, D. (1992). Determination of the critical concentration of inhibitory products in a repeated fed-batch culture. *Biotechnol Tech*, **6**(1), 33-8.

Van Ginkel, S., Logan, B.E. (2005). Inhibition of biohydrogen production by undissociated acetic and butyric acids. *Environ Sci Technol*, **39**(23), 9351-6.

Vanlier, J.B., Grolle, K.C.F., Frijters, C.T.M.J., Stams, A.J.M., Lettinga, G. (1993). Effects of acetate, propionate, and butyrate on the thermophilic anaerobic degradation of propionate by methanogenic sludge and defined cultures. *Appl Environ Microbiol*, **59**(4), 1003-11.

Vignais, P.M., Billoud, B., Meyer, J. (2001). Classification and phylogeny of hydrogenases. *Fems Microbiol Rev*, **25**(4), 455-501.

Vignais, P.M., Colbeau, A. (2004). Molecular biology of microbial hydrogenases. *Curr Iss Mol Biol*, **6**, 159-88.

Voordouw, G. (1992). Evolution of Hydrogenase Genes. *Adv Inorg Chem*, **38**, 397-422.

Wang, G., Wang, D.I.C. (1984). Elucidation of growth inhibition and acetic acid production by *Clostridium thermoaceticum*. *Appl Environ Microbiol*, **47**(2), 294-8.

Wang, J., Liu, H., Fu, B., Xu, K.W., Chen, J. (2013). Trophic link between syntrophic acetogens and homoacetogens during the anaerobic acidogenic fermentation of sewage sludge. *Biochem Eng J*, **70**, 1-8.

Wang, J.L., Wan, W. (2008). Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. *Int J Hydrogen Energy*, **33**(12), 2934-41.

Wang, J.L., Wan, W. (2009). Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy*, **34**(2), 799-811.

Wang, M.Y., Tsai, Y.L., Olson, B.H., Chang, J.S. (2008). Monitoring dark hydrogen fermentation performance of indigenous *Clostridium butyricum* by hydrogenase gene expression using RT-PCR and qPCR. *Int J Hydrogen Energy*, **33**(18), 4730-8.

Wang, X., Monis, P.T., Saint, C.P., Jin, B. (2009). Biochemical kinetics of fermentative hydrogen production by *Clostridium butyricum* W5. *Int J Hydrogen Energy*, **34**(2), 791-8.

Welte, C., Deppenmeier, U. (2011). Membrane bound electron transport in *Methanosaeta thermophila*. *J Bacteriol*, **193**(11), 2868-70.

Yokoi, H., Maki, R., Hirose, J., Hayashi, S. (2002). Microbial production of hydrogen from starch manufacturing wastes. *Biomass Bioenergy*, **22**(5), 389-95.

Yokoyama, H., Moriya, N., Ohmori, H., Waki, M., Ogino, A., Tanaka, Y. (2007). Community analysis of hydrogen-producing extreme thermophilic anaerobic microflora enriched from cow manure with five substrates. *Appl Microbiol Biotechnol*, **77**(1), 213-22.

Zindel, U., Freudenberg, W., Rieth, M., Andreesen, J.R., Schnell, J., Widdel, F. (1988). *Eubacterium acidaminophilum* sp. nov., a versatile amino acid-degrading anaerobe producing or utilizing H_2 or formate - Description and enzymatic studies. *Arch Microbiol*, **150**(3), 254-66.

CHAPTER 7: EFFECTS OF ADDING LINOLEIC ACID (LA) AND HYDRAULIC RETENTION TIME (HRT) ON BIOHYDROGEN PRODUCTION FROM GLUCOSE USING MIXED CULTURES IN ANAEROBIC SEQUENCING BATCH REACTORS

7.1. Introduction

Biohydrogen (Bio-H_2) production by dark fermentation is affected by a wide variety of process parameters, such as pH, temperature, seed sludge, hydraulic retention time (HRT), inhibitors, reactor design and the means of lowering H_2 partial pressure (Li and Fang, 2007) and performance parameters (e.g. hydrogen production rate and conversion efficiency). Among them, pH, temperature, HRT and substrate concentration are considered the most important parameters affecting fermentative H_2 production (Wu et al., 2010). The operating pH plays an important role in controlling the production of H_2 because of its effects on hydrogenase activity (Dabrock et al., 1992), metabolic pathways (Lay, 2000) and microbial communities (Fang and Liu, 2002). A pH range of 4.5 to 6.0 is favourable for H_2 production with optimum pH of approximately 5.5 (Fang et al., 2006; Hawkes et al., 2007). Temperature also plays an important role in H_2 evolution because the activity of hydrogenase enzyme depends upon temperature. Many fermentative H_2 production studies have been performed using temperature between 30 and 40 °C (Fang and Liu, 2002; Hawkes et al., 2007; Lin and Chang, 1999; Van Ginkel and Logan, 2005). HRT has a major influence on H_2 production during continuous operation. Higher H_2 yields are associated with low HRTs (Wu et al., 2009) because of alteration in fermentation patterns and the suppression of methanogenic growth. Since most of the mesophilic fermentative H_2 production studies have been conducted at HRT's varying from 6 h- 32 h (Fang and Liu, 2002; Hawkes et al., 2007; Lay et al., 2005), the experimental HRT's (37.5 h, 17.5 h, 10.8 h, 7.5 h and 3.3 h HRT) was chosen for this current study.

Biohydrogen production by mixed cultures fed simple carbohydrates (e.g. glucose, xylose, sucrose and starch) in combination with mineral nutrients to mimic carbohydrate-rich wastewater (Li and Fang, 2007) has been the focus of study in recent years. The theoretical maximum yield of H_2 from glucose upon complete conversion to H_2 and carbon dioxide is 12 mol mol^{-1} glucose. However, this theoretical maximum yield cannot be achieved in practice because the reaction is not thermodynamically feasible (Westermann et al., 2007). Moreover, H_2 production is usually accompanied by the production of volatile fatty acids (VFAs) and/or alcohols, but never as a single, reduced compound. The theoretical maximum H_2 yield of 4 mol H_2 mol^{-1} glucose can be obtained if the dark fermentation process follows the acetic acid pathway (Nandi and Sengupta, 1998). The stoichiometric maximum H_2 yield of 8 mol H_2 mol^{-1} sucrose can be obtained if sucrose is used as the substrate for dark fermentation. Batch studies using *Thermotoga maritime* have reported yields close to 4.0 mol H_2 mol^{-1} glucose (Schroder et al., 1994). High H_2 yields are associated with employing pure strains of individual bacterial cultures because no syntrophic relationship exists. With mixed cultures, H_2 yields are affected because of syntrophic association between H_2 consumers and H_2 producers. Hydrogenotrophic methanogens and homoacetogens are two major H_2 consumers affecting the H_2 yield in the absence of sulfate reducers.

Mixed cultures from wastewater treatment facilities can be employed for H_2 production. Another advantage of using mixed culture is their ability to utilize non-sterile feed stocks. Because of the ease of availability and ability to use non-sterile feedstock, many researchers have focused their efforts on developing fermentative H_2 production technologies using mixed cultures.

In recent years, H_2 production using mixed cultures pretreated with heat or acid has been studied (Oh et al., 2003; Tommasi et al., 2008). Heat pretreatment affects the composition of the microbial community is by inactivating non-spore formers, particularly H_2-consuming hydrogenotrophic methanogens (Lay et al., 1999) as well as non-spore H_2 producers such as *Enterobacter* (Zhang et al., 2011). Several chemicals, such as BES (Zinder et al., 1984), acetylene (Sprott et al., 1982) and long chain fatty acids (LCFA) (Lalman and Bagley, 2002), are effective in controlling the growth of H_2-consuming

methanogens. Linoleic acid (LA), an unsaturated LCFA (C18:2), is a promising candidate for diverting the electron fluxes towards H_2 production (Chowdhury et al., 2007). LA inhibits hydrogenotrophic methanogens, aceticlastic methanogens, and may possibly affect homoacetogens. The effect of LCFAs on H_2 production has been reported previously (Chowdhury et al., 2007; Lalman and Bagley, 2002). To date, the exact mechanism of action of the LCFAs is not well understood, but LCFAs adsorbed on the surface of the organisms could prevent the transport of substrates and nutrients required for microbial growth (Demeyer and Henderickx, 1967; Wiegant and Lettinga, 1985). Another advantage to using LCFAs rather than other chemicals is that LCFAs are biodegradable, and are produced from renewable resources.

Continuous stirred tank reactors (CSTR) and up-flow sludge blanket reactors (UASBRs) have been widely used in laboratory scale H_2 production studies (Carrillo-Reyes et al., 2012; Kim et al., 2005; Kotsopoulos et al., 2009; Shiue-Lin et al., 2011). Operating CSTRs and UASBRs is complex and expensive because the reaction vessel and settling tank are separated. An alternative emerging technology is the anaerobic sequencing batch reactor (ASBR). The major advantages of using ASBRs include higher retention of solids,; a higher degree of process flexibility with respect to changes in organic loading rate (OLR); a single reaction vessel for operation and settling (i.e., separate equipment is not needed for clarification); relative ease of operation; and lower capital costs (Arooj et al., 2008). According to Wu et al. (2009), the semi-continuous mode of operation of ASBRs is feasible for potential practical applications and commercialization.

Recent studies (Arooj et al., 2008; Chen et al., 2009; Kim et al., 2010; Sreethawong et al., 2010; Venkata Mohan et al., 2007; Wu et al., 2009) using ASBRs have focused on assessing the effects of operational parameters, such as pH, HRT, OLR and the C: N ratio, on biohydrogen production. All of these studies considered using a variety of pre-treatment methods to suppress non-spore formers. Pretreatment options which have been considered can be grouped as non-specific (heat, acid and alkali) (Ren et al., 2008) and specific (LCFAs, BES and acetylene) (Liu et al., 2011; Shanmugam et al., 2014). Many laboratory scale reactor H_2 fermentative studies have employed heat, acid, alkali and BES inoculum pretreatment to increase H_2 yields; however, data using LCFAs in continuous

or semi-continuous flow reactors is unavailable. Hence, the objectives of this study were as follows: 1. Examine effects of LA pretreatment and HRT on fermentative H_2 production in ASBRs; 2. Investigate microbial community composition changes for both initial inoculum (37.5 h HRT) and inoculum from lower HRT (3.3 h) for the control cultures.

7.2 Materials and methods:

7.2.1 Inoculum Source

Experiments were conducted using mixed anaerobic cultures procured from wastewater treatment facilities located at a brewery (Guelph, ON). The volatile suspended solids (VSS) concentration of the inoculum was approximately 40,000 mg L^{-1}.

7.2.2 Experimental Procedures

Examination of the effects of HRT on H_2 production was performed in 6 L ASBRs with a 5 L working volume. The mixed cultures were acclimated at 37 °C with glucose (5 g L^{-1}). The VSS concentration was 10,000 mg L^{-1}. All the experimental conditions (Table 7.1) were examined in duplicate reactors operating at pH 5.5 and 37°C. The ASBR filling, settling and decant periods were 10 min, 25 min and 10 min, respectively. The react period used was based on the HRT (Table 7.2).

Table 7.1 Experimental design matrix to study the effects of HRT and LA on bio-H_2 production in ASBRs using glucose as substrate.

Experiment No.	HRT (h)	LA addition (2 g L^{-1})	Substrate glucose (5 g L^{-1})	Metabolite analysis	
1	37.5	X	√	Gas (H_2, CH_4, CO_2)	Liquid (VFAs, alcohols, glucose)
2	17.5	X	√		
3	10.8	X	√		
4	7.5	X	√		
5	3.3	X	√		
6	37.5	√	√		
7	17.5	√	√		
8	7.5	√	√		

Note: LA = linoleic acid; H_2 = hydrogen; CH_4 = methane; VFAs = volatile fatty acids

For the control experiments (i.e., without the addition of LA), the initial loading rate was increased incrementally by reducing the HRT (from 37.5 h > 17.5 h > 10.8 h > 7.5 h > 3.3 h; Table 7.1). For LA pretreatment experiments, only HRT conditions of 37.5 h, 17.5 h and 7.5 h were considered. In control studies, pretreatment was not utilized and glucose was fed to the cultures under a range of HRT conditions. LA studies were conducted after completing the control experiments with only a glucose feed. The experimental design for LA treatment studies were similarly to the control studies.

For each HRT condition with culture pretreatment, fresh inoculum acclimated to glucose was fed 2 g L^{-1} LA for 24 h at pH 5.5. The LA stock solution was prepared according to the procedures described in section 3.8. The LA concentration and LA inhibition period were based on previous studies (Lalman and Bagley, 2002; Saady et al., 2012). After 24 h, glucose was added and the reactor was operated at different HRT conditions (Table 7.1).

The volume of gas produced was measured using a tipping bucket gas meter (Speece, 1976). The biogas composition was determined by GC according to methods described in section 3.7.2. The pH was controlled at 5.5 using 3 N HCl or 3 M KOH. Before the start of each cycle, N_2 gas was purged into the reactors for 3 minutes to ensure anaerobic conditions. The liquid sample at the end of each cycle was collected and analyzed using IC according to the methods described in section 3.7.1. FBA was used to measure the flux of electrons diverted towards acetogenesis and methanogenesis. The fermentation metabolites obtained under different HRTs were used as the input for the FBA. Flux balance was conducted according to the method described in section 3.11.

Table 7.2 ASBR operation.

HRT (h)	Cycle time (h)	Feed (min)	React (h)	Settle (min)	Decant (min)
37.5	18.75	10	18	25	10
17.5	875	10	8	25	10
10.8	5.67	10	5	25	10
7.5	3.75	10	3	25	10
3.3	1.75	10	1.5	25	10

The microbial community analysis using T-RFLP was performed for the samples collected at the end of each HRT in the control studies. T-RFLP analysis was conducted only for the control experiments. The T-RFLP analyses were performed according to the protocols described in section 3.8. In addition to the genomic analysis using T-RFLP, pyrosequencing was performed for the inoculum before the start of the experiment and for the inoculum sample collected at 3.3 h HRT which yielded the maximum H_2 production rate.

Pyrosequencing was conducted performed according to the protocol optimized by Dr. Subba Rao Chaganti. Multiplex amplicon sequencing was performed on all the samples following DNA isolation. The V5 and V6 regions of bacterial 16S rRNA genes were amplified using the fusion primers E786F (5'-GATTAGATACCCTGGTAG-3'), E1063R (5'- CTCACGRCACGAGCTGACG -3'), containing the 454 FLX adaptors and a sample-specific multiplex identifier (Andersson et al., 2008). PCR mixtures conducted in 25 µL volumes included the following: 10.2 mM Tris buffer, 50 mM KCl, 2% 2.3 mM $MgCl_2$, DMSO, 0.2 mM of each dNTP, 5 mg BSA, 0.2 mM of each primer, 1 µL of DNA template (20 ng μL^{-1}) and 1.0 U of AmpliTaq DNA polymerase (Applied Biosystems, Carlsbad, CA) were cycled as follows: 95 °C for 5 min followed by; 35 cycles of 94 °C for 1 min, 48 °C for 30 s, 72 °C for 1 min, with a final elongation step of 72 °C for 7 min. Triplicate PCR products for each sample were pooled, purified using the 1 % low meting agarose gel followed by QIA quick gel extraction kit (Qiagen) and quantified using Nano vue. Equal amounts of the bar-coded PCR products were mixed and submitted to the EnGenCore LLC, (University of South Carolina, Columbia, USA) for pyrosequencing on a 454 GS20 FLX platform. Prior to sequencing, all amplicon types were assessed for fragment size distribution and DNA concentration using a Bioanalyser 2100 (Agilent Technologies, USA).

The pyrosequencing data obtained after the initial process by the GL FLX software, RDP pyrosequencing pipeline were used to sort by tag sequence, trim the 16S primers and filter out additional sequences of low-quality. The high quality reads were clustered to OTUs, with a complete linkage algorithm on a 97 % sequence identity level. The

taxonomic affiliation of the OTUs was determined using a naive Bayesian rRNA Classifier (Wang et al., 2007) and at a confidence threshold of 80 %.

7.3. Results and discussion

7.3.1 Hydrogen and methane production

A 48 h HRT was selected to operate the reactor before selecting any of the HRTs in Table 7.1. At the 48h HRT, glucose (5 g L^{-1}) was fed to the culture and the biogas composition was analyzed until attaining a pseudo-steady state condition. The pseudo-steady state condition was establish when the H_2 content of the biogas composition < 10% (data not shown). After achieving the pseudo-steady state condition, the HRT was gradually reduced in increments from 37.5 h to 3.3 h in the control studies (without the addition of LA).

Five to eight experiments at each HRT (37.5 h, 17.5 h, 10.8 h, 7.5 h and 3.3 h) were performed using two ASBRs. Hydrogen production in the control cultures increased with decreasing HRT (Figure 7.1). The H_2 yield increased from 0.24±0.02 mol mol^{-1} glucose at 37.5 h HRT to 2.02±0.05 mol mol^{-1} glucose at 7.5 h HRT and then decreased to 1.79±0.06 mol mol^{-1} glucose at 3.3 h HRT (Table 7.3). At low HRT conditions, microorganism's washout is the cause for the decline in H_2 yield at the 3.3 h HRT (data not shown). These results are comparable to H_2 production studies by Chang and Lin (2004), who used sucrose as the substrate. Chang and Lin (2004) reduced the HRT from 24 h to 4 h and observed a maximum H_2 yield (1.6 mol mol^{-1}sucrose) at 8 h HRT. As the HRT was lowered from 8 h to 4 h, the H_2 yield decreased to 0.9 mol mol^{-1} sucrose due to the washout of the microorganisms. Similar findings of increasing H_2 yields at lower HRTs have been described by many researchers (Chen and Lin, 2003; Fan et al., 2006; Zhang et al., 2006). These researchers concluded that HRT affected the microbial community composition which in turn impacted H_2 production. Selectively enriching dominant H_2-producing populations by lowering the HRT is another reason for the observed high H_2 yields

Methane (CH_4) production with changing HRT is shown in Figure 7.1. The highest CH_4 yield of 0.94±0.05 mol mol^{-1} glucose was observed at 37.5 h HRT. The declining CH_4 yields with decreasing HRT's are due to the wash-out of methanogenic archaea.

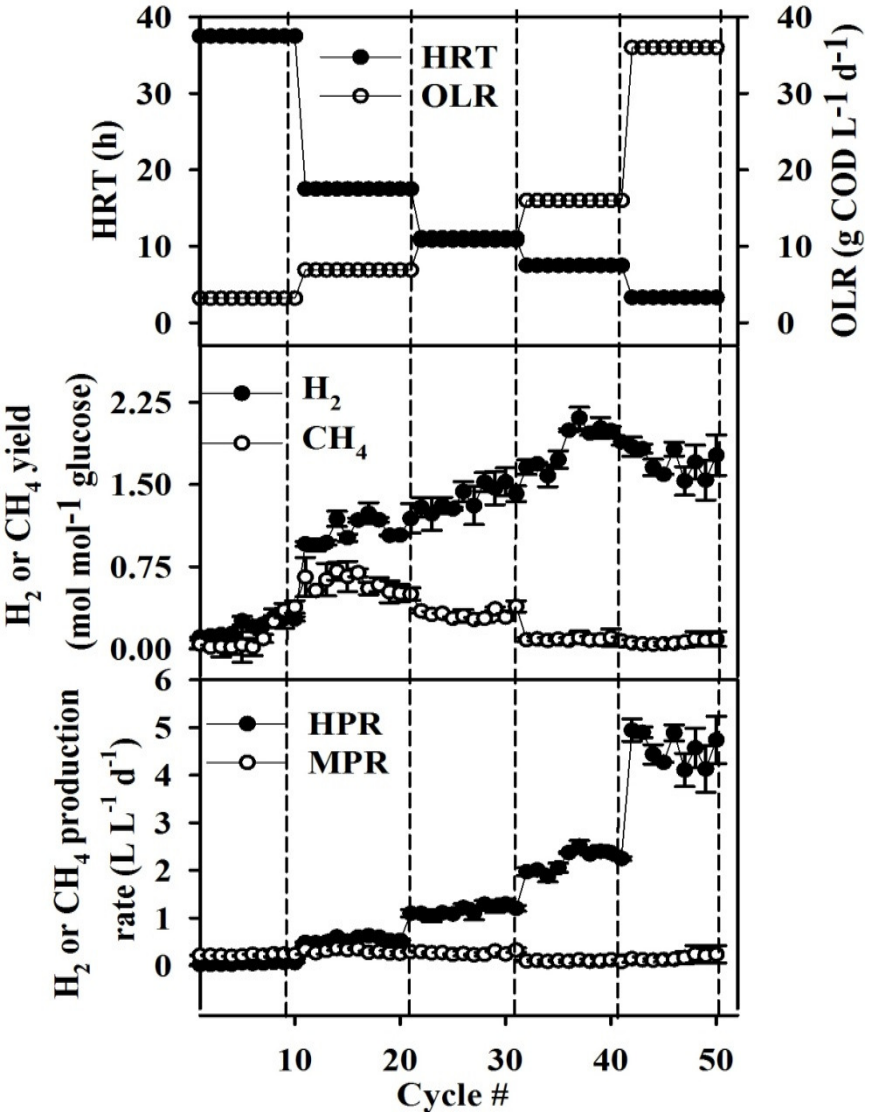

Figure 7.1 Hydrogen (H_2) or methane (CH_4) production profiles for the untreated control cultures at various HRT conditions.

Notes: 1. HPR = hydrogen production rate; MPR = methane production rate; OLR = organic loading rate; HRT = hydraulic retention time. 2. The error bars in this figure represents the standard deviation for n = 4.

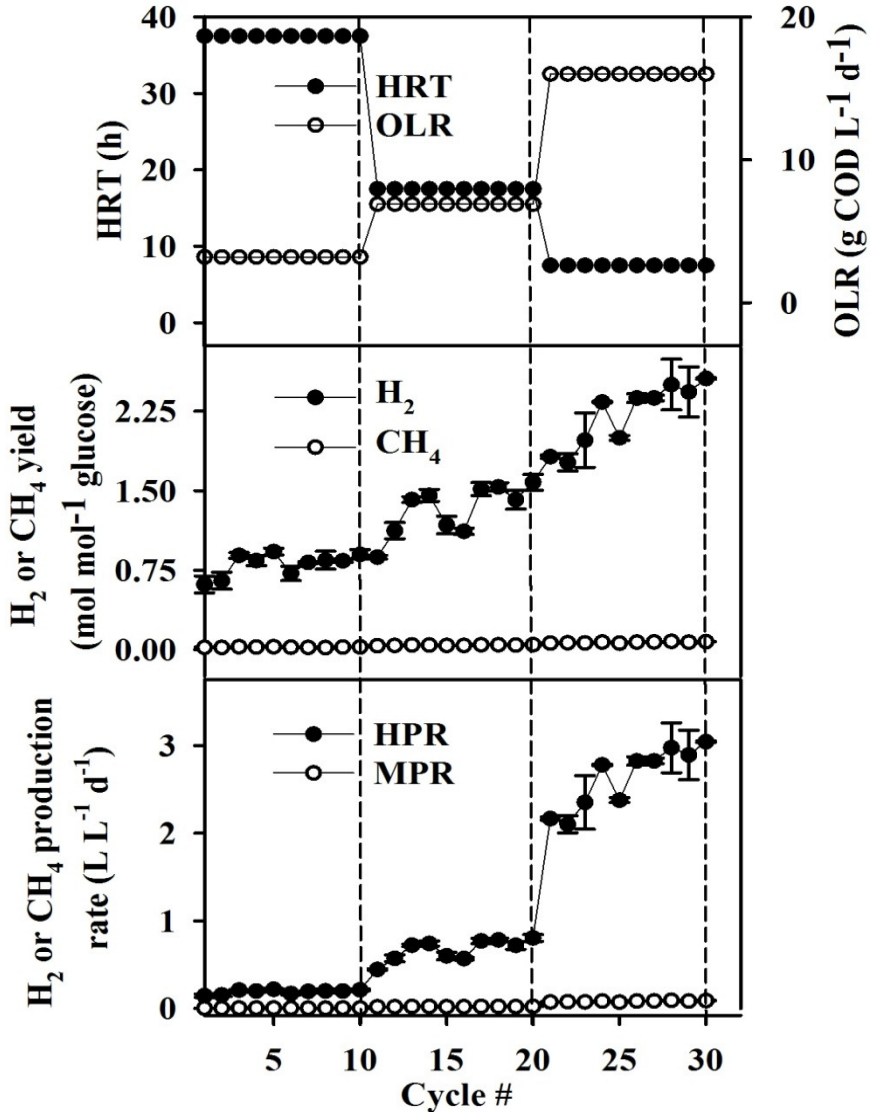

Figure 7.2 Hydrogen or methane production profiles for the LA-treated cultures at various HRTs.

Notes: 1. H_2 = hydrogen; CH_4 = methane; OLR = organic loading rate; HRT = hydraulic retention time.

2. Steady state values under each HRT were considered for the analysis. The error bars in this figure represents the standard deviation for n = 4

Note in the control studies, the higher H_2 yields were observed at a 7.5 h HRT when compared to 10.8 h HRT. This was basis for omitting the 10.8 h HRT condition from the LA pretreatment studies. In addition, as washout of the biomass (data not shown) occurred at 3.3 h HRT in the control studies, this condition was no considered in the LA pretreatment study. The HRT conditions examined with the addition of LA were 37.5 h, 17.5 h and 7.5 h.

Table 7.3 Summary of hydrogen and liquid metabolites produced at various HRTs.

	HRT (h)	Major liquid metabolites formed (mol mol^{-1} hexose$_{consumed}$)				H_2 (%)	H_2 yield (mol mol^{-1} glucose)	Et-OH / HAc ratio	HAc / HBu ratio
		HAc	HPr	HBu	Et-OH				
Control	37.5	0.55 ± 0.06^{a}	0.44 ± 0.04^{a}	0.14 ± 0.01^{b}	$0.27\pm0.02^{b,c}$	12±1	0.24 ± 0.02^{f}	0.48	3.98
	17.5	0.3 ± 0.04^{b}	0.29 ± 0.03^{b}	$0.08\pm0.01^{b,c}$	0.48 ± 0.05^{a}	40±2	$1.19\pm0.03^{c,d}$	1.57	3.95
	10.8	0.30 ± 0.02^{b}	0.24 ± 0.05^{c}	0.08 ± 0.01^{c}	0.41 ± 0.07^{b}	57±1	$1.44\pm0.05^{b,c}$	1.46	3.95
	7.5	0.28 ± 0.01^{b}	0.25 ± 0.03^{b}	0.04 ± 0.01^{d}	0.32 ± 0.06^{a}	62±1	2.02 ± 0.05^{b}	2.22	6.52
	3.3	0.24 ± 0.03^{b}	0.24 ± 0.03^{b}	0.05 ± 0.01^{d}	0.57 ± 0.01^{a}	61±2	1.79 ± 0.06^{b}	2.35	4.75
LA	37.5	0.32 ± 0.01^{b}	0.32 ± 0.00^{e}	0.48 ± 0.01^{a}	0.09 ± 0.01^{d}	44±2	0.84 ± 0.04^{d}	0.27	0.65
	17.5	0.20 ± 0.01^{b}	$0.01\pm0.00^{d,e}$	0.49 ± 0.03^{a}	0.20 ± 0.01^{c}	53±2	1.50 ± 0.12^{b}	0.99	0.42
	7.5	0.22 ± 0.01^{b}	0.02 ± 0.00^{d}	0.49 ± 0.02^{a}	0.23 ± 0.01^{c}	58±3	2.45 ± 0.15^{a}	1.07	0.37

Notes: 1. Superscripts $^{a, b, c, d}$ and ^{e} indicate statistically different means within the same column (Tukey's test; $\alpha = 0.05$). No comparisons should be made between columns.

2. LA = linoleic acid; HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; Et-OH = ethanol.

3. Biogas composition for both control and LA-treated cultures at various HRT's are provided in Appendix Fig. C.2.

4. The values in the table represent mean ± standard deviation of triplicate samples.

In LA-treated cultures, increasing H_2 yields with decreasing HRTs was similar to the control experiments (Figure 7.2). The highest H_2 yield of 2.45±0.15 mol mol^{-1} glucose was observed at 7.5 h HRT. Compared to the control cultures, higher H_2 yields of 0.84±0.04 and 1.50±0.12 mol mol^{-1} glucose at 37.5 h HRT and 17.5 h HRT, respectively, were observed in the LA-treated cultures (Table 7.3). This corresponds to a relative increase (compared to the corresponding control yield) of approximately 250±2 % at a 37.5 h HRT and 26±2 % at a 17.5 h HRT in H_2 yields for the LA-treated cultures. In contrast, the CH_4 yields were reduced in LA-treated cultures, showing that the addition of LA had an inhibitory effect on methanogens. These results provide evidence that the addition of LA combined with shorter HRTs has a synergistic effect on the methanogens in mixed culture. Average CH_4 yields (mol mol^{-1} glucose) were 0.11±0.01 at 37.5 h, 0.04±0.01 at 17.5 h, and 0.02±0.01 at 7.5 h HRTs (Figure 7.2).

Previous research on H_2 production from different types of substrates also indicated that lowering the HRT increased the H_2 yield, and inhibited CH_4 production (Chang and Lin, 2004; Fan et al., 2006; Hussy et al., 2003; Oh et al., 2004; Ueno et al., 1996). Optimizing the HRT can provide favourable conditions for H_2 production and may also divert the metabolic pathway towards the acid-forming pathway and away from the solvent-forming pathway (Han and Shin, 2004). Operation of reactors at lower HRTs provides some major advantages, such as the need for smaller reactor volumes. In addition, the reactors can also handle increased organic loading rates. Hussy et al. (2003) observed a 26 % increase in the H_2 yield obtained when the HRT was reduced from 24 h to 12 h HRT.

Variations in volumetric H_2 production rate (HPR) ($LH_2\ L^{-1}\ d^{-1}$) at different HRTs are shown in Figures 7.1 and 7.2. The volumetric HPRs also tend to increase with decreasing HRTs in both control and LA-treated cultures. The HPRs in control cultures at 37.5 h and 17.5 h HRTs were lower than the HPRs in LA-treated cultures. A maximum HPR of 4.89±0.15 $LH_2\ L^{-1}\ d^{-1}$ was obtained at 3.3 h, and was followed by a HPR of 2.4±0.06 $LH_2\ L^{-1}\ d^{-1}$ at 7.5 h HRTs in control cultures. The HPR results obtained in this study are similar to the findings of Arooj et al. (2008), who reported a HPR of 4.2 $LH_2\ L^{-1}\ d^{-1}$ at 6

h HRT in an ASBR fed with the substrate, corn starch. In studies conducted with the addition of LA, a maximum HPR of 2.9±0.13 $LH_2\ L^{-1}\ d^{-1}$ was observed at 7.5 h HRT.

7.3.2 Soluble metabolite profiles at various HRTs

The VFAs and alcohols profiles can be used to understand the metabolic pathways and their impact on H2 production (Table 7.3). The type of fermentation pathway followed by the microorganisms can be identified according to the dominant liquid metabolites formed. Acetic acid (HAc), butyric acid (HBu), propionic acid (HPr) and ethanol (Et-OH) are the major soluble metabolites observed in control studies. Propionic acid formation declined with decreasing HRTs indicating that

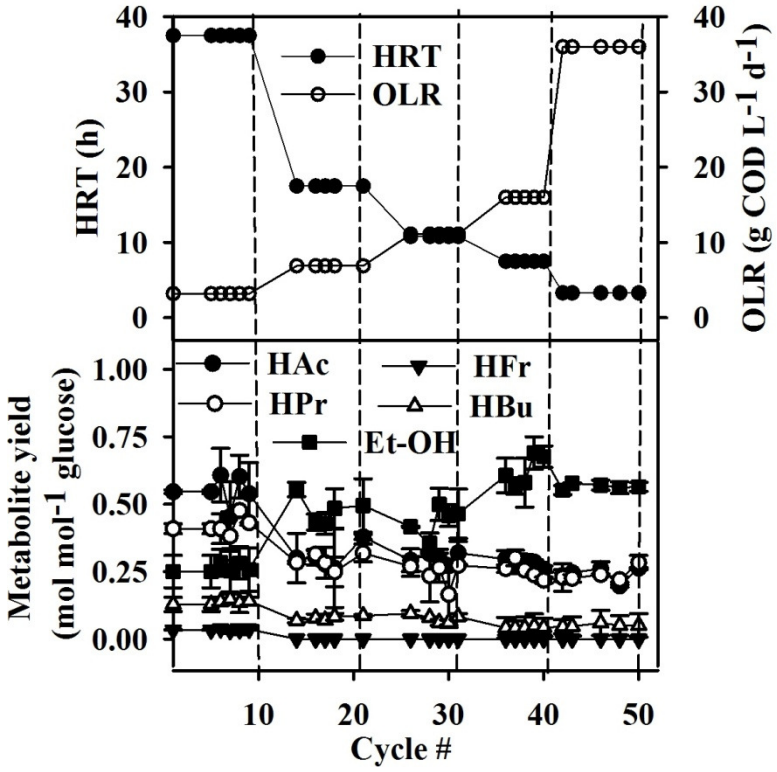

Figure 7.3 Liquid metabolite profiles for control cultures at various HRTs.
Notes: 1. HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; Et-OH = ethanol; 2. The error bars in this figure represents the standard deviation for n = 4.

Propionic acid forming bacteria (H_2 consumers) could have been washed out and hence, resulting in increased H_2 yields at shorter HRTs. Similar findings of decreasing HPr concentration with decreasing HRT were reported by Arooj et al. (2008). Hussy et al. (2004) also observed a 70 % reduction in HPr production when the HRT was lowered

from 24 h HRT to 12 h HRT. In LA-treated cultures, a negligible amount of HPr was produced at 17.5 h and 7.5 h HRT. Higher levels of H_2 production in LA-treated cultures were associated with elevated HBu levels. Badiei et al. (2011) reported a similar pattern of higher levels of H_2 production were associated with low levels of HPr and high levels of HBu. HBu production is limited by the substrate concentration and HPr production is limited by the H_2 concentration (Dinamarca and Bakke, 2012). HPr produced by H_2-consuming HPr-producing bacteria, is linked to lower H_2 yields. HBu-producing and HPr-producing bacteria are present in close association in mixed anaerobic culture (Kendall et al., 2006). Successful washout of the HPr-forming bacteria (with shorter HRTs) combined with LA treatment resulted in greater H_2 production as the HPr-forming bacteria did not consume the H_2 produced as a byproduct by the HBu-producing bacteria. In general, HPr production is negatively correlated with H_2 production and HBu and H_2 production are positively correlated (Dinamarca and Bakke, 2012). Similar correlations in metabolite formation have been reported previously (Arooj et al., 2008; Badiei et al., 2011; Cohen et al., 1985). The outcomes of the current study indicate that the experiments involving LA treatment resulted in the HBu-HAc type of fermentation (Yu and Fang, 2000), and the control experiments resulted in the mixed acid type of fermentation (Ren et al., 2007).

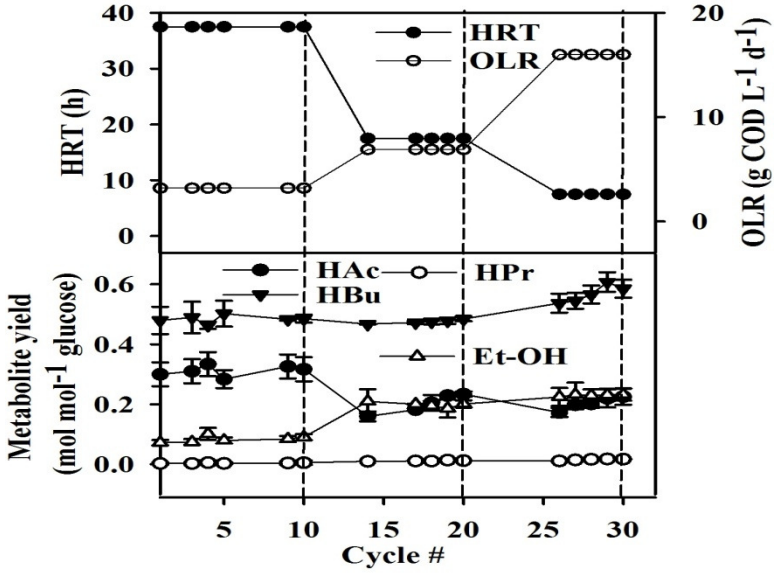

Figure 7.4 Liquid metabolites profile for the LA-treated cultures at various HRTs.
Notes: HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; Et-OH = ethanol. 2. The error bars in this figure represents the standard deviation for n = 4.

7.3.3 Flux balance analysis (FBA)

FBA was used to predict the internal metabolic fluxes based on the levels of the external metabolites. Gas (H_2, CH_4 and CO_2) and liquid metabolites (VFAs and alcohols) were given as the inputs for the flux model. Homoacetogenesis and hydrogenotrophic methanogenesis were reported as the primary mechanism of H_2 loss at long HRTs (1.7 days) in cultures fed glucose and xylose in an ASBR (Chaganti et al., 2013).

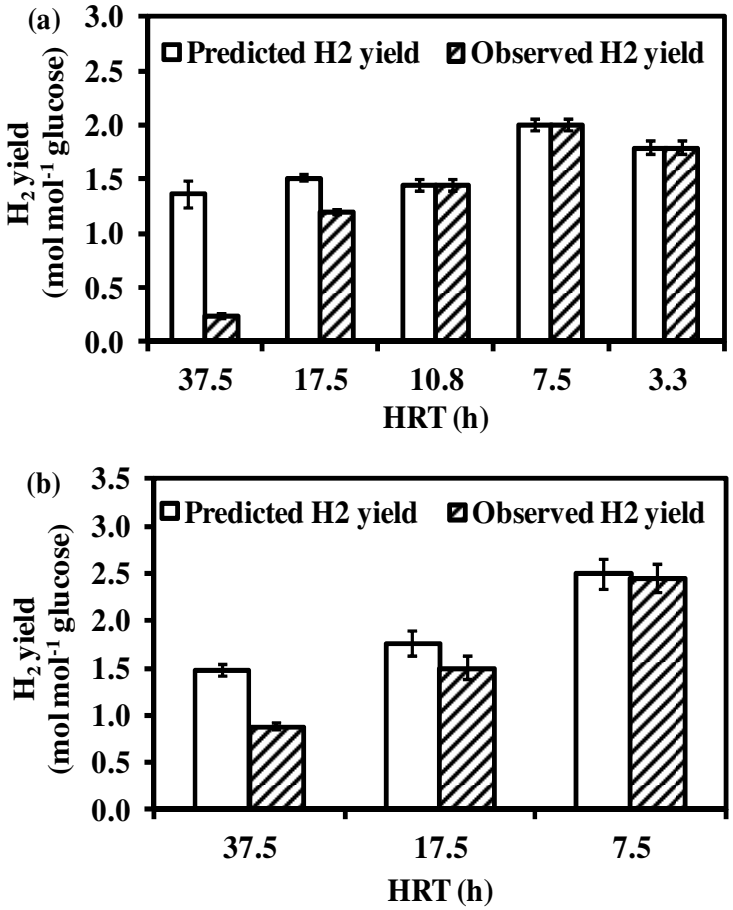

Figure 7.5 Comparison of the flux model's predicted and the experimental H_2 yields. (a) Control cultures; (b) LA treated cultures. Note: 1. Predicted H_2 yields were calculated based on the flux of ferredoxin Fd^{2+} reduction to H_2 using the MFA (from R12 flux described in Table 3.4). 2. The error bars in this figure represents the standard deviation for n = 10

In control experiments, aceticlastic and hydrogenotrophic methanogens accounted for 70 % and 30 %, respectively, of the CH_4 production at 37.5 h HRT (data not shown). As the HRT is reduced, hydrogenotrophic methanogenic activity is lost, and aceticlastic

methanogens are responsible for 100 % of the CH_4 production. No detectable homoacetogenic activity was observed at 37.5 h, 10.8 h, 7.5 h and 3.3 h HRTs in the control experiments. In LA-treated cultures, aceticlastic methanogen activity was observed at 17.5 h and 7.5 h HRTs, and hydrogenotrophic methanogen activity was observed at 37.5 h HRT. The predicted H_2 yields were calculated from the MFA that was based on the flux of electrons directed towards H_2 production through ferredoxin (Fd^{2+}) reduction (Figure 7.5). The experimental H_2 yield was then compared with the flux model's predicted H_2 yield in order to study the characteristics of the H_2 loss observed under each experimental condition. Plots of the predicted and observed H_2 yields are shown in Figure 7.5. At both 37.5 h HRT and 17.5 h HRT the experimental observations for the controls showed the loss of H_2 to H_2 consumers, whereas at 10.8 h, 7.5h and 3.3 h HRTs, the experimental data correlated well with the H_2 yields predicted by the model, indicating minimal H_2 loss to the H_2 consumers present in the mixed anaerobic culture.

Table 7.4 Distribution of H_2 flux among the major H_2 consumers.

HRT (h)	Homoacetogenesis[2] (mol mol^{-1} glucose)		Hydrogenotrophic methanogenesis[3] (mol mol^{-1} glucose)		H_2 consumed[1] (mol mol^{-1} glucose)	
	Control	LA	Control	LA	Control	LA
37.5	0.00±0.00	0.20±0.01	1.12±0.17	0.44±0.02	1.12±0.17	0.64±0.03
17.5	0.32±0.04	0.18±0.00	0.00±0.00	0.00±0.00	0.32±0.04	0.26±0.02
10.8	0.00±0.00	-	0.00±0.00	-	0.00±0.00	-
7.5	0.00±0.00	0.04±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.04±0.00
3.3	0.00±0.00	-	0.00±0.00	-	0.00±0.00	-

Notes: [1]H_2 consumed was calculated by subtracting the model's predicted (reaction R 12 from Table 3.4) and the experimental H_2 yields (Table 7.3) from flux balance analysis.
[2]Homoacetogenic activity was calculated using the reaction, R 17 (Table 3.4), from the flux balance analysis.
[3]Hydrogenotrophic methanogen activity was calculated using the reaction, R 29 (Table 3.4), from the flux balance analysis.
4. LA = linoleic acid-treated cultures.
5. Sample calculations are shown in Appendix B. The values shown in the table represents mean ± standard deviation of sample size n = 10.

Approximately, 1.12±0.17 mol H_2 mol^{-1} glucose was consumed at a 37.5 h HRT in the control experiment (Figure 7.5a). This was mainly due to hydrogenotrophic methanogens

activity in the mixed culture (Table 7.4). With decreasing HRTs, this activity was lost because of possible washout of methanogens that have longer doubling times than acidogens (Chynoweth and Isaacson, 1987). The observed reduction in the H_2 yield (0.32±0.04 mol mol^{-1} glucose) at a 17.5 h HRT was mainly due to the activity of homoacetogens. In LA-treated cultures, the loss of H_2 to H_2 consumers decreased with decreasing HRTs. At a 37.5 h HRT, there was an approximately 50 % reduction in electron loss to H_2 consumers in LA-treated cultures compared to the loss in control cultures (Figure 7.5b). This is the reason for the increased H_2 yields (0.88±0.04 mol mol^{-1} glucose) obtained at 37.5 h HRT in the LA-treated cultures. At 17.5 h HRT, 0.26 mol mol^{-1} glucose were consumed by the LA-treated cultures. Homoacetogens were responsible for 0.18±0.00 mol mol^{-1} glucose of the H_2 consumed, and the remaining 0.08±0.02 mol mol^{-1} glucose of H_2 consumed was utilized for the synthesis of iso-propanol (data not shown).

7.3.4 Principal component analysis

The PCA is helpful in extracting meaningful data and patterns from the large number of input variables (fermentation metabolites) in the original dataset. The PCA provides a clearer understanding of the similarities between the control and the stressed (LA-treated) cultures at different HRTs based on their grouping within the PCA bi-plot. The PCA was conducted using the fermentation metabolites data for both the control and the LA-treated cultures as the inputs. Principal component 1 (PC 1) and principal component 2 (PC 2) accounted for more than 94 % of the variation in the dataset (Figure 7.6). The untreated control cultures were grouped in separately, apart from the LA-treated cultures. PC 1 was positively correlated with CH_4 (0.4794), HAc (0.4568), HPr (0.4943) and Et-OH (0.1587) (Figure. 7.7a), whereas PC 2 was positively correlated with H_2 (0.4114), HFr (0.2130) and Et-OH (0.6665) (Figure. 7.7b). This is evident in the loading plots for PC 1 and PC 2, respectively.

The PCA bi-plot clearly distinguishes the metabolite patterns of cultures observed at different HRTs, both in the presence and the absence of LA. This is clearly seen in the variation in loading values (Fig. 7.7) that is illustrated in the PCA bi-plot (Fig. 7.6). The

lengths of the vectors in the bi-plot of PC 1 and PC 2 indicate the degree of variance of that particular metabolite within the dataset.

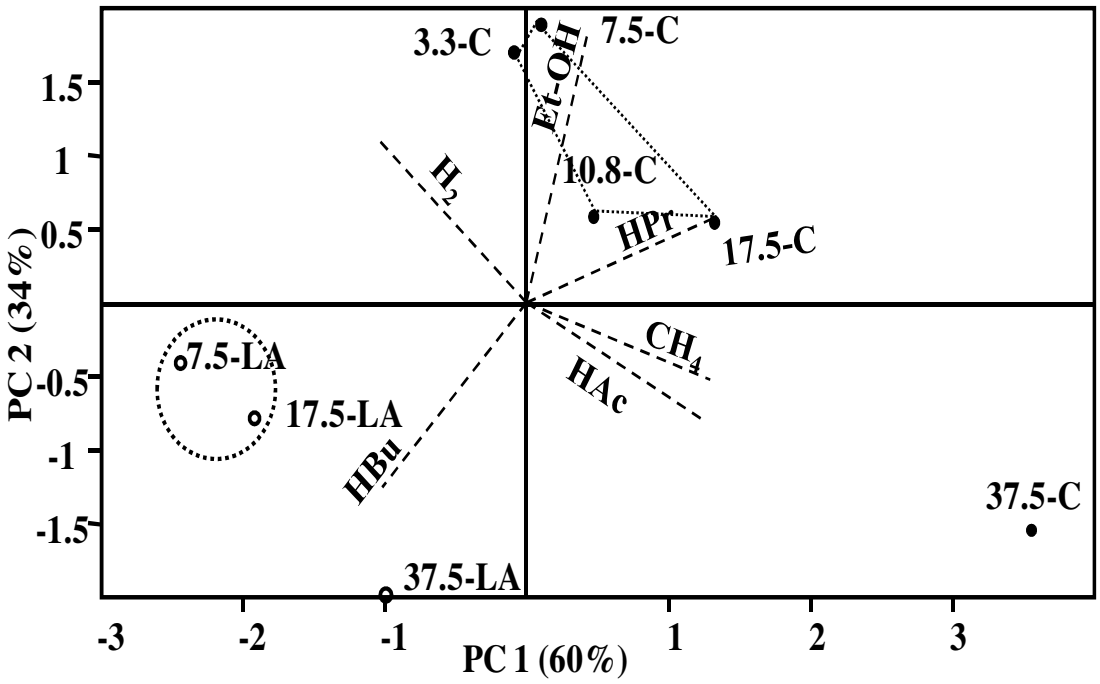

Figure 7.6 PCA bi-plot of fermentation metabolites at various HRTs.
Notes: 1. C = control culture; LA = Linoleic acid (LA)-treated culture.
2. HBu = butyric acid; HAc = acetic acid; H_2 = hydrogen; CH_4 = methane; HPr = propionic acid; Et-OH = ethanol.

For example, higher variance in both HPr and Et-OH levels at all HRTs was observed for the untreated control (without LA) cultures (Figure 7.6). However, there is a slight decrease in HPr levels (from 0.44±0.04 mol mol^{-1} glucose to 0.29±0.03 mol mol^{-1} glucose) when the HRT was decreased from 37.5 h HRT to 17.5 h HRT (Table 7.3). Similarly, as the HRT was decreased from 37.5 h HRT to shorter HRTs, there was an increase in Et-OH levels. Et-OH production increased from 0.27±0.02 mol mol^{-1} glucose at 37.5 h HRT to 0.57±0.01 mol mol^{-1} glucose at 3.3 h HRT in control cultures. In the PCA bi-plot, higher Et-OH concentrations resulted in the close association of the control cultures maintained at HRTs ranging from 17.5 h HRT to 3.3 h HRT (upper-right quadrant of the PCA bi-plot Fig. 7.6). Lower levels of HBu were observed in the untreated control cultures. In LA-treated cultures, negligible levels of HPr were produced under the conditions studied, but many of the electrons from the substrate, glucose, were

diverted towards HBu formation. This is also evident in the PCA bi-plot, which shows a clear association of HBu with LA-treated cultures (Figure 7.6). Low levels of Et-OH (0.09±0.01 mol mol^{-1} glucose) were observed at 37.5 h HRT in LA-treated cultures, and decreasing the HRT increased the flux of electrons diverted towards Et-OH production. Nevertheless, Et-OH production in the LA-treated cultures at 7.5 h HRT was 269 % lower than Et-OH production in the control cultures (Table 7.3).

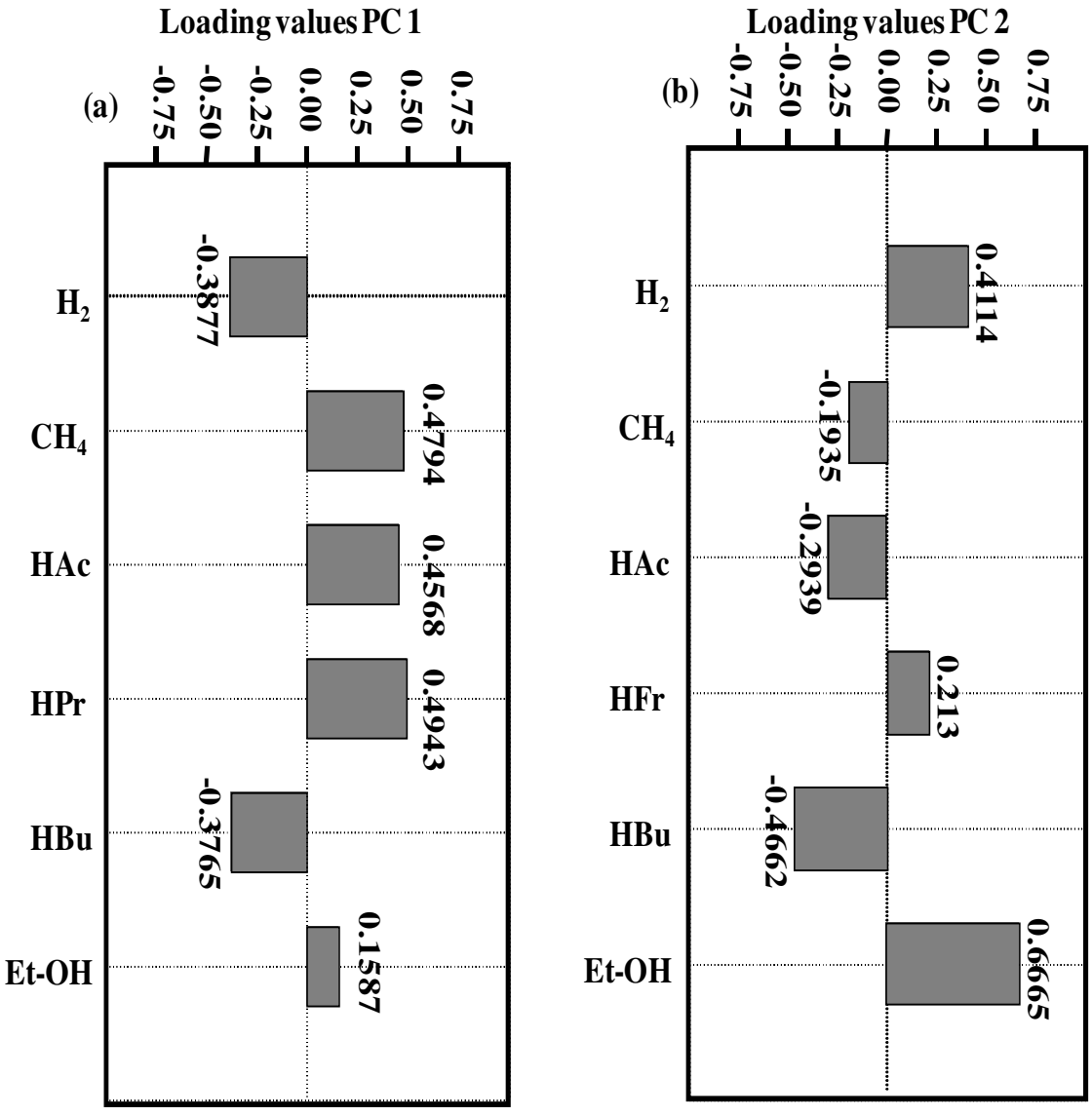

Figure 7.7 Loading values of the principal components of the PCA bi-plot (Figure 7.6). (a) PC 1; (b) PC 2.
Note: 1. PC = principal component.

7.3.5 Genomic analysis

7.3.5.1 T-RFLP analysis

The microbial community composition under different HRTs was analyzed using 16S rRNA gene-based T-RFLPs. A strong band indicating abundant *Lactococcus lactis* was detected in the initial inoculums, but declined to undetectable levels by the end of the 37.5 h HRT. This band was not detected at HRT values of 17.5h, 10.8h, 7.5h and 3.3h. According to Sikora et al. (2013), *Lactobacillus lactis* is mainly responsible for lactic acid production. The absence of lactic acid production under all of the HRTs conditions in the control experiments was likely due to absence of *Lactococcus lactis*. In the case of the methanogens, intense bands representing *Methylomicrobium* sp. and *Methylophaga* sp. were detected in the inoculum samples taken before start-up of the ASBRs. With decreasing HRT, methanogenic activity disappeared and the bands related to these organisms were no longer detected. This indicates that shortening the HRT is unfavourable for methanogenic growth but instead favourable for growth of H_2 producers.

A weak band of *Eubacterium* sp. was detected at 37.5h, 17.5h, 10.8h and 7.5h HRTs, but was not detected for the 3.3h HRT. Also, bands related to *Moorella thermoacetica* were detected in the initial inoculum but they were not present at the end of the cycle for all HRTs. The flux balance analysis revealed that homoacetogenesis was not detectable at 10.8h, 7.5h and 3.3h HRT's (Table 7.4) indicating that these homoacetogens might have been washed out at low HRT conditions. Both *Moorella thermoaceti*ca and *Eubacterium* sp. are homoacetogenic bacteria capable of growing with H_2 (Henderson et al., 2010; Mechichi et al., 1998). The same type of *Eubacterium* sp. was detected in mesophilic biogas digesters treating piggery waste (Liu et al., 2009). Bands related to *Geobacter* sp. were present only in the initial inoculums, but were not detected at other HRTs. Bands related to *Clostridium* sp., *Bacillus* sp., *Dethiosulfovibrio* sp., *Desulfomicrobium* sp., and *Alkaliphilus metalliredigens* were detected under all of the HRTs examined. In general, the initial inoculum showed the presence of lactic acid producing bacteria, methanogens and homoacetogenic bacteria but with decrease HRTs, these organisms were washed out

and the microbial population which was enriched with H_2 producing *Clostridium* sp. and *Bacillus* sp. resulted in higher H_2 yields.

7.3.5.2 Pyrosequencing

Pyrosequencing of the initial inoculum showed greater abundance of organisms belonging to the class, *Firmicutes* (56 %), followed by *Bacteroidetes* (38 %) (Figure 7.8a). The phylum, *Firmicutes*, includes organisms with low G+C content. The cell morphology of *Firmicutes* is either rod-shaped (bacillus) or coccoid-shaped. *Firmicutes* have the ability to produce endospores, which are resistant to stressful conditions. This class includes organotrophs, hydrolytic strains capable of degrading proteins, lipids and carbohydrates (Lynd et al., 2002). This group was reported to be the dominant population in biogas-producing reactors (Lee et al., 2008; Leven et al., 2007; Schluter et al., 2008; Wang et al., 2009). The phylum, *Bacteroidetes*, consists of non-spore-forming anaerobic, rod-shaped organisms present in soil, sediments and in the gut of animals. This class of microorganisms includes the genus, *Bacteroides*. *Firmicutes* and *Bacteroidetes* were the dominant in anaerobic biogas-producing reactors (Sundberg et al., 2013). Organisms related to the phyla, *Synergistetes*, *Proteobacteria*, *Fusobacteria*, *Chloroflexi*, *Actinobacteria*, *Arthropoda*, and uncultured organisms account for the remaining 6 % in the initial inoculum collected before start-up of the ASBR.

Pyrosequencing of microbial samples taken at 3.3 h HRT also indicated that the phylum, *Firmicutes* which was the dominant microbial population accounted for 91 % of the total population (Figure 7.8b). The *Bacteroidetes* population decreased to 2 % of the total population at 3.3h HRT compared to 38 % observed in the initial inoculum. At the family level, the initial inoculum which consisted of *Carnobacteriaceae*, accounted for 45 % of the 56 % of the population comprised of *Firmicutes* (Figure 7.8a). According to Machado et al. (2013), *Carnobacteriaceae* belongs to the class of gram positive lactic acid bacteria. A band related to *Lactobacillus lactis* was detected in the initial inoculum. In comparison, *Ruminococcaceae* accounted for 65 % of the total *Firmicutes* population at 3.3h HRT. This family of microorganisms was dominated by *Ethanoligenens.*

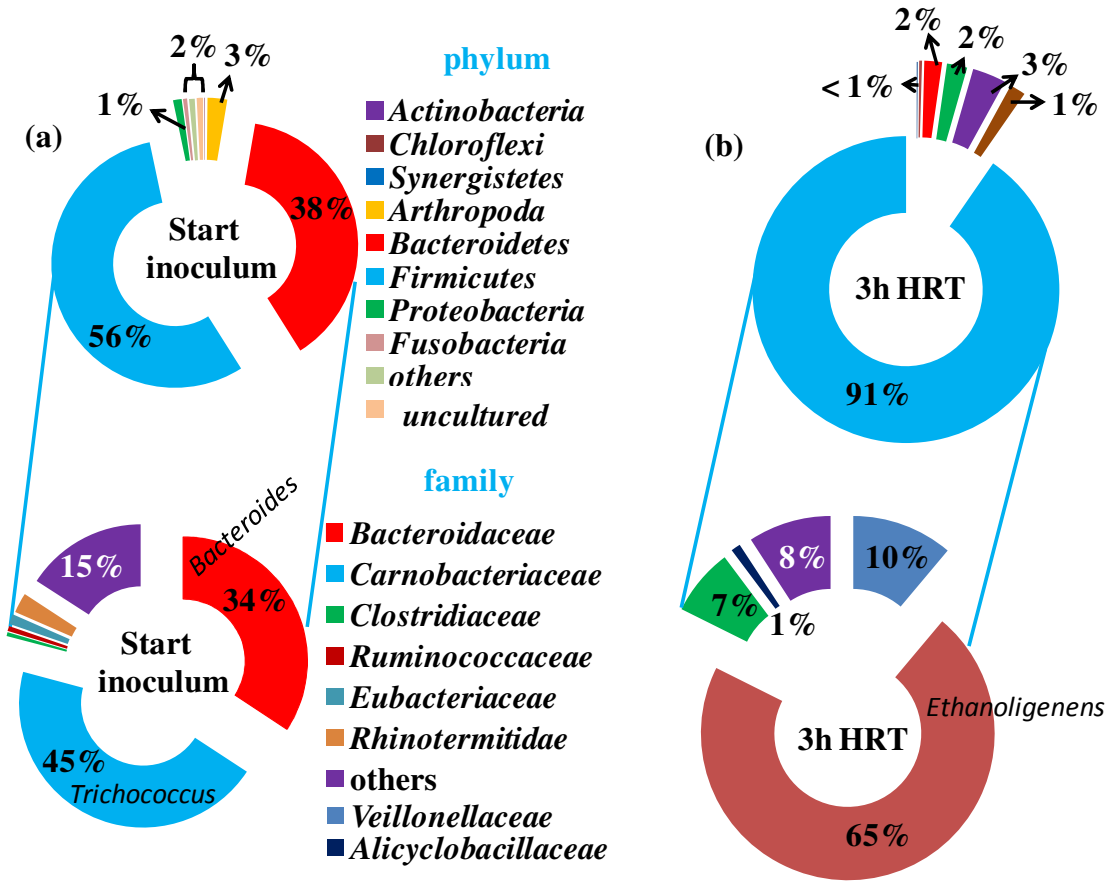

Figure 7.8 Pyrosequencing of the microbial samples (a) Initial inoculum and (b) 3.3 h HRT culture.

This family of organisms has the ability to utilize monomeric and polymeric sugars to produce acetic acid, ethanol, H_2 and CO_2 (Abreu et al., 2011). The higher HPR obtained at 3.3h HRT might be due to the activity of this family of organisms (Table 7.3). Reactor operation at 3.3h HRT also resulted in greater production of ethanol (0.57±0.01 mol mol^{-1} glucose) (Table 7.3), which might be due to the presence of *Ethanoligenens*. According to Xu et al. (2010), these organisms are able to ferment acetic acid to ethanol. *Eubacteriaceae and Clostridiaceae* accounted for 10 % and 7 %, respectively, of the *Firmicutes* population (Figure 7.8b).

7.4 Conclusions

The effect of HRT on biohydrogen production was investigated using an ASBR at 37 °C with glucose as the substrate. The major conclusions of this study are as follows:

1. Decrease in HRT showed an increase in H_2 yield due to the washout of non-spore forming methanogens.
2. Control cultures also showed higher HPR of 4.89±0.15 L H_2 L^{-1} d^{-1} at 3.3 h HRT. Adding LA resulted in 72±2 % and 25±2 % relative increase in H_2 yields at 37.5 h and 17.5 h HRT's. Negligible CH_4 production was observed in cultures treated with LA.
3. Liquid metabolite analysis revelaed negligible HPr production in LA fed cultures. HPr production also decreased with decreasing HRT.
4. FBA showed decreasing homoacetogenic activity (R17 flux) in LA treated cultures with decreasing HRT. In comparison, control cultures showed R17 flux only at 17.5 h HRT. Hydrogenotrophic methanogen activity (R29 flux) was observed in control cultures only at 37.5 h HRT.
5. Bi-plot PCA showed distinct grouping of LA treated cultures away from the control cultures due to distinct fermentation metabolite pattern.
6. T-RFLP analysis with the start inoculum showed the presence of HLa-producing bacteria, methanogens and homoacetogens. However, methanogens and HLa-producers were not observed with decreasing HRT. In addition, bands related to *Clostridium* sp., *Bacillus* sp., *Dethiosulfovibrio* sp., *Desulfomicrobium* sp., and *Alkaliphilus metalliredigens* were detected under all the experimental conditions.
7. Pyrosequencing analysis revealed that phylum Firmicutes accounted for 56% of the total bacterial population of which 46% comprised of HLa-producing bacteria. At 3.3 h HRT, there is a 38% increase in Firmicutes population and 95% decrease in Bacteroidetes population.
8. The results from this study indicate that proper control of HRT is sufficient to eliminate methanogens and HLa-producers. However, addition of LA is required to maximize H_2 yields at longer HRT's and minimize CH_4 production.

7.5 References

Abreu, A.A., Alves, J.I., Pereira, M.A., Sousa, D.Z., Alves, M.M. (2011). Strategies to suppress hydrogen-consuming microorganisms affect macro and micro scale structure and microbiology of granular sludge. *Biotechnol Bioeng*, **108**(8), 1766-75.

Andersson, A.F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P., Engstrand, L. (2008). Comparative analysis of human gut microbiota by barcoded pyrosequencing. *Plos One*, **3**(7), e2836.

Arooj, M.F., Han, S.K., Kim, S.H., Kim, D.H., Shin, H.S. (2008). Effect of HRT on ASBR converting starch into biological hydrogen. *Int J Hydrogen Energy*, **33**(22), 6509-14.

Badiei, M., Jahim, J.M., Anuar, N., Rozaimah, S., Abdullah, S. (2011). Effect of hydraulic retention time on biohydrogen production from palm oil mill effluent in anaerobic sequencing batch reactor. *Int J Hydrogen Energy*, **36**(10), 5912-9.

Carrillo-Reyes, J., Celis, L.B., Alatriste-Mondragón, F., Razo-Flores, E. (2012). Different start-up strategies to enhance biohydrogen production from cheese whey in UASB reactors. *Int J Hydrogen Energy*, **37**(7), 5591-601.

Chaganti, S.R., Pendyala, B., Lalman, J.A., Veeraoalli, S.S., Heath, D.D. (2013). Influence of linoleic acid, pH and HRT on anaerobic microbial populations and metabolic shifts in ASBRs during dark hydrogen fermentation of lignocellulosic sugars. *Int J Hydrogen Energy*, **38**(5), 2212-20.

Chang, F.Y., Lin, C.Y. (2004). Biohydrogen production using an up-flow anaerobic sludge blanket reactor. *Int J Hydrogen Energy*, **29**(1), 33-9.

Chen, C.C., Lin, C.Y. (2003). Using sucrose as a substrate in an anaerobic hydrogen producing reactor. *Adv Environ Res*, **7**(3), 695-9.

Chen, W.H., Sung, S., Chen, S.Y. (2009). Biological hydrogen production in an anaerobic sequencing batch reactor: pH and cyclic duration effects. *Int J Hydrogen Energy*, **34**(1), 227-34.

Chowdhury, N., Lalman, J.A., Seth, R., Ndegwa, P. (2007). Biohydrogen production by mesophilic anaerobic fermentation of glucose in the presence of linoleic acid. *J Environ Eng*, **133**(12), 1145-52.

Chynoweth, D.P., Isaacson, R. (1987). Anaerobic digestion of biomass. Elsevier Applied Science Publishers, Ltd. , New York, NY. p.279.

Cohen, A., Distel, B., Vandeursen, A., Breure, A.M., Vanandel, J.G. (1985). Role of anaerobic spore forming bacteria in the acidogenesis of glucose - Changes induced by discontinuous or low rate feed supply. *A Van Leeuw J Microb*, **51**(2), 179-92.

Dabrock, B., Bahl, H., Gottschalk, G. (1992). Parameters affecting solvent production by *Clostridium pasteurianum*. *Appl Environ Microbiol*, **58**(4), 1233-9.

Demeyer, D.I., Henderickx, H.K. (1967). The effect of C_{18} unsaturated fatty acids on methane production *in vitro* by mixed rumen bacteria. *BBA-Lipid Lipid Met*, **137**(3), 484-97.

Dinamarca, C., Bakke, R. (2012). Simultaneous hydrogen production and consumption in anaerobic mixed culture fermentation *Int J Energy Environ*, **3**(3), 323-32.

Fan, K.S., Kan, N.R., Lay, J.J. (2006). Effect of hydraulic retention time on anaerobic hydrogenesis in CSTR. *Bioresour Technol*, **97**(1), 84-9.

Fang, H.H.P., Li, C., Zhang, T. (2006). Acidophilic biohydrogen production from rice slurry. *Int J Hydrogen Energy*, **31**(6), 683-92.

Fang, H.H.P., Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresour Technol*, **82**(1), 87-93.

Han, S.K., Shin, H.S. (2004). Biohydrogen production by anaerobic fermentation of food waste. *Int J Hydrogen Energy*, **29**(6), 569-77.

Hawkes, F.R., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D.L. (2007). Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. *Int J Hydrogen Energy*, **32**(2), 172-84.

Henderson, G., Naylor, G.E., Leahy, S.C., Janssen, P.H. (2010). Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by analysis of formyltetrahydrofolate synthetase sequences from ruminants. *Appl Environ Microbiol*, **76**(7), 2058-66.

Hussy, I., Hawkes, F.R., Dinsdale, R., Hawkes, D.L. (2003). Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. *Biotechnol Bioeng*, **84**(6), 619-26.

Kendall, M.M., Liu, Y., Boone, D.R. (2006). Butyrate- and propionate-degrading syntrophs from permanently cold marine sediments in Skan Bay, Alaska, and description of *Algorimarina butyrica* gen. nov., sp. nov. *FEMS Microbiol Lett*, **262**(1), 107-14.

Kim, D.H., Kim, S.H., Kim, K.Y., Shin, H.S. (2010). Experience of a pilot-scale hydrogen-producing anaerobic sequencing batch reactor (ASBR) treating food waste. *Int J Hydrogen Energy*, **35**(4), 1590-4.

Kim, S.H., Han, S.K., Shin, H.S. (2005). Performance comparison of a continuous-flow stirred-tank reactor and an anaerobic sequencing batch reactor for fermentative hydrogen production depending on substrate concentration. *Water Sci Technol*, **52**(10-11), 23-9.

Kotsopoulos, T.A., Fotidis, I.A., Tsolakis, N., Martzopoulos, G.G. (2009). Biohydrogen production from pig slurry in a CSTR reactor system with mixed cultures under hyper-thermophilic temperature (70 °C). *Biomass Bioenerg*, **33**(9), 1168-74.

Lalman, J., Bagley, D.M. (2002). Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. *Water Res*, **36**(13), 3307-13.

Lay, J.J. (2000). Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol Bioeng*, **68**(3), 269-78.

Lay, J.J., Lee, Y.J., Noike, T. (1999). Feasibility of biological hydrogen production from organic fraction of municipal solid waste. *Water Res*, **33**(11), 2579-86.

Lay, J.J., Tsai, C.J., Huang, C.C., Chang, J.J., Chou, C.H., Fan, K.S., Chang, J.I., Hsu, P.C. (2005). Influences of pH and hydraulic retention time on anaerobes converting beer processing wastes into hydrogen. *Water Sci Technol*, **52**(1-2), 123-9.

Lee, C., Kim, J., Shin, S.G., Hwang, S. (2008). Monitoring bacterial and archaeal community shifts in a mesophilic anaerobic batch reactor treating a high-strength organic wastewater. *Fems Microbiol Ecol*, **65**(3), 544-54.

Leven, L., Eriksson, A.R.B., Schnurer, A. (2007). Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. *Fems Microbiol Ecol*, **59**(3), 683-93.

Li, C., Fang, H.H.P. (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Cri Rev Environ Sci Technol*, **37**(1), 1-39.

Lin, C.Y., Chang, R.C. (1999). Hydrogen production during the anaerobic acidogenic conversion of glucose. *J Chem Tech Biotechnol*, **74**(6), 498-500.

Liu, F.H., Wang, S.B., Zhang, J.S., Zhang, J., Yan, X., Zhou, H.K., Zhao, G.P., Zhou, Z.H. (2009). The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis. *J Appl Microbiol*, **106**(3), 952-66.

Liu, H., Wang, J., Wang, A., Chen, J. (2011). Chemical inhibitors of methanogenesis and putative applications. *Appl Microbiol Biotechnol*, **89**(5), 1333-40.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol Mol Biol Rev*, **66**(3), 506-77.

Machado, A., Almeida, C., Carvalho, A., Boyen, F., Haesebrouck, F., Rodrigues, L., Cerca, N., Azevedo, N.F. (2013). Fluorescence *in situ* hybridization method using a peptide nucleic acid probe for identification of *Lactobacillus* spp. in milk samples. *Int J Food Microbiol*, **162**(1), 64-70.

Mechichi, T., Labat, M., Woo, T.H.S., Thomas, P., Garcia, J.L., Patel, B.K.C. (1998). *Eubacterium aggregans* sp. nov., a new homoacetogenic bacterium from olive mill wastewater treatment digestor. *Anaerobe*, **4**(6), 283-91.

Nandi, R., Sengupta, S. (1998). Microbial production of hydrogen: An overview. *Crit Rev Microbiol*, **24**(1), 61-84.

Oh, S.E., Lyer, P., Bruns, M.A., Logan, B.E. (2004). Biological hydrogen production using a membrane bioreactor. *Biotechnol Bioeng*, **87**(1), 119-27.

Oh, S.E., Van Ginkel, S., Logan, B.E. (2003). The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ Sci Technol*, **37**(22), 5186-5190.

Ren, N.Q., Chua, H., Chan, S.Y., Tsang, Y.F., Wang, Y.J., Sin, N. (2007). Assessing optimal fermentation type for bio-hydrogen production in continuous-flow acidogenic reactors. *Bioresour Technol*, **98**(9), 1774-80.

Ren, N.Q., Guo, W.Q., Wang, X.J., Xiang, W.S., Liu, B.F., Wang, X.Z., Ding, J., Chen, Z.B. (2008). Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. *Int J Hydrogen Energy*, **33**(16), 4318-24.

Saady, N.M.C., Chaganti, S.R., Lalman, J.A., Veeravalli, S.S., Shanmugam, S.R., Heath, D.D. (2012). Effects of linoleic acid and its degradation by-products on mesophilic hydrogen production using flocculated and granular mixed anaerobic cultures. *Int J Hydrogen Energy*, **37**(24), 18747-60.

Schluter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.H., Krahn, I., Krause, L., Kromeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Puhler, A., Runte, K.J., Szczepanowski, R., Tauch, A., Tilker, A., Viehover, P., Goesmann, A. (2008). The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *J Biotechnol*, **136**(1-2), 77-90.

Schroder, C., Selig, M., Schonheit, P. (1994). Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium thermotoga maritima: Involvement of the Embden-Meyerhof pathway. *Archiv Microbiol*, **161**(6), 460-70.

Shanmugam, S.R., Chaganti, S.R., Lalman, J.A., Heath, D.D. (2014). Effect of inhibitors on hydrogen consumption and microbial population dynamics in mixed anaerobic cultures. *Int J Hydrogen Energy*, **39**(1), 249-57.

Shiue-Lin, L., Jian-Sheng, L., Yu-Hsuan, W., Ze-Kun, L., Shih-Chiang, K., Cheng, T.I., Sheng-Shung, C. (2011). Strategy of controlling the volumetric loading rate to promote hydrogen-production performance in a mesophilic-kitchen-waste fermentor and the microbial ecology analyses. *Bioresour Technol*, **102**(18), 8682-7.

Sikora, A., Zielenkiewicz, U., Błaszczyk, M., Jurkowski, M. (2013). *Lactic acid bacteria in hydrogen producing consortia: On purpose or by coincidence?*

Speece, R.E. (1976). Gas Flow Totalizer. US Patent 4064750 A.

Sprott, G.D., Jarrell, K.F., Shaw, K.M., Knowles, R. (1982). Acetylene as an inhibitor of methanogenic bacteria. *J Gen Microbiol*, **128**(10), 2453-62.

Sreethawong, T., Chatsiriwatana, S., Rangsunvigit, P., Chavadej, S. (2010). Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor: Effects of operational parameters, COD:N ratio, and organic acid composition. *Int J Hydrogen Energy*, **35**(9), 4092-102.

Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sørensen, S.J., Karlsson, A. (2013). 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *Fems Microbiol Ecol*, **85**(3), 612-26.

Tommasi, T., Sassi, G., Ruggeri, B. (2008). Acid pre-treatment of sewage anaerobic sludge to increase hydrogen producing bacteria HPB: Effectiveness and reproducibility. *Water Sci Technol*, **58**(8), 1623-8.

Ueno, Y., Otsuka, S., Morimoto, M. (1996). Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. *J Ferment Bioeng*, **82**(2), 194-7.

Van Ginkel, S., Logan, B.E. (2005). Inhibition of biohydrogen production by undissociated acetic and butyric acids. *Environ Sci Technol*, **39**(23), 9351-6.

Venkata Mohan, S., Lalit Babu, V., Sarma, P.N. (2007). Anaerobic biohydrogen production from dairy wastewater treatment in sequencing batch reactor (AnSBR): Effect of organic loading rate. *Enzyme Microb Technol*, **41**(4), 506-15.

Wang, H., Lehtomaki, A., Tolvanen, K., Puhakka, J., Rintala, J. (2009). Impact of crop species on bacterial community structure during anaerobic co-digestion of crops and cow manure. *Bioresour Technol*, **100**(7), 2311-5.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*, **73**(16), 5261-7.

Westermann, P., Jørgensen, B., Lange, L., Ahring, B.K., Christensen, C.H. (2007). Maximizing renewable hydrogen production from biomass in a bio/catalytic refinery. *Int J Hydrogen Energy*, **32**(17), 4135-41.

Wiegant, W.M., Lettinga, G. (1985). Thermophilic anaerobic digestion of sugars in upflow anaerobic sludge blanket reactors. *Biotechnol Bioeng*, **27**(11), 1603-7.

Wu, X., Yang, H., Guo, L. (2010). Effect of operation parameters on anaerobic fermentation using cow dung as a source of microorganisms. *Int J Hydrogen Energy*, **35**(1), 46-51.

Wu, X., Zhu, J., Dong, C., Miller, C., Li, Y., Wang, L., Yao, W. (2009). Continuous biohydrogen production from liquid swine manure supplemented with glucose

using an anaerobic sequencing batch reactor. *Int J Hydrogen Energy*, **34**(16), 6636-45.

Xu, J.F., Ren, N.Q., Su, D.X., Qiu, J. (2010). Bio-hydrogen production from acetic acid steam-exploded corn straws by simultaneous saccharification and fermentation with *Ethanoligenens harbinense* B49. *Int J Energy Res*, **34**(5), 381-6.

Yu, H.Q., Fang, H.H.P. (2000). Thermophilic acidification of dairy wastewater. *Appl Microbiol Biotechnol*, **54**(3), 439-44.

Zhang, C., Lv, F.X., Xing, X.H. (2011). Bioengineering of the *Enterobacter aerogenes* strain for biohydrogen production. *Bioresour Technol*, **102**(18), 8344-9.

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T., Lee, D.J., Jiang, W.J. (2006). Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. *Process Biochem*, **41**(10), 2118-23.

Zinder, S.H., Anguish, T., Cardwell, S.C. (1984). Selective inhibition by 2-bromoethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor. *Appl Environ Microbiol*, **47**(6), 1343-5.

CHAPTER 8: STATISTICAL OPTIMIZATION OF HYDROGEN PRODUCTION FROM A STEAM EXPLODED CORN STALK HYDROLYSATE USING A MIXED CULTURE IN AN ANEROBIC SEQUENCING BATCH REACTOR

8.1 Introduction

The global energy economy is heavily dependent a plentiful supply of fossil fuels. Significant global research efforts is focused on utilizing fuels produced from renewable feedstocks because their impact on the environment is less when compared to non-renewable fossil fuels (Cheng et al., 2011). Among the alternative renewable fuels, hydrogen (H_2) is emerging as a promising energy carrier because it is carbon neutral, recyclable, efficient and can be directly used in fuel cells for electricity generation (Das and Veziroglu, 2001). In addition, H_2 is used in a wide range of industrial applications such as ammonia, alcohol and aldehyde synthesis as well as the hydrogenation of vegetable oils, petroleum and coal. The high cost associated with the production of biological H_2 is a major obstacle preventing commercialization (Cheng et al., 2011) and hence, significant efforts are focused on developing economical H_2 production technologies using lignocellulosic feedstocks.

A strategy in developing an economical source of H_2 is to utilize low value lignocellulosic feedstocks. Lignocellulosic materials such as agricultural residues (corn stover, cereal straw, and sugarcane bagasse), forest residues (hardwood and softwood chips) and dedicated crops (switchgrass and sorghum) are abundant and easily available. In Canada, greater than 4 MT (million tonnes) of cereal straw and corn stover are produced annually with a gross energy potential of approximately 92 million GJ per year (Helwig et al., 2002) or 17 million BOE (barrel of oil equivalent). Because of the availability of lignocellulosics, current research interests are focused on the microbial conversion of these materials into H_2 and other energy fuels.

Many past H_2 production studies have utilized pure carbohydrates such as glucose, xylose and starch to produce H_2 either by employing pure cultures or mixed cultures (Fan et al., 2006). However, recent trends have shown research activities are beginning to focus on converting renewable lignocellulosic biomass residues to H_2 by mixed cultures (Fan et al., 2006). Employing pure cultures to degrade complex substrates such as starch and cellulose is problematic because they have limited metabolic capabilities (Argun and Kargi, 2009) and they can be easily contaminated by H_2 consuming microorganisms (Bartacek et al., 2007). In comparison, mixed cultures are suitable for fermenting complex substrates such as those derived from energy crops (Hallenbeck and Ghosh, 2009).

Pretreatment using enzyme, chemical or thermal methods are utilized to convert lignocellulosics into hemicellulose and cellulose monomers plus furans and their derivatives (Kumar et al., 2009). Steam explosion employs a combination of temperature and high-pressure saturated steam, which is suddenly reduced to undergo rapid decompression. Steam explosion is generally initiated at temperatures ranging between 160 and 260°C and pressures ranging from 0.69 to 4.85 MPa. Addition of dilute sulfuric acid, sulfur dioxide or carbon dioxide in the steam explosion process often results in lowering the reaction temperature and residence time. This improves the hydrolysis process, decreases the production of inhibitory compounds and increases hemicellulose recovery (Ballesteros et al., 2006; Stenberg et al., 1998). Only a few studies have described H_2 production using liquors derived from steam exploded lignocellulosic biomass.

Hydrogen fermentation is affected by factors such as pH, temperature, HRT, substrate concentration, partial pressure of H_2 (pH_2) and inoculum concentration. Among the various factors, pH, temperature and HRT are important in controlling the fermentation process (Appels et al., 2008). Previous work on H_2 production from corn stover have used a single factor approach to assess the impact each variable on H_2 production (Datar et al., 2007; Wang et al., 2012; Wang et al., 2010; Zhang et al., 2007). Conducting studies to optimize H_2 production from lignocellulosics such as corn stover using a 'one-factor at a time' approach will often lead to misinterpretation of data, especially when the

interaction effects from other variables are not considered in the data analysis. Using a statistical approach to examine the effect of multiple process variables on the H_2 yield has been reported by (Ghosh and Hallenbeck, 2010; Hallenbeck, 2009).

Response surface methodology (RSM), a collection of mathematical and statistical techniques used in the development of adequate functional relationship between the response variables and a number of associated control variables. A few studies have reported using RSM to model H_2 production from a variety of substrates (Cao et al., 2010; Costa et al., 2011; Ghosh and Hallenbeck, 2010; Lay, 2000; Sun et al., 2010). Moreover, studies concerning the optimization of H_2 production from lignocellulosic materials are sparse. Hence, the first objective of this study was to develop models which can predict H_2 and CH_4 production from a corn stover steam exploded liquor over a range of temperature, pH and HRT conditions using a mixed anaerobic culture. The second objective of this study was to investigate the microbial diversity analysis of cultures subjected to different experimental conditions. The third objective is to compare the specific methanogenic activity (SMA) of the cultures fed with pure sugars (glucose: xylose, 50:50) and to those fed with corn stalk liquor

8.2 Materials and methods

The procurement of corn stalks (CS) and its compositional analysis were carried out according to the methods described in sections 3.1, 3.6 and 3.7. The cellulose, hemicellulose and lignin content of the CS used in this study are described in Table 8.1. The elemental composition of raw CS, steam exploded CS and its liquor are described in Table 8.2. The culture source used in this study was described in section 3.2. The initial VSS measurement for the raw sludge collected from the UASBr reactor was calculated according to the method described in section 3.7.3. Then, three mother reactors (10 L) were constructed with a working volume of 8 L for operation with a VSS concentration of 20,000 mg L^{-1} at three different temperatures (21 °C, 37 °C and 53 °C). These cultures were fed a mixture of glucose (G) and xylose (X) in the ratio of 1:1 on a regular basis. The feed concentration was 5 g COD L^{-1}. These cultures were maintained at these temperatures in order to acclimatize the culture and to enrich the dominant

microorganisms present at these temperatures. Gas production was monitored using a biogas meter and GC (gas chromatography) was used to measure the composition of the headspace biogas produced in each of the reactors. Before the start of the experimental studies, two CSTRs (continuous stirred tank reactors) were washed and the fresh culture was inoculated into the two reactors. The initial VSS concentration in the CSTRs was 10,000 mg L^{-1}. The reactors were operated in a sequential batch mode.

Table 8.1 Compositional analysis of raw corn stalk (CS) residues and steam exploded CS liquor.

Composition analysis (Raw CS)	**Composition (g 100 g^{-1} dry biomass)**
Cellulose	36.3±1.5
Hemicellulose	34.1±0.8
Lignin[a]	20.0±0.5
Ash	5.5±0.2
Crude protein[b]	4.6±0.3
Total volatile solids (TVS)	90
Steam exploded CS liquor	**Concentration (g L^{-1})**
Total reducing sugars[c]	18.00±0.50
Glucose	4.62±0.09
Xylose	11.01±0.27
Arabinose	1.38±0.08
Galactose	0.73±0.05
Mannose	0.23±0.01
Phenol	0.32±0.05
Furfural	2.42±0.03
5-hydroxy methyl furfural	0.26±0.03
Acetic acid	2.10±0.29
Soluble COD	28.34±0.50

Notes: 1. Values are expressed as mean ± standard deviation of triplicate samples.
2. [a]Total lignin content measured using the sum of acid soluble lignin + acid insoluble lignin (Klason lignin). Acid soluble lignin = 5.9±0.09; Klason lignin = 14.0±0.10
3. [b]Crude protein is determined using the % N estimated using CHNS analysis. Crude protein = % N * 6.25.
4. [c]Total reducing sugars were estimated using DNS method (Miller, 1959).

The CSTRs were fed pure G and X (1:1 ratio, 5 g COD L^{-1}) in each experimental condition (Table 8.3), and the biogas production was monitored. After stable gas

production was observed under each experimental condition, the feed for the reactors was switched from the G: X mixture to CS liquor (5 g COD L^{-1}).

Table 8.2 Elemental analysis of raw corn stalk, steam exploded corn stalk and steam exploded liquor.

Sample	Sample mass taken (mg)	% C	% H	% N	% S	% O[1]	HHV[2] (MJ/kg)
Raw corn stalk	8.12	52.87	5.03	0.73	1.13	40.24	20.03
Steam exploded corn stalk	9.30	44.09	4.51	0.47	1.04	49.89	17.21
Steam exploded liquor[3]	1.89	40.57	6.20	1.79	1.55	49.89	17.29

Notes: [1]% oxygen (O) content was estimated by summing the % composition due to carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) and subtracting the total from 100; i.e., O = (100 - C - H - N – S). All the samples were analyzed in triplicate. The error in the experimental analysis was less than 5 %.

[2]HHV = high heating value of the material studied expressed in mega Joules (MJ) per kilogram (kg) of the dry weight of the sample. HHV was calculated using the following equation developed by Sheng and Azevedo (2005).

HHV (MJ/kg) = -1.3675 + (0.3137*C) + (0.7009*H) + (0.0318*O). The units of C, H, and O are percentages.

[3]The steam exploded liquor was left to undergo vaporization @ 70 °C so that it would condense into a thick paste. This sample was then analyzed.

Before feeding the CS liquor, microbial samples were collected in 160 mL serum bottles and purged with N_2 to ensure proper anaerobic conditions. These serum bottle reactors were then incubated at the experimental temperature corresponding to the temperature that the CSTRs were maintained at for each experimental condition (Table 8.3). After completion of each cycle, both gas samples and liquid samples were collected for analysis. The quantity of biogas produced was measured using a tipping bucket gas meter (US patent #4064750 A) (Speece, 1976).

Table 8.3 Fractional factorial ($3^{(3-1)}$) experimental design with three independent variables and the response (H_2 and CH_4) yields of triplicate samples.

Exp. No.	Experimental variables						Response variables			
	Temperature (°C)		pH		HRT (h)		H_2 yield (mL g^{-1} TVS)		CH_4 yield (mL g^{-1} TVS)	
	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	Experimental	Predicted	Experimental	Predicted
1	21	-1	4.5	-1	6	-1	8.17±1.26	3.45	2.59±0.82	4.86
2	37	0	5.5	0	12	0	65.00±3.61	60.28	10.52±3.61	12.79
3	53	+1	6.5	+1	18	+1	75.00±1.50	70.29	6.28±0.13	8.55
4	21	-1	5.5	0	18	+1	0.03±0.01	5.42	15.45±1.49	12.99
5	37	0	6.5	+1	6	-1	21.67±1.61	38.16	18.09±0.73	15.63
6	53	+1	4.5	-1	12	0	96.17±10.28	101.55	0.04±0.02	0
7	21	-1	6.5	+1	12	0	0.21±0.02	0	12.14±0.93	12.32
8	37	0	4.5	-1	18	+1	38.83±0.58	27.05	13.50±0.58	13.68
9	53	+1	5.5	0	6	-1	97.00±4.77	96.32	0.02±0.02	0.20

Each HRT cycle was configured as follows: 8 min fill time, 30 min settling time, 7 min decant time and with varying reaction times of 2.25 h, 5.25 h and 8.25 h for 6 h, 12 h and 18 h HRT's, respectively. The volume decanted per cycle was constant at 2.5 L. Effluent liquid samples were collected at the decant stage of each cycle. Fresh inoculum was replaced before the beginning of each experiment. The number of cycles of operation varied for each experimental condition after feeding the CSTR with CS liquor. Each HRT was continued until there was minimum variation in the analysis of the headspace gas from both of the reactors.

All of the samples were analyzed in triplicate. The GC (gas chromatography), IC (ion chromatography) and HPLC (high-performance liquid chromatography) analyses were conducted according to the methods described in section 3.7. The reduction in COD for each experimental condition was determined by performing a COD analysis according to the methods described in section 3.6.2.2. Before the end of each experiment, samples for microbial (T-RFLP) analysis were collected and stored at -4 °C (in order to prevent further microbial growth). The cultures were also collected in 15 mL plastic centrifuge tubes. At the end of each experiment in ASBR fed CS liquor microbial cultures were collected in 160 mL serum bottles and purged with N_2 and incubated at corresponding experimental temperature in an orbital shaker. Similarly, batch reactors (160 mL) inoculated with either sugar-fed or CS liquor-fed cultures were collected over the course of the entire study, and their VSS content was quantified.

Table 8.4 Experimental design matrix for the specific methanogenic activity (SMA) analysis.

Exp #	Temperature (°C)	pH	Culture A	Culture B	Bottle preparation (time = -24 h)	Substrate (3000µmol H_2) addition (time = 0, 4, 8, 22, 48, 72 h)	Liquid metabolite (VFAs and alcohol) analysis (t = 72 h)
10	21	4.5	√	√			
11	21	5.5	√	√			
12	21	6.5	√	√			
13	37	4.5	√	√			
14	37	5.5	√	√			
15	37	6.5	√	√			
16	53	4.5	√	√			
17	53	5.5	√	√			
18	53	6.5	√	√			

Note: 1. Cultures A and B represent "pure sugar-fed" and "liquor-fed" cultures, respectively.

Specific methanogenic activity (SMA) was studied in the cultures fed (G: X) and CS liquor. Batch reactors (160 mL) were prepared in an anaerobic glove box with the cultures collected at the end of each experiment, and incubated at the temperature corresponding to the experimental temperatures in ASBR. These cultures were centrifuged and the supernatant was discarded in order to remove any remaining substrate or its degradation by-products before preparing the bottles. The pellet was resuspended in

fresh basal medium (described in section 3.3). The final operating VSS concentration in the batch reactors was adjusted to 500 mg L^{-1} using the basal medium. The pH of each batch reactor was adjusted according to the pH level specified for the particular experimental condition (Table 8.4) with the addition of 1M KOH or 1 N HC1. Both the control (G:X fed) cultures and CS liquor fed cultures were incubated at appropriate experimental temperatures (Table 8.4) in an orbital shaker for performing the SMA tests. After 24 h, 3000 µmol of H_2 was injected into the headspace of each batch reactor and the H_2 consumption was monitored at regular time intervals using GC. Liquid samples were collected before H_2 addition and at the end of the experiment, and analyzed for VFAs (HAc) production.

8.2.1 Hydrogen optimization study

The range of factors selected for the optimization study was based on data from published reports. The optimal pH for H_2 production using mixed anaerobic cultures is dependent on numerous factors and many studies have examined a pH range from 5 to 7 (Pakarinen, 2011; Valdez-Vazquez and Poggi-Varaldo, 2009). Hence, pH 4.5, 5.5 and 6.5 were selected to cover the range for optimum H_2 production. Typically, H_2 production is favorable at shorter HRT because the growth rates of acidogenic H_2 producing bacteria are larger when compared to H_2 consuming methanogens (Pakarinen, 2011; Valdez-Vazquez and Poggi-Varaldo, 2009).Based on previously reported studies (Valdez-Vazquez and Poggi-Varaldo, 2009), the selected HRT values were 6 h, 8 h and 12 h (Table 8.3). Three temperature conditions considered included 21 °C, 37 °C and 53 °C because greater H_2 yields are observed with increasing temperature conditions.

A $3^{(3-1)}$ fractional factorial experimental design was used to optimize the experimental factors (Table 8.3). The design matrix for the coded and uncoded variables plus the model predicted and experimental responses (H_2 and CH_4 yields) are shown in Table 8.3. The response values were used for further analysis in fitting of quadratic polynomial equations (equations 1 and 2) to correlate the relationship between the experimental and the response variables. An analysis of variance (ANOVA) was also conducted to evaluate the model. The Minitab (Version 16, Minitab Inc., State College, PA) was used for

analysis of design, generating the interaction plots and the contour plots. An analysis of residuals was conducted using the Anderson Darling (AD) statistic. The AD test was used to predict whether the residual values were normally distributed. An algorithm from MINITAB 16, a statistical software package, was used to compute the D-optimality values for the three experimental factors (temperature, initial pH and HRT) and the levels for each experimental factor which yields a maximum response (maximum H_2 yield and minimum CH_4 yield). The D-optimality criterion minimizes the variance of the response surface model by maximizing the factor levels (Titterington, 1975).

8.2.2 Genomic analysis

The diversity of different microbial communities in the mixed culture subjected to different pretreatment conditions was determined using nested polymerase chain reaction (PCR) of the 16S rRNA gene followed by terminal restriction fragment length polymorphism (T-RFLP) analysis. The microbial samples collected at the end of the experiment were used for the community analysis. Details for DNA isolation, PCR amplification and T-RFLP methods were conducted according to the methods described in section 3.10. The data sets obtained from the T-RFLP analysis comprised the peaks reflecting the size of terminal restriction fragments (T-RFs) in base pairs (bp) together with the area of each peak measured in fluorescence units. For the T-RFs generated in the current study, a phylogenetic assignment was performed using a modified database generated for T-RFs which were previously described in section 3.10.

8.2.3 Flux balance analysis

The flux balance analysis (FBA) was conducted using the fermentation metabolite data obtained under different experimental conditions (Table 8.3). The metabolic reactions network and the reaction stoichiometries were previously in section 3.11. The FBA was conducted using the MetaFluxNet Software, Version 1.8.6.2. Flux analysis was conducted to estimate the H_2 consuming electron fluxes (homoacetogenic, hydrogenotrophic and propanol) under different experimental conditions.

8.2.4 Multivariate analysis

The interrelationships between multiple dependent variables and independent variables can be studied using a multivariate statistical model such as canonical correspondence analysis (CCA). The CCA was conducted using the PAST software package (Hammer et al., 2001). The relationship between the microbial community compositions (species), byproduct (environmental variables) distributions under different experimental conditions were determined using the CCA. The CCA tri-plot shows the effect of the experimental variables (pH, temperature and HRT) on the microbial population. The length of the vector implies the relative importance of a factor in the CCA tri-plot. The relative closeness of the relationship between the vector and nearest axis can be calculated using the angle between them. Principal component analysis (PCA) was performed using the fermentation metabolites according to the methods described previously in section 3.12.

8.3 Results and discussion

8.3.1 Experimental design analysis

The experimental responses (H_2 yield and CH_4 yield) obtained under varying levels of the selected factors are shown in Table 8.3. The H_2 and CH_4 yields range from 0.03±0.01 to 97.00±4.77 mL g^{-1} TVS and 0.02±0.02 to 18.09±0.73 mL g^{-1} TVS, respectively. Variation in experimental responses with varying temperature, pH and HRT supports the need to select fermentation conditions to optimize H_2 production. The quadratic polynomial equations relating the experimental factors and the response variables are shown in equations 8.1 and 8.2.

$$H_2\ yield\ (mL\ g^{-1}\ TVS) = 60.29 + 43.29 * (Temperature) - 7.72 * (pH) - 2.16 * (HRT) + 4.26 * (Temperature)^2 - 14.00 * (pH)^2 - 13.66 * (HRT)^2 \quad (8.1)$$

$$CH_4\ yield\ (mL\ g^{-1}\ TVS) = 12.80 - 3.97 * (Temperature) + 3.40 * (pH) + 2.42 * (HRT) - 7.95 * (Temperature)^2 + 0.11 * (pH)^2 + 1.75 * (HRT)^2 \quad (8.2)$$

8.3.2 Significance of ANOVA

The statistical significance of the experimental responses was examined using an analysis of variance (ANOVA). The ANOVA was performed to test the significance of fit for the reduced quadratic model (Table 8.5).

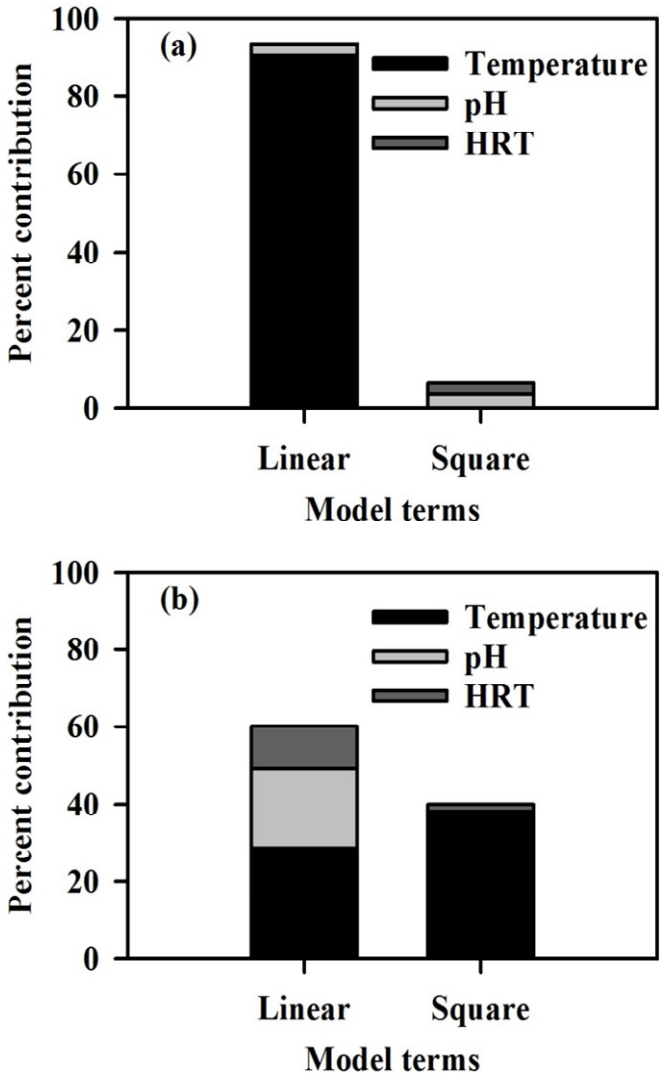

Figure 8.1 Factor contribution plots for the experimental responses (a) H_2 yield; (b) CH_4 yield.
Note: Linear and square terms for the regression models were provided in Table 8.5.

Table 8.5 ANOVA for experimental responses (H_2 and CH_4 yields) at different factor levels.

Source	Degrees of Freedom	Sequential Sum of Squares		Mean Square		*F-value*		*p-value*	
		H_2	CH_4	H_2	CH_4	H_2	CH_4	H_2	CH_4
Regression	6	37301.30	995.34	6216.90	165.89	163.31	29.69	0.000	0.000
Linear	3	34893.70	597.41	11631.20	199.14	305.54	35.64	0.000	0.000
Temperature (X_1) (°C)	1	33738.10	284.17	33738.10	284.17	886.26	50.86	0.000	0.000
pH (X_2)	1	1071.50	207.67	1071.50	207.67	28.15	37.17	0.000	0.000
HRT (X_3) (h)	1	84.10	105.56	84.10	105.56	2.21	18.89	0.153*	0.000
Square	3	2407.60	397.93	802.50	132.64	21.08	23.74	0.000	0.000
X_1*X_1	1	109.00	379.43	109.00	379.43	2.86	67.91	0.106*	0.000
X_2*X_2	1	1176.70	0.07	1176.70	0.07	30.91	0.01	0.000	0.912*
X_3*X_3	1	1122.00	18.43	1122.00	18.43	29.47	3.30	0.000	0.084*
Residual error	20	761.40	111.74	38.10	5.59				
Lack-of-Fit	2	465.20	101.02	232.60	50.51	14.14	84.77	0.000	0.000
Pure Error	18	296.20	10.73	16.50	0.60				
Total	26	38062.70	1107.08						

Notes: 1. $F_{(critical)\ 0.05,6,27} = 3.40$; 2.* denotes *p-values* which are insignificant at $\alpha = 0.05$

This technique subdivides the total variation in a set of data into component parts associated with specific sources of variation and it also helpful in testing hypotheses on the model parameters (Kim et al., 2003). *p*-values were used to determine the significance (*p*-value < 0.05) of the linear (Temperature (X_1), pH (X_2) and HRT (X_3)) and quadratic terms (X_1^2, X_2^2 and X_3^2). In general, variables with greater F-values and smaller *p*-values are termed significant (Montgomery, 2012).

Note, the *F-statistic* for the experimental H_2 and CH_4 response yields were 163.3 and 29.7, respectively. The *F-statistic* for the regression model was greater than the critical *F-value* of 3.40 at a 5 % level of significance. The larger *F-values* obtained for both H_2 and CH_4 yields suggest that variation in the experimental response can be explained by the regression equation.

Linear and quadratic terms *p*-values less than 0.05 indicate that the model fit is statistically significant. The variation due to model inadequacy is indicated by the lack-of-fit term. The *p*-value for the lack-of-fit term (less than 0.05) indicates that there is sufficient evidence to suggest that the model adequately fit the data. In simple terms, the model derived clearly explains the relationship between the experimental and the response variables. The percent contribution of the linear and square model terms of the response variables were shown in Figure 8.1. The linear terms (temperature (90 %), pH (3 %) and HRT (0.2 %)) accounted for the largest percent contribution for the H_2 yield in the regression model (93.2 %) (Figure 8.1a). Similarly, linear terms accounted for 61 % for the CH_4 yield (Figure 8.1b). The percent contributions from the square terms for the H_2 and CH_4 response functions were 6.8 % and 39 %, respectively. The results show the first-order linear terms were associated with the highest level of significance.

8.3.3 Effects of experimental variables on the response variables

8.3.3.1 Main effects plot

The linear effect of the experimental variables (temperature, pH and HRT) on the response variables was evaluated using the main effects plot (Figure 8.2). The average response for each experimental variable without considering the effects of other variables is shown in the main effects plots. The main effects plots should be examined with caution because they do not consider the effects of other variables. The mean response values for H_2 and CH_4 yields are 44.7 and 8.6 mL g^{-1} TVS, respectively.

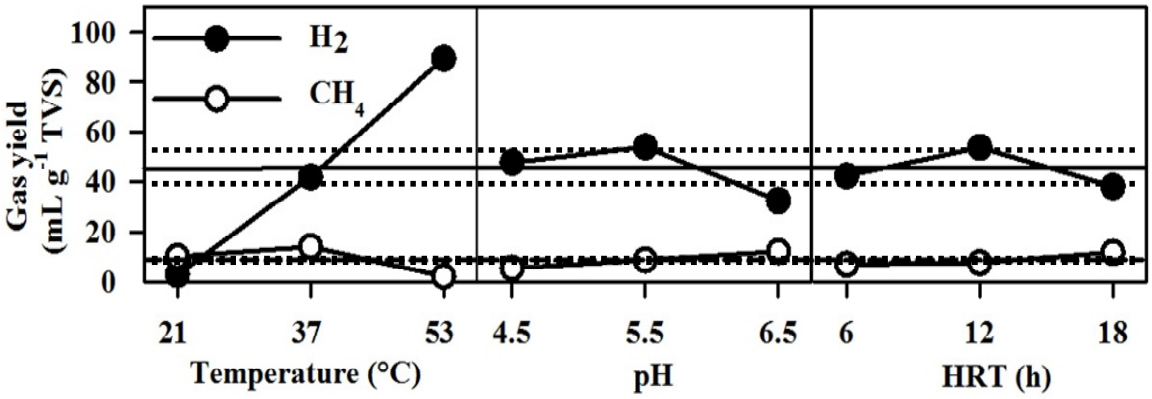

Figure 8.2 Main effect plots of the experimental variables on the response variables.
Notes: 1. Average values are shown. The dotted lines above the mean represent the standard error of the mean (n = 27).

2. H_2 yield; CH_4 yield.

The impact of temperature on the response variables (H_2 and CH_4 yields) is shown in Figure 8.2. The mean H_2 yield increased from 2.8 mL g^{-1} TVS at 21 °C to 41.8 mL g^{-1} TVS at 37 °C. As the temperature was increased to 53 °C, the mean H_2 yield reached 89.3 mL g^{-1} TVS (Figure 8.2). Note over a temperature rise of 16 ^{o}C, the percent H_2 increased was approximately 40 mL g^{-1} TVS. The greater mean CH_4 yield at 37 °C (14.0 mL g^{-1} TVS) when compared to 53 °C (2.1 mL g^{-1} TVS) indicate increasing temperatures are unfavorable for methanogenic growth (Figure 8.2, Fig. 8.4 d, e) under the conditions examined. A similar trend illustrating peak methanogenic activity for aceticlastic and hydrogenotrophic methanogens at different temperatures has also been reported by Patel et al. (1990). According to these researchers, peak methanogenic activity for *Methanobacterium espanolae* sp., a mesophilic and acidophilic hydrogenotrophic methanogens, was observed at approximately 35 ^{o}C and pH 6. In comparison, evidence by Goldberg and Cooney (1981) for mixed anaerobic cultures utilizing H_2 plus CO_2 show an increase in acetogenesis with a rise in temperature from 44 to 56 ^{o}C. In studies conducted by Zeikus and Winfrey (1976), peak hydrogenotrophic methanogenic activity at approximately 40 ^{o}C was detected in an enriched methanogenic culture.

pH is an important factor regulating the metabolic shift towards acidogenesis and solventogenesis (Abreu et al., 2012). pH could be used as a operating variable in controlling H_2 production in mixed culture systems due to its effect on methanogens. A

small increase in the H_2 yield with rising pH values from 4.5 to 5.5 was observed with a subsequent decreased at 6.5 (Figure 8.2). This suggests that H_2 production is more favorable at pH 5.5. A maximum mean H_2 yield (54.0 mL g^{-1} TVS) was obtained at pH 5.5 while in comparison, CH_4 production showed a linear increase with increasing pH (Figure 8.2). Van Haandel and Lettinga (1995) reported that a higher mean CH_4 yield (12.2 mL g^{-1} TVS) is due to the favorable operating pH range (pH 6.3 to 7.8) for methanogens (Van Haandel and Lettinga, 1995). Past studies with varying operating pH also confirm that optimum H_2 production is favorable between pH 4.5-5.5 (favorable range for spore-forming H_2 producers *Clostridium* sp.) while CH_4 production is favorable closer to neutral pH range (Bartacek et al., 2007; Kim et al., 2004; Ray et al., 2010).

The H_2 yield as a function of the HRT is shown in Fig. 1. Notice the mean H_2 yield increasing from 42.3 mL g^{-1} TVS to 53.8 mL g^{-1} TVS with increasing HRT from 6 h to 12 h (Figure 8.2). At an 18 h HRT, the mean H_2 yield decreased to 37.9 mL g^{-1} TVS. Decreasing the HRT to a threshold value likely caused washing-out methanogens and hence, an increase in the H_2 yields. In general, HRT ranging between 0.5 and 12 h can be used to control CH_4 production (Valdez-Vazquez and Poggi-Varaldo, 2009). This is mainly attributed to lower methanogens slower specific growth rates in comparison to acidogenic H_2 producing bacteria (Valdez-Vazquez and Poggi-Varaldo, 2009). In general, microorganisms with growth rates less than the HRT will be washed-out from the reactor. The higher mean CH_4 yield (11.7 mL g^{-1} TVS) observed at 18 h HRT compared to 6 h (6.7 mL g^{-1} TVS) and 12 h HRT's (7.6 mL g^{-1} TVS) indicate longer HRTs enables methanogenic growth and hence, support methane production. These results clearly suggest that the most important factor to control in full-scale facilities is temperature. Note HRT and pH affect H_2 levels; however, controlling these factors is not as important in affecting H_2 yields when compared to temperature.

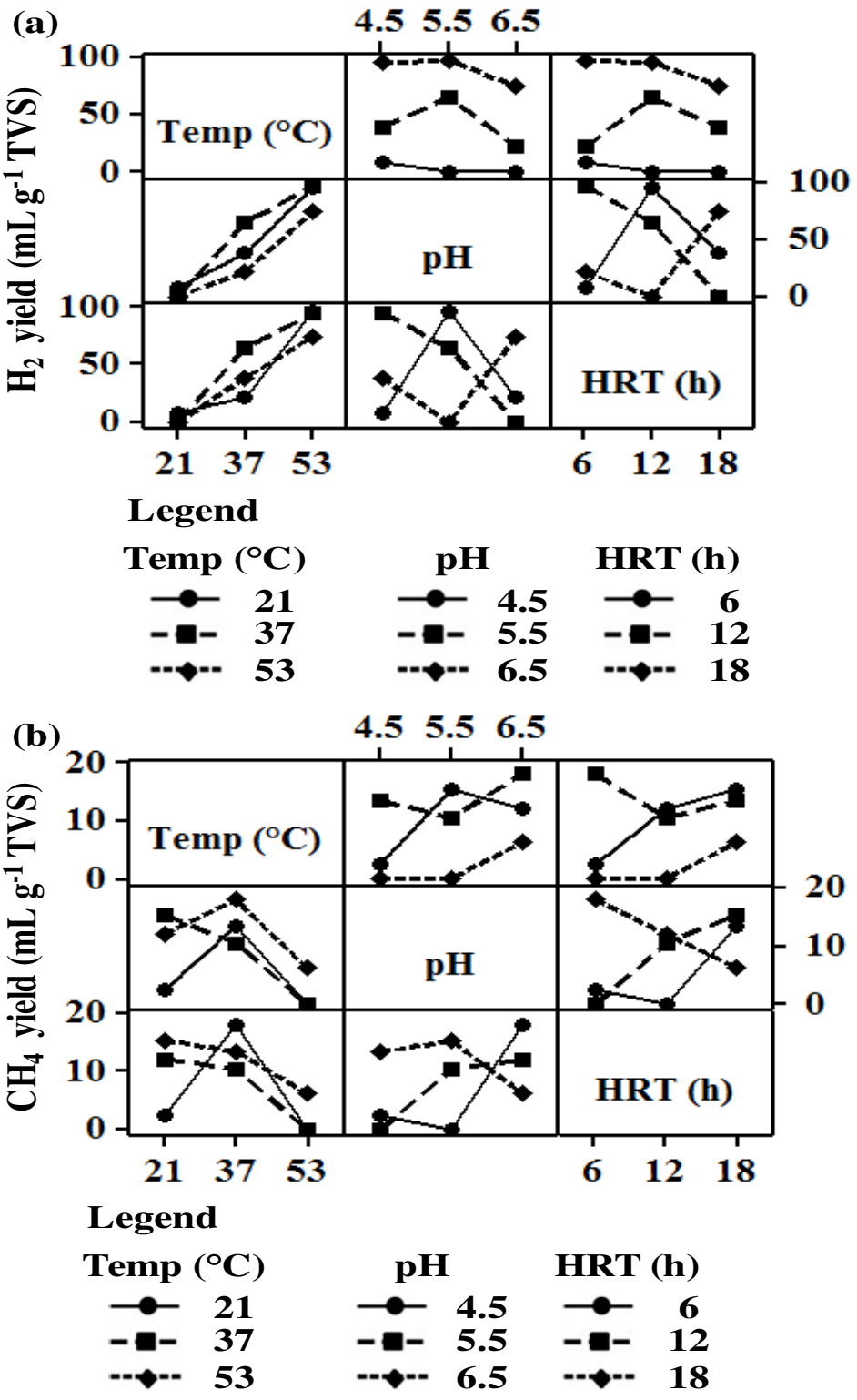

Figure 8.3 Two factor interaction plots for the response variables. (a) H_2 yield; (b) CH_4 yield.

8.3.3.2 Two factor interaction plots

The relationship between the experimental variables is shown in the linear two factor interaction plots (Figure 8.3). Response lines parallel to the X-axis indicates no interaction between the experimental variables. The combined effect of two process variables on H_2 and CH_4 yields are shown in Figure 8.3a and 8.3b, respectively. Notice a linear increase in H_2 yield with increasing temperature at varying pH and HRT levels suggest temperature had the most significant effect on H_2 production (Figure 8.3a). A negligible difference in H_2 production was observed at pH from 4.5 to 5.5 at 53 °C. As the pH is further increased to 6.5 (53 °C), the H_2 yield showed a significant decrease compared to pH 5.5. Higher H_2 yields coupled with lower CH_4 production were observed at pH 4.5 for cultures operated with a 12 h HRT. Note the responses at pH 5.5 coupled with a 6 h HRT indicate that both pH and HRT significantly influenced the response variables (Figure 8.3a and 8.3b).

8.3.3.3 Surface plots

The optimum level of experimental variable and their interaction on both response variables are shown in the surface plots (Figure 8.4 a-c). These surface plots were generated by varying two experimental variables while the other experimental variable is held constant at an optimum level (see equations 8.1 and 8.2). Higher H_2 yields and lower CH_4 yields were observed in cultures at operating at an HRT less than 12 h, at temperatures greater than 40 °C and at a constant pH of 5.5 (Figure 8.4a and 8.4d). The surface plot show conditions for maximum H_2 production (H_2) yield between pH 5.0-5.5 and 10-13 h HRT (Figure 8.4c) at 37 °C is > 60 mL g^{-1} TVS. Notice, lower CH_4 yields (< 10 mL g^{-1} TVS) are located at pH 4.5-4.8 and at an HRT 6-13 h (Figure 8.4e). At a constant 12 h HRT, optimum conditions for higher H_2 yields and lower CH_4 yields were observed for cultures operating in the thermophilic range (45 °C to 55 °C) and at pH < 5.5 (Figure 8.4b). The low methanogenic activity with increasing temperature is likely attributed to a combination of low pH and low HRT (Figure 8.4 d, e). The optimum pH for growing most methanogenic pure cultures range between 6.0 and 8.0, with a majority of cultures operating close to an optimum at pH 7.0 (Williams and Crawford, 1984).

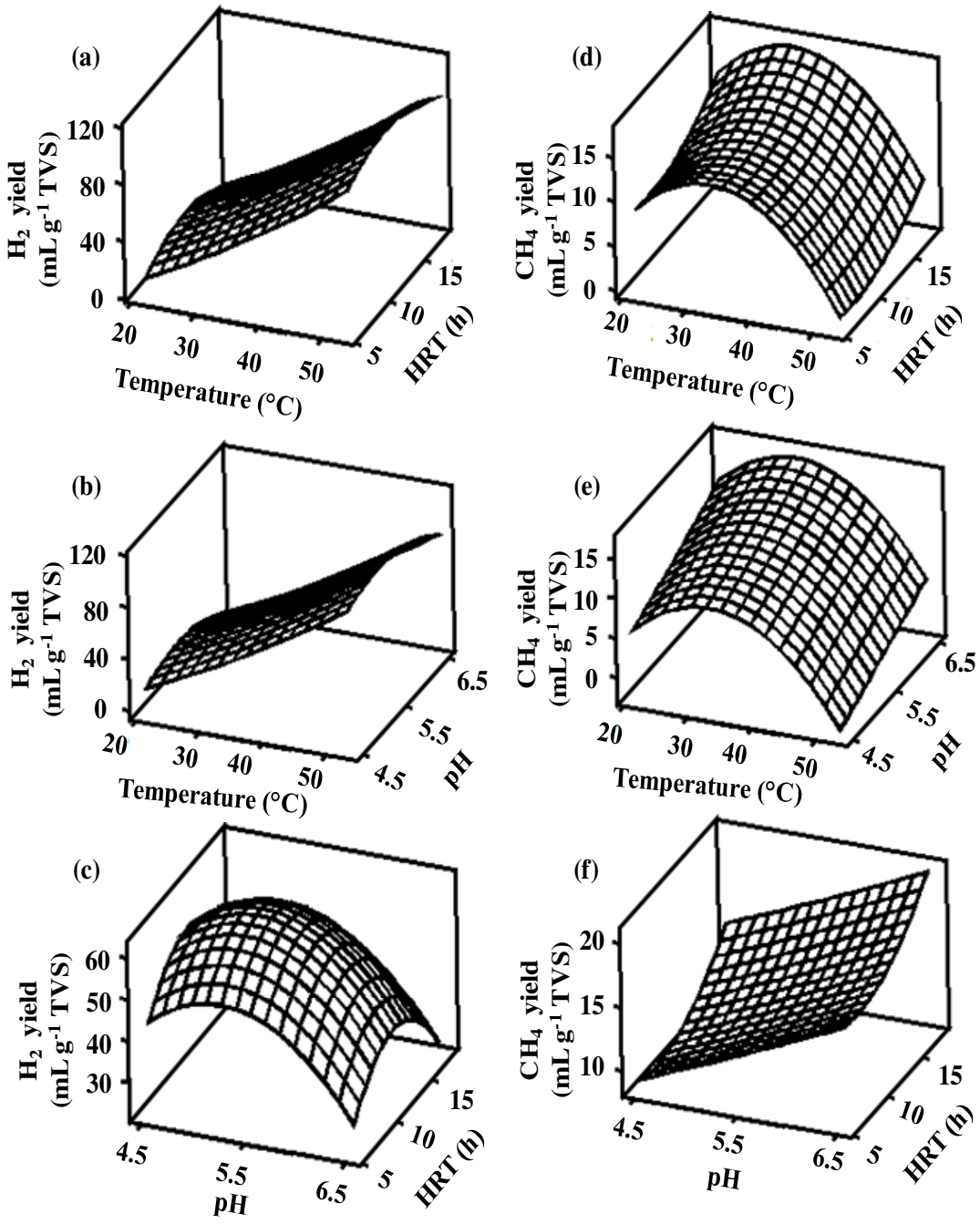

Figure 8.4 Surface plots for the experimental responses: H_2 yield (a, b, c) and CH_4 yield (d, e, f).

Notes:

1. Temperature versus HRT [at constant pH = 5.5]
2. Temperature versus pH [at constant HRT = 12 h]
3. pH versus HRT [at constant temperature = 37 °C]

Using a single factor experimental design, Lay et al. (2005) examined the impact of pH as well as HRT on H_2 production. According to these researchers, peak H_2 production was observed at pH approximately 6.0 for HRT values ranging from 12 h to 36 h.

8.3.4 Model verification

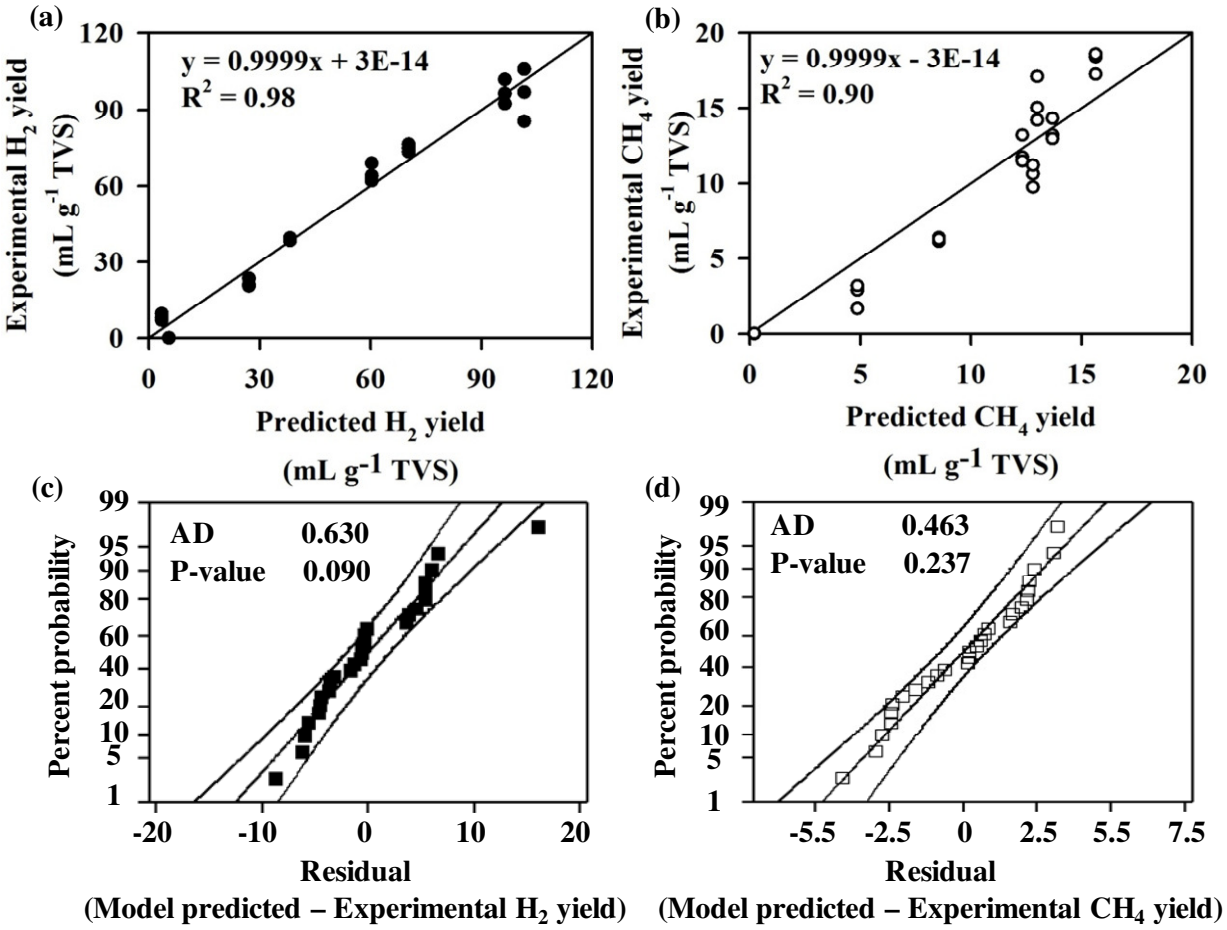

Figure 8.5 Assessment of the accuracy of the response surface model. Plot of the model predicted and experimental responses; (a) H_2 yield and (b) CH_4 yield. Anderson–Darling (A-D) normal plot of residuals; (c) H_2 yield and (d) CH_4 yield.

The experimental responses (H_2 and CH_4 yields) computed by the model (Eqn. 1 and 2) correlated reasonably well with the experimental values. The response variables coefficient of determination (R^2) was 0.98 and 0.90 for the H_2 and CH_4; models, respectively (Figure 8.5a and 8.5b). These values imply that the quadratic model is able to reliably predict the experimental response values.

The Anderson-Darling (AD) statistic was used to confirm the normal distribution of the residuals. The calculated AD statistic for H_2 (0.630) and CH_4 (0.463) was lower than the critical value of the statistic (0.752) for a sample size of 27 and at a 5 % level of significance (Figure 8.5c and 8.5d). Corresponding *p*-values of 0.090 and 0.237, greater than 0.05 for H_2 and methane responses (Eq. 1 and 2) confirms a normal distribution of the residuals (data not shown). This again suggests that model predicted response values correlated reasonably well with the experimental variables within the varying factor levels under consideration.

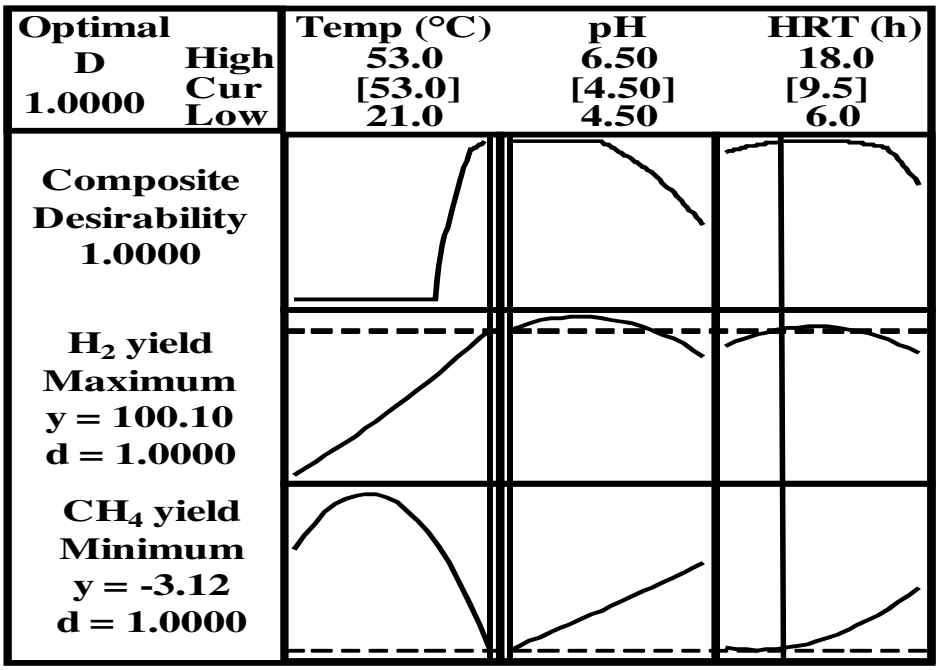

Figure 8.6 D-optimality plot.

The optimal experimental outcome (i.e., maximum H_2 yield and minimum CH_4 yield) using these independent variables was determined using the D-optimality index. This index uses the concept of a desirability function. The D-optimality index is varied between 0.0 and 1.0, with values closer to 1.0 indicating completely desirable solution. The individual desirability of each experimental variable is combined together to obtain a

composite desirability plot (Figure 8.6). This plot determines the optimal operating conditions of each experimental variable. The profiles in the plot show the composite desirability and the experimental response as a function of each other. A D-optimality value of 1.0000 was obtained with a maximum H_2 yield of 100 mL g^{-1} TVS at 53 °C, pH 4.5 and 9.5 h HRT. The D-optimality value under similar conditions showed negligible CH_4 yield (Figure 8.6). The observed experimental response values for cultures operated at 53°C, pH 4.5 and 12 h HRT (Expt. # 7, Table 8.3) were 96.2±10.3 mL H_2 g^{-1} TVS and 0.0±0.0 mL CH_4 g^{-1} TVS. The maximum H_2 yield observed in this study is comparable with the results from previously reported data for corn stalk (Table 8.6).

Table 8.6 Comparison of maximum H_2 yields obtained from fermentation of corn stalk residues.

S.No.	Inoculum type	Reactor type	Temperature (°C)	pH	Substrate	Pretreatment method	Maximum H_2 yield	Reference
1	Mixed culture	Batch	36±1	7	Corn stalk	Acid hydrolysis	149.7 mL g^{-1} TVS	Zhang et al. (2007)
2	Mixed culture	Batch	35	5.5	Corn stover	Steam explosion	3.0 mol H_2 mol^{-1} hexose	Datar et al. (2007)
3	Mixed culture	Batch	36	7	Corn stalk	Acid + enzyme hydrolysis	146.9 mL g^{-1} TS	Wang et al. (2010)
4	Mixed culture	Batch	38±1	7	Corn stalk	Ultrasonic-assisted acid hydrolysis	142.6 mL g^{-1} CS	Wang et al. (2012)
5	Mixed culture	ASBR	53±0.1	5.5	Corn stover	Steam explosion	87.3±0.1 mL g^{-1} CS[a]	This study

Notes: 1. ASBR = anaerobic sequencing batch reactor; TS = total solids; TVS = Total volatile solids; CS = corn stalk
[a] 2.26±0.01 mol mol^{-1} hexose equivalents.

8.3.5 Model validation

A validation study was performed for each of the three independent variables. In the validation study, the optimum values for the model obtained from the D-optimality procedure were compared with the experimental values. The predicted optimum conditions for the maximum H_2 and minimum CH_4 yields were 53 °C, a pH of 4.5 and a 9.5 h HRT. Additional experiments were conducted to validate the response under this optimum condition. A response of 98±2 mL H_2 g^{-1} TVS together with a methane yield of 0±0 mL CH_4 was attained under the optimum condition (data not shown). The experimental H_2 yield under optimum conditions at pH 4.5, a 9.5 h HRT and 53 ^{o}C is 2 % less than the model predicted response value.

8.3.6 Soluble metabolites formation

Liquid metabolites (VFAs, alcohols, furans, furan derivatives and residual sugars), gas metabolites (H_2 and CH_4) and biomass, were used as inputs to calculate the mass balance on a COD basis (Table 8.7). Larger amounts of undegraded sugars were observed cultures at 21 °C when compared to those maintained at 53 °C. Approximately 1.15±0.25 g COD L^{-1} in the feed sugars were not consumed during fermentation at 21 °C, whereas at 53 °C less than 0.24±0.02 g COD L^{-1} remained undegraded. At 21 °C, lactic acid (HLa) production ranging from 0.33±0.06 to 0.71±0.10 g COD L^{-1} of the substrate resulted in simultaneous reduction in the H_2 yield. Greater than 0.7±0.03-1.6±0.13 g COD L^{-1} of the substrate were diverted towards ethanol (EtOH) and iso-propanol (i-PrOH) production at 21 °C and 37 °C irrespective of the operating pH and HRT. In similar work reported by Veeravalli et al. (2013) for batch anaerobic mixed cultures fed a mixture containing glucose, furfural, hydroxymethylfurfural (HMF) and linoleic acid (LA) at pH 5.5 and maintained at 37 ^{o}C, the major byproducts detected depended on the culture source, the furans level and the LA content. In cultures fed LA plus furans, the major byproduct detected included EtOH and i-PrOH while in cultures fed the same substrates and inhibitors, the major byproducts included acetic acid (HAc) and propionic acid (HPr) (Veeravalli et al., 2013).

Table 8.7 Percent mass (COD) balance of the fermentation metabolites under different experimental conditions.

Metabolites conc. (g COD L^{-1})	Exp #								
	1	2	3	4	5	6	7	8	9
H_2	0.10±0.04	0.72±0.00	0.86±0.03	0.00±0.00	0.24±0.00	0.89±0.14	0.00±0.00	0.42±0.00	1.10±0.11
CH_4	0.06±0.03	0.44±0.27	0.17±0.06	0.61±0.09	0.75±0.14	0.00±0.00	0.46±0.03	0.55±0.15	0.00±0.00
HLa	0.71±0.10	0.24±0.05	0.08±0.05	0.52±0.11	0.24±0.05	0.10±0.00	0.33±0.06	0.24±0.05	0.09±0.05
HAc	0.55±0.08	0.95±0.12	0.98±0.25	0.82±0.03	0.67±0.05	2.15±0.25	0.41±0.04	0.77±0.03	1.56±0.05
HBu	0.45±0.07	1.10±0.08	1.27±0.09	0.41±0.04	0.25±0.03	1.15±0.04	0.35±0.03	0.70±0.04	1.33±0.10
EtOH	0.82±0.09	0.90±0.07	0.07±0.01	0.58±0.03	1.36±0.10	0.07±0.01	1.09±0.28	0.84±0.03	0.08±0.01
PrOH	0.39±0.05	0.18±0.00	0.07±0.02	0.13±0.00	0.24±0.03	0.06±0.01	0.22±0.02	0.28±0.02	0.06±0.01
Furfural	0.01±0.00	0.03±0.01	0.00±0.00	0.01±0.00	0.03±0.00	0.01±0.01	0.02±0.00	0.04±0.00	0.00±0.00
Fu-Ac	0.01±0.00	0.03±0.00	0.21±0.01	0.01±0.00	0.04±0.00	0.11±0.01	0.02±0.00	0.03±0.00	0.07±0.02
FuOH	0.00±0.00	0.01±0.00	0.02±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.05±0.01
Rem. Sugar	1.40±0.04	0.07±0.02	0.09±0.00	0.91±0.00	0.68±0.05	0.24±0.02	1.22±0.01	0.26±0.02	0.08±0.01
Biomass	0.40±0.04	0.29±0.07	0.19±0.17	0.35±0.09	0.26±0.08	0.20±0.11	0.31±0.10	0.33±0.07	0.23±0.11
Total COD (g L^{-1})	4.90±0.53	4.95±0.62	4.00±0.55	4.35±0.31	4.75±0.44	4.98±0.48	4.42±0.47	4.47±0.36	4.67±0.36
COD balance (%)	98±11	99±12	80±11	87±06	95±09	100±10	88±09	89±07	93±07

Notes:

1. Exp # refers to the conditions described in Table 8.3.
2. HLa = lactic acid; HAc = acetic acid; HBu = butyric acid; EtOH = ethanol; i-PrOH = iso-propanol; HFu = furoic acid; FuOH = furfuryl alcohol; Rem. Sugar = remaining sugar; H_2 = hydrogen; CH_4 = methane.
3. Biomass was calculated based using the conversion formula 1.42 g COD g^{-1} biomass.
4. COD balance (%) = (Total COD (g L^{-1})/ (Influent COD (5 g L^{-1}))) * 100.
5. The values a ± b represents mean ± standard deviation of triplicate samples.

Results from this work indicate mixed anaerobic cultures are able to shift their metabolic pathways to solvent production in order to avoid the toxicity caused by stressing agents such as elevated H_2 partial pressures, furans and furan derivatives. The larger amounts of butyric acid (HBu) and HAc observed at 53 °C clearly suggest that H_2 production followed from the HAc-HBu fermentation pathway. The formation of reduced carbon byproducts from oxidized carbon compounds is a mechanism of reducing the H_2 partial pressure and forcing the reactions to proceed with a negative free energy. Furoic acid was produced in cultures operating at 53 °C and at pH values of 5.5 and 6.5. At pH 5.5, furfuryl alcohol was produced at 53 °C. Less than 1.11±0.09 g COD L^{-1} of the substrate electrons were recovered as gaseous byproducts under all of the experimental conditions examined (Table 8.7).

8.3.7 Principal component analysis

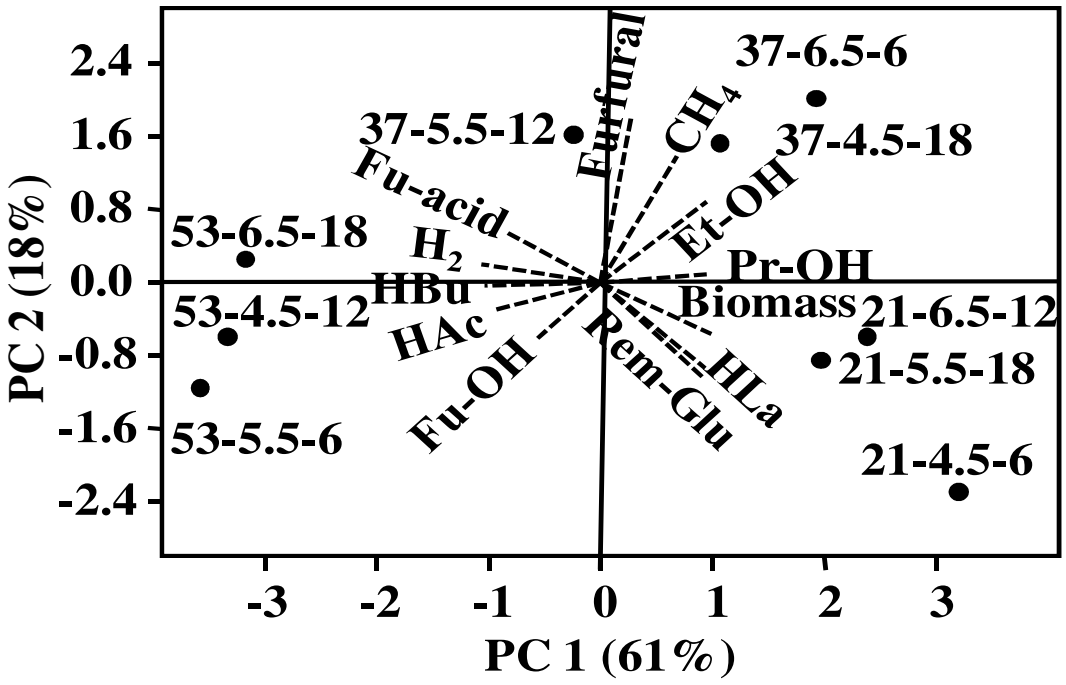

Figure 8.7 PCA bi-plot of fermentation metabolites under different experimental conditions (shown in Table 8.3).
Notes: 1. the numbers identifying each point in the bi-plot represents the experimental condition in the following order 'temperature – pH - HRT'.

A principal component analysis (PCA) was performed to simplify the large set of data (fermentation metabolites) and to obtain a more meaningful understanding of the patterns within the experimental data. Together, principal component 1 (PC 1) and principal component 2 (PC 2) accounted for more than 79 % of the variation in the total data set (Figure 8.7). PC 1 is positively correlated with lactic acid (HLa), ethanol (Et-OH), propanol (Pr-OH), biomass, remaining glucose (Rem-Glu) and methane (CH_4) production, whereas PC 2 is positively correlated with Et-OH, Pr-OH, furfural, furoic acid (Fu-Ac), H_2 and CH_4. The loading values for PC 1 are HLa (0.309), Et-OH (0.314), Pr-OH (0.315), furfural (0.091), Rem-Glu (0.301), biomass (0.327) and CH_4 (0.226). The loading values for PC 2 are Et-OH (0.291), Pr-OH (0.029), furfural (0.607), Fu-Ac (0.180), H_2 (0.065) and CH_4 (0.456). The PCA bi-plot of PC 1 and PC 2 indicates that cultures incubated at different temperatures are clustered together in the bi-plot, away from the other incubation temperatures indicating the influence of temperatures on metabolic pathways (during biohydrogen fermentation from corn stalk hydrolysate) (Figure 8.7).

Cultures operated at 53 °C were clustered together in the left side quadrant in the PCA bi-plot. These cultures correlated positively with the loading vector for H_2, indicating that higher H_2 yields were obtained at these experimental conditions (Exp # 3, 6 and 9; Table 8.3). The placement of cultures incubated at 37 °C correlated with the vectors for Et-OH, Pr-OH, furfural and CH_4 on the upper right side of the PCA bi-plot. The presence of HLa (21 °C) was associated with the decreased H_2 yields obtained at these experimental conditions.

8.3.8 Species-environment correlation

A CCA was conducted to examine the relationships between the microbial species and the environment variables (fermentation metabolites). The first two CCA axes on the tri-plot (Figure 8.8) explained 48 % of the species-environment variance. The length of the lines in the CCA plot indicates the degree of variance of the particular variable within the

data set. Cultures operating at 53 °C were clustered together and associated with both HBu and H_2 production.

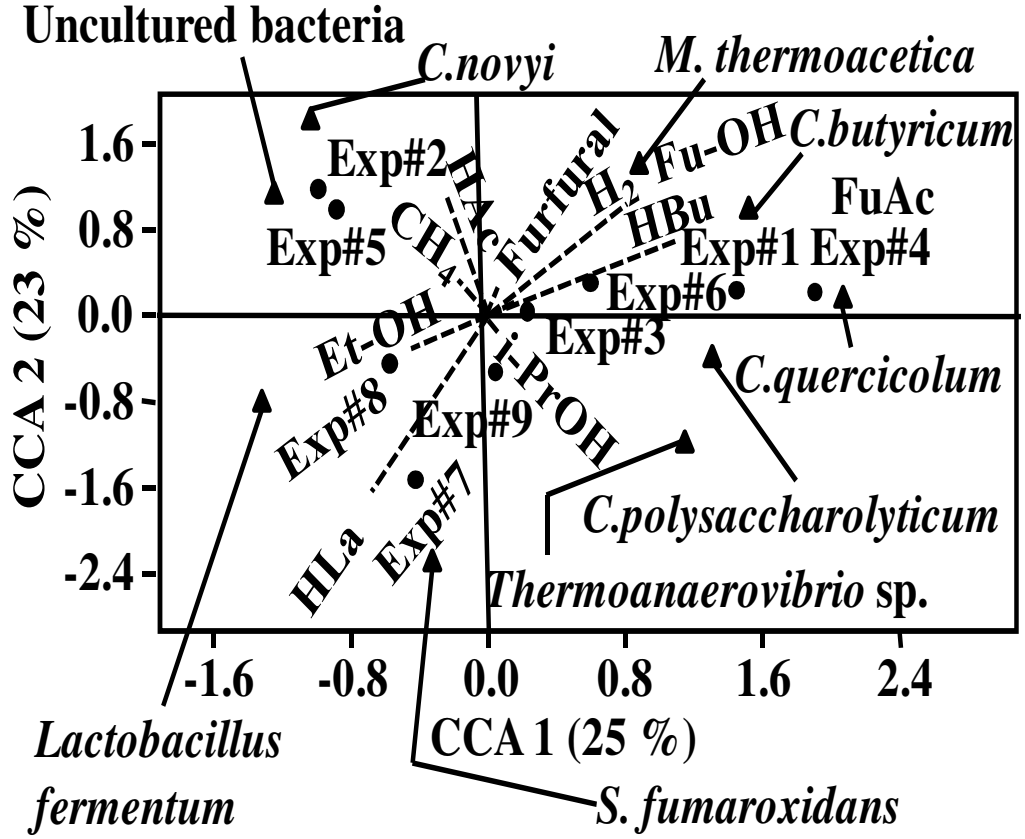

Figure 8.8 Canonical Correspondence analysis.
Notes: 1. 'Exp #" denotes the corresponding experiments mentioned in Table 8.3.
2. HAc = acetic acid; HBu = butyric acid; HLa = lactic acid; Et-OH = ethanol; i-PrOH = iso-propanol; Fu-OH = furyl alcohol; FuAc = Furoic acid; H_2 = hydrogen; CH_4 = methane.
3. *C. thermoaceticum* was renamed as *M. thermoacetica*.

The CCA results showed a total inertia (sum of all Eigen values or variance) of 3.238 (Table 8.8). The first CCA axis, with an eigenvalue of 0.813, accounted for the largest variance compared to the other axes. The first axis, explained 25.1 % variance of the species data. Taken together, the 1st and the 2nd axis of the data set explained 48 % of the total inertia. The CCA plot showed a clear pattern of species distribution associated with the metabolite data. High species-environment correlations (≥0.60) were observed in all the five axes (Table 8.8). Higher H_2 yields together with HBu production were associated with a greater abundance of *Clostridium* sp. such as *C. polysaccharolyticum*, *C.*

quercicolum and *C. butyricum* in addition to *Thermoanaerovibrio* sp. High levels of HLa production was due to the presence of *Lactobacillus fermentum* whereas HBu production was associated with *C. butyricum* while HAc production was associated with the presence of *Moorella thermoacetica*.

Table 8.8 Summary of Eigen values and percentage variance of species data calculated using Canonical correspondence analysis.

	1	**2**	**3**	**4**	**5**	**6**	**Total inertia**
Eigen values	0.8130	0.7342	0.7006	0.5987	0.3913	0.0003	3.238
Species-environment correlations (SEC)	0.782	0.970	0.663	0.683	0.594	0.168	*
Cumulative percentage variance of species data	25.10	47.78	69.42	87.90	99.99	100.00	*

8.3.9 Quantification of hydrogen consuming electron fluxes

A flux analysis was conducted to predict the internal fluxes based on the metabolites. Both the liquid (VFA's, alcohols and residual sugars) and the gaseous (H_2 and CH_4) byproducts levels were used as inputs into the flux model. The results from the flux analysis are shown in Table 8.9. The model predicted H_2 yield (hydrogenase activity) was based on R12 ($2Fd^+ + 2H^+ \rightarrow 2\ Fd^{2+} + H_2$) flux (Chaganti et al., 2011). The experimental H_2 yield was compared against this model predicted H_2 yield to measure the H_2 consumption activity (Table 8.9). This loss in H_2 yield can be attributed to the formation of acetic acid (R17), i-propanol (R21) and CH_4 (R28 and R29). Cultures operating at 21 °C and 37 °C showed higher H_2 consumption compared to those at 53 °C except Expt. # 3 (Table 8.9). Hydrogen consumption via the R21 flux was observed under all the experimental conditions. In cultures at lower temperature conditions (Expt. # 1, 4, 7), more than 61 % of the H_2 consumption occurred via homoacetogenesis (R17 flux; Table 8.9). This indicates that homoacetogens are more active and able to out-compete hydrogenotrophic methanogens for H_2 at lower temperatures.

Table 8.9 Analysis of homoacetogenic and methanogenic electron fluxes under different experimental conditions.

Expt. #	Hydrogenase flux (R12) (mol mol^{-1} hexose)	Experimental H_2 yield (mol mol^{-1} hexose)	Total H_2 consumed[1] (mol mol^{-1} hexose)	H_2 consumed via homoacetogenesis (R17 flux) (mol mol^{-1} hexose)	H_2 consumed via methanogenesis (R29 flux) (mol mol^{-1} hexose)	H_2 consumed via propanol formation (R21 flux) (mol mol^{-1} hexose)	Aceticlastic methanogenesis (R28 flux) (mol mol^{-1} hexose)
1	1.18 ± 0.04^{d}	$0.22\pm0.01^{d, e}$	0.96 ± 0.04^{c}	0.59 ± 0.05^{b}	0.28 ± 0.01^{d}	0.09 ± 0.00^{a}	0.00 ± 0.00^{d}
2	1.80 ± 0.00^{c}	1.74 ± 0.00^{c}	0.06 ± 0.00^{e}	0.02 ± 0.00^{d}	0.00 ± 0.00^{e}	0.04 ± 0.00^{e}	$0.28\pm0.08^{a, b}$
3	3.12 ± 0.10^{a}	$2.01\pm0.06^{b, c}$	1.11 ± 0.04^{b}	0.41 ± 0.10^{c}	$0.68\pm0.04^{b, c}$	0.02 ± 0.00^{g}	0.00 ± 0.00^{d}
4	1.43 ± 0.00^{d}	0.00 ± 0.00^{e}	1.43 ± 0.00^{a}	1.40 ± 0.05^{a}	0.00 ± 0.00^{e}	0.03 ± 0.00^{f}	0.41 ± 0.04^{a}
5	1.60 ± 0.00^{d}	0.58 ± 0.00^{d}	$1.02\pm0.00^{b, c}$	$0.00\pm0.00^{d, e}$	0.96 ± 0.13^{a}	0.06 ± 0.00^{c}	0.24 ± 0.03^{b}
6	$2.58\pm0.36^{b, c}$	2.57 ± 0.35^{b}	$0.01\pm0.00^{e, f}$	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	$0.01\pm0.00^{g, h}$	0.00 ± 0.00^{d}
7	1.40 ± 0.01^{d}	0.01 ± 0.00^{e}	1.39 ± 0.01^{a}	1.34 ± 0.06^{a}	0.00 ± 0.00^{e}	0.05 ± 0.00^{d}	0.33 ± 0.01^{a}
8	$1.73\pm0.00^{c, d}$	$1.04\pm0.00^{c, d}$	0.69 ± 0.01^{d}	0.00 ± 0.00^{e}	0.62 ± 0.09^{c}	0.07 ± 0.00^{b}	0.21 ± 0.03^{c}
9	2.61 ± 0.29^{b}	2.60 ± 0.29^{a}	0.01 ± 0.00^{f}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.01 ± 0.00^{h}	0.00 ± 0.00^{d}

Notes:

1. Total H_2 consumed is the difference between theoretical H_2 yield and experimental H_2 yield. Note, 1 g of dry biomass = 0.90 g TVS and 1 g TVS = 0.32 g COD. For example, 97 ± 4.77 mL g^{-1} TVS = 303.13 ± 14.90 mL g^{-1} COD = 2.69 ± 0.29 mol mol^{-1} hexose.
2. The flux analysis was conducted using the model previously developed by Chaganti et. al (2011). The stoichiometric equations for R12, R17, R21, R28 and R29 were reported by Chaganti et. al (2011). The error bars shown in this table represents the standard deviation for n= 3.

Flux Reaction No.	Stoichiometric Reaction
R12	$2\ Fd^{+} + 2\ H^{+} \rightarrow 2\ Fd^{2+} + H_2$
R17	$4\ H_2 + 2\ CO_2 \rightarrow HAc + 2\ H_2O$
R21	$Acetone + H_2 \rightarrow PrOH$
R28	$HAc \rightarrow CH_4$
R29	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$

3. [a, b, c, d, e, f, g] and [h] indicate statistically different means in the same column. No comparison should be made between columns.

In comparison, hydrogenotrophic methanogenesis (R29 flux) accounted for approximately 90 % of H_2 consumption in cultures at 37 °C (Expt. # 4, 7; Table 8.9). The low H_2 yields obtained at 21 °C and 37 °C (Table 8.8) were mainly due to homoacetogenic and hydrogenotrophic methanogenic activities. Methane production via aceticlastic methanogenesis (R28 flux) were observed in Expt. # 2, 4, 5, 7 and 8 (Table 8.9). Similar results for both HAc and CH_4 production via homoacetogenesis at 21 °C and hydrogenotrophic methanogenesis 37 °C, respectively, were observed in batch cultures fed H_2/CO_2 (Table 4.3). Homoacetogenesis was observed in heat-treated mixed anaerobic cultures maintained at 35 °C (pH 5.3) and fed a starch containing wastewater (Arooj et al., 2008). These researchers observed an increase in acetic acid production as a result of homoacetogenesis with decreasing HRT's (from 18 h to 6 h). In comparison, the results from this study show a decrease in homoacetogenic flux (R17) with decreasing HRT (Table 8.9).

One possible reason for low homoacetogenic activity could be attributed to the presence of furan derivatives in CS liquor which might have an inhibitory effect on homoacetogens. Studies conducted by Veeravalli et al. (2013) with varying furan (furfural + HMF) levels indicates that furan derivatives are capable of inhibiting H_2 consumers (methanogens in particular) at threshold levels. Low operational pH (≤5.0) are not favorable for homoacetogenesis (Rogers and Gottschalk, 1993), because of the fact that acetic acid behaves as an uncoupler in this pH region. Results from this study also support this theory which is evident from negligible R17 flux in cultures at pH 4.5 (Expt. # 6 and 8) except Expt. # 1 (Table 8.9). Cultures maintained at high pH (6.5) showed higher methanogenic activities irrespective of the operational HRT and temperature (Table 8.9). This is clearly evident from the CH_4 yields obtained under these experimental conditions (Expt. # 3, 5 and 7; Table 8.3 and 8.6). A FBA under these experimental conditions (Expt. # 3 and 7) indicate that both homoacetogens and methanogens are active at high fermentation pH (Table 8.9). Cultures operated with a combination of low pH (4.5) and HRT (12 h) (Expt. # 6) and mid-level pH (5.5) and low HRT (6 h) (Expt. # 9) showed minimum H_2 consumption (0.01±0.00 mol mol^{-1} hexose; Table 8.9) and hence, higher H_2 yields. In comparison, in cultures at pH 6.5 and an

elevated HRT of 18 h (Expt. # 3), the H_2 consumption of approximately 0.41±0.10 mol mol^{-1} hexose resulted in lower H_2 yields and higher CH_4 yields when compared to Expt. # 6 and 9 (Table 8.9). These results point out that H_2 production is affected either by a combination of low pH and high temperature (53 °C) or by low HRT and high temperature (53 °C) (Table 8.9).

8.3.10 Microbial community analysis

Genomic analysis using T-RFLP revealed the presence of microorganisms, which are closely associated with the metabolites. Thermophilic microorganisms such as *T. acidaminovorans* are closely associated with cultures operating at 53 °C. *T. acidaminovorans* is a gram-negative obligate anaerobe and has the ability to utilize glucose and produce H_2, CO_2, HAc and EtOH as the major end products (Zavarzina et al., 2000). HAc production is associated with the presence of organisms related to *M. thermoacetica* (Pierce et al., 2008). Higher levels of HLa production at 21 °C and 37 °C were due to the presence of *Lactobacillus fermentum*. *Lactobacillus* sp. generally exerts a tolerance towards lower pH (pH 3.0). The presence of *Lactobacillus* sp. is responsible for the lower H_2 yields obtained at 21 °C. Similar reports on the presence of lactic acid producing bacteria together with lower H_2 yields have been reported (Kim et al., 2006; Saraphirom and Reungsang, 2011).

The presence of *Clostridium* sp. such as *C. polysaccharolyticum* in cultures at 53 °C might be responsible for the high H_2 yields. *C. polysaccharolyticum*, formerly known as *Fusobacterium polysaccharolyticum* has the ability to degrade polysaccharides. *F. polysaccharolyticum* had been reported to produce EtOH, HBu and H_2 as end products from polysaccharides such as cellobiose (Varel et al., 1995). Other *Clostridium* sp., such as *C. butyricum*, *C. novyi* NT, *C. quercicolum* was detected in cultures at 37 °C and 53 °C. The presence of *C. butyricum* was likely responsible for HBu production (Cai et al., 2013). The data indicates that spore forming *Clostridium* sp. survived the stress treatment imposed by furan derivatives.

The Archaeal *Hae* III enzyme digests of the 16S rRNA samples detected the presence of organisms related to *Methanothermobacter marburgensis. M. marburgensis,* a hydrogenotrophic methanogen and a member of the family *Methanobacteriales* (Liesegang et al., 2010), was detected in cultures at 53 °C and at pH 6.5. The presence of this organism in cultures operating at 53 °C (pH 6.5) is likely the main reason for CH_4 formation under this experimental condition (Table 8.3). Supporting data from the flux analysis (R29 flux; Table 8.9) also showed hydrogenotrophic methanogenic activity under the same condition. However, at lower pH values of 4.5 and 5.5 and at 53 °C, this organism was not detected. The negligible amounts of CH_4 detected at 53 °C at all the pH conditions indicate that the low activity of *M. marburgensis* might be due to chemical inhibition or washing-out because of the lower HRT conditions.

An Archaeal rumen clone was detected in cultures maintained at 21 °C and 37 °C. According to Pei et al. (2010), this clone is associated with *Methanobrevibacter ruminatum*. Aceticlastic methanogens closely related to *Methanosaeta concilii*, *Methanosarcina mazei* and *Methanospirillum* sp. have been observed in cultures at low temperature conditions (Diaz et al., 2006). The high methane levels suggest that both aceticlastic and hydrogenotrophic methanogens were active at low temperatures; however, their absence or suppression correlated well with the low CH_4 yields at the higher temperature condition (Table 8.7).

8.3.11 Specific methanogenic activity

The concentrations of intermediate metabolites in the anaerobic digestion process play a vital role in the control of biogas digesters that are degrading complex organic matter. However, these concentrations do not provide useful information about the activity of different trophic groups involved in the digestion process. Therefore, measurement of the specific bio-activity of the trophic groups involved in the anaerobic digestion process will enable better understanding of reactor performance. Another major advantage to conducting these activity tests is that the outcomes facilitate the determination of the most suitable inoculum to use for degrading specific types of wastes. The activity of

different trophic groups of microorganisms is measured by adding specific substrates to different batches of inoculum and then monitoring the subsequent biogas production rate.

Granular sludge is unique in that the active cells appear together in a floc that is separated from the liquid phase and is suspended within a medium of a particular chemical composition. On the other hand, sludge from conventional biogas digesters appear as slurry of active cells mixed with dissolved organic and inorganic matter. Therefore, care must be taken to measure the background level of substrate in the biomass being tested prior to conducting the experiment.

Table 8.10 Methane and acetic acid production by cultures A and B.

Exp #	Culture A[3, a]	Culture B[3, a]	H_2 consumed – culture A (%)[3]	H_2 consumed-culture B (%)[3]	HAc produced (mg COD L^{-1}) – culture A[3]	HAc produced (mg COD L^{-1})-culture B[3]
10	0.54±0.00	0.32±0.06	100±0	76±1	ND	ND
11	0.47±0.04	0.34±0.02	100±0	86±1	ND	ND
12	0.42±0.02	0.38±0.03	100±0	82±1	8±0	ND
13	0.46±0.03	0.10±0.02	100±0	89±1	ND	179±25
14	0.47±0.03	0.20±0.04	100±0	100±1	ND	25±10
15	0.51±0.02	0.47±0.02	100±0	100±0	ND	ND
16	0.09±0.00	0.00±0.00	64±0	25±1	ND	ND
17	0.51±0.04	0.00±0.00	99±8	26±1	17±5	25±10
18	0.50±0.01	0.02±0.00	100±5	50±2	ND	10±14

Notes: 1. ND = not detected
2. Cultures 'A' and 'B' denote cultures fed 'pure sugar' (glucose: xylose; 1:1) and cultures fed 'corn stalk liquor', respectively.
3. The values shown are mean ± standard deviation of the triplicate samples.
4. [a]represents specific methanogenic activity (SMA) expressed in mmol g^{-1} VSS h^{-1}.

This baseline assessment helps to ensure the accurate measurement of the specific microbial activity associated with the test substrate used. The SMA of cultures fed different feedstocks (culture A [fed pure sugars] and culture B [fed CS liquor]; Table 8.10) were studied to examine the effects of toxic inhibitors on the H_2 consumers present in mixed anaerobic culture. H_2 (3000 µmol) was used as the substrate for these experiments. The experimental design matrix for the SMA tests is shown in Table 8.4.

The resulting activity levels varied from 0.09±0.00 to 0.54±0.00 mmol g^{-1} VSS h^{-1} for culture A under all of the experimental conditions investigated, (Table 8.10).

Similar results in the SMA (from 11.1 (0.49 mmol g^{-1} VSS h^{-1}) to 12.8 (0.57 mmol g^{-1} VSS h^{-1}) mL CH4 g^{-1} VSS h^{-1}) were reported previously using granular anaerobic sludge treating dairy effluents (Coates et al., 1996). (Note: Conversion from mmol g^{-1} VSS h^{-1} to mL g^{-1} VSS h^{-1} was based on 1 mmol of gas @ STP occupies a volume of 22.4 mL). Culture A showed higher SMA than culture B under all of the experimental conditions. The SMA of culture B at 21 °C and at lower pH (4.5) was 40 % less than the SMA of culture A under these conditions (Exp # 10) (Table 8.10). At pH 5.5 and 6.5 (Exp # 11 and 12), the difference in SMA of culture A and B were 28 % and 10 %, respectively (Figure 8.9).

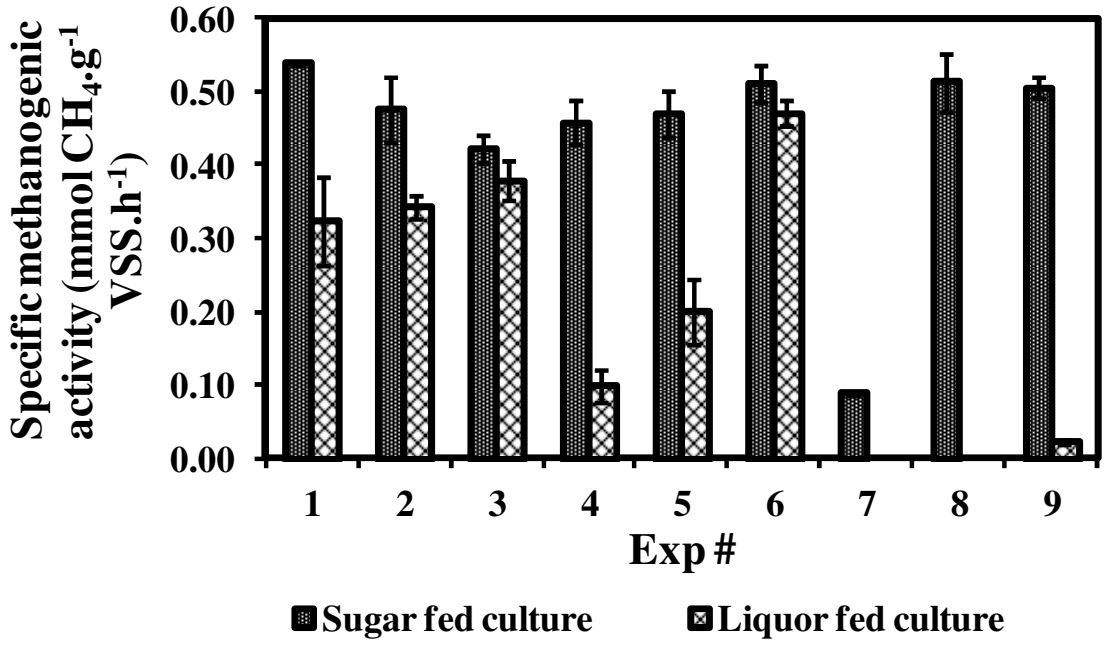

Figure 8.9 Specific methanogenic activity levels of sugar-fed and CS liquor-fed cultures. Note: the error bars shown in this figure represents the standard deviation for n= 3.

The SMA observed for culture A was 0.54±0.001 mmol g^{-1} VSS h^{-1} at pH 4.5 and at a temperature of 21 °C. These results indicate that lower pH alone is not sufficient to inhibit the methanogens present in the mixed anaerobic culture. The SMA levels at pH 5.5 and 6.5 were 0.47±0.04 mmol g^{-1} VSS h^{-1} and 0.42±0.02 mmol g^{-1} VSS h^{-1},

respectively, at 21 °C (Exp # 11 and 12) (Table 8.10). There was a 78 % relative decrease in the SMA of culture B at pH 4.5 at 37 °C compared to culture A under same the operating condition (Exp # 13). At higher pH values of 5.5 and 6.5 (Exp # 14 and 15), culture B showed minimal loss in SMA compared to culture A under the same experimental conditions. The SMA levels at both 21 °C and 37 °C indicate that methanogens are active before the start of the experiment and at the end of the experiment indicating that methanogenic activity is not completely lost. The lower H_2 yields obtained at these fermentation temperatures together with the higher CH_4 yields are consistent with the results obtained for SMA at these temperatures. There is a loss of 50 % of the SMA at pH values 4.5 (37 °C) and 5.5 (37 °C) which resulted in higher H_2 yields (Exp # 8 and 2; Table 8.7) under these conditions. At thermophilic temperature and a lower operating pH of 4.5, the SMA of culture A showed a loss in activity of almost 80 % compared to the SMA for culture A at thermophilic temperature when operated at pH 5.5 and 6.5. In comparison, culture B lost almost 100 % of SMA at pH values of 5.5 and 6.5 at 53 °C.

The SMA levels at pH 5.5 (53 °C) and 6.5 (53 °C) were 0.51±0.04 and 0.50±0.01 mmol g^{-1} VSS h^{-1} (Exp # 17 and 18; Table 8.10) for culture A. Higher temperatures combined with the fermentation inhibitors present in the CS liquor inhibited the methanogens present in culture B at these pH values. Culture A incubated at 21 °C, 37 °C and 53 °C showed 100 % H_2 consumption, with the exception of culture A incubated at 53 °C (pH 4.5). This particular set of conditions resulted in culture A showing only 64±0 % H_2 consumption (Table 8.10). The H_2 consumption of culture B incubated at 53 °C at pH 6.5 was 50±2 % less than the consumption of culture A incubated at the same conditions. Liquor-fed cultures (culture B) showed almost 100 % H_2 consumption at 37 °C at pH values of 5.5 and 6.5, but approximately 80 % H_2 consumption was observed in cultures incubated at 21 °C. Lower levels of SMA in cultures incubated at pH 6.5 (21 °C) are due to the formation of acetic acid (8±0 mg COD L^{-1}). Acetic acid (HAc) production was observed in cultures A and B with initial pH 5.5 and incubated at 53 °C. Similarly, higher HAc levels were seen in culture B incubated at 37 °C at pH values of 4.5 and 5.5. However, HAc production was not detected in culture A under the same operating conditions. The main reason for the absence of HAc production in culture A might be the

presence of aceticlastic methanogens, which could consume the acetic acid and produce CH_4. This might account for the higher SMA in culture A compared to culture B. In culture B, the finding that HAc production was observed in combination with lower SMA indicates that the inhibitors present in the CS hydrolysate inhibited the methanogens. These results also indicate that when methanogens are inhibited, homoacetogens become active and HAc formation is evident under such conditions.

8.4 Conclusions

Semi-continuous fermentative H_2 production using steam exploded corn stalk (CS) liquor was investigated using a fractional factorial design. The factors considered in this study included temperature, pH and HRT. The results suggest that steam exploded CS liquor is a potential substrate for biological H_2 production. The conclusions of this study based on the range of experimental factors are as follows:

1. All the experimental variables had an effect on the experimental responses (both H_2 and CH_4 yields); however, the impact of temperature was greater than pH and HRT. This is clearly evident from the increase in H_2 yield of approximately 40 mL g^{-1} TVS every 16 °C over a temperature rise from 21 °C to 53 °C.
2. Normal distribution of the residuals indicated good correlation between the model predicted and the experimental variables.
3. The observed maximum H_2 yield of 98±2 mL g^{-1} TVS together 0 mL CH_4 g^{-1} TVS were similar to the model predicted responses (using the D-optimality procedure) under optimal conditions at 53 °C, a pH at 4.5 and a 9.5 h HRT.
4. Within the range of experimental conditions examine, the surface plots clearly indicate that in full-scale operation, the most important parameter to control is temperature.
5. Analysis of liquid metabolites revealed that both acetic acid and butyric acid were the dominant metabolites associated with the H_2 production. Cultures incubated at lower temperatures (both 21 °C and 37 °C) showed higher HLa levels as well as increasing solvent (EtOH and i-PrOH) production. This might be the possible reason for lower H_2 yields at these temperatures.

6. The CCA tri-plot showed good species-environment correlation. The fermentation metabolites HLa, HBu, HAc and H_2 are associated with the presence of microorganisms capable of producing them. For example, spore forming H_2 producing *Clostridium* sp. and *Thermoanaerovibrio* sp. detected at 53 °C correlated with the higher H_2 yields. *L. fermentum* detected in cultures maintained at 21 °C and 37 °C correlated with HLa production.
7. The flux analysis revealed negligible homoacetogenic activity in cultures operating at 53 °C at low pH (≤5.5). Both homoacetogenic (R17) and methanogenic (R28 and R29) fluxes accounted for more than 68-90 % of the H_2 consumption in cultures at low temperatures.
8. The microbial diversity analysis showed a higher abundance of *Clostrdium* sp. in cultures maintained at 53 °C. In comparison, CH_4 production was linked to the presence of hydrogenotrophic methanogens (*M. marburgensis* and *M. ruminatum*) and aceticlastic methanogens (*Methanosaeta* sp. and *Methanosarcina* sp.) in cultures maintained at lower temperatures.
9. Examination of SMA indicated that pure sugar-fed cultures showed higher levels of SMA compared to liquor-fed cultures. More than 80 % of the injected H_2 was consumed within 72 h by liquor-fed cultures (culture B) at 21 °C and 37 °C, regardless of initial fermentation pH. This indicates that H_2 consumers are active under these conditions, which also resulted in lower H_2 yields. Almost 100 % loss of SMA was observed in liquor-fed cultures incubated at 53 °C with pH values of 4.5, 5.5 or 6.5, which indicates the influence of stressing agents (furan aldehydes) on methanogens at higher temperatures. Since acetic acid production was observed at pH 5.5 and 6.5 in culture B incubated at 53 °C, indicating that homoacetogenesis is not completely inhibited under these experimental conditions.

8.5 References

Abreu, A.A., Karakashev, D., Angelidaki, I., Sousa, D.Z., Alves, M.M. (2012). Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. *Biotechnol Biofuels*, **5**(6), 1-12.

Appels, L., Baeyens, J., Degreve, J., Dewil, R. (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Prog Energ Combust Sci*, **34**(6), 755-81.

Argun, H., Kargi, F. (2009). Effects of sludge pre-treatment method on bio-hydrogen production by dark fermentation of waste ground wheat. *Int J Hydrogen Energ*, **34**(20), 8543-8.

Arooj, M.F., Han, S.K., Kim, S.H., Kim, D.H., Shin, H.S. (2008). Continuous biohydrogen production in a CSTR using starch as a substrate. *Int J Hydrogen Energy*, **33**(13), 3289-94.

Ballesteros, I., Negro, M.J., Oliva, J.M., Cabañas, A., Manzanares, P., Ballesteros, M. (2006). Ethanol production from steam-explosion pretreated wheat straw. *Appl Biochem Biotechnol*, **130**(1-3), 496-508.

Bartacek, J., Zabranska, J., Lens, P.N.L. (2007). Developments and constraints in fermentative hydrogen production. *Biofuel Bioprod Bior*, **1**(3), 201-14.

Cai, G.Q., Jin, B., Monis, P., Saint, C. (2013). A genetic and metabolic approach to redirection of biochemical pathways of *Clostridium butyricum* for enhancing hydrogen production. *Biotechnol Bioeng*, **110**(1), 338-42.

Cao, G.L., Ren, N.Q., Wang, A.J., Guo, W.Q., Yao, J., Feng, Y.J., Zhao, Q.L. (2010). Statistical optimization of culture condition for enhanced hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. *Bioresource Technol*, **101**(6), 2053-8.

Chaganti, S.R., Kim, D.H., Lalman, J.A. (2011). Flux balance analysis of mixed anaerobic microbial communities: Effects of linoleic acid (LA) and pH on biohydrogen production. *Int J Hydrogen Energ*, **36**(21), 14141-52.

Cheng, C.L., Lo, Y.C., Lee, K.S., Lee, D.J., Lin, C.Y., Chang, J.S. (2011). Biohydrogen production from lignocellulosic feedstock. *Bioresource Technol*, **102**(18), 8514-23.

Coates, J.D., Coughlan, M.F., Colleran, E. (1996). Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. *J Microbiol Meth*, **26**(3), 237-46.

Costa, J.B., Rossi, D.M., De Souza, E.A., Samios, D., Bregalda, F., Peralba, M.D.R., Flores, S.H., Ayub, M.A.Z. (2011). The optimization of biohydrogen production by bacteria using residual glycerol from biodiesel synthesis. *J Environ Sci Heal A*, **46**(13), 1461-8.

Das, D., Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. *Int J Hydrogen Energ*, **26**(1), 13-28.

Datar, R., Huang, J., Maness, P.C., Mohagheghi, A., Czemik, S., Chornet, E. (2007). Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. *Int J Hydrogen Energ*, **32**(8), 932-9.

Diaz, E.E., Stams, A.J.A., Amils, R., Sanz, J.L. (2006). Phenotypic properties and microbial diversity of methanogenic granules from a full-scale upflow anaerobic sludge bed reactor treating brewery wastewater. *Appl Environ Microbiol*, **72**(7), 4942-9.

Fan, Y.T., Zhang, Y.H., Zhang, S.F., Hou, H.W., Ren, B.Z. (2006). Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. *Bioresource Technol*, **97**(3), 500-5.

Ghosh, D., Hallenbeck, P.C. (2010). Response surface methodology for process parameter optimization of hydrogen yield by the metabolically engineered strain *Escherichia coli* DJT135. *Bioresource Technol*, **101**(6), 1820-5.

Goldberg, I., Cooney, C.L. (1981). Formation of short-chain fatty acids from H_2 and CO_2 by a mixed culture of bacteria. *Appl Environ Microbiol*, **41**(1), 148-54.

Hallenbeck, P.C. (2009). Fermentative hydrogen production: Principles, progress, and prognosis. *Int J Hydrogen Energ*, **34**(17), 7379-89.

Hallenbeck, P.C., Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? *Trends Biotechnol*, **27**(5), 287-97.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. (2001). Past: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, **4**(1), XIX-XX.

Helwig, T., Jannasch, R., Samson, R., DeMaio, A., Caumartin, D. (2002). Agricultural biomass residue inventories and conversion systems for energy production in Eastern Canada. Accessed on 1st July 2013:<http://reap-canada.com/online_library/feedstock_biomass/>.

Kim, H.K., Kim, J.G., Cho, J.D., Hong, J.W. (2003). Optimization and characterization of UV-curable adhesives for optical communications by response surface methodology. *Polym Test*, **22**(8), 899-906.

Kim, I.S., Hwang, M.H., Jang, N.J., Hyun, S.H., Lee, S.T. (2004). Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process. *Int J Hydrogen Energ*, **29**(11), 1133-40.

Kim, S.H., Han, S.K., Shin, H.S. (2006). Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. *Process Biochem*, **41**(1), 199-207.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res*, **48**(8), 3713-29.

Lay, J.J. (2000). Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol Bioeng*, **68**(3), 269-78.

Lay, J.J., Tsai, C.J., Huang, C.C., Chang, J.J., Chou, C.H., Fan, K.S., Chang, J.I., Hsu, P.C. (2005). Influences of pH and hydraulic retention time on anaerobes converting beer processing wastes into hydrogen. *Water Sci Technol*, **52**(1-2), 123-9.

Liesegang, H., Kaster, A.K., Wiezer, A., Goenrich, M., Wollherr, A., Seedorf, H., Gottschalk, G., Thauer, R.K. (2010). Complete genome sequence of *Methanothermobacter marburgensis*, a methanoarchaeon model organism. *J Bacteriol*, **192**(21), 5850-1.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*, **31**(3), 426-8.

Montgomery, D.C. (2012). Design and analysis of experiments. 8th ed. Wiley, New York.

Pakarinen, O. (2011). Methane and hydrogen production from crop biomass through anaerobic digestion. Ph. D. thesis: Department of Biological and Environmental Science, University of Jyväskylä.

Patel, G.B., Sprott, G.D., Fein, J.E. (1990). Isolation and characterization of *Methanobacterium espanolae* sp.nov, a mesophilic, moderately acidiphilic methanogen. *Int J Syst Bacteriol*, **40**(1), 12-8.

Pei, C.X., Mao, S.Y., Cheng, Y.F., Zhu, W.Y. (2010). Diversity, abundance and novel 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium fractions of Jinnan cattle. *Animal*, **4**(1), 20-9.

Pierce, E., Xie, G., Barabote, R.D., Saunders, E., Han, C.S., Detter, J.C., Richardson, P., Brettin, T.S., Das, A., Ljungdahl, L.G., Ragsdale, S.W. (2008). The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). *Environ Microbiol*, **10**(10), 2550-73.

Ray, S., Reaume, S.J., Lalman, J.A. (2010). Developing a statistical model to predict hydrogen production by a mixed anaerobic mesophilic culture. *Int J Hydrogen Energ*, **35**(11), 5332-42.

Rogers, P., Gottschalk, G. (1993). Biochemistry and regulation of acid and solvent production in *Clostridia*. In: Woods DR, editor. The Clostridia and Biotechnology. p.25-50.

Saraphirom, P., Reungsang, A. (2011). Effect of organic loading rate on bio-hydrogen production from sweet sorghum syrup by anaerobic mixed cultures in anaerobic sequencing batch reactor. *The 6th IASME / WSEAS international conference on Energy and Environment (EE'11)*, Cambridge, UK. pp. 128-33.

Sheng, C.D., Azevedo, J.L.T. (2005). Estimating the higher heating value of biomass fuels from basic analysis data. *Biomass Bioenerg*, **28**(5), 499-507.

Speece, R.E. (1976). Gas Flow Totalizer. US Patent 4064750 A.

Stenberg, K., Tengborg, C., Galbe, M., Zacchi, G. (1998). Optimisation of steam pretreatment of SO_2-impregnated mixed softwoods for ethanol production. *J Chem Technol Biot*, **71**(4), 299-308.

Sun, Q., Xiao, W., Xi, D., Shi, J.P., Yan, X., Zhou, Z.H. (2010). Statistical optimization of biohydrogen production from sucrose by a co-culture of *Clostridium acidisoli* and *Rhodobacter sphaeroides*. *Int J Hydrogen Energ*, **35**(9), 4076-84.

Titterington, D.M. (1975). Optimal design - some geometrical aspects of D-optimality. *Biometrika*, **62**(2), 313-20.

Valdez-Vazquez, I., Poggi-Varaldo, H.M. (2009). Hydrogen production by fermentative consortia. *Renew Sust Energ Rev*, **13**(5), 1000-13.

Van Haandel, A.C., Lettinga, G. (1995). Anaerobic sewage treatment: A practical guide for regions with hot climate. 1st ed. Wiley. New York.

Varel, V.H., Tanner, R.S., Woese, C.R. (1995). *Clostridium herbivorans* sp.nov, a cellulolytic anaerobe from the pig intestine. *Int J Syst Bacteriol*, **45**(3), 490-4.

Veeravalli, S.S., Chaganti, S.R., Lalman, J.A., Heath, D.D. (2013). Effect of furans and linoleic acid on hydrogen production. *Int J Hydrogen Energ*, **38**(28), 12283-93.

Wang, H., Zhi, Z.L., Wang, J., Ma, S.H. (2012). Comparison of various pretreatment methods for biohydrogen production from cornstalk. *Bioproc Biosyst Eng*, **35**(7), 1239-45.

Wang, Y., Wang, H., Feng, X.Q., Wang, X.F., Huang, J.X. (2010). Biohydrogen production from cornstalk wastes by anaerobic fermentation with activated sludge. *Int J Hydrogen Energ*, **35**(7), 3092-9.

Williams, R.T., Crawford, R.L. (1984). Methane production in Minnesota peatlands. *Appl Environ Microbiol*, **47**(6), 1266-71.

Zavarzina, D.G., Zhilina, T.N., Tourova, T.P., Kuznetsov, B.B., Kostrikina, N.A., Bonch-Osmolovskaya, E.A. (2000). *Thermanaerovibrio velox* sp. nov., a new anaerobic, thermophilic, organotrophic bacterium that reduces elemental sulfur, and emended description of the genus *Thermanaerovibrio*. *Int J Syst Evol Microbiol*, **50**, 1287-95.

Zeikus, J.G., Winfrey, M.R. (1976). Temperature limitation of methanogenesis in aquatic sediments. *Appl Environ Microbiol*, **31**(1), 99-107.

Zhang, M.L., Fan, Y.T., Xing, Y., Pan, C.M., Zhang, G.S., Lay, J.J. (2007). Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures. *Biomass Bioenerg*, **31**(4), 250-4.

CHAPTER 9: SUMMARY AND CONCLUSIONS

9.1 Summary and conclusions

Understanding the characteristics and activity of H_2-consumers is required to maximize H_2 production via dark fermentation. The main focus of this thesis is to examine engineering, chemical and physical factors affecting the activities of methanogens and homoacetogens in mixed anaerobic cultures in batch and semi-continuous reactors. Factors affecting H_2 production such as pH (Abreu et al., 2012), HRT (Valdez-Vazquez and Poggi-Varaldo, 2009), temperature (Wang and Wan, 2008) was taken into consideration together with microbial growth inhibitors such as LA (Lalman and Bagley, 2002), BES (Pendyala et al., 2012), furfural (Veeravalli et al., 2013) in elucidating the behaviour of H_2 consumers. Previous research on microbial H_2 consumption (by methanogens and homoacetogens) in mixed anaerobic culture has examined the impact of different stressing treatments (Luo et al., 2011; Oh et al., 2003). Stressing agents employed in inhibiting/suppressing H_2 consumption are variable in their chemical composition and in their mode of action on H_2 consumers (Liu et al., 2011). Various chemical stressing treatments reported in the literature include heat, acid, alkali, loading shock and BES treatment (Luo et al., 2011; Pendyala et al., 2012). Stressing agents are able to impair methanogenic growth; however, homoacetogens can survive and proliferate (Argun and Kargi, 2009). Although heat treatment can successfully enhanced H_2 production under experimental conditions, the application of this treatment in large-scale processes is not economically viable because of the energy costs associated with heating. Using chemical inhibitors to enhance H_2 production is limited because releasing chemicals such as BES into the environment can lead to severe damage of ecosystems. In comparison, pre-treatment with long chain fatty acids (LCFAs) is a promising alternative to many specific and non-specific stress treatments because they are renewable and they can selectively inhibit acetogens and methanogens at threshold levels. When compared to BES, LCFAs are relatively cheap and environmentally friendly. The degree of inhibition exerted by a particular LCFA depends mainly upon the number of carbon-carbon unsaturated bonds and also on the length of the carbon chain. When considering the various factors into consideration, the experiments described in this thesis were designed

to study the impact of different types of stressing agents on the H_2 consumers in mixed anaerobic cultures. In particular, experiments were designed to examine the impact of various factors affecting H_2 production. The series of experiments under consideration were divided into five phases:

Phase I was focused on assessing the effects of temperature, pH and LA concentration on H_2 consumption in mixed cultures. The data from phase 1 demonstrated that LA inhibited H_2 consumption. Approximately 100% H_2 consumption was observed in the controls regardless of the fermentation pH and temperature (Table 4.3). An ANOVA conducted on the experimental response (% H_2 consumed) indicated that the BBD model linear, square and interaction terms were significant contributors to the response. Model verification conducted using the Anderson-Darling (A-D) statistic indicated the normal distribution of residuals. Model predicted and experimental response values had a correlation coefficient of $R^2 = 0.92$, which indicates that the experimental results closely approximated the model's predicted values (Figure 4.4). Using the D-optimality procedure to optimize the experimental conditions for minimum H_2 consumption indicate that at 39.7 °C and pH 5.5 for mixed culture fed 1394 mg L^{-1} LA, a response value of 1% H_2 consumed was observed (Figure 4.6). The validation experiment conducted under the optimum conditions yielded a response value of 0 % H_2 consumption which is close to the model's predicted optimum value. The low levels of CH_4 observed under favourable thermodynamic conditions (Table 4.5) indicated the impact of LA on methanogens. Acetic acid and propionic acid were the two major by-products produced. Propionate production was detected under all the experimental conditions studied. The free energy values (Table 4.5) indicated that propionogenesis was possible using H_2/CO_2 and also from acetic acid.

The dendrogram constructed using the Jaccard similarity index for both archaeal and bacterial *Hae* III enzyme digested T-RFs showed that microbial cultures fed 2 g L^{-1} LA and incubated at 37 °C were clustered together in a separate clade. *M. taiwanensis* and *M. halotolerans* were detected under all of the experimental conditions, except for the cultures incubated at 53 °C with an initial pH of 4.5. *M. alaskense* was only present in the control cultures incubated at 37 °C with an initial pH of 7.5. The addition of LA or

lowering the pH to 4.5 inhibited these organisms at 37 °C. The T-RFs from bacterial *Hae* III enzyme digestion showed greater abundance of *Clostridium* sp. These results imply that LA treatment selectively enriched *Clostridium* sp. under the temperature conditions examined. In addition to *Clostridium* sp., syntrophic organisms related to *S. aciditrophus* were detected in LA-treated cultures. Control cultures incubated at 37 °C showed the presence of homoacetogens, such as *Eubacterium* sp., that were not detected in cultures fed 2 g L^{-1} LA. The results from phase I concludes that LA can be used as an effective inhibitor to minimize H_2 consumption in mixed anaerobic culture together with operating at pH 5.5.

In phase II, the comparative effects of different stressing agents such as furfural, BES, lauric acid (LUA) (C12:0), LA (C 18:2) and fish oil (containing docosahexanoic acid (DHA), (C 22:6) and eicosapentanoic acid (EHA), (C 20:5)) on H_2 consumption were studied at a mesophilic temperature at an initial pH 5.5. The H_2 consumption results indicated that all of the stressing agents inhibited H_2 consumption. The lowest level of H_2 consumption was detected in the LUA treated cultures followed by BES. Cultures treated with LA, fish oil or furfural showed similar levels of inhibition. The differences between these treatments were insignificant (according to the post hoc Tukey's analysis at a significance level of $\alpha = 0.05$). This outcome implies that furfural can also be used as an effective H_2 consumption inhibitor. LUA produced the strongest inhibitory effect which is evident from the longer half-life values for LUA compared to LA and fish oil-treated cultures. A major problem associated with using LUA as a H_2 consumption inhibitor in H_2 production studies is that LUA can also impair the activity of H_2 producers. Similar levels of H_2 consumption by LA and fish oil treated cultures indicate that as the degree of unsaturation increases, lower concentrations may be sufficient to obtain similar levels of inhibition on H_2 consumers.

Different stress treatments resulted in the production of a wide array of by-products from glucose degradation. Methane and acetic acid production in control (untreated) cultures indicated that both acetogenesis and methanogenesis are possible under the experimental conditions described in Phase 2. In fish oil-treated cultures, higher levels of formic acid

were produced (Table 5.2). In the case of LUA and BES treatment, similar formic levels were produced.

Negligible amounts of acetic acid production together with the propionate production observed in cultures treated with LA or LUA suggest the possibility of conversion of acetic acid into propionic acid by the LA treated cultures. The results obtained from the Phase 1 experiments (described in chapter 4) also showed no indication of acetic acid production in cultures at 37 °C and fed 2000 mg L^{-1} of LA. Propionic acid production was observed only in the LA and LUA treated cultures. Fish oil-treated cultures showed no propionic acid production, which suggests the possibility of inhibition of propionogenesis by the fish oil treatment. Microbial diversity analysis using the Shannon Weiner diversity index (Table 5.4) and rarefaction analysis (Figure 5.5) found lower levels of microbial species diversity in both the LA and LUA treated cultures.

LCFA treated cultures showed higher levels of diversity of *Clostridium* sp. when compared to cultures exposed to other stress treatments. Control cultures showed the presence of both aceticlastic and hydrogenotrophic methanogens. These organisms were not detected in cultures treated with chemical stressors clearly indicating their inhibitory effects on methanogens. The presence of propionate producing bacteria, such as *Propionobacterium acnes* and *Clostridium propionicum*, in the LA treated cultures also validates the possibility of propionate formation in LA and LUA treated cultures. In comparison, fish oil treated cultures did not show the presence of propionate producing species. This finding also supports the hypothesis that LCFAs with a higher degree of unsaturation inhibit propionogenesis. Fish oil-treated cultures showed the highest levels of species diversity, followed by BES and furfural treated cultures. *Eubacteria* sp. (homoacetogenic bacteria) was dominant in fish oil-treated cultures. In comparison, *Clostridium* sp., *Eubacteria* sp., and *Bacteroides* sp. were the dominant organisms in the furfural fed cultures. The PCA bi-plot showed clustering of the stressor treated cultures separately from the control cultures (Figure 5.3). Microbial cluster analysis using the Kulczynski similarity index grouped the LA and fish oil treated cultures together in Clade I, implied that microbial communities exposed to LCFAs showed more similarity

(approximately 25%; Figure 5.4). Control cultures were grouped apart from the cultures treated with chemical stressors.

The results from phase I and phase II indicate that propionate formation becomes favorable under treatment with LA addition using H_2 as substrate. The T-RFLP results from phase I and phase II also indicated that LA treatment resulted in the selective enrichment of *Clostridium* sp. and organisms related to *propionobacterium acnes* and *Clostridium propionicum*. In phase II, the different chemical stressors had an inhibitory effect on the H_2 consumption. Hence, the experiments in phase III were designed to compare the effectiveness of chemical stress treatments with other type of stress treatments such as heat, acid, alkali, loading shock. The conditions under which BES and LA inhibited H_2 consumption in phase II were selected for this study.

The results from phase III indicated that inoculum pretreatment is necessary to selectively inhibit the H_2 consumers in order to maximize the H_2 yields. Mesophilic fermentation temperatures (37 °C) were more favourable for producing H_2 when compared to thermophilic fermentation temperatures (55 °C). This is evident from the higher H_2 yields obtained following different stress treatments after feeding glucose 5 times (Figure 6.1). In comparison, control cultures from the 5^{th} glucose addition maintained at 55 °C showed higher H_2 yields than control cultures maintained at 37 °C. Low methane levels were observed over long periods at 37 °C or 55 °C following any of the stress treatments. Low levels of H_2 consumption in stressor-treated cultures (H_2 fed cultures) were observed in cultures maintained at 55 °C compared to cultures maintained at 37 °C. Control cultures (fed H_2 and incubated at 37 °C) showed 100±2 % H_2 consumption compared to only 28±7 % H_2 consumption in the control cultures incubated at 55 °C (Figure 6.2). Loading shock (LS) pretreatment resulted in low levels of H_2 consumption (39±2 %), followed by the consumption levels observed in LA and alkali-treated cultures (Figure 6.2).

A FBA of the fermentation metabolites from cultures fed 5 times with glucose indicated that CH_4 production in control mesophilic cultures was due to aceticlastic methanogenesis. No detectable hydrogenotrophic methanogenic activity was observed in all experiments subjected to 5 glucose additions. Homoacetogenic activity (R17 flux; Table 3.4) was observed in heat, acid, alkali and BES treated cultures incubated at

mesophilic temperature (Figure 6.8a). The model predicted H_2 yield (hydrogenase activity) was based on R12 ($2Fd^+ + 2H^+ \rightarrow 2\ Fd^{2+} + H_2$) flux. In LS pretreated cultures incubated at mesophilic and thermophilic temperatures, the homoacetogenic (R17) flux was not detected after successively feeding glucose. In comparison, at 55 °C, the heat, alkali, LA and BES treated cultures showed notable homoacetogenic R17 flux after feeding glucose 5 times (Figure 6.8b). Note the decrease in experimental H_2 yield in control, LS and acid treated cultures incubated at 55 °C (after 5 glucose feedings) could be due to H_2 consumption via iso-propanol formation (R21 flux; Figure 6.8).

The PCA biplot (PC 1 and PC 2) of cultures incubated at 37 °C showed clustering of the pretreated cultures separately from the control cultures compared to the PCA bi-plot of cultures incubated at 55 °C (Figure 6.9). This indicates that differences in the fermentation pattern are apparent at different incubation temperatures. In the PCA biplot for the cultures operating at 55 °C, the first two principal components (PC 1 and PC 2) accounted for only 47 % of the variance in the dataset, whereas more than 64 % of the variance is explained by PC 1 and PC 2 in the cultures operated at 37 °C (Figure 6.9).

Control cultures showed higher uptake hydrogenase activity (UHA) compared to the stress treated cultures after 5 glucose additions (Figure 6.7). This indicates that H_2 consumers are more active in control cultures which are clearly evident from the lower H_2 yields. In comparison, there was negligible difference in evolution hydrogenase activity (EHA) between the control cultures compared to the stress treated cultures (Figure 6.6). Moreover, cultures incubated at thermophilic temperature showed lower hydrogenase activities compared to the cultures incubated at mesophilic temperature. This might be attributed to the higher concentration of un-ionized VFAs which resulted in decreasing the pH to 4.5. At pH below the optimum pH range (around neutral pH) for this enzyme, hydrogenase activity decreases.

T-RFs generated using archaeal *Hha* I enzyme digestion showed fewer archaeal bands after repeated batch cultivations (successive glucose additions) and archaeal genes were detected only under a few of the experimental conditions. These findings imply that repeated batch cultivations eliminated the presence of methanogens from the microbial community. In LA-treated cultures fed glucose, no methanogens were detected after the

5^{th} glucose feeding. The dendrogram constructed using the Bray-Curtis similarity index showed a Cophenetic score above 0.80, which indicates that a good correlation was obtained through the analysis (Figure 6.10).

Homoacetogens and methanogens were detected in control cultures fed repeatedly fed 5 times with glucose. However, they were not detected or were present in smaller quantities in pre-treated cultures incubated at 37 °C. Some of the pre-treatments resulted in selective enrichment of the spore-forming *Clostridium* sp. at 37 °C. The presence of *Clostridium* sp. was also detected in pre-treated cultures incubated at 55 °C, but these microbial communities were primarily dominated by *Flavobacterium* sp., *Bacillus* sp., *Thermoanaerobacter* sp., *Bacteroides* sp., and *Thioalkalivibrio* sp.

Biohydrogen production was examined using anaerobic sequencing batch reactors (ASBRs) in phases IV and V. Control (untreated and without LA) experiments showed lower H_2 yields compared to the yields from LA treated cultures at all the HRTs conditions (Table 7.3). As the HRT decreased, the H_2 yield increased along with decreasing CH_4 yields. The maximum H_2 yield of 2.02±0.05 mol mol^{-1} glucose was attained at a 7.5 h HRT in the untreated controls (Table 7.3). As the HRT is further decreased from 7.5 h to 3.3 h, no further increase in H_2 yield was observed. At a 3.3 h HRT, a shift in the metabolic pathway towards solvent (ethanol) production and washout of the biomass occurred. The CH_4 yield observed under the same conditions (7.5 h HRT in the control cultures), was 0.09±0.05 mol mol^{-1} glucose (Figure 7.1). As the HRT decreased from 37.5 h HRT to 3.3 h HRT, reduction in CH_4 yield (from 0.92±0.07 to 0.07±0.04 mol mol^{-1} glucose) was observed (Figure 7.1). Since the maximum level of H_2 production occurred at 7.5 h HRT, studies examining the addition of LA reduced HRT to a minimum level of 7.5 h HRT. Adding LA caused the electron flux from glucose towards H_2 production to increase by 72±2 % and by 25±2 % at 37.5 h and 17.5 h HRT, respectively. There was also a 17±1 % increase in H_2 yield at a 7.5 h HRT (Table 7.3). These studies clearly indicate LA culture treatment changes the microbial population and diverted the electron fluxes to H_2 production.

Notable differences in the fermentation pathways were observed in LA treated cultures compared to control cultures. Negligible amounts of propionate production were

observed in the LA treated cultures. In comparison, propionate production in the control cultures decreased when HRT was reduced from 37.5 h to 17.5 h (Table 7.3). Any further decreases in HRT did not alter propionate production. In general, control experiments showed evidence of the mixed acid type of fermentation. LA treated cultures showed higher butyric acid levels than the control cultures (Table 7.3). In general, fermentation in the LA treated cultures followed the acetic acid-butyric acid type pathway. Low levels of ethanol were observed in LA treated cultures at 37.5 h HRT and 17.5 h HRT. However, as the HRT was decreased to 7.5 h HRT, more ethanol was produced. FBA of the fermentation metabolites detected considerable homoacetogenic activity in the LA treated cultures at all of the HRTs examined (Table 7.4). The H_2 consumption flux was lower in LA treated cultures at 37.5 h and 17.5 h HRT compared to the levels in control cultures under similar operating conditions. The LA treated cultures clustered together separately from the control cultures in the PCA bi-plot (Figure 7.6). Moreover, more than 90 % of the variation in the dataset was explained by PC 1 and PC 2. The hydrogen yields and butyric acid yields were associated with LA treated cultures in the bi-plot.

Phase V experiments were conducted to examine the feasibility of using lignocellulosic biomass (corn stalk (CS) residues) to produce H_2. Temperature adjustment affected both the H_2 and CH_4 yields when using the CS liquor. Less variation was observed in the experimental responses observed following changes in pH and HRT compared to temperature. Lower pH (4.5) favoured H_2 production when compared to a higher pH value at 6.5. Similarly, lower HRT (6 h) resulted in higher H_2 yields than higher HRT (18 h) (Figure 8.1). This is clearly shown in the main effects plots. The mean H_2 yield of approximately 89.3 mL g^{-1} TVS was observed in cultures operated at 53 °C (Figure 8.1). In comparison, lower H_2 yields were obtained from cultures fermented at lower temperatures. On the other hand, CH_4 yields showed an opposite trend to that of H_2 production. Lower CH_4 yields were observed at 53 °C than at 37 °C or 21 °C. An analysis of variance of the experimental response indicated that several of the terms (i.e., the linear and square terms) in the predictive fractional factorial model were statistically significant ($P < 0.05$; Table 8.5). This implied that these terms contribute significantly to the response outcome. The model which was verified by comparing predicted observations to the experimental response values showed a strong correlation ($R^2 > 0.90$).

Optimizing the experimental conditions for maximum H_2 production and minimum CH_4 production from fermentation of CS liquor was performed using a MINITAB 16 algorithm (D-optimality). The D-optimality model predicted maximum H_2 production of 100 mL g^{-1} TVS with 0 mL g^{-1} TVS CH_4 production at 53 °C with a pH of 4.5 and a 6 h HRT (Figure 8.6). Similar H_2 yields of 96.17±10.28 mL g^{-1} TVS together with negligible CH_4 production were observed experimentally at 53 °C, pH 4.5 and 12 h HRT (Table 8.3). This experimental response is very close to the D-optimality model's predicted optimum value for these temperature and pH conditions, although the HRT condition was not the same. Liquid metabolite analysis revelaed that more than 14-32 % of the electrons present in the substrate (5 g COD L^{-1}) was diverted towards solvent (ethanol and propanol) production (Table 8.7). In order to avoid the toxicity of the fermentation inhibitors in the CS liquor, the microorganisms shifted their metabolic pathway towards solvent production. The lower H_2 yields at 21 °C were mainly attributed to higher lactic acid and ethanol production. Cultures maintained at 53 °C were clustered separately from the 37 °C and 21 °C cultures in the PCA bi-plot. Greater than 79 % of the variation in the dataset was explained by PC 1 and PC 2 (Figure 8.7). The bi-plot shows a clear association of cultures maintained at 53 °C cultures with both H_2 and butyric acid production. A similar type of association was observed in the LA treated cultures (chapter 7).

The H_2 yields obtained by using glucose (1.44±0.05 mol mol^{-1} glucose) at a 10.8 h HRT and cultures maintained at 37 °C (Chapter 7; Table 7.3) were comparable with the results obtained using the CS liquor (1.74±0.00 mol mol^{-1} glucose equivalent (Table 8.9); obtained at 12h HRT) at the same temperature and pH condition. The presence inhibitors in the CS liquor coupled with lactic acid production is likely the reasons affecting H_2 production at 21 °C. The SMA assessment showed higher levels of methanogenic activity in pure sugar fed cultures compared to that of CS liquor fed cultures which was maintained at three temperatures. Cultures maintained at 21 °C and 37 °C showed more than 80 % H_2 consumption under all the pH conditions examined (Table 8.10). These results indicate that inhibition of methanogens by furan derivatives resulted in lower SMA. Almost 100 % loss in SMA was observed in liquor fed cultures maintained at 53 °C compared to the control cultures. This result provides evidence for higher H_2 yields at

53 °C (Table 8.10). Also, low acetic acid levels were detected in cultures maintained at 53 °C at pH levels of 5.5 and 6.5 in both sugar and CS liquor fed cultures (Table 8.10). Note no acetic acid was detected in cultures maintained at 53 °C and at a pH of 4.5. These results indicated that CS residues could be used for H_2 production.

Comparison of microbial diversity

Genomic analysis using T-RFLP revealed the presence of microorganisms, which are closely associated with the metabolites produced under each experimental condition. The following section will be a brief summary of the microbial diversity (Archaeal and bacterial population's) detected using T-RFLP analysis in both control and stress treated cultures across different chapters.

a) Control cultures

In general, control cultures showed different microbial composition compared to the stress treated cultures and are dominated by the presence of major H_2 consuming bacteria such as homoacetogens and hydrogenotrophic methanogens. In addition, aceticlastic methanogens and non-H_2 producing bacteria such as lactic acid producers, propionate producers were also present in the control (untreated) cultures. Control cultures (Chapter 4) incubated at 37 °C was dominated by *Eubacterium* sp., a homoacetogen. Methanogens utilizing H_2/CO_2 (*Methanomicrobiales* (hydrogenotrophic methanogen)) or acetic acid (*Methanosarcinales* (aceticlastic methanogen)) were present in the control cultures at pH 7.5. The T-RFs from untreated control cultures (Chapter 5) also confirmed the presence of both aceticlastic methanogens, such as *Methanosarcina* sp. and *Methanosaeta* sp. as well as hydrogenotrophic methanogens, such as *Methanococcus* sp. These microorganisms were absent in all cultures treated with chemical inhibitors. Microbial community analysis in control cultures received 5 glucose feedings (Chapter 6) revealed the presence of *Moorella thermoacetica* (homoacetogen accounting for 14 % of the total bacterial population), *Methanosarcina mazei* (aceticlastic methanogen) and *Methanosaeta thermophila* (aceticlastic methanogen).

Analysis of the archaeal community structure for cultures fed repeatedly with glucose 5 times did not show the presence of hydrogenotrophic methanogens under all of the

experimental conditions. Methanogens isolated from mesophilic anaerobic butyric acid-degrading reactors were detected in mesophilic control cultures (Chapter 6) fed H_2/CO_2. In comparison, methanogens were not detected in thermophilic control cultures (Chapter 6) fed H_2/CO_2. A strong band indicating abundant *Lactococcus lactis* was detected in the initial inoculums, but they declined to undetectable levels by the end of the 37.5 h HRT (Chapter 7). This band was not detected at HRT values of 17.5 h, 10.8 h, 7.5 h and 3.3 h. In the case of the methanogens, intense bands representing *Methylomicrobium* sp. and *Methylophaga* sp. were detected in the inoculum samples taken before start-up of the ASBRs (Chapter 7). With decreasing HRT, methanogenic activity disappeared and the bands related to these organisms were no longer detected. A weak band of *Eubacterium* sp. (homoacetogen) was detected at 37.5 h, 17.5 h, 10.8 h and 7.5 h HRTs, but was not detected for the 3.3 h HRT. Also, bands related to *Moorella thermoacetica* (homoacetogen) were detected in the initial inoculum but they were not present at the end of the cycle for all HRTs ($\leq$ 10.8 h). In general, the initial inoculum showed the presence of lactic acid producing bacteria, methanogens and homoacetogenic bacteria but with decrease HRTs, these organisms were washed out and the microbial population which was enriched with H_2 producing *Clostridium* sp. and *Bacillus* sp. resulted in higher H_2 yields.

The Archaeal *Hae* III enzyme digests of the 16S rRNA samples detected the presence of organisms related to *Methanothermobacter marburgensis*. *M. marburgensis*, a hydrogenotrophic methanogen and a member of the family *Methanobacteriales* was detected in cultures at 53 °C and at pH 6.5 (Chapter 8). An Archaeal rumen clone (associated with *Methanobrevibacter ruminatum*) was detected in cultures maintained at 21 °C and 37 °C (Chapter 8). Aceticlastic methanogens closely related to *Methanosaeta concilii*, *Methanosarcina mazei* and *Methanospirillum* sp. have been observed in cultures at low temperature conditions resulting in high methane levels at low temperatures.

b) Stress treated cultures

In general, stress treated cultures are enriched with spore forming H_2 producing bacteria majorly related to *Clostridium* sp. In LA treated cultures (Chapter 4), the abundance of *Clostridium* sp. closely related to *C. novyi* (at 37 °C), *C. sporogenes* and *C. beijerinckii*

(at 21 °C), and *C. algidicarnis*, *C. innocuum* (at 53 °C) was observed. Also, the diversity of H_2 producers varied according to the type of stress treatment employed. For example, the treatment with long chain fatty acids such as LA and LUA showed higher levels of *Clostridium* sp. diversity compared to the other types of chemical stressors (Chapter 5). In comparison, treatment with BES, furfural or fish oil showed abundance of *Eubacteria* sp. and are responsible for producing HFr.

Similar to Chapter 4 and 5, high levels of *Clostridium* sp. were detected in mesophilic (37 °C) cultures treated with various stressors (fed repeatedly with glucose 5 times) when compared to the thermophilic (55 °C) cultures. Note variation in *Clostridium* sp. was a function of the pretreatment method. For example, *C. sporogenes* (60 %) and *C. bifermentans* (10.3 %) were abundant in cultures pre-treated with alkali while in acid-treated cultures *C. acidiuric* (30 %), *C. beijerinckii* (11 %), *C. argentinense* (9 %) and C. bifermentans (11.5 %) were abundant. In the mesophilic LA treated cultures; *C. sporogenes* (> 95 % relative abundance) was detected after 5 repeated glucose feedings. Microbial analyses of thermophilic cultures fed repeatedly with glucose were associated with the presence of *Lactobacillus ruminis* and *L. salivarius*. In the repeatedly fed thermophilic cultures, *Flavobacterium* sp., *Bacillus* sp., *Thermoanaerobacter* sp., *Bacteroides* sp., *Thioalkalivibrio* sp., and several species of *Clostridium*, such as *C. septicum*, *C. carnis* and *C. botulinum* were detected in stress-treated cultures with low abundance of *Clostridium* sp. Organisms related to *Bacteroides* sp. and *Propionibacterium acnes* were detected in control- (15 %), acid- (6 %) and LA- (6 %) treated cultures. *Thioalkalivibrio* sp. was found to be the dominant population (87 %) in alkali treated thermophilic cultures whereas *Moorella thermoacetica* was found to be dominant (50 %) in BES treated thermophilic cultures (Chapter 6).

The data from Chapter 8 also indicated that spore forming *Clostridium* sp. survived the stress treatment imposed by furan derivatives. The presence of *Clostridium* sp. such as *C. polysaccharolyticum* in cultures at 53 °C might be responsible for the high H_2 yields. Other *Clostridium* sp., such as *C. butyricum*, *C. novyi* NT, *C. quercicolum* was detected in cultures at 37 °C and 53 °C. The presence of *C. butyricum* was likely responsible for HBu production. Thermophilic microorganisms such as *T. acidaminovorans* are closely

associated with cultures operating at 53 °C. HAc production is associated with the presence of organisms related to *M. thermoacetica*. Higher levels of HLa production at 21 °C and 37 °C were due to the presence of *Lactobacillus fermentum*. *Lactobacillus* sp. generally exerts a tolerance towards lower pH (pH 3.0). The presence of *Lactobacillus* sp. is responsible for the lower H_2 yields obtained at 21 °C.

In conclusion, the microbial diversity observed across different experimental conditions mainly depends upon the type of stress treatments. Control cultures resulted in high CH_4 and HAc production (using both H_2/CO_2 and sugars) mainly due to the presence of methanogens (both hydrogenotrophic and aceticlastic) and homoacetogens. In contrast, stress treated cultures resulted in high H_2 production due to the abundance of spore forming bacteria mainly related to *Clostridium* sp.

9.2 References

Abreu, A.A., Karakashev, D., Angelidaki, I., Sousa, D.Z., Alves, M.M. (2012). Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. *Biotechnol Biofuels*, **5**(6), 1-12.

Argun, H., Kargi, F. (2009). Effects of sludge pre-treatment method on bio-hydrogen production by dark fermentation of waste ground wheat. *Int J Hydrogen Energy*, **34**(20), 8543-8.

Lalman, J., Bagley, D.M. (2002). Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. *Water Res*, **36**(13), 3307-13.

Liu, H., Wang, J., Wang, A.J., Chen, J.A. (2011). Chemical inhibitors of methanogenesis and putative applications. *Appl Microbiol Biotechnol*, **89**(5), 1333-40.

Luo, G., Karakashev, D., Xie, L., Zhou, Q., Angelidaki, I. (2011). Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol Bioeng*, **108**(8), 1816-27.

Oh, S.E., Van Ginkel, S., Logan, B.E. (2003). The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ Sci Technol*, **37**(22), 5186-90.

Pendyala, B., Chaganti, S.R., Lalman, J.A., Shanmugam, S.R., Heath, D.D., Lau, P.C.K. (2012). Pretreating mixed anaerobic communities from different sources: Correlating the hydrogen yield with hydrogenase activity and microbial diversity. *Int J Hydrogen Energy*, **37**(17), 12175-86.

Valdez-Vazquez, I., Poggi-Varaldo, H.M. (2009). Hydrogen production by fermentative consortia. *Renew Sust Energ Rev*, **13**(5), 1000-13.

Veeravalli, S.S., Chaganti, S.R., Lalman, J.A., Heath, D.D. (2013). Effect of furans and linoleic acid on hydrogen production. *Int J Hydrogen Energy*, **38**(28), 12283-93.

Wang, J., Wan, W. (2008). Effect of temperature on fermentative hydrogen production by mixed cultures. *Int J Hydrogen Energy*, **33**(20), 5392-7.

CHAPTER 10: ENGINEERING SIGNIFICANCE AND SUGGESTIONS FOR FUTURE RESEARCH

10.1 Engineering Significance and Future Research

The use of fossil fuels as the primary energy source is projected to end in the near future. Over the past century, our society's dependency on fossil fuel sources to meet all our energy needs caused incalculable amount of damage to both humans and our ecosystem. Alternative renewable sources of energy are developed and considerable research activities have been carried out to replace fossil fuels. Possible renewable energy alternatives include bioethanol, biodiesel and biohydrogen (bio-H_2). Among them, bio-H_2 is a promising alternative because of its high energy content compared to other hydrocarbon fuels and its carbon neutral nature. Fuel cell vehicles (FCV) have the potential to significantly reduce the harmful greenhouse gas emissions by employing pure H_2 as fuel instead of gasoline. Already the technology in terms of proton exchange membrane (PEM) fuel cells has shown the ability to convert H_2 to power automobiles. The other issues for employing H_2 in FCV includes lack of H_2 filling stations and the need for a safe on-vehicle H_2 storage tank.

There are several methods (such as electrolysis, catalytic steam reforming, biological methods, etc) of generating H_2. Among the various biological methods, H_2 generation via dark fermentation is most preferred because of its higher H_2 production rate. Also, employing mixed microbial cultures from wastewater reactors for H_2 generation from organic substrates was found to be more promising due to the presence of complex of microorganisms capable of fermenting a wide variety of substrates. However, the difficulty associated in employing mixed microbial cultures for large scale H_2 production is the presence of H_2 consumers which often limit the H_2 yields obtained by this process. Selective inhibition/suppression of these H_2 consumers using eco-friendly stress treatments will prevent the loss of substrate derived electrons to these H_2 consumers and also maximizes the H_2 yield obtained. Therefore, this research aims in understanding the role/activities of H_2 consumers subjected to different stress treatments.

Utilization of lignocellulosic materials as feed stock for fermentative H_2 production is gaining widespread attention. Employing pure glucose as substrate for H_2 production in large scale reactors is not economical from a practical perspective. Waste agricultural lignocellulosic residues such as cornstalks, wheat straw, etc are rich sources of sugars (stored as cellulose and hemicellulose) and could be exploited for their energy value. Upon hydrolysis, both celluloses and hemicelluloses degrade into fermentable sugars such as glucose and xylose which serves as a promising feed stock for bio-H_2 production. In Canada, approximately 12.9 million tonnes of agricultural crop residues are generated each year (Levin et al., 2007). If all these biomass residues are utilized for H_2 generation it could provide a heating value of 16.6 million GJ per year or 2.8 million barrel of oil equivalent (BOE) (assuming 1 BOE = 5.8 GJ energy).

According to Statistics Canada (http://www.statcan.gc.ca/pub/11-526-s/2010001/part-partie1-eng.htm), an average Canadian household uses about 130 GJ of energy (for four person households) in 2007. Therefore, utilizing all the available energy in these crop residues could provide sufficient energy for approximately 127,692 households. In simple terms, this amount of biomass could provide sufficient energy for Essex County (Windsor, ON) to all the households.

The *Archaea* colonizing the rumen has gained considerable attention since they emit the greenhouse gas methane (CH_4) which is contributing to our global warming (Hook et al., 2010). Methane production through enteric fermentation is of concern worldwide for its contribution towards the accumulation of greenhouse gas in the atmosphere; as well as its waste of fed energy for the animal (Hook et al., 2010). Methane is produced in animals by a domain of *Archaea* collectively known as methanogens, which belong to the phylum *Euryarchaeota*. Globally, 50-60 % of CH_4 emissions are from agricultural sector, specifically from livestock production operations (Ellis et al., 2007; Hook et al., 2010). Domesticated animals such as cattle, sheep, and goats produce as much as 86 million Tonnes (Tg) of CH_4 per year (McMichael et al., 2007). As this CH_4 is exhaled through the mouth and nose into the atmosphere, the ruminant suffers a loss of feed-derived energy of approximately 2-12 % depending upon the diet (Johnson and Ward, 1996). There has been recent interest in the presence of methanogens in the intestine of humans.

Archaea was found to be more abundant in the large intestine of obese individuals using 454 pyrosequencing analyses (Zhang et al., 2009). Real time PCR (RT-PCR) has been used to detect *Methanobrevibacter smithii* and *Methanosphaera stadmanae* from human faeces (Dridi et al., 2009). Methanogens such as *Methanobrevibacter gottschalkii*, *Methanobrevibacter thaueri*, *Methanobrevibacter woesei*, and *Methanobrevibacter wolinii* are isolated from the faeces of horse, cow, goose, and sheep, respectively [25]. *Methanobrevibacter oralis* was isolated in sub-gingival sites of patients with periodontal disease (Miller and Lin, 2002). Current research interests have been directed towards CH_4 abatement strategies to be used in ruminants and other animals (Buddle et al., 2011; Kumar et al., 2009; Shibata and Terada, 2010). Fish oil was found to be effective against H_2 consumers (methanogens in particular). Fish oil is consumed by humans for supplementing their omega-3 fatty acids. This finding could be used more useful in quantification of methanogens and also could possibly help in abatement of CH_4 production in humans and ruminants.

Methanogens are known to have symbiotic relationship with other rumen microorganisms for interspecies H_2 transfer (Sharp et al., 1998). They are found to be associated intra-cellularly or extra-cellularly. Methanogens are also found to exhibit symbiotic association with anaerobic fungi, such as *Neocallimastix frontalis* involving interspecies H_2 transfer where by fungi's enzymatic activity was found to be increased and its metabolic activity shifted towards acetic acid production (Bauchop and Mountfort, 1981; Joblin et al., 2002). Methane mitigation is effective in two ways. First type, involves direct effect on the methanogens and in the second type, limiting the substrate availability for methanogenesis via affecting other microbiome present in the rumen (Hook et al., 2010). Both these approaches are proven to be effective against the methanogens *in vivo* and *in vitro*.

Lipids such as fatty acids and oils are used as feed supplements and their effect on ruminal methanogen inhibition was examined both *in vitro* and *in vivo*. Increased lipid content was found to be effective against methanogenesis through inhibition of protozoa, increased production of propionic acid and by biohydrogenation of unsaturated fatty acids (Johnson and Johnson, 1995). These fatty acids inhibit methanogens directly by

binding to the cell membrane and interrupting in membrane transport (Dohme et al., 2001). In another study by Kong et al. (2010) supplementing dairy cows with flaxseed oil, affected the activity of methanogens instead of quantity of methanogens, indicating that fatty acid inhibition was bacteriostatic. *In vitro* studies have found that fatty acids when used in combination have grater methane suppressing effect due to a synergistic effect (Dohme et al., 2001; Soliva et al., 2004). It is likely that oil supplementation would provide a more dramatic depression of CH_4 production than individual fatty acids (Soliva et al., 2004).

Linseed oil supplementation to dairy cows resulted in a 56% reduction in grams of CH_4 produced per day (Martin et al., 2008). Coconut oil is most popular for its use in methane abatement experiments. Although the extent of CH_4 reduction varies between 13-73%, depending upon the inclusion level, diet, ruminant species used (Jordan et al., 2006; Machmuller and Kreuzer, 1999; Machmuller et al., 2000). The ratio of C12:0:C14:0 in coconut oil was found to be (2.6:1.0) Similar to the effective ratios of CH_4 abatement reported in literature (Dohme et al., 2001). Palm kernel oil (with the ratio of C12:0:C14:0, 3:1) was found to be more effective compared to coconut oil (Dohme et al., 2000). Dietary fats rich in saturated medium chain fatty acids (MCFA) have shown to suppress the CH_4 formation *in vivo* (Machmuller and Kreuzer, 1999). Dohme et al. (2001) investigated the anti-methanogenic effect of both lauric (C12:0) and myristic (C14:0) acids at 39 °C.

In metabolic reactions involved in lignocellulose degradation by termite hindguts H_2 appears to be the key intermediate during the fermentative breakdown of carbohydrates (sugars) favoring methanogenesis or reductive acetogenesis (homoacetogenesis). In the absence of other electron acceptors, CO_2 is the terminal electron sink (acceptor) in anaerobic environments. In addition to H_2, formic acid is also produced in the fermentative degradation of organic compounds. Methanogenesis (with higher Gibb's free energy value; $\Delta G^{o'}$ = -135 kJ/mol) was normally expected to outcompete homoacetogens (with lower Gibb's free energy; $\Delta G^{o'}$ = -104 kJ/mol).

However, at lower temperatures and also in slightly acidic environments, methanogens may not be as competitive as homoacetogens for H_2 reduction (Schink, 1997; Stams,

1994). The intestinal tracts of animals (ruminants) are generally characterized by the coexistence of both these H_2 consuming microorganisms. In principle, homoacetogenesis is considered to be more advantageous to the host microorganism which uses acetic acid as the carbon/energy source (Breznak and Kane, 1990). Although, the *in situ* rates of homoacetogens are found to be lower in ruminal samples and pig hindgut, they exhibit increased activities when methanogenesis is inhibited by bromoethanesulfonate (BES) or under elevated H_2 partial pressure (Degraeve et al., 1994; Nollet et al., 1997). The different types of chemical stressors (LCFAs, fish oil and furfural) could be used instead of BES in both soil and wood feeding termites to examine their effectiveness against methanogens.

LCFAs and fish oils are eco-friendly and can be produced from terrestrial and aquatic plants. LUA is present in coconut oil\ while LA is abundant in both safflower and sunflower oil. Furfural could also serve as a H_2 consumption inhibitor at a threshold level. Also, different stress treatments also resulted in enrichment of selective microorganisms which is clearly evident from the formation of specific fermentation by-products. This approach could be very helpful in isolating the organisms responsible for specific product formation. The different fermentation metabolites (VFAs) produced under different stress treatments has promising application in microbial fuel cells (MFCs) or microbial electrolysis cells (MECs).

This research is a culmination of work which provides data describing the effects of different stressing agents on H_2 metabolism in mixed anaerobic cultures. Almost all of the stressing agents used showed promising results with selective methanogen inhibition. Using thermodynamics, hydrogenase assay, flux analysis, statistical methods combined together with the genomic methods provided more insights in understanding the role of methanogens and homoacetogens exposed to different fermentation conditions. This multidisciplinary approach in using these methods to this particular research is unique to this study. Understanding the microbial community structure will be more helpful to better understand the role of microorganisms in large-scale anaerobic reactors.

The following the lists of recommendations for future research work:

1. The results from Phase I of this study indicated the flux of electrons was diverted towards propionic acid formation under suppressed methanogenesis and homoacetogenesis. Propionic acid degradation is very difficult under standard conditions and requires lower H_2 partial pressures. A proper understanding of propionic acid formation will assist in developing operating protocol for minimizing the propionic acid formation. Results from phase I and phase II predicted the formation of propionic acid from H_2/CO_2 and acetic acid using Gibb's free energy values. However, more clear insight on propionic acid formation must be elucidated using radio-labeled carbon isotopes to understand the role of propionic acid formation using these above mentioned substrates.
2. Similar to fish oil, other types of vegetable oils such as safflower oil and sunflower oil, corn oil, coconut oil, etc could be tested for their effectiveness against H_2 consumers. Another set of experiments using these types of oils in the presence of both pure glucose and lignocellulosic hydrolysate should be performed to investigate the effectiveness of these oils on H_2 production.
3. The results from phase V indicated that lignocellulosic biomass such as corn stalk (CS) residues could serve as a promising source for H_2 production at higher temperature (53 °C). Resin treatment of CS liquor to remove furan inhibitors is necessary to prevent their inhibitory effects on both H_2 producers and H_2 consumers. The inhibitory effect of furfural on H_2 consumers varied with the type of substrate. In the presence of sugars, microorganisms are able to evade the inhibitory effect of furfural by diverting the flux of electrons towards ethanol or lactic acid production. Some of the organisms also possess the ability to degrade the furan derivatives to furoic acid and furfuryl alcohol by using the NADH derived during glycolysis. The inhibition of non-H_2 producing microorganisms such as lactic acid bacteria is necessary to re-direct the electrons towards H_2 production.
4. Evaluation of specific methanogenic activities (SMA) was a useful tool in quantifying the activity of methanogens under different stress conditions. The SMA using H_2/CO_2 was examined in this study. More clear idea on SMA of stress treated (CS liquor fed) cultures using a wide range of methanogenic substrates

(acetic acid, formic acid, etc) needs to be conducted for better understanding the activity of methanogens.

10.2 References

Bauchop, T., Mountfort, D.O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl Environ Microbiol*, **42**(6), 1103-10.

Breznak, J.A., Kane, M.D. (1990). Microbial H_2/CO_2 acetogenesis in animal guts - nature and nutritional significance. *FEMS Microbiol Lett*, **87**(3-4), 309-13.

Buddle, B.M., Denis, M., Attwood, G.T., Altermann, E., Janssen, P.H., Ronimus, R.S., Pinares-Patino, C.S., Muetzel, S., Wedlock, D.N. (2011). Strategies to reduce methane emissions from farmed ruminants grazing on pasture. *Vet J*, **188**(1), 11-7.

Degraeve, K.G., Grivet, J.P., Durand, M., Beaumatin, P., Cordelet, C., Hannequart, G., Demeyer, D. (1994). Competition between reductive acetogenesis and methanogenesis in the pig large-intestinal flora. *J Appl Bacteriol*, **76**(1), 55-61.

Dohme, F., Machmuller, A., Wasserfallen, A., Kreuzer, M. (2001). Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. *Lett Appl Microbiol*, **32**(1), 47-51.

Dohme, F., Machmuller, A., Wasserfallen, A., Kreuzer, W. (2000). Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with RUSITEC. *Can J Anim Sci*, **80**(3), 473-82.

Dridi, B., Henry, M., El Khechine, A., Raoult, D., Drancourt, M. (2009). High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *Plos One*, **4**(9), 1-6.

Ellis, J.L., Kebreab, E., Odongo, N.E., McBride, B.W., Okine, E.K., France, J. (2007). Prediction of methane production from dairy and beef cattle. *J Dairy Sci*, **90**(7), 3456-66.

Hook, S.E., Wright, A.D.G., McBride, B.W. (2010). Methanogens: Methane producers of the rumen and mitigation strategies. *Archaea*, 1-11.

Joblin, K.N., Matsui, H., Naylor, G.E., Ushida, K. (2002). Degradation of fresh ryegrass by methanogenic co-cultures of ruminal fungi grown in the presence or absence of *Fibrobacter succinogenes*. *Curr Microbiol*, **45**(1), 46-53.

Johnson, D.E., Ward, G.M. (1996). Estimates of animal methane emissions. *Environ Monit Assess*, **42**(1-2), 133-141.

Johnson, K.A., Johnson, D.E. (1995). Methane emissions from cattle. *J Anim Sci*, **73**(8), 2483-92.

Jordan, E., Lovett, D.K., Monahan, F.J., Callan, J., Flynn, B., O'Mara, F.P. (2006). Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heffers. *J Anim Sci*, **84**(1), 162-70.

Kong, Y.H., He, M.L., McAlister, T., Seviour, R., Forster, R. (2010). Quantitative fluorescence in situ hybridization of microbial communities in the rumens of cattle fed different diets. *Appl Environ Microbiol*, **76**(20), 6933-8.

Kumar, S., Puniya, A.K., Puniya, M., Dagar, S.S., Sirohi, S.K., Singh, K., Griffith, G.W. (2009). Factors affecting rumen methanogens and methane mitigation strategies. *World J Microb Biot*, **25**(9), 1557-66.

Levin, D.B., Zhu, H.G., Beland, M., Cicek, N., Holbein, B.E. (2007). Potential for hydrogen and methane production from biomass residues in Canada. *Bioresource Technol*, **98**(3), 654-60.

Machmuller, A., Kreuzer, M. (1999). Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. *Can J Anim Sci*, **79**(1), 65-72.

Machmuller, A., Ossowski, D.A., Kreuzer, M. (2000). Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. *Anim Feed Sci Tech*, **85**(1-2), 41-60.

Martin, C., Rouel, J., Jouany, J.P., Doreau, M., Chilliard, Y. (2008). Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *J Anim Sci*, **86**(10), 2642-50.

McMichael, A.J., Powles, J.W., Butler, C.D., Uauy, R. (2007). Food, livestock production, energy, climate change, and health. *Lancet*, **370**(9594), 1253-63.

Miller, T.L., Lin, C.Z. (2002). Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. *Int J Syst Evol Microbiol*, **52**, 819-22.

Nollet, L., Demeyer, D., Verstraete, W. (1997). Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. *Appl Environ Microbiol*, **63**(1), 194-200.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol R*, **61**(2), 262-80.

Sharp, R., Ziemer, C.J., Stern, M.D., Stahl, D.A. (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol Ecol*, **26**(1), 71-8.

Shibata, M., Terada, F. (2010). Factors affecting methane production and mitigation in ruminants. *Anim Sci J*, **81**(1), 1-10.

Soliva, C.R., Meile, L., Cieslak, A., Kreuzer, M., Machmuller, A. (2004). Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. *Brit J Nutr*, **92**(4), 689-700.

Stams, A.J.M. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Anton Leeuw Int J G*, **66**(1-3), 271-94.

Zhang, H.S., DiBaise, J.K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y.S., Parameswaran, P., Crowell, M.D., Wing, R., Rittmann, B.E., Krajmalnik-Brown, R. (2009). Human gut microbiota in obesity and after gastric bypass. *P Natl Acad Sci USA*, **106**(7), 2365-70.

APPENDIX A : CALIBRATION CURVES

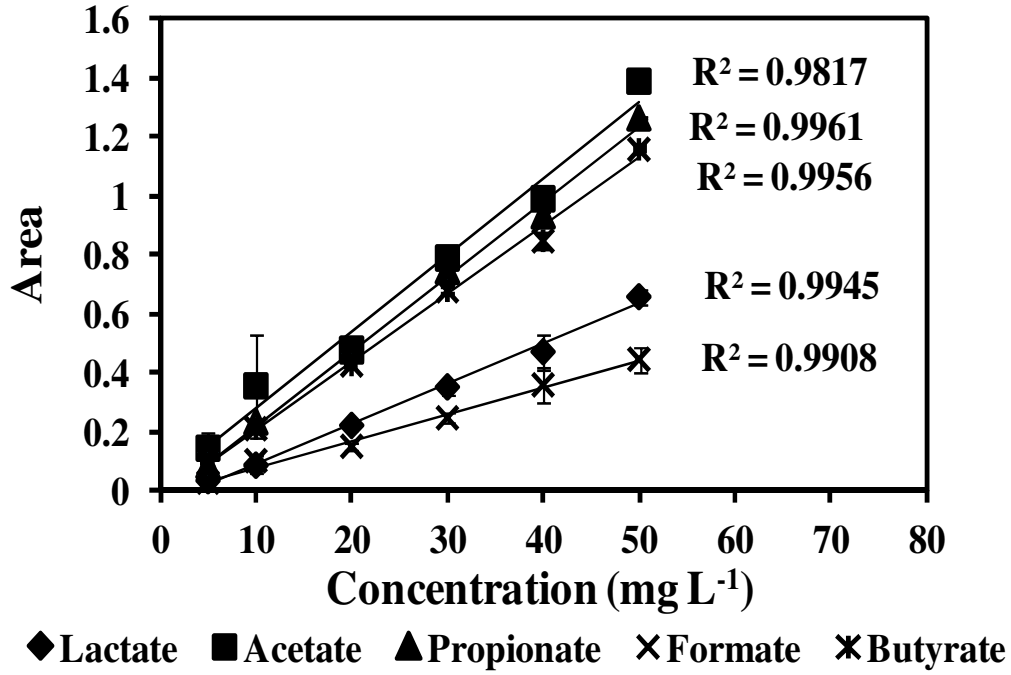

Figure A.1 Volatile fatty acids calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

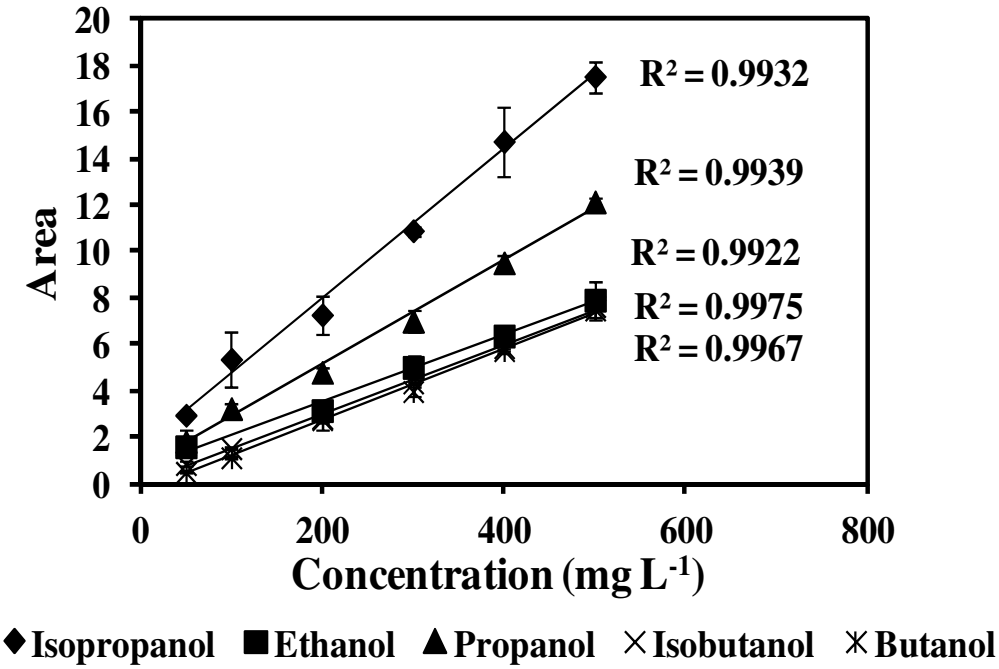

Figure A.2 Alcohols calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

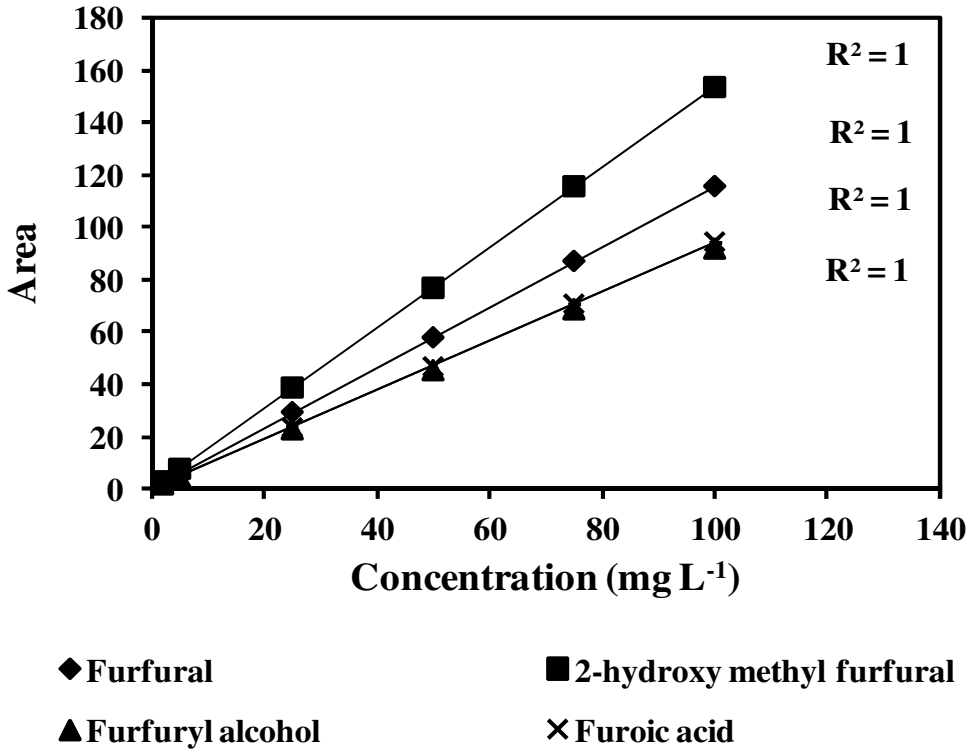

Figure A.3 Furans calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

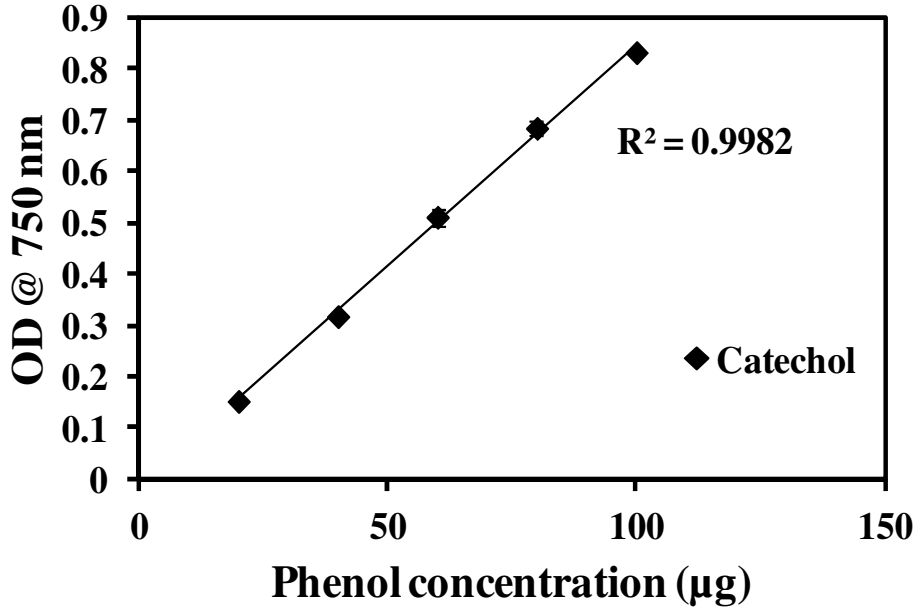

Figure A.4 Phenol calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

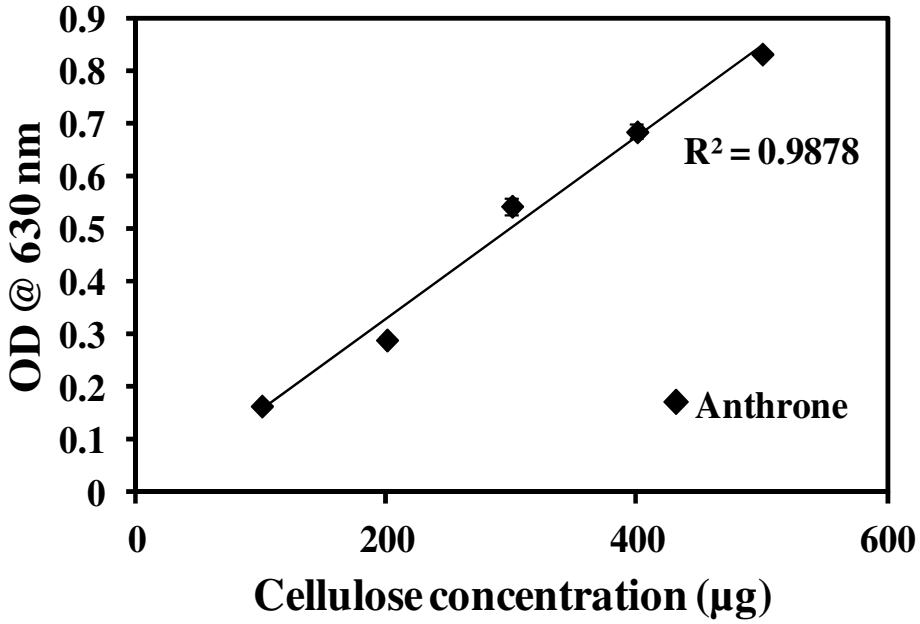

Figure A.5 Cellulose calibration curve (Anthrone method). Note: Error bars in this figure represent standard deviation for n = 3**.**

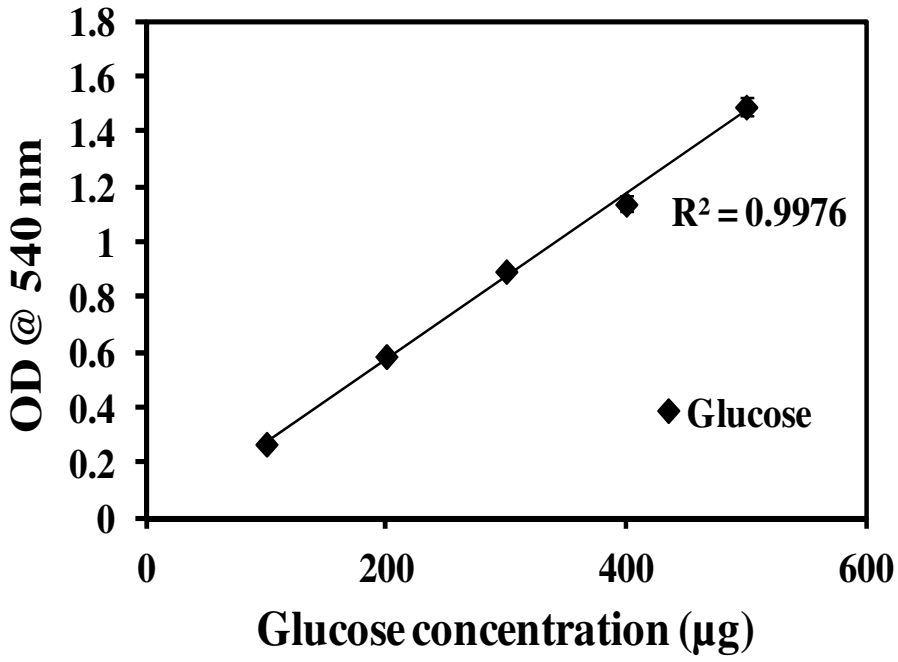

Figure A.7 Glucose calibration curve (DNS method). Note: Error bars in this figure represent standard deviation for n = 3**.**

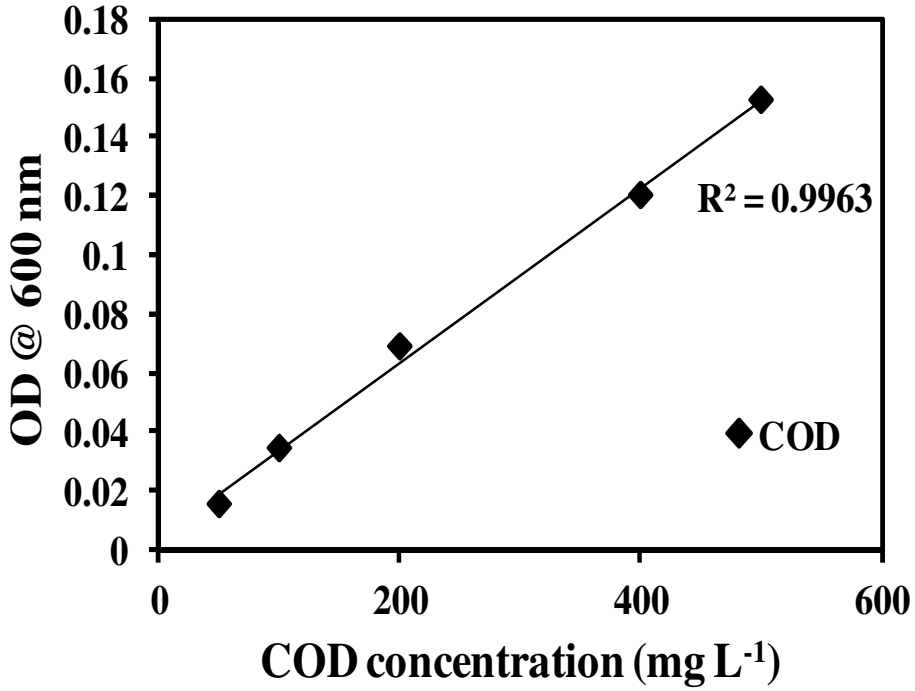

Figure A8. COD calibration curve Note: Error bars in this figure represent standard deviation for n = 3**.**

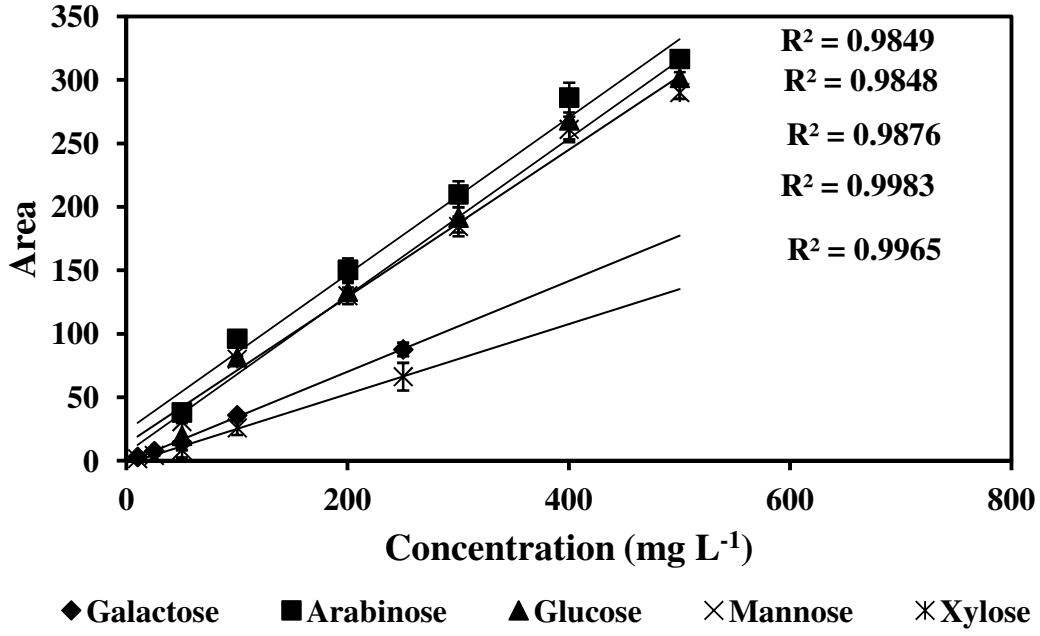

Figure A9. Sugar mix calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

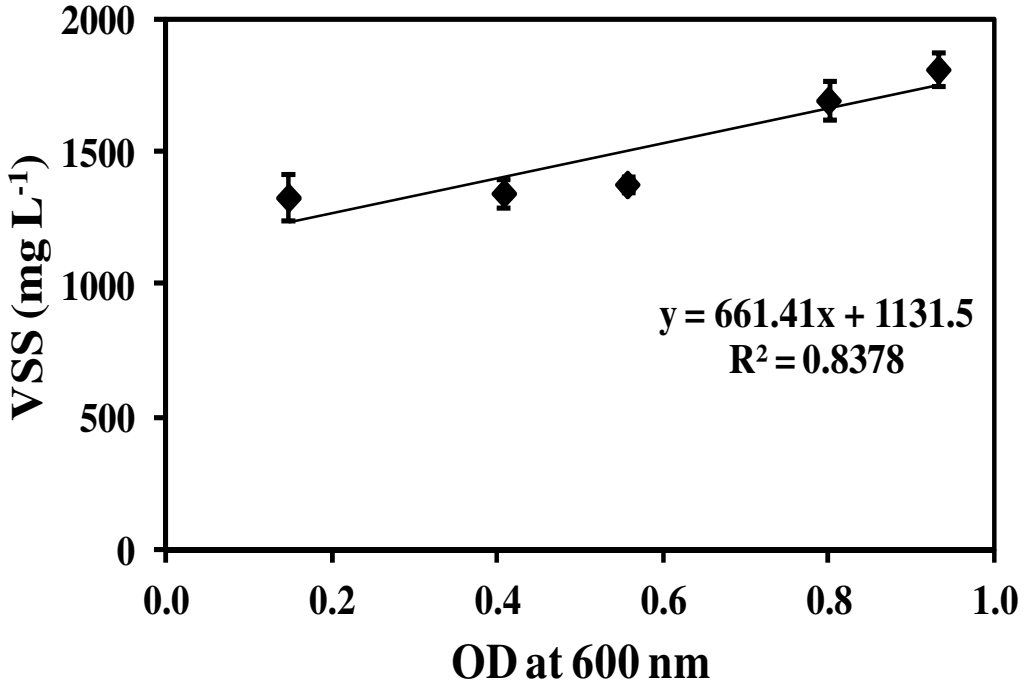

Figure A.10 OD Vs VSS calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

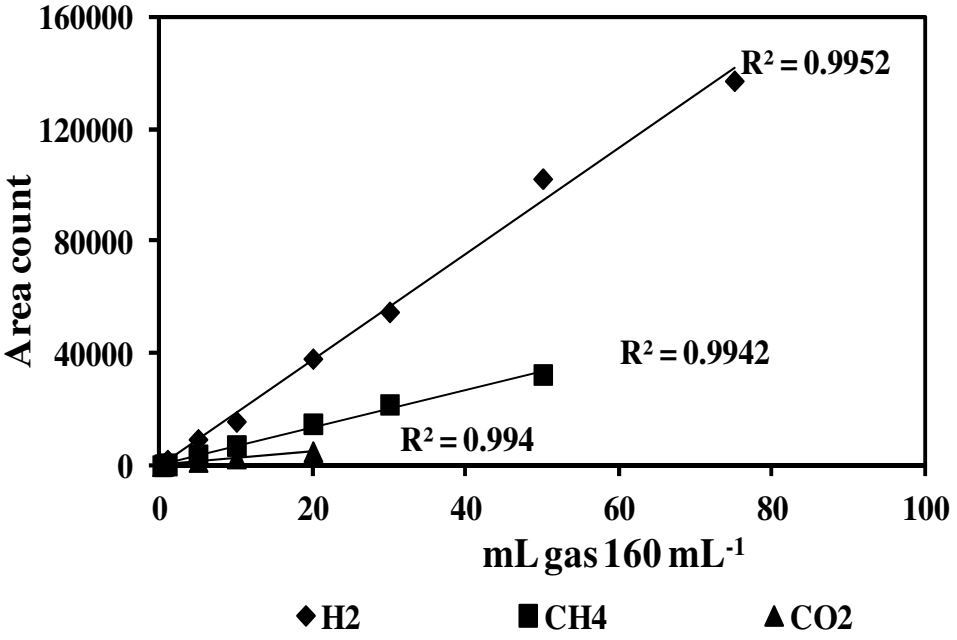

Figure A.11 Gas calibration curve. Note: Error bars in this figure represent standard deviation for n = 3**.**

APPENDIX B : SAMPLE CALCULATIONS

VSS/TSS calculation:

$$\textbf{Total suspended solids (TSS, mg L}^{-1}\textbf{)} = \frac{\textbf{mass@105°C (g)} - \textbf{empty mass (g)}}{\textbf{volume (mL)}} \textbf{ X } 10^6$$

$$\textbf{Volatile suspended solids (VSS, mg L}^{-1}\textbf{)} = \frac{\textbf{mass@105°C (g)} - \textbf{mass@550°C (g)}}{\textbf{volume (mL)}} \textbf{ X } 10^6$$

Empty mass (g) = mass of aluminium pan + mass of 0.45 µm glass fibre filter paper

Volume (mL) = amount of sample added to be filtered

mass@105°C (g) = mass of aluminium pan + filter paper + sample after 1 h in 105°C oven

mass@550°C (g) = mass of aluminium pan + filter paper + sample after 1 h in 550°C muffle furnace

Example:

Empty mass (g)	*Volume (mL)*	*mass@105°C (g)*	*mass@550°C (g)*
0.8825	5	0.8957	0.8839

$$\textbf{TSS (mg L}^{-1}\textbf{)} = \frac{(0.8957 - 0.8825)}{5} \textbf{ X } 10^6 = 2640$$

$$\textbf{VSS (mg L}^{-1}\textbf{)} = \frac{(0.8957 - 0.8839)}{5} \textbf{ X } 10^6 = 2360$$

Note: All TSS and VSS measurements were made in triplicates.

Stock Preparation:

1. **Glucose**

Glucose stock solution was prepared in quantities of 120 mL with final concentration (100,000 mg L^{-1})

100,000 mg L^{-1} = x / 0.120 L

X = 100,000 mg/L * 0.120 L = 12,000 mg = 12 g

Amount of Milli-Q (MQ) water to be added:

120 mL * 0.9979 g mL^{-1} = 119.748 g (Note: Assume density of water@22°C = 0.9979 g mL^{-1})

119.748 g = 12 g glucose + X g MQ water

X = 119.748 g – 12 g = 107.748 g MQ water

Therefore, 12 grams of glucose was added to 107.748 g MQ water to make 120 mL of 100,000 mg L^{-1} glucose stock.

2. BES

BES stock solution was prepared in quantities of 20 mL and in concentrations of 1000 mM

50 mM = 1 M = 1 mol L^{-1}

1 mol L^{-1} = X / 0.05 L

X = 1 mol L^{-1} * 0.02 L = 0.02 mol BES

0.02 mol * 211.0057 g mol^{-1} = 4.22 g BES

Amount of MQ water to be added: Assuming density of water@22°C = 0.9979 g mL^{-1}

20 mL * 0.9979 g mL^{-1} = 19.958 g

19.958 g = 4.22 g BES + X g MQ water

X = 19.958 g – 4.22 g = 15.738 g MQ water

Therefore, 4.22 g BES was added to 15.738 g MQ water to make 50 mL of 1 M BES stock.

3. LCFA (LUA, LA)

LCFA stock solutions were prepared in quantities of 20 mL with concentration 50,000 mg L^{-1}

50,000 mg L^{-1} = X / 0.02 L

X = 0.02 L * 50,000 mg L^{-1} = 1,000 mg = 1 g

Amount of MQ water to be added: Assuming density of water@22°C = 0.9979 g mL^{-1}

20 mL * 0.9979 g mL^{-1} = 19.958 g

19.958 g = 1 g LCFA + *y* g NaOH + *x* g MQ water

x = 19.958 g – 1 g LCFA – 0.142 g NaOH = 18.816 g MQ water

Note: *y* for LA and LUA are 0.142 g and 0.200 g NaOH respectively.

Therefore, 1 g of LA was added to 18.816 g MQ water and 0.142 g NaOH to make 20 mL of 50,000 mg L^{-1} LA stock. Similarly, 1 g LUA was added to 18.578 g MQ water and 0.200 g NaOH to make 20 mL of 50,000 mg L^{-1} LUA stock solution.

Culture preparation calculation:

The final operating concentration in 160 mL batch reactor = 2000 mg L^{-1}

Example: VSS of culture from mother reactor = 10,000 mg L^{-1}.

Amount of substrate (glucose) to be added = 5 g L^{-1} = 5000 mg L^{-1}

Amount of LA to be added = 2000 mg L^{-1}

Note: Glucose stock solution was prepared to a final concentration of 100,000 mg L^{-1}

LA stock solution was prepared to a final concentration of 50,000 mg L^{-1}

Amount of glucose to be added (mL) = Liquid volume / (stock concentration of glucose. desired glucose concentration in $bottle^{-1}$)

Amount of glucose to be added (mL) = 50 mL / (100,000 mg L^{-1}. 5,000 mg L^{-1}) = 2.5 mL

Amount of LA to be added (mL) = Liquid volume / (LA stock concentration. desired LA $concentration^{-1}$)

Amount of glucose to be added (mL) = 50 mL / (5,000 mg L^{-1}. 2000 mg L^{-1}) = 2.0 mL

Amount of culture to be added (mL) = Liquid volume / (mother reactor VSS. desired VSS^{-1})

Amount of glucose to be added (mL) = 50 mL / (10,000 mg L^{-1}. 2000 mg L^{-1}) = 10.0 mL

Therefore, final reactor volume (50 mL) = Amount of glucose + amount of inhibitor (LA) + amount of culture + amount of basal media.

Thus, the final bottle volume was attained by adding the following:

10.0 mL culture + 2.5 mL glucose stock +2.0 mL LA stock +35.5 mL basal media = 50 mL total volume.

VFAs: Peak Area (µS.min) to concentration (mg L^{-1})

Example for Acetic acid (peak area of 2.1589 µS.min)

The calibration curve equation for acetic acid is: $y = 0.1029\ x$

y (µS.min) = peak area

x (mg L^{-1}) = concentration

Concentration (mg L^{-1}) = (Peak area (μS. min) / slope of calibration curve) * dilution factor

Dilution factor for VFAs = 20

Concentration (mg L^{-1}) = (2.1589 / 0.1029) * 20 = 419.61

Alcohols: Peak area (nC.min) to concentration (mg L^{-1})

Example for ethanol (peak area of 0.3014 nC. min)

The calibration curve equation for ethanol is $y = 0.0055\ x$

y (nC. min) = peak area

x (mg L^{-1}) = Concentration

Concentration (mg L^{-1}) = (Peak area (nC. min) / slope of calibration curve) * dilution factor

Dilution factor for alcohols = 5

Concentration (mg L^{-1}) = (0.3014 / 0.0055) * 5 = 274

Glucose: Peak area (nC. min) to concentration (mg L^{-1})

Example for glucose (Peak area of 67.1598 nC.min)

The calibration curve equation for glucose is: $y = 2.5942\ x$

y (nC. min) = peak area

x (mg L^{-1}) = Concentration

Concentration (mg L^{-1}) = (Peak area (nC. min) / slope of calibration curve) * dilution factor

Dilution factor for alcohols = 10

Concentration (mg L^{-1}) = (67.1598 / 2.5942) * 10 = 258.88

Gas calculation: Peak area count to number of mol

Example for H_2 (area count of 178,650 and pressure of 30.5 psi)

The calibration curve equation for H_2 is: $y = 340000000000\ x$

y = area count

x (mL / 160 mL bottle) = concentration

Note: Gas calibrations were conducted by injecting known volumes of individual gas (H_2, CO_2, and CH_4) in 160 mL serum bottles pressurized to 1 atm with N_2. The GC area count

is a function of mL of gas per 160 mL bottle. Each bottle prepared contained a liquid volume of 50 / 75 mL in a 160 mL serum bottle. Therefore, headspace correction factor must be incorporated in order to account for the difference between experiments and calibrations.

Headspace correction = (headspace volume / Gas injection volume)

Headspace correction = 85 mL / 0.025 mL = 3400

Note: Headspace volume for 50 mL liquid volume reactor is 110 mL.

Pressure (atm) = (Pressure (psi) + 14.7 (psi)) / 14.7 (psi)

Note: 14.7 psi was added to the pressure gauge reading in addition to the reading measured from each bottle. This value is divided by 14.7 psi in order to convert the pressure to atm. The pressure gauge was calibrated to atmospheric pressure which reads zero at room pressure.

Pressure (atm) = (30.1 + 14.7) / 14.7 = 3.04 atm

Number of moles (n) = (Pressure (atm) * volume (L)) / (R (L. atm.mol^{-1}.K^{-1}) * T (K))

Note: The values of R = 0.08205 L. atm. mol^{-1}.K^{-1} and temperature = 298 K are already incorporated in the gas calibration slope.

Therefore, n = (3.04) * (4400)*(178,650 / 340,000,000,000) = 0.00543 mol = 5430 µmol

Yield calculation:

5 g L^{-1} glucose was injected into each batch reactor with a working volume of 75 mL.

Assuming, 0.00543 mol H_2 have been calculated.

Amount of glucose (g) = volume (L) * glucose (g L^{-1}) = 0.075 * 5 = 0.375 g

Moles of glucose added into each bottle (mol) = Weight of glucose added (g) / molecular weight of glucose (g mol^{-1})

Moles of glucose = 0.375 / 180 = 0.002083 mol

H_2 yield = moles of H_2 / moles of glucose = 0.00543 / 0.002083 = 2.61 mol H_2.mol^{-1} glucose.

Similarly, the CH_4 yield was calculated using the appropriate calibration curve for CH_4.

Model electron balance

The electron (e-) mass balance takes into account the substrate (H_2 or glucose) electron equivalents and the electrons in the fermentation by-products. Electron equivalents in LCFA fed cultures does not include the parent (LCFA) compound or its degradation products in Chapters 5, 6 and 7. In Chapter 8, COD balance was performed similar to the electron mass balance by converting the electrons equivalents to COD equivalents. The electron balance was conducted based on the assumption that 10% of the substrate derived electrons are utilized for biomass synthesis. The number of electron equivalents (e^- equiv) available from glucose and by-products were determined from the half reactions:

Formic acid: $^1/_2\ HCOO^- + {}^1/_2\ H_2O \leftrightarrow {}^1/_2\ HCO_3^- + H^+ + e^-$

Acetic acid: $^1/_8\ CH_3COO^- + {}^3/_8\ H_2O \leftrightarrow {}^1/_8\ HCO_3^- + H^+ + e^-$

Propionic acid: $^1/_{14}\ CH_3CH_2COO^- + {}^5/_{14}\ H_2O \leftrightarrow {}^1/_{14}\ HCO_3^- + {}^1/_7\ CO_2 + H^+ + e^-$

Hydrogen: $^1/_2\ H_2 \leftrightarrow H^+ + e^-$

Methane: $^1/_8\ CO_2 + H^+ + e^- \leftrightarrow {}^1/_8\ CH_4 + {}^1/_4\ H_2O$

Table B.1 Electron balance for BES treated cultures fed H_2 (Chapter 5).

Metabolite	Concentration (mmol)	e- equiv per mol metabolite	e- equiv in	e- equiv out
H_2	3.300	2	6.60	5.14
CH_4	0.000	8		0.00
CH_3COO^-	0.056	8		0.44
$HCOO^-$	0.073	2		0.15
$CH_3CH_2COO^-$	0.000	14		0.00
Biomass				0.66
Total			6.60	6.24
% balance = (6.24 / 6.60) *100 = 95 %				

One mole of propionic acid gives 14 e^- equiv, one mole of hydrogen gives 2 e^- equiv and one mole of methane and acetic acid gives 8 e^- equiv, one mole of formic acid gives 2 e^- equiv, and so on. The substrate and product concentrations in mg L^{-1} were converted to e^- equiv based on the half reactions as mentioned above. The electrons distributed in by-products were added and then compared to the initial substrate electrons in order to calculate the electron mass balance (%).

$$Electron\ mass\ balance\ (\%) = \frac{e^-\ in\ byproducts + \ e^-\ in\ biomass + \ remaining\ substrate\ e^-}{e^-\ in\ substrate} * 100$$

COD mass balance

Table B.2 COD mass balance for CS liquor fed culture (Exp # 6 -Table 8.7; Chapter 8).

Metabolite	metabolite concentration (g L^{-1})	g COD per g metabolite	g COD in	g COD out
H_2	0.14	8		1.09
CH_4	0.00	4		0.00
Lactic acid	0.09	1.067		0.10
Acetic acid	2.01	1.067		2.15
Butyric acid	0.66	1.81		1.15
Ethanol	0.03	2.08		0.07
Iso-Propanol	0.03	2.4		0.06
Furfural	0.01	1.67		0.01
HMF	0.00	1.52		0.00
Furoic acid	0.08	1.28		0.11
Furfuryl alcohol	0.00	1.79		0.00
Glucose	4.69	1.067	5	0.24
Biomass				0.20
Total				5.19
% balance = (5.19 / 5) * 100 = 104 %				

The COD mass balance was conducted in a similar manner to electron mass balance. The only difference though is balance was performed based on COD equivalents instead of electron equivalents. The amount of COD equivalents diverted towards biomass synthesis

is calculated using the conversion formula 1.42 g COD g^{-1} VSS. The COD equivalents of the fermentation metabolites (both gas and liquid) were mentioned in the Table B.2.

Sample Flux Balance Analysis calculation (for LA treated cultures- Chapter 7)

The FBA was conducted to quantify the H_2 consuming internal electron fluxes based on the external fluxes (fermentation metabolite data). It was assumed that 10 % of the initial substrate derived electrons is utilized for biomass synthesis. Sample calculations on H_2 consuming electron fluxes in LA (2 g L^{-1}) treated cultures at 37.5 h HRT is shown here. The yields of the fermentation metabolites in (mol metabolite mol^{-1} glucose) was calculated and used as the input for the FBA model. Model predicted H_2 yield was calculated using R12 flux (Table 3.4). Experimental H_2 yield (R13 flux; Table 3.4) was then compared against this model predicted value and their difference shows the total H_2 consumed under particular experimental condition. This consumed H_2 could be utilized due to homoacetogenesis (R17 flux), iso-propanol formation (R21 flux) and hydrogenotrophic methanogenesis (R29 flux). The results from FBA are as follows: R12 flux = 1.48±0.06 mol H_2 mol^{-1} glucose; R13 flux = 0.84±0.04 mol H_2 mol^{-1} glucose; R17 flux = 0.05±0.002 mol H_2 mol^{-1} glucose; R21 flux = 0.00±0.00 mol H_2 mol^{-1} glucose and R29 flux = 0.11±0.004 mol H_2 mol^{-1} glucose. The difference between R 12 and R13 flux (i.e., the total H_2 consumed) = 1.48±0.06 – 0.84±0.04 = 0.64±0.03 mol H_2 mol^{-1} glucose reported in Table 7.4.

Since 4 mol H_2 are utilized for the formation of 1 mol of HAc and CH_4, respectively the H_2 flux due to homoacetogenesis (R 17 flux) is equal to 0.20±0.01 mol H_2 mol^{-1} glucose and H_2 flux due to CH_4 formation (R29 flux) is equal to 0.44±0.02 mol H_2 mol^{-1} glucose.

APPENDIX C : ADDITIONAL FIGURES AND TABLES

Table C.1 Hydrogen yields (in mol mol^{-1} glucose) for the cultures incubated at mesophilic temperature.

Batch	Day	Control	Heat	Acid	Alkali	BES	Linoleic acid	Loading shock
1	1	0.03±0.02	2.32±0.07	1.97±0.02	1.99±0.01	1.94±0.08	1.85±0.03	0.14±0.06
	2	0.00±0.00	2.66±0.03	2.28±0.01	2.09±0.04	2.00±0.06	1.75±0.09	1.08±0.05
	3	0.00±0.00	2.16±0.04	1.52±0.05	1.21±0.09	1.60±0.11	1.46±0.11	1.71±0.10
	4	0.00±0.00	2.25±0.11	1.75±0.03	1.65±0.10	1.73±0.03	1.40±0.04	2.11±0.02
2	1	0.25±0.11	2.17±0.13	2.13±0.01	1.76±0.06	2.39±0.04	2.10±0.12	2.93±0.05
	2	0.00±0.00	2.30±0.09	2.44±0.12	1.92±0.08	2.47±0.03	2.30±0.23	2.96±0.12
	3	0.00±0.00	2.03±0.08	1.95±0.09	1.68±0.12	2.12±0.02	2.63±0.19	2.88±0.05
	4	0.00±0.00	2.05±0.08	1.55±0.15	1.72±0.17	1.20±0.06	2.49±0.10	1.31±0.14
3	1	1.49±0.05	2.77±0.09	2.69±0.00	1.07±0.00	2.40±0.01	2.65±0.06	2.79±0.07
	2	0.99±0.03	2.57±0.01	2.57±0.14	1.90±0.19	2.59±0.16	2.68±0.07	2.47±0.09
	3	0.38±0.01	2.22±0.07	2.07±0.14	1.51±0.04	1.69±0.16	2.42±0.08	2.39±0.15
	4	0.03±0.00	2.08±0.07	1.42±0.06	1.42±0.06	1.34±0.06	2.26±0.15	1.47±0.46
4	1	1.44±0.14	2.54±0.07	2.46±0.07	2.59±0.04	2.33±0.05	1.54±0.08	2.03±0.05
	2	0.71±0.13	2.16±0.07	2.08±0.06	2.39±0.12	2.30±0.09	1.50±0.14	2.33±0.10
	3	0.00±0.01	2.02±0.03	1.95±0.07	2.17±0.22	1.84±0.06	1.39±0.08	2.03±0.06
	4	0.00±0.00	1.79±0.00	1.93±0.01	1.76±0.00	1.19±0.03	1.49±0.13	1.90±0.08
5	1	1.35±0.27	2.48±0.07	2.59±0.04	2.28±0.00	2.29±0.04	2.40±0.05	2.33±0.06
	2	0.09±0.06	1.92±0.19	1.95±0.04	1.57±0.06	2.07±0.12	1.75±0.12	2.04±0.00
	3	0.00±0.00	2.10±0.03	1.85±0.07	1.68±0.12	1.79±0.14	2.00±0.11	2.25±0.06
	4	0.00±0.00	1.88±0.09	2.00±0.03	1.55±0.15	1.64±0.04	1.80±0.11	2.12±0.08

Note: a ± b represents mean ± standard deviation of triplicate samples.

Table C.2 Hydrogen yields (in mol mol^{-1} glucose) for the cultures incubated at thermophilic temperature.

Batch	Day	Control	Heat	Acid	Alkali	BES	Linoleic acid	Loading shock
1	1	0.00±0.00	1.32±0.05	1.99±0.01	0.28±0.05	1.60±0.01	0.00±.00	0.27±0.02
	2	0.00±0.00	1.30±0.19	1.46±0.36	1.02±0.03	1.19±0.05	1.97±0.31	0.47±0.10
	3	0.02±0.01	1.74±0.29	1.35±0.26	1.56±0.06	0.08±0.19	2.47±0.19	2.03±0.05
	4	0.01±0.00	1.16±0.15	1.30±0.41	1.99±0.23	1.14±0.15	2.58±0.10	2.16±0.13
2	1	1.19±0.15	1.47±0.04	1.65±0.12	0.72±0.00	1.40±0.08	0.47±0.13	0.21±0.02
	2	1.53±0.17	1.69±0.01	1.93±0.12	1.58±0.16	2.02±0.08	0.97±0.01	0.41±0.04
	3	1.65±0.20	1.54±0.01	1.44±0.02	1.72±0.09	2.17±0.21	0.88±0.04	0.58±0.07
	4	1.81±0.13	1.41±0.02	1.50±0.06	2.16±0.05	2.23±0.12	1.05±0.00	0.77±0.16
3	1	0.60±0.07	0.99±0.00	0.26±0.08	1.55±0.08	0.97±0.11	0.74±0.10	1.15±0.08
	2	0.99±0.05	1.32±0.11	1.14±0.02	2.14±0.15	1.35±0.11	0.93±0.15	1.40±0.00
	3	1.16±0.05	1.25±0.01	1.23±0.04	1.96±0.15	1.25±0.10	1.02±0.17	1.33±0.04
	4	0.96±0.08	1.13±0.02	1.15±0.07	1.79±0.15	1.22±0.08	0.59±0.16	1.22±0.02
4	1	0.22±0.02	0.93±0.06	1.93±0.10	0.52±0.11	0.55±0.04	0.87±0.06	1.43±0.07
	2	0.37±0.00	1.26±0.02	1.61±0.16	0.78±0.08	0.76±0.10	1.13±0.13	1.40±0.03
	3	0.63±0.05	1.12±0.02	1.57±0.01	0.87±0.05	0.88±0.19	1.53±0.07	1.40±0.08
	4	0.89±0.08	1.47±0.02	1.65±0.12	0.92±0.08	0.64±0.05	1.60±0.03	1.38±0.08
5	1	0.39±0.02	0.35±0.02	0.23±0.02	0.27±0.07	0.32±0.05	0.63±0.03	0.44±0.04
	2	1.00±0.06	0.87±0.02	1.00±0.09	0.49±0.04	0.77±0.06	0.92±0.02	0.81±0.07
	3	1.08±0.13	0.81±0.02	1.19±0.04	0.61±0.08	0.97±0.18	1.21±0.01	1.29±0.03
	4	1.18±0.04	0.76±0.02	1.30±0.04	0.65±0.08	0.92±0.12	1.48±0.04	0.62±0.14

Note: a ± b represents mean ± standard deviation of triplicate samples.

Table C.3 Methane yields (in mol mol^{-1} glucose) for the cultures incubated at mesophilic temperature.

Batch	Day	Control	Heat	Acid	Alkali	BES	Linoleic acid	Loading shock
1	1	0.93±0.17	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.01
	2	1.14±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.00
	3	0.83±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
	4	0.97±0.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
2	1	0.62±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01
	2	0.72±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.04±0.01
	3	0.68±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.05±0.01
	4	0.72±0.07	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.06±0.02
3	1	0.33±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01
	2	0.42±0.15	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01
	3	0.51±0.14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01
	4	0.54±0.07	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01
4	1	0.32±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
	2	0.49±0.19	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
	3	0.56±0.06	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
	4	0.56±0.07	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
5	1	0.30±0.09	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	0.60±0.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	3	0.59±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	4	0.58±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Note: a ± b represents mean ± standard deviation of triplicate samples.

Table C.4 Methane yields (in mol mol^{-1} glucose) for the cultures incubated at thermophilic temperature.

Batch	Day	Control	Heat	Acid	Alkali	BES	Linoleic acid	Loading shock
1	1	0.72±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.02
	2	0.74±0.12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.05±0.01
	3	0.66±0.09	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.05±0.02
	4	0.58±0.13	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.04±0.01
2	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
4	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
5	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Note: a ± b represents mean ± standard deviation of triplicate samples.

Table C.5 Percent electrons diverted towards acetic acid and methane production.

Temperature (°C)	Exp. Set	% Electrons diverted towards methane production [a]	% Electrons diverted towards acetic acid production [b]
37	Control	49±5	19±2
	Heat	54±3	31±2
	Acid	46±2	26±1
	Alkali	42±3	36±3
	BES	41±1	36±1
	LA	64±7	23±2
	LS	0±0	85±1
53	Control	0±0	80±1
	Heat	0±0	80±6
	Acid	0±0	76±2
	Alkali	0±0	91±3
	BES	0±0	20±2
	LA	0±0	18±0
	LS	0±0	18±2

Note: [a and b] represents the percent of consumed substrate (H_2) electrons diverted towards methane and acetic acid formation. The values shown in this table represent mean ±standard deviation of triplicate samples.

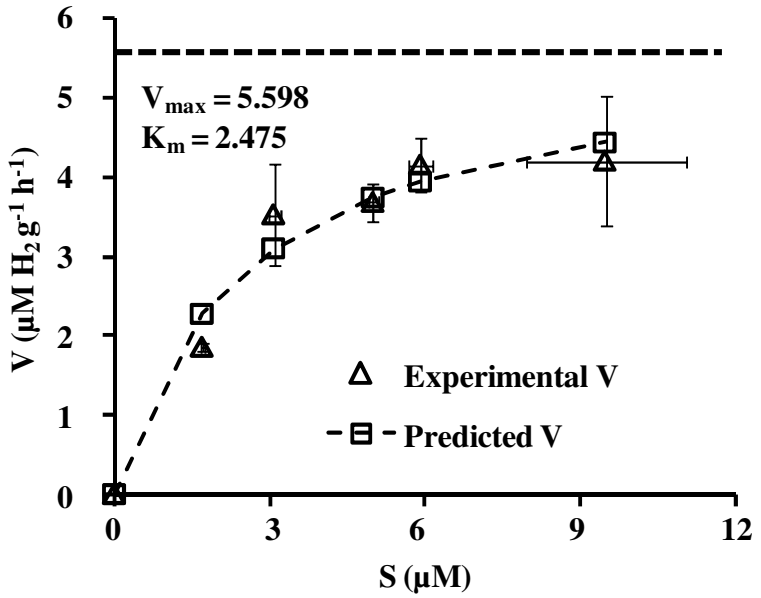

Figure C.1 Hydrogen uptake kinetics.
Note: The error bars shown in this figure represents mean ± standard deviation of triplicate samples.

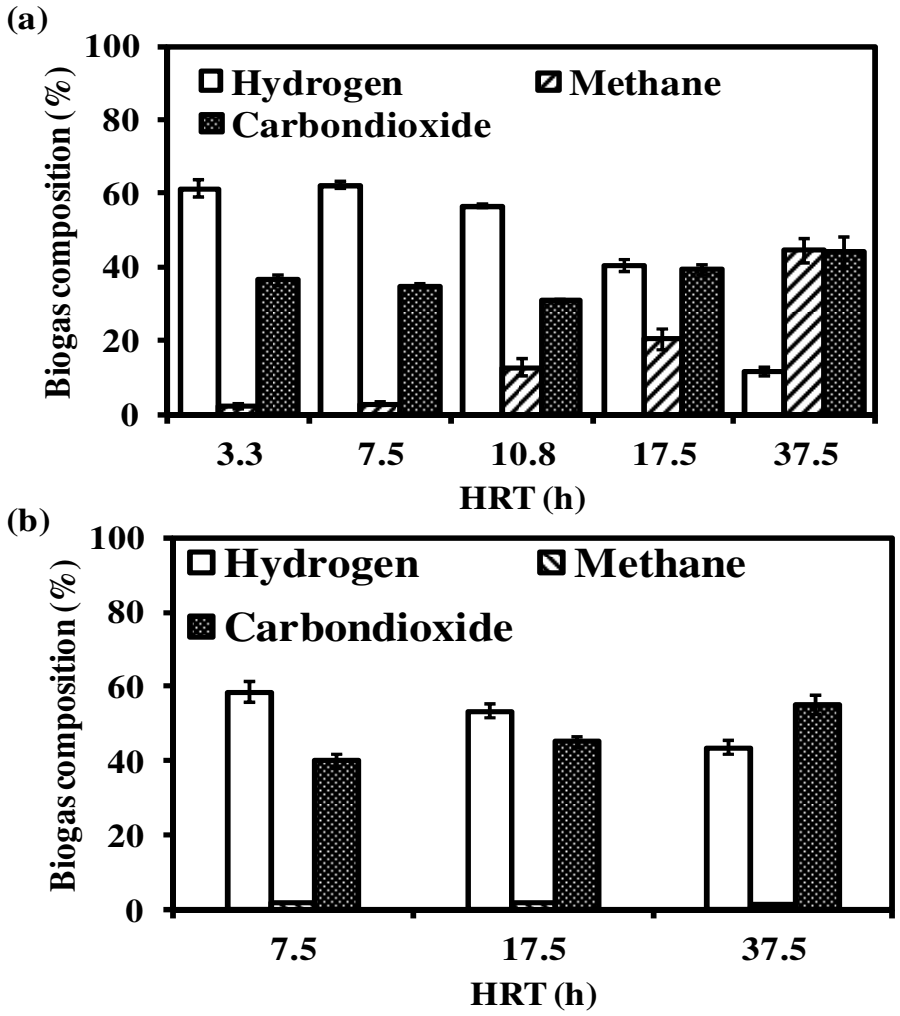

Figure C.2 Composition of biogas in both control (a) and LA-treated (b) cultures at various HRT's.
Note: The error bars shown in this figure represents mean ± standard deviation of triplicate samples.

APPENDIX D : QUALITY ASSURANCE AND QUALITY CONTROL PROCEDURES

The Quality assurance and Quality control (QA/QC) protocols were followed during the experimental analysis to ensure accuracy and the precision in the data obtained using both chemical and analytical methods.

- All the glass wares used in the experimental analysis were washed and cleaned by soaking them in a hypochlorite containing soap solution for at least 24 hours. Thereafter, the glasswares were washed with tap water followed by rinsing in distilled water, and were dried at the 180 ^{o}C for 3 hours. (Notable exceptions are plastic materials such as IC vials which are air dried at room temperature after rinsing with DI water, HPLC vials were ultra-sonicated for 15 min to clean and remove dirt inside the vials).

- All the chemicals used for the standards were of HPLC grade (> 98 % purity). All other chemicals used for basal media preparation (including pure sugars- glucose and xylose) were of analytical grade.

- Disposable glass Pasteur pipettes (VWR, Canada) were used for weighing the chemicals in preparing the stock solutions. The stock solutions (VFAs, Alcohols) were prepared in either 20 mL or 160 mL serum vials/bottles and sealed tightly using an aluminium cap along with Teflon-lined rubber septa to prevent loss via evaporation. Individual dilutions of stock solutions were prepared using separate plastic syringes (with varying volumes- 10 mL, 5 mL and 1 mL) to avoid cross contamination. New needles were replaced before the next usage. The stock solutions were stored in the refrigerator (at 4 °C) till use.

- Refrigerated stock solutions were used in preparation of varying concentrations of standards and all the dilutions from this stock solution were carried out using Milli-Q (MQ) water or diluted medium (basal medium and MQ) in a clean 5 or 20 mL serum vials.

- Stock solutions of the standards were prepared freshly for the chemical methods and analytical methods, the stocks were prepared for every 3-4 months. The quality of the standard stock was checked by loading a few standards on IC and HPLC to compare the relative change in the area with the original area of the standards ran at the time of preparation of the stock solution. If the difference is greater than 5% then fresh stock solutions were used.

- Alternatively, the chemical oxygen demand (COD) method was used in checking the quality of standards. The quality of the standard is estimated using the knowing theoretical oxygen demand for the standard.

- Calibration curves prepared for each set of analysis such as analytical methods (VFAs, alcohols, sugars, etc.) and chemical methods (COD, DNSA, anthrone methods, etc.) were established in the range of actual sample concentration observed with dilution. All calibration curves were established with minimum three replicates of each standard.

- The HPLC and IC reliability was checked before analyzing the samples for each phase of the experiments conducted. Blanks (MQ) and calibration standards were run at the beginning of each analysis of the samples in the instrument. (Note: For chemical methods such as DNSA, COD and anthrone tests, calibration curve was run at each time of reagent preparation.)

- Spike standards or internal standards in case of chemical methods were run periodically to validate and assess the instrumental accuracy and reliability in the solutions/reagents used in the method. The results are expressed as "mean ± standard deviation" of triplicate samples.

- Gas standards for the gas chromatograph (GC) analysis were prepared each time (i.e., during the time of calibration and the detoriation of the chromatographic column or worsening of the condition at injection of the samples due to dirt or moisture in the column are identified by a change in retention time, tailing of the peak or improper conductivity signal in the output). The column is cleaned by baking the column oven at 120 °C overnight. Then using a fresh set of standards,

three different known compositions are analyzed to check the quality of the column by comparing the deviation in slope obtained with the previous slope. A slope correction was made to ensure accurate analysis.

- The detection limits for the instrumental methods of analysis for each analytes were analyzed by preparing standards of lower concentrations. The detection limits for each instrument are given in the following section (QA/QC results).

- The pH probe was calibrated on a daily basis before measuring the pH of the samples. Fresh pH buffers (pH 4.0, pH 7.0 and pH 10.0) were used for calibrating the pH meter.

- The glass cuvette used for the spectrophotometer were cleaned with MQ water and wiped with Kimwipes® disposable wipers. The cuvettes (both before and after usage) were placed in iso-propanol solution to prevent bacterial contamination on the cuvette walls.

- All the batch (serum bottle; 160 mL) experiments were conducted in triplicate and the lab-scale reactor (5.0 L) experiments were conducted in replicates, in order to test the reproducibility associated with the process and to determine the errors associated with the sampling and analysis.

QA/QC RESULTS

D.1 Sample precision analysis and accuracy

The sample precision analysis was carried by running duplicate samples, injection of standards before the sample and/or spiking of the samples. Analytical precision was carried out by comparing the previous calibration with the new calibration standards. Precision implies the degree of similarity among measurements taken from two or more subsamples of a particular sample or from two or more repeated samples collected in close proximity i.e., both time and space (replicates). Accuracy is a measure of the agreement of a value of a variable in a sample with a known value (concentration). Accuracy refers to both random (random variation in precision of data) and systematic (values which are consistently lower or higher than the true value) errors during

sampling. The precision analysis is expressed in terms of relative percent difference (RPD) and accuracy is expressed in terms of percent recovery (% R). The detection limits (DL) i.e., sensitivity of the instruments used for experimental analysis was carried out by lowering the concentrations of the standards. The data for precision analysis, recovery analysis together with the lower detection limits of each instrument was provided in this Appendix.

$$RPD = \frac{(A_1 - A_2)}{B} * 100 \qquad (D.1.1)$$

Where A_1, A_2 represent the observed sample values; B represents the mean of the observed sample values

$$\% R = \frac{(A_p - B)}{A_k} * 100 \qquad (D.1.2)$$

Where A_p represent the measured value (area) of spiked sample; B represents the mean of the observed sample values and A_k represent the known value of the spiked standard.

Notes:

1. A shift in calibration curve and detection limits was observed, with change in column or detector etc., of the instrument over the duration of experiment. Accordingly, different calibration curves have been used for calculating the concentration in the samples. However, only one set of calibration curve and QA/QC for each analyte/instrument have been shown in Appendix A and B, respectively.

2. The injection volume used for finding the detection limits were 25 µL for IC and GC instruments and 10 µL for the HPLC.

D.2 Elemental CHNS analysis

The elemental analysis for the corn stalk was carried out at Department of Chemistry and Bio-Chemistry, University of Windsor, Ontario. The instrument specifications list: accuracy ≤ 0.3% and precision ≤ 0.2% using Helium as carrier gas. The detection limits were less than 0.5% for CHNS with a sample volume of 2 mg.

D.3 Gas Chromatograph

The calibration curve for the experimental standards had less than 5% deviation over the period of research conducted. The detection limits of H_2 and CH_4 were 0.0032 kPa (5 µL per 160 mL) and 0.0064 kPa (10 µL per 160 mL), respectively.

B.4 High Performance liquid chromatograph (HPLC)

Table D.1 HPLC-VFA method - QA/QC results

VFAs	Spiked Conc. (mg L^{-1})	RPD (%)	Recovery (%)	Detection Limit (mg L^{-1})
Lactic acid	30.0	4.83	87.34	2.0
Acetic acid	30.0	1.27	102.22	2.0
Propionic acid	30.0	5.35	98.96	2.0
Formic acid	30.0	11.29	101.03	2.0
Butyric acid	30.0	0.32	101.27	2.0

Table D.2 HPLC-Furan method - QA/QC results

Furans	Spiked Conc. (mg L^{-1})	RPD (%)	Recovery (%)	Detection Limit (mg L^{-1})
Furfural	25.0	3.59	101.70	1.0
5-Hydroxymethyl furfural	25.0	3.45	101.64	1.0
2-Furoic acid	25.0	3.94	100.86	1.0
Furfuryl alcohol	25.0	3.63	101.18	1.0

D.5 Ion-exchange chromatograph (IC)

Table D.3 IC-Alcohol method - QA/QC results

Alcohols	Spiked Conc. (mg L^{-1})	RPD (%)	Recovery (%)	Detection Limit (mg L^{-1})
Iso-propanol	50.0	2.88	97.63	10.0
Ethanol	50.0	7.86	113.86	10.0
Propanol	50.0	5.18	121.09	10.0
Iso-butanol	50.0	7.54	104.41	10.0
Butanol	50.0	8.81	80.86	10.0

Table D.4 IC-Sugar method - QA/QC results

Sugars	Spiked Conc. (mg L^{-1})	RPD (%)	Recovery (%)	Detection Limit (mg L^{-1})
Glucose	100.0	1.30	98.12	5.0
Xylose	100.0	0.17	98.95	5.0
Arabinose	100.0	0.10	97.86	5.0
Mannose	100.0	7.82	95.93	5.0
Galactose	100.0	4.38	106.08	5.0

D.6 Chemical methods

Table D.5 Chemical methods - QA/QC results

Analysis	Spiked Conc. (mg L^{-1})	RPD (%)	Recovery (%)	Detection Limit (mg L^{-1})
DNS (reducing sugar)	100.0	5.24	100.37	50.0
Anthrone (Total carbohydrate sugar)	100.0	7.93	100.30	10.0
COD (glucose)	100.0	1.46	100.15	100.0
Phenol	60.0	6.62	99.61	10.0

APPENDIX E : CLUSTER ANALYSIS

The enormous amount of data generated across the life sciences due to the recent advances in experimental methods have made clustering and classification techniques the most widely used tool which are once predominantly used only by ecologists (Fielding, 2007). Cluster analysis is an approach that finds structure in data by identifying groupings in the data (Fielding, 2007). In many multivariate analyses the first step is to measure the ecological distance between data sets (Shi, 1993). All the similarity functions could be used in the same way. The species occurrences are arranged in the same way for each site, and that any absent species are represented by a zero. The species estimator functions could be broadly grouped into two categories based on the usage of occurrence data (i.e., presence or absence of T-RF bands) or abundance data (T-RF band intensity). It is possible to use the occurrence based measures with the abundance data where the abundance matrix is converted to an occurrence matrix by the function. Jaccard similarity measure is one of the oldest and the best known occurrence measure (Jaccard, 1912). It is also known as coefficient of community (Shi, 1993). Extensive usage of this measure is largely due to its simplicity and intuitiveness (Shi, 1993). Similar to Jaccard similarity index, Sorenson measure also known as Dice, Czekanowski or Coincidence index is also common in use. It focuses more on shared species present rather than unshared. Kulczynski similarity index is also occasionally used as alternative to these two of the above mentioned indices. The most widely used abundance based measure is the Bray-Curtis similarity measure (Bray and Curtis, 1957; Clarke, 1993; Faith et al., 1987; Minchin, 1987). This measure is an equivalent of Sorenson measure when used as a similarity measure using the occurrence data. The formula used for calculating these indices are as follows:

$$Dice = \frac{2C}{N1 + N2}$$

$$Kulczynski = \frac{C(N1 + N2)}{2(N1 * N2)}$$

$$Jaccard = \frac{C}{N1 + N2 - C}$$

Where, C = species present in both areas; N1 = total number of species in first area; N2 = total number of species in second area

References:

Bray, J.R., Curtis, J.T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr*, **27**(4), 326-49.

Clarke, K.R. (1993). Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol*, **18**(1), 117-43.

Faith, D.P., Minchin, P.R., Belbin, L. (1987). Compositional dissimilarity as a robust measure of ecological distance. *Vegetatio*, **69**(1-3), 57-68.

Fielding, A.H. (2007). *Cluster and classification techniques for the biosciences. Cambridge university press, New York*.

Jaccard, P. (1912). The distribution of the flora in the alpine zone. *New Phytol*, **11**(2), 37-50.

Minchin, P.R. (1987). An evaluation of the relative robustness of techniques for ecological ordination. *Vegetatio*, **69**(1-3), 89-107.

Shi, G.R. (1993). Multivariate data analysis in paleoecology and paleobiogeography - a review. *Palaeogeogr Palaeocl*, **105**(3-4), 199-234.

APPENDIX F : TESTING FOR ROBUSTNESS USING PCA DATA

The bi-plot PCA (Figure 4.7) shown in Chapter 4 was tested for robustness using the Box-plot. The Box-plot indicated that Exp # 3 (21-7.5-1) was the outlier. Since the data set contains many zeros (where negligible H_2 consumption occurs), log transformation of the original data set was performed in STATISTICA version 8.0. The PCA was then performed using the STATISTICA software. The score plot (Figure 1) and loading plot (Figure 2) of the log transformed PCA variables was shown below.

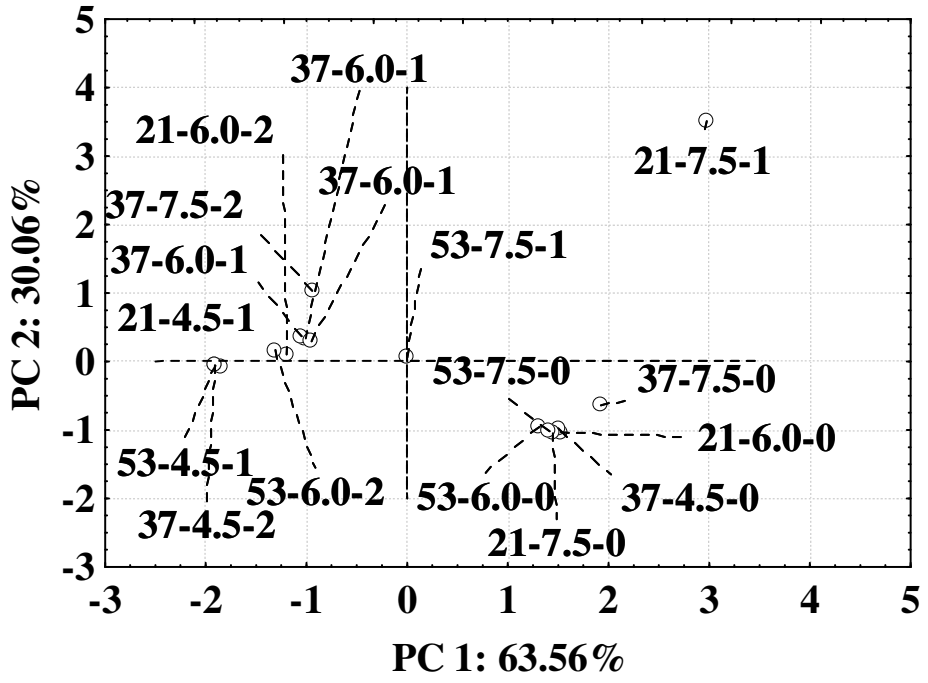

Figure F.1 Score plot using the log transformed PCA data (Chapter 4).
Note: Labels indicate fermentation temperature-pH-LA concentration. For example, 37-7.5-2 represents the fermentation temperature of 37 °C, pH of 7.5 and 2 g L^{-1} LA concentration.

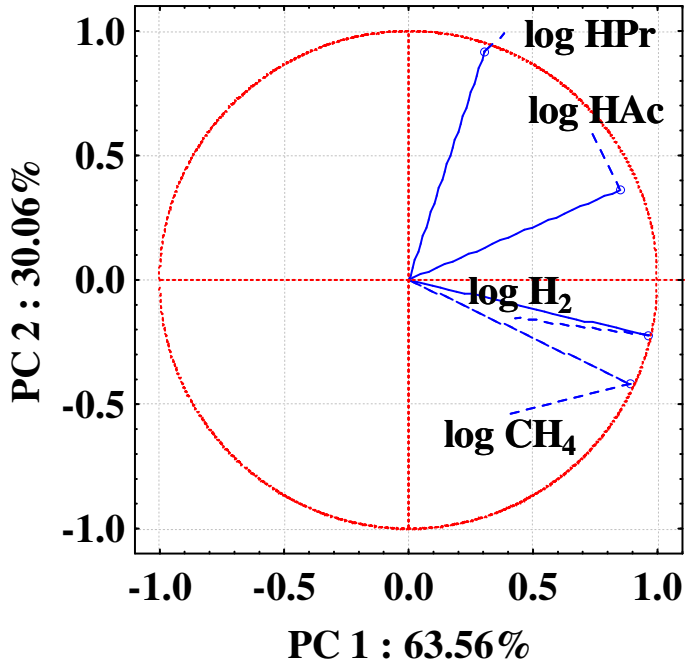

Figure F.2 Loading map of the log transformed PCA data (Chapter 4).

Table F.1 Principal Component loadings of the log transformed PCA.

Factor Loadings		
Variable	**PC-I**	**PC-II**
Log H_2	0.9579	-0.2561
Log CH_4	0.8825	-0.4444
Log HAc	0.8437	0.3815
Log HPr	0.3509	0.8995
Explained Variance	2.5311	1.2178
Proportion of total Variance	0.6328	0.3044
Cumulative Variance explained	63.2781	93.7221

Note: Only the data for first two principal components are shown.

The factor scores of principal components (PC 1 and PC 2) having the highest variance (Table 1) was then checked for the outlier using the Box-plot. Again the data shows that Exp # 3 as outlier. Hence, removal of the outlier was carried out. The results (factor and loading scores) obtained using the outlier removal procedure was shown in Figure 3 and 4.

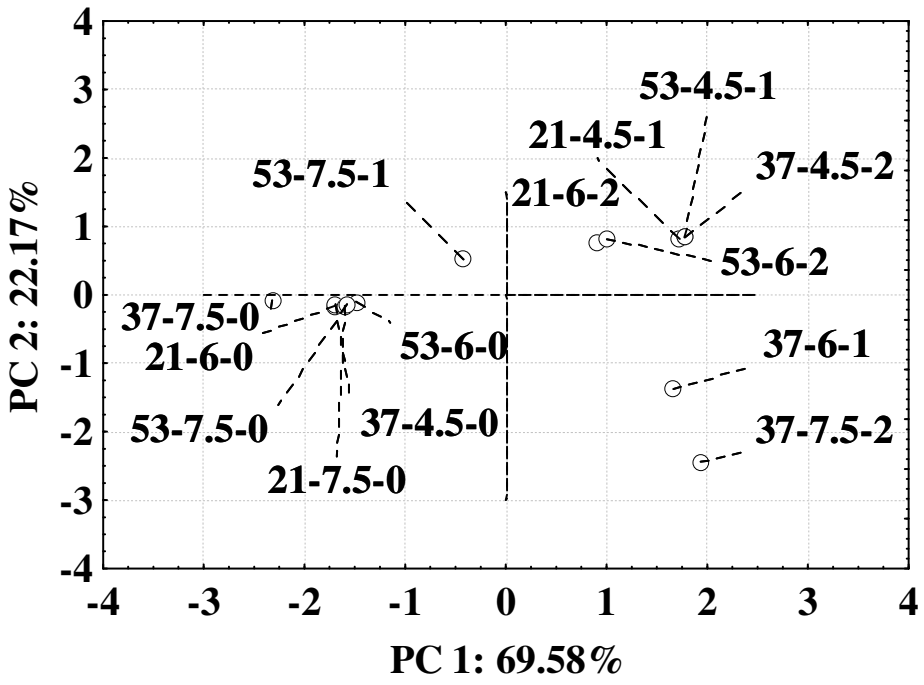

Figure F.3 Score plot using the log transformed PCA data (Chapter 4) with outlier term removed. Note: Labels indicate fermentation temperature-pH-LA concentration. For example, 37-7.5-2 represents the fermentation temperature of 37 °C, pH of 7.5 and 2 g L^{-1} LA concentration.

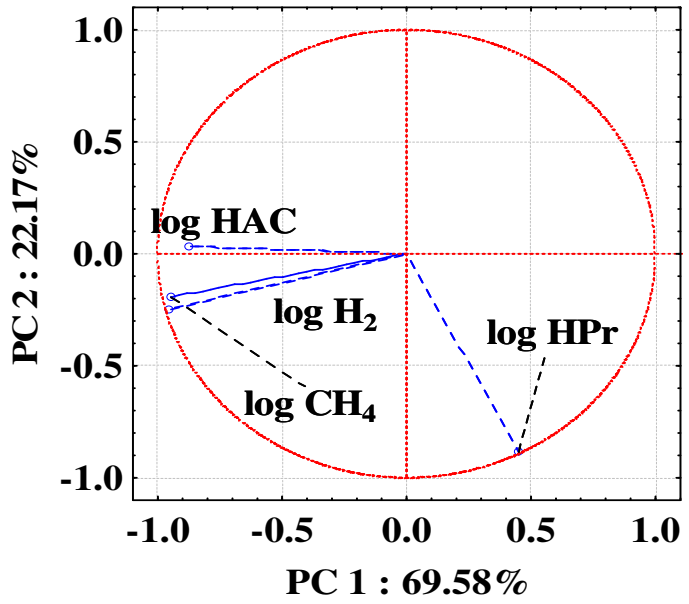

Figure F.4 Loading map of the log transformed PCA data (Chapter 4) with outlier term removed.

Table F.2 Principal Component loadings of the log transformed PCA.

Factor Loadings		
Variable	**PC-I**	**PC-II**
Log H_2	-0.9575	-0.2513
Log CH_4	-0.9463	-0.1948
Log HAc	-0.8762	0.0294
Log HPr	0.4506	-0.8858
Explained Variance	2.7830	0.8866
Proportion of total Variance	0.6957	0.2217
Cumulative Variance explained	69.5759	91.7421

Note: Only the data for first two principal components are shown.

Robustness testing has been carried out for this outlier removed data which passed the robustness test (i.e., doesn't show any outlier). The ANOVA for the log transformed PCA factor scores with both outlier terms not removed and removed are provided in Table F.3 and F.4 respectively.

Table F.3 ANOVA for the log transformed principal component factor scores (Figure F.1) at different factor levels.

Source	DF	Seq SS		MS		F-value		*p-value*	
		Factor score 1	**Factor score 2**	**Factor score 1**	**Factor score 2**	**Factor score 1**	**Factor score 2**	**Factor score 1**	**Factor score 2**
Regression	9	38.534	36.782	4.282	4.087	24.770	23.644	0.000	0.000
Linear	3	32.248	19.347	10.749	6.449	62.187	37.309	0.000	0.000
Temp (X1)	1	1.939	3.394	1.939	3.394	11.218	19.636	0.002	0.000
pH (X2)	1	10.024	7.351	10.024	7.351	57.992	42.529	0.000	0.000
LA (X3)	1	20.285	8.602	20.285	8.602	117.352	49.762	0.000	0.000
Square	3	3.253	9.806	1.084	3.269	6.273	18.911	0.002	0.000
X1^2	1	0.417	0.433	0.417	0.433	2.414	2.507	0.058*	0.136*
X2^2	1	0.682	1.977	0.682	1.977	3.945	11.438	0.031	0.002
X3^2	1	2.154	6.659	2.154	6.659	12.458	38.526	0.001	0.000
Interaction	3	3.033	7.629	1.011	2.543	5.849	14.712	0.002	0.000
X1*X2	1	2.919	7.335	2.919	7.335	16.886	42.435	0.000	0.000
X1*X3	1	0.007	0.000	0.007	0.000	0.043	0.002	0.838*	0.962*
X2*X3	1	0.107	0.294	0.107	0.294	0.619	1.698	0.437*	0.218*
Residual error	35	6.050	6.520	0.173	0.186				
Lack-of Fit	3	5.775	5.396	1.925	1.799	224.078	51.220	0.000	0.000
Pure error	32	0.275	1.124	0.009	0.035				
Total	44	44.584	43.302						

Notes: 1. $F_{(critical)\ 0.05,9,45} = 2.10$; 2. * denotes *p-values* which are insignificant at $\alpha = 0.05$.

Table F.4 ANOVA for the log transformed principal component factor scores (Figure F.3) at different factor levels.

Source	DF	Seq SS		MS		F-value		*p-value*	
		Factor score 1	**Factor score 2**	**Factor score 1**	**Factor score 2**	**Factor score 1**	**Factor score 2**	**Factor score 1**	**Factor score 2**
Regression	9	106.757	32.257	11.862	3.584	55.502	20.870	0.000	0.000
Linear	3	80.134	9.364	25.648	2.158	120.009	12.570	0.000	0.000
Temp (X1)	1	0.038	0.324	0.432	0.510	2.022	2.970	0.165*	0.094*
pH (X2)	1	6.792	8.098	2.159	5.528	10.102	32.190	0.003	0.000
LA (X3)	1	73.305	0.942	73.305	0.942	342.998	5.490	0.000	0.026
Square	3	24.961	14.220	8.734	3.915	40.869	22.790	0.000	0.000
X1^2	1	1.966	12.275	0.859	10.580	4.021	61.600	0.053*	0.000
X2^2	1	0.482	1.785	0.026	1.044	0.120	6.080	0.731*	0.019
X3^2	1	22.513	0.160	23.636	0.380	110.595	2.210	0.000	0.147*
Interaction	3	1.661	8.673	0.554	2.891	2.591	16.830	0.070	0.000
X1*X2	1	1.268	0.595	1.268	0.595	5.933	3.460	0.021	0.072
X1*X3	1	0.031	0.000	0.031	0.000	0.145	0.001	0.706*	0.974*
X2*X3	1	0.362	8.078	0.362	8.078	1.694	47.040	0.202*	0.000
Residual error	32	6.839	5.496	0.214	0.172				
Lack-of Fit	2	5.903	5.305	2.952	2.652	94.599	416.607	0.000	0.000
Pure error	30	0.936	0.191	0.031	0.006				
Total	41	113.596	37.753						

Notes: 1. $F_{(critical)\ 0.05,9,42} = 2.10$; 2.* denotes *p-values* which are insignificant at $\alpha = 0.05$.

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